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Abstract

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

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

1 Introduction

Bacillus cereus is a foodborne pathogen and an endospore-forming bacteria causing diarrheal or emetic food poisoning (Ehling-Schulz et al., 2004; Stenfors Arnesen et al., 2008; Forghani et al., 2014). Because its spores survive a wide range of heat-treatments, (Luu-Thi et al., 2014), *B. cereus* represents a particular risk for cooked or pasteurised food products. It has been reported as the second leading cause of foodborne outbreaks in France (Santé_publique_France, 2019) since 2012. In EU the second leading cause of foodborne outbreaks corresponds to the "toxin producing bacteria" that includes *B. cereus*, *Staphylococcus aureus* and *Clostridium* spp other than *C. botulinum* (EFSA and ECDC, 2018). *B. cereus* foodborne outbreaks are mostly associated to consumption of foods of non-animal origin, including vegetables and vegetables purées (Lund et al., 2000; Cadel Six et al., 2012; EFSA, 2012; Bennett et al., 2013; EFSA, 2013; Cadel Six et al., 2014; EFSA, 2014, 2015). *B. cereus sensu lato* was divided into seven phylogenetic groups in function of their optimal growth temperature (Guinebretière et al., 2008). In particular, it includes groups of psychrotolerant strains which spores can germinate and grow in foods stored at refrigeration temperatures. Spores of *B. cereus* are particularly prevalent in vegetables and heat processed, non-sterilized, foods containing vegetables (Choma et al., 2000; Del Torre et al., 2001; Valero et al., 2002; Daelman et al., 2013). The capacity of psychrotolerant *B. cereus* strains to grow at temperatures of refrigeration in vegetables was reported (Valero et al., 2000; Valero et al., 2003; Valero et al., 2007; Samapundo et al., 2011; De Sarrau et al., 2013a). However, heat processed, non-sterilized, foods containing vegetables are stored under refrigeration and can be packaged without oxygen or under air. The aim of the present study was to determine the growth capacity of psychrotolerant *B. cereus* in a vegetable-based medium, at cold temperature, in presence or absence of oxygen. This study was performed with a 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 ± 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 Amsko 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 ± 0.2 were inoculated with 100 µl 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 µl 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

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

2.3 Microscopy and image analysis

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

2.4 Cell viability

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

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 µl of a Nile red solution prepared by dissolving 0.5 mg/ml Nile red
144 (Sigma) in absolute ethanol, were added to 100 µl 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 µl of phosphate buffer saline (PBS)
147 with Tween 80. This step was performed twice and cells were finally suspended in 100 µl 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

decline in number observed for 4 batches of carrots, cells grew in carrot broth (Fig. 2A) and *B. weihenstephanensis* reached a maximal population (N_{max}) of approximately 8.2 $\log_{10}$ CFU/ml, followed by a rapid decline (Fig. 2A).

B. weihenstephanensis behaved similarly in carrot broth boiled in air before tyndallisation in air (condition 2 in Fig. 1) and in carrot broth directly tyndallized in air, without boiling (condition 1 in Fig. 1), with an initial reduction in counts upon inoculation and no growth (or delayed growth depending on carrot batches) at 8 °C (data not shown).

When carrot broth was prepared and tyndallized anaerobically (conditions 3 and 4 in Fig. 1) we enumerated the expected 10^3 CFU/ml inoculated at time zero (Fig. 2A and 2B), showing that the lethal effect did not occurred or was abolished in this condition. This result was observed in both anaerobic broths kept anaerobic (condition 4 in Fig. 1, Fig. 2B) and aerated (condition 3 in Fig. 1, Fig. 2A), after tyndallization. In these carrot broths prepared according to conditions 3 and 4, *B. weihenstephanensis* multiplied at 30 °C and at 8 °C, and reached a N_{max} from 7.5 to 8 $\log_{10}$ CFU/ml (Fig. 2A and B).

Because *B. weihenstephanensis* can be present in foods as spores, we then tested inoculations with suspension of spores at 10^3 CFU/ml and 10^6 CFU/ml in carrot broth in tubes tyndallized with oxygen (condition 1 in Fig. 1) (Table 1). We recovered the expected populations at time zero, suggesting that unlike vegetative cells spores were not killed shortly after inoculation. When tubes inoculated with spores were incubated at 30 °C, numbers of CFU remained stable for 2 h before starting to increase and reached the same N_{max} as with a vegetative cells inoculum (data not shown).

3.2 Microscopic observations of B. weihenstephanensis cells in carrot broths

Morphology of *B. weihenstephanensis* KBAB4 cells changed with the different culture 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 % ± 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.

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

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

B. weihenstephanensis KBAB4 cells were able to grow aerobically in carrot broth at 30 °C with a N_{max} around 8 $\log_{10}$ CFU/ml. Apart of the initial decline, no difference was observed in growth kinetic and in cell morphology in carrot-broth tyndallized with or without oxygen, suggesting that the effect of the lethal compound(s) present in carrot-broth tyndallized aerobically was rather short in these conditions. It is possible that the compounds were inactivated while reacting with the bacterial cells after inoculation. In contrast, no growth (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 $\log_{10}$ CFU/ml after 6 days and had regular shapes with no

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

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

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288 **References**

289 Abbas, A.A., Planchon, S., Jobin, M., Schmitt, P., 2014. Absence of oxygen affects the
290 capacity to sporulate and the spore properties of *Bacillus cereus*. Food Microbiol. 42, 122-
291 131. 10.1016/j.fm.2014.03.004.
292 Babic, I., Nguyen-the, C., Amiot, M.J., Aubert, S., 1994. Antimicrobial activity of shredded
293 carrot extracts on food-borne bacteria and yeast. J. Appl. Bacteriol. 76, 135-141.
294 10.1111/j.1365-2672.1994.tb01608.x.
295 Bennett, S.D., Walsh, K.A., Gould, L.H., 2013. Foodborne Disease Outbreaks Caused by
296 *Bacillus cereus*, *Clostridium perfringens*, and *Staphylococcus aureus*-United States, 1998-
297 2008. Clin. Infect. Dis. 57, 425-433. 10.1093/cid/cit244.
298 Beuchat, L.R., Brackett, R.E., 1990. Inhibitory effects of raw carrots on *listeria-*
299 *monocytogenes*. Appl. Env. Microbiol. 56, 1734-1742.
300 Bressuire-Isoard, C., Bornard, I., Henriques, A.O., Carlin, F., Broussolle, V., 2016.
301 Sporulation Temperature Reveals a Requirement for CotE in the Assembly of both the Coat
302 and Exosporium Layers of *Bacillus cereus* Spores. Appl. Environ. Microbiol. 82, 232-243.
303 10.1128/aem.02626-15.
304 Cadel Six, S., De Buyser, M.L., Vignaud, M.L., Dao, T.T., Messio, S., Pairaud, S.,
305 Hennekinne, J.A., Pihier, N., Brisabois, A., 2012. Toxi-infections alimentaires collectives à
306 *Bacillus cereus*: Bilan de la caractérisation des souches de 2006 à 2010. BEH, 45-49.
307 Cadel Six, S., Herbin, S., Vignaud, M.L., Chretien, R., Messio, S., Pairaud, S., Prigent, L.,
308 Hennekinne, J.A., Brisabois, A., 2014. Investigation d'une toxi-infection alimentaire
309 collective (TIAC) à *Bacillus cereus* producteurs d'entérotoxines. Bull. épidémiol., santé anim.
310 et alim. 63, 20-23.
311 Chen, H.J., Tsai, T.K., Pan, S.C., Lin, J.S., Tseng, C.L., Shaw, G.C., 2010. The master
312 transcription factor Spo0A is required for poly(3-hydroxybutyrate) (PHB) accumulation and

313 expression of genes involved in PHB biosynthesis in *Bacillus thuringiensis*. Fems Microbiol.
314 Lett. 304, 74-81. 10.1111/j.1574-6968.2010.01888.x.
315 Choma, C., Guinebretière, M.H., Carlin, F., Schmitt, P., Velge, P., Granum, P.E., Nguyen-
316 The, C., 2000. Prevalence, characterization and growth of *Bacillus cereus* in commercial
317 cooked chilled foods containing vegetables. J. Appl. Microbiol. 88, 617-625. 10.1046/j.1365-
318 2672.2000.00998.x.
319 Daelman, J., Membré, J.-M., Jacxsens, L., Vermeulen, A., Devlieghere, F., Uyttendaele, M.,
320 2013. A quantitative microbiological exposure assessment model for *Bacillus cereus* in
321 REPFEDs. Int. J. Food Microbiol. 166, 433-449.
322 http://dx.doi.org/10.1016/j.ijfoodmicro.2013.08.004.
323 De Sarrau, B., Clavel, T., Bornard, I., Nguyen-the, C., 2013b. Low temperatures and
324 fermentative metabolism limit peptidoglycan digestion of *Bacillus cereus*. Impact on colony
325 forming unit counts. Food Microbiol. 33, 213-220.
326 http://dx.doi.org/10.1016/j.fm.2012.09.019.
327 De Sarrau, B., Clavel, T., Zwickel, N., Despres, J., Dupont, S., Beney, L., Tourdot-Marechal,
328 R., Nguyen-the, C., 2013a. Unsaturated fatty acids from food and in the growth medium
329 improve growth of *Bacillus cereus* under cold and anaerobic conditions. Food Microbiol. 36,
330 113-122. 10.1016/j.fm.2013.04.008.
331 Degirmenci, H., Karapinar, M., Karabiyikli, S., 2012. The survival of *E. coli* O157:H7, *S.*
332 *Typhimurium* and *L. monocytogenes* in black carrot (Daucus carota) juice. Int. J. Food
333 Microbiol. 153, 212-215. 10.1016/j.ijfoodmicro.2011.11.017.
334 Del Torre, M., Della Corte, M., Stecchini, M.L., 2001. Prevalence and behaviour of *Bacillus*
335 *cereus* in a REPFED of Italian origin. Int. J. Food Microbiol. 63, 199-207.
336 http://dx.doi.org/10.1016/S0168-1605(00)00421-9.

337 EFSA, 2012. Scientific report of EFSA and ECDC - The European Union summary report on
338 trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2010. EFSA J.
339 10, 2597. 10.2903/j.efsa.2012.2597.
340 EFSA, 2013. Scientific report of EFSA and ECDC - The European Union summary report on
341 trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2011. EFSA J.
342 11, 3129. 10.2903/j.efsa.2013.3129.
343 EFSA, 2014. Scientific report of EFSA and ECDC - The European Union summary report on
344 trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2012. EFSA J.
345 12, 3547. 10.2903/j.efsa.2014.3547.
346 EFSA, 2015. Scientific report of EFSA and ECDC - The European Union summary report on
347 trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2013. EFSA J.
348 13, 3991. 10.2903/j.efsa.2015.3991.
349 EFSA, ECDC, 2018. The European Union summary report on trends and sources of zoonoses,
350 zoonotic agents and food-borne outbreaks in 2017. EFSA Journal 16, 5500, 5262 pp.
351 https://doi.org/10.2903/j.efsa.2018.5500.
352 Ehling-Schulz, M., Fricker, M., Scherer, S., 2004. *Bacillus cereus*, the causative agent of an
353 emetic type of food-borne illness. Mol. Nutr. Food Res. 48, 479-487.
354 10.1002/mnfr.200100055.
355 Forghani, F., Kim, J.-B., Oh, D.-H., 2014. Enterotoxigenic Profiling of Emetic Toxin- and
356 Enterotoxin-Producing *Bacillus cereus*, Isolated from Food, Environmental, and Clinical
357 Samples by Multiplex PCR. J. Food Sci., 2288-2293. 10.1111/1750-3841.12666.
358 Garton, N.J., Christensen, H., Minnikin, D.E., Adegbola, R.A., Barer, M.R., 2002.
359 Intracellular lipophilic inclusions of mycobacteria in vitro and in sputum. Microbiology 148,
360 2951-2958. doi:10.1099/00221287-148-10-2951.

Guérin, A., Dargaignaratz, C., Broussolle, V., Clavel, T., Nguyen-the, C., 2016. Combined effect of anaerobiosis, low pH and cold temperatures on the growth capacities of psychrotrophic *Bacillus cereus*. Food Microbiol. 59, 119-123.

Guinebretière, M.H., Thompson, F.L., Sorokin, A., Normand, P., Dawyndt, P., Ehling-Schulz, M., Svensson, B., Sanchis, V., Nguyen-The, C., Heyndrickx, M., De Vos, P., 2008. Ecological diversification in the *Bacillus cereus* Group. Env. Microbiol. 10, 851-865. 10.1111/j.1462-2920.2007.01495.x.

Guinebretiere, M.H., Velge, P., Couvert, O., Carlin, F., Debuyser, M.L., Nguyen-The, C., 2010. Ability of *Bacillus cereus* group strains to cause food poisoning varies according to phylogenetic affiliation (groups I to VII) rather than species affiliation. J. Clin. Microbiol. 48, 3388-3391.

Lopez, J.A., Naranjo, J.M., Higuita, J.C., Cubitto, M.A., Cardona, C.A., Villar, M.A., 2012. Biosynthesis of PHB from a new isolated *Bacillus megaterium* strain: Outlook on future developments with endospore forming bacteria. Biotechnol. Bioprocess Eng. 17, 250-258. 10.1007/s12257-011-0448-1.

Lund, T., De Buyser, M.-L., Granum, P.E., 2000. A new cytotoxin from *Bacillus cereus* that may cause necrotic enteritis. Mol. Microbiol. 38, 254-261. 10.1046/j.1365-2958.2000.02147.x.

Luu-Thi, H., Khadka, D.B., Michiels, C.W., 2014. Thermal inactivation parameters of spores from different phylogenetic groups of *Bacillus cereus*. Int. J. Food Microbiol. 189, 183-188. http://dx.doi.org/10.1016/j.ijfoodmicro.2014.07.027.

Nguyen-the, C., Lund, B.M., 1991. The lethal effect of carrot on listeria species. J. Appl. Bacteriol. 70, 479-488. 10.1111/j.1365-2672.1991.tb02744.x.

Nguyen-the, C., Lund, B.M., 1992. An investigation of the antibacterial effect of carrot on *listeria-monocytogenes*. J. Appl. Bacteriol. 73, 23-30. 10.1111/j.1365-2672.1992.tb04964.x.

386 Noriega, E., Newman, J., Saggers, E., Robertson, J., Laca, A., Diaz, M., Brocklehurst, T.F.,
387 2010. Antilisterial activity of carrots Effect of temperature and properties of different carrot
388 fractions. Food Res. Int. 43, 2425-2431. 10.1016/j.foodres.2010.09.012.
389 Obruca, S., Sedlacek, P., Mravec, F., Samek, O., Marova, I., 2016. Evaluation of 3-
390 hydroxybutyrate as an enzyme-protective agent against heating and oxidative damage and its
391 potential role in stress response of poly(3-hydroxybutyrate) accumulating cells. Appl.
392 Microbiol. Biot. 100, 1365-1376. 10.1007/s00253-015-7162-4.
393 Pandiani, F., Brillard, J., Bornard, I., Michaud, C., Chamot, S., Nguyen-the, C., Broussolle,
394 V., 2010. Differential involvement of the five RNA helicases in adaptation of *Bacillus cereus*
395 ATCC 14579 to low growth temperatures. Appl. Environ. Microbiol. 76, 6692-6697.
396 Samapundo, S., Everaert, H., Wandutu, J.N., Rajkovic, A., Uyttendaele, M., Devlieghere, F.,
397 2011. The influence of headspace and dissolved oxygen level on growth and haemolytic BL
398 enterotoxin production of a psychrotolerant *Bacillus weihenstephanensis* isolate on potato
399 based ready-to-eat food products. Food Microbiol. 28, 298-304.
400 http://dx.doi.org/10.1016/j.fm.2010.04.013.
401 Santé_publique_France, 2019. Surveillance des toxi-infections alimentaires collectives -
402 Données de la déclaration obligatoire, 2017. http://invs.santepubliquefrance.fr/Dossiers-
403 thematiques/Maladies-infectieuses/Maladies-a-declaration-obligatoire/Toxi-infections-
404 alimentaires-collectives/Donnees-epidemiologiques
405 Sorokin, A., Candelon, B., Guilloux, K., Galleron, N., Wackerow-Kouzova, N., Ehrlich, S.D.,
406 Bourguet, D., Sanchis, V., 2006. Multiple-locus sequence typing analysis of *Bacillus cereus*
407 and *Bacillus thuringiensis* reveals separate clustering and a distinct population structure of
408 psychrotrophic strains. Appl. Env. Microbiol. 72, 1569-1578. 10.1128/aem.72.2.1569-
409 1578.2006.

410 Stenfors Arnesen, L.P., Fagerlund, A., Granum, P.E., 2008. From soil to gut: *Bacillus cereus*
411 and its food poisoning toxins. Fems Microbiol. Rev. 32, 579-606. 10.1111/j.1574-
412 6976.2008.00112.x.
413 Valero, M., Fernández, P.S., Salmerón, M.C., 2003. Influence of pH and temperature on
414 growth of *Bacillus cereus* in vegetable substrates. Int. J. Food Microbiol. 82, 71-79.
415 http://dx.doi.org/10.1016/S0168-1605(02)00265-9.
416 Valero, M., Hernandez-Herrero, L.A., Fernandez, P.S., Salmeron, M.C., 2002.
417 Characterization of *Bacillus cereus* isolates from fresh vegetables and refrigerated minimally
418 processed foods by biochemical and physiological tests. Food Microbiol. 19, 491-499.
419 10.1006/yfmic.507.
420 Valero, M., Hernández-Herrero, L.A., Giner, M.J., 2007. Survival, isolation and
421 characterization of a psychrotrophic *Bacillus cereus* strain from a mayonnaise-based ready-to-
422 eat vegetable salad. Food Microbiol. 24, 671-677. http://dx.doi.org/10.1016/j.fm.2007.04.005.
423 Valero, M., Leontidis, S., Fernández, P.S., Martínez, A., Salmerón, M.C., 2000. Growth of
424 *Bacillus cereus* in natural and acidified carrot substrates over the temperature range 5–30°C.
425 Food Microbiol. 17, 605-612. http://dx.doi.org/10.1006/fmic.2000.0352.
426 Vilas-Boas, G., Sanchis, V., Lereclus, D., Lemos, M.V.F., Bourguet, D., 2002. Genetic
427 differentiation between sympatric populations of *Bacillus cereus* and *Bacillus thuringiensis*.
428 Appl. Env. Microbiol. 68, 1414-1424.
429 Wang, Q., Yu, H.M., Xia, Y.Z., Kang, Z., Qi, Q.S., 2009. Complete PHB mobilization in
430 *Escherichia coli* enhances the stress tolerance: a potential biotechnological application.
431 Microb. Cell Fact. 8. 47
432 10.1186/1475-2859-8-47.

433 Wu, D.D., He, J., Gong, Y.H., Chen, D.J., Zhu, X.L., Qiu, N., Sun, M., Li, M.S., Yu, Z.N.,
434 2011. Proteomic analysis reveals the strategies of *Bacillus thuringiensis* YBT-1520 for
435 survival under long-term heat stress. Proteomics 11, 2580-2591. 10.1002/pmic.201000392.
436 Zhao, Y.H., Li, H.M., Qin, L.F., Wang, H.H., Chen, G.Q., 2007. Disruption of the
437 polyhydroxyalkanoate synthase gene in *Aeromonas hydrophila* reduces its survival ability
438 under stress conditions. Fems Microbiol. Lett. 276, 34-41. 10.1111/j.1574-
439 6968.2007.00904.x.
440
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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[a]	10^3	10^6	10^3	10^6
With oxygen	Aerobiosis	< 10 [b]	< 10	10^3	10^6
Without oxygen	Aerobiosis	10^3	NT [c]	NT	NT
Without oxygen	Anaerobiosis	10^3	NT	NT	NT

447

448 [a] Inoculation was done at room temperature with cells or spores

449 [b] Limit of detection was 10 CFU/ml.

450 [c]NT: not tested.

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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 with10^3 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 µm.

479

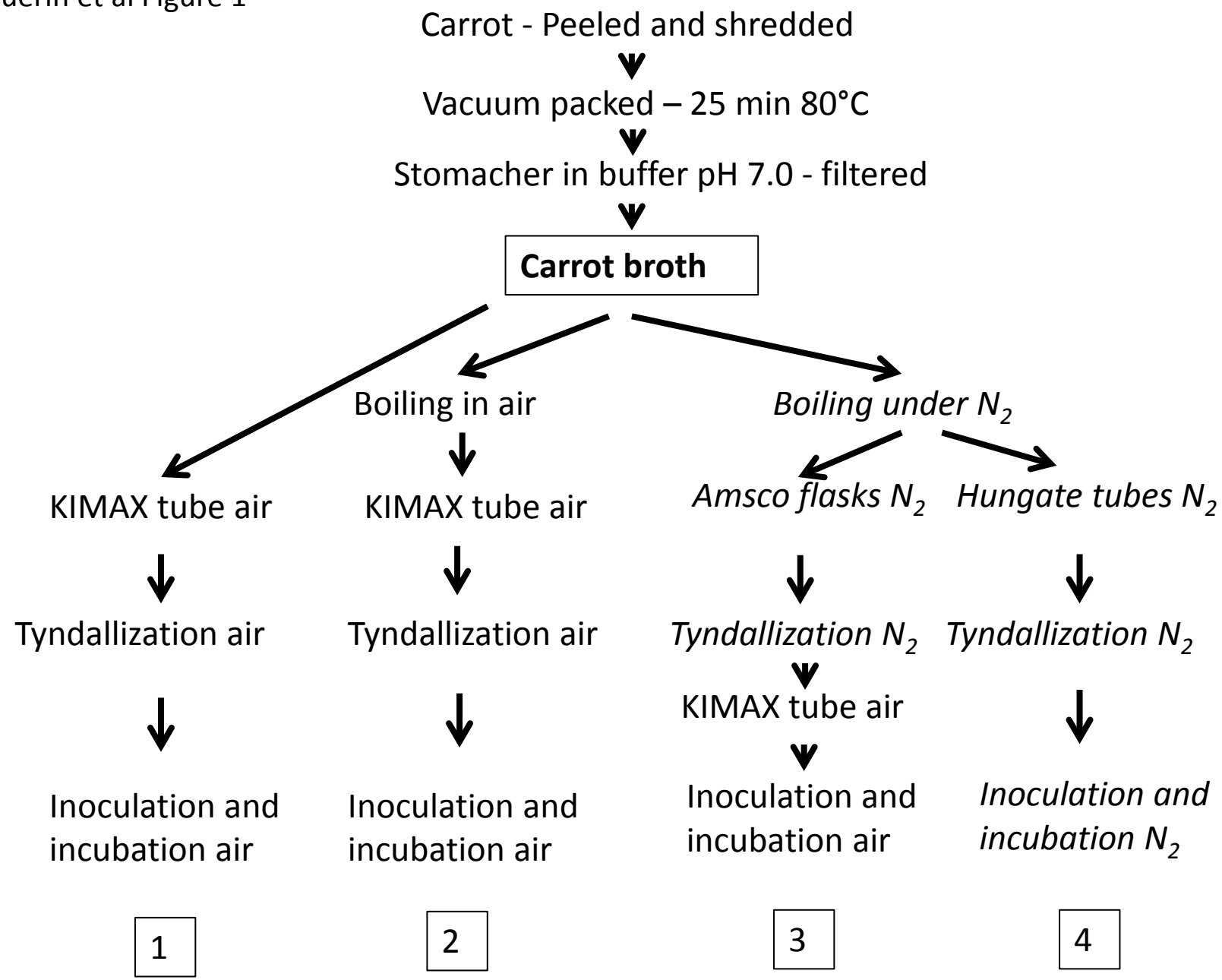
480 Fig. 4. Microscopic observations of *B. weihenstephanensis* KBAB4 under phase contrast (A

481 and C) and epifluorescence (B and D) (X 1,000). KBAB4 cells were cultivated aerobically for

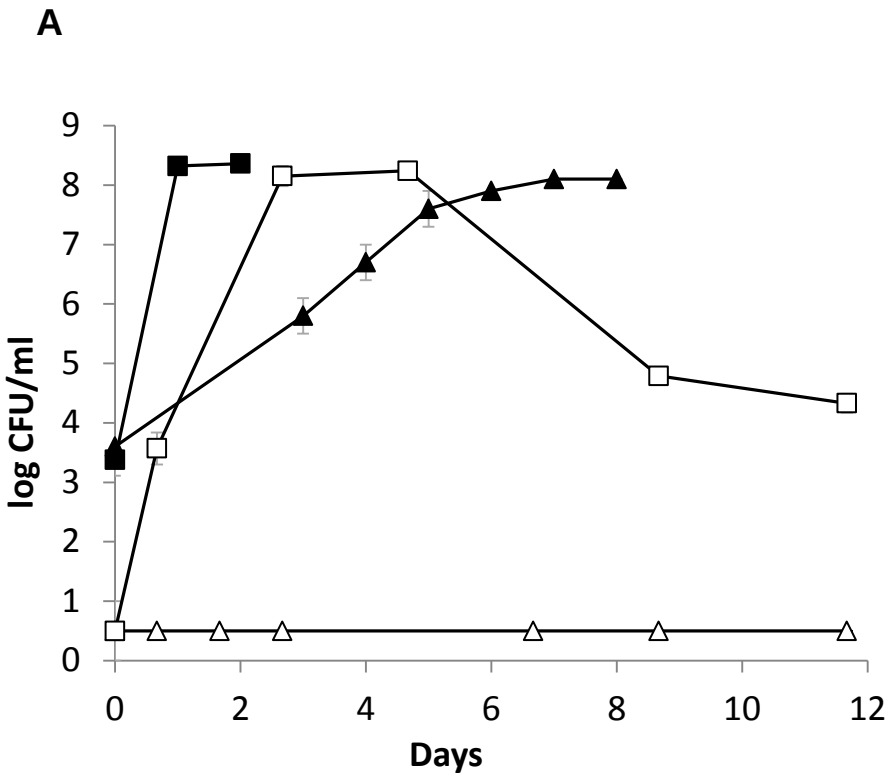
482 4 days at 30 °C in carrot broth (winter carrots) tyndallized without oxygen (condition 3 in

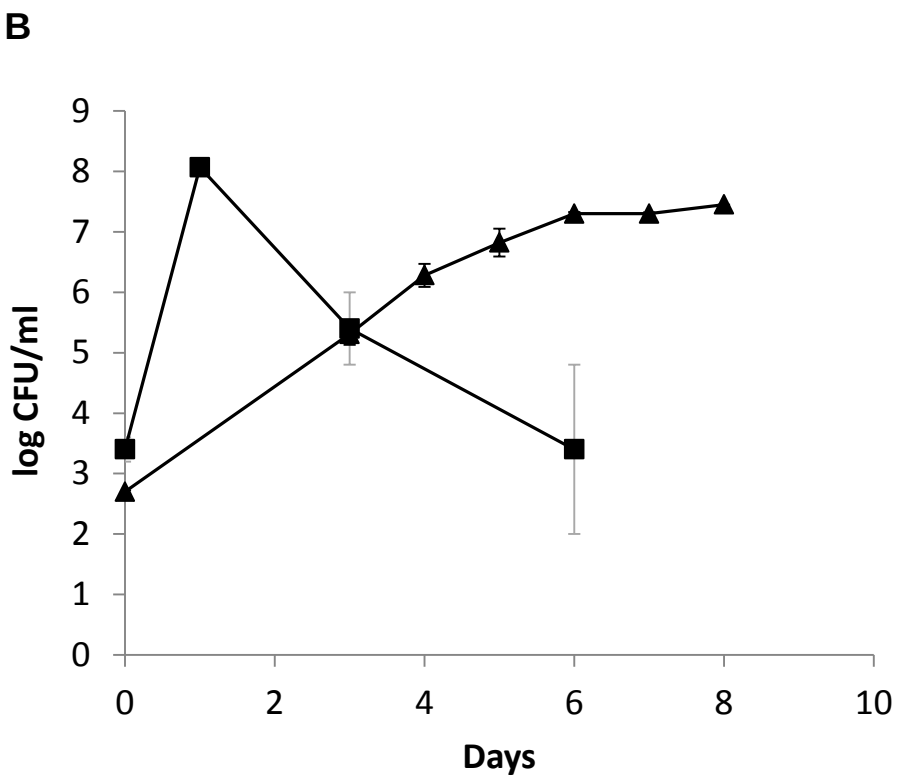
Fig. 1) (A and C) or in BHI during 2 days (B and D) and stained with Nile red. White (in panels B and D) and black (in panels A and C) arrows show lipid inclusions, fluorescing in panel B and D.

Guerin et al Figure 1

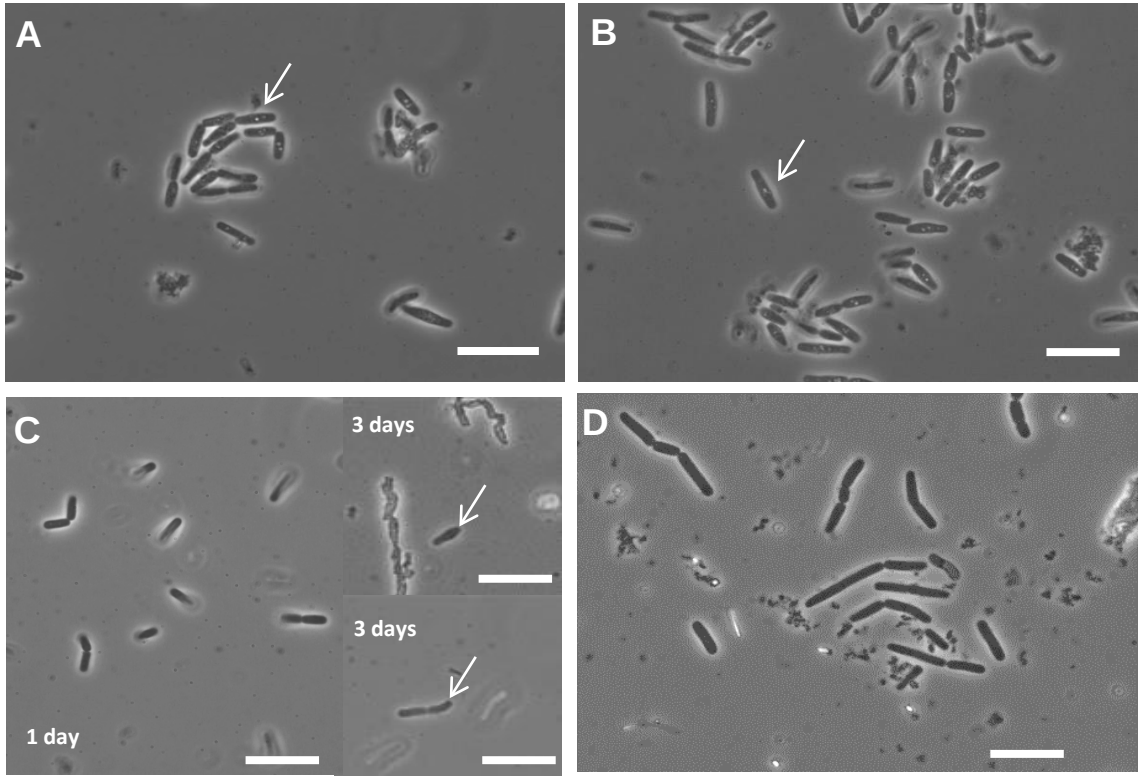


Guerin et al Figure 2





Guerin et al Figure 3



Guerin etal Figure 4