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

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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

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

2 Materials and methods

2.1 Strain and carrot broth preparation

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

Carrot broth was prepared with Nantaise carrots from France. Various types of carrots were used, two different batches of “winter carrots” purchased at the end of their storage period (beginning of spring), and three batches of “spring carrots” purchased at the beginning of summer. Carrots were washed, peeled, grated and vacuum -packed into plastic pouches (between 100 g and 150 g per plastic pouches) (Fig. 1). These pouches were heated 25 min at 80 °C in a water bath. Grated carrots were then weighted, transferred into a stomacher bag with filter (< 100 g/stomacher bag), and stomached during 2 min after addition of phosphate buffer pH 7.0 (100 ml of buffer for 50 g of carrots) to achieve a pH of 7.0 ± 0.2 for the tyndallized carrot broths. The filtered carrot broth was poured from the stomacher bag into a sterile flask and dispensed into KIMAX tubes, or into Hungate tubes (Dutscher) (10 ml/tube) or in Amsco flasks. Carrot broth was directly dispensed in KIMAX tubes under air (condition 1 in Fig. 1), whereas oxygen was eliminated from carrot broth by boiling under a flow of nitrogen passed through a Hungate column (Guérin et al., 2016), before dispensing anaerobically into Amsko flasks and Hungate tubes (conditions 3 and 4 in Fig. 1, respectively). To assess the effect of boiling independently of anaerobic conditions, carrot broth was also boiled without nitrogen flow and dispensed in KIMAX tubes with air (condition 2 in Fig. 1). All tubes and Amsco flasks were then sterilized following a tyndallization protocol, consisting in heating the carrot broth tubes and flasks three times at 80 °C during 1 h in a water bath, with a 24 h-storage at room temperature in the dark between each heat treatment. The sterile tubes and flasks were finally stored at room temperature in the dark before use.

2.2 *Growth conditions*

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

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

2.6 Statistical analysis

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

3 Results

3.1 Growth of B. weihenstephanensis *in carrot broths*

In broth prepared with the two batches of winter carrots, an immediate death of *B. weihenstephanensis* cells upon inoculation was observed in the broth tyndallized in presence of oxygen (condition 1 in Fig. 1). At time zero, we did not recover the 10^3 CFU/ml inoculated in the broth, when incubated with oxygen and whatever the temperature of growth subsequently applied (Fig. 2A). No CFU were recovered upon inoculation of a higher inoculum of 10^6 CFU/ml (Table 1). No growth was subsequently observed at 8 °C and in aerobiosis (condition 1 in Fig. 1) during the 12 d of incubation (Fig. 2A). With 2 batches of spring carrots, an initial reduction of 2 log cycles was observed while no reduction in counts was observed for a third batch. In these three batches, no increase in numbers of cells was 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 inclusions when grown in aerobiosis with or without oxygen during tyndallization (conditions 1 and 3 in Fig. 1) (white arrows in Fig. 3A and B). In contrast, cells exhibited classical shape, and no inclusion was observed when grown in anaerobiosis at 30 °C (condition 4 in Fig. 1) (Fig. 3C, white arrow) for both young cells (1 day) and cells from declining population (3 days). Cells grown at 8 °C in anaerobiosis (condition 4 in Fig. 1) were longer with no inclusion (Fig 3D). As we hypothesised that these inclusions could be lipid granules, we performed a Nile red coloration of cells cultivated 3 days at 30 °C in aerobiosis in a carrot broth tyndallized without oxygen (condition 3 in Fig. 1) (Fig. 4A and B). In fluorescence microscopy, several inclusions appeared in each Nile red-stained cells (white arrow in enlarged Fig. 4B and same cells are shown under phase contrast microscopy in Fig. 4A). A Live/Dead coloration of the same cultures as in Fig 4A and Fig. 4B showed that dead cells represented 49 % ± 15 % of total cells (n=632 cells), irrespective of the presence of inclusions. When grown at 30 °C in aerobiosis in BHI medium, cells also contained lipid inclusions but less than in cells cultivated in carrot broth (Fig. 4C and D).

4 Discussion

The presence of oxygen during tyndallization of carrot-broth (conditions 1 and 2 in Fig. 1) seemed to create lethal compounds for *B. weihenstephanensis* cells because immediately (e.g. within 5 min) upon inoculation, the vegetative cells were no longer recovered on medium plates. In contrast, spores were resistant to this lethal effect as the expected concentration of spores was recovered after their inoculation in tyndallized carrot broth, as observed in previous studies (Valero et al., 2000; Valero et al., 2003; Valero et al., 2007). These authors did not test inoculation with vegetative cells and logically did not report any lethal effect from 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, in carrot broth tyndallized in presence of oxygen, suggesting that the cells may not recover (winter carrots) or needed 3-4 days to recover (spring carrots) at 8 °C from the effect of the lethal compound(s) of the aerobically tyndallized carrot broth. Lethal effect of fresh carrots on various bacterial species has previously been described (Beuchat and Brackett, 1990; Nguyen-the and Lund, 1991, 1992; Babic et al., 1994; Noriega et al., 2010; Degirmenci et al., 2012), although not on species of the *B. cereus* group. As in the present study, an antimicrobial effect of fresh carrot was previously observed on *Listeria monocytogenes,* generated upon de-structuration of carrot tissue, short-lived, and active only in presence of oxygen (Nguyen-the and Lund, 1991). However, this antimicrobial effect on *L. monocytogenes* was shown to be heat labile (Beuchat and Brackett, 1990; Nguyen-the and Lund, 1991) and is therefore presumably different from the one observed in the present study.

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

We observed that when *B. weihenstephanensis* cells grew without oxygen at 8 °C in carrot-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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Table 1. Counts of *B. weihenstephanensis* KBAB4 vegetative cells 5 min after inoculation in carrot broth.

		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

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

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

[c] NT: not tested.

Legend of figures

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

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

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

Numbers referred to conditions cited in the text.

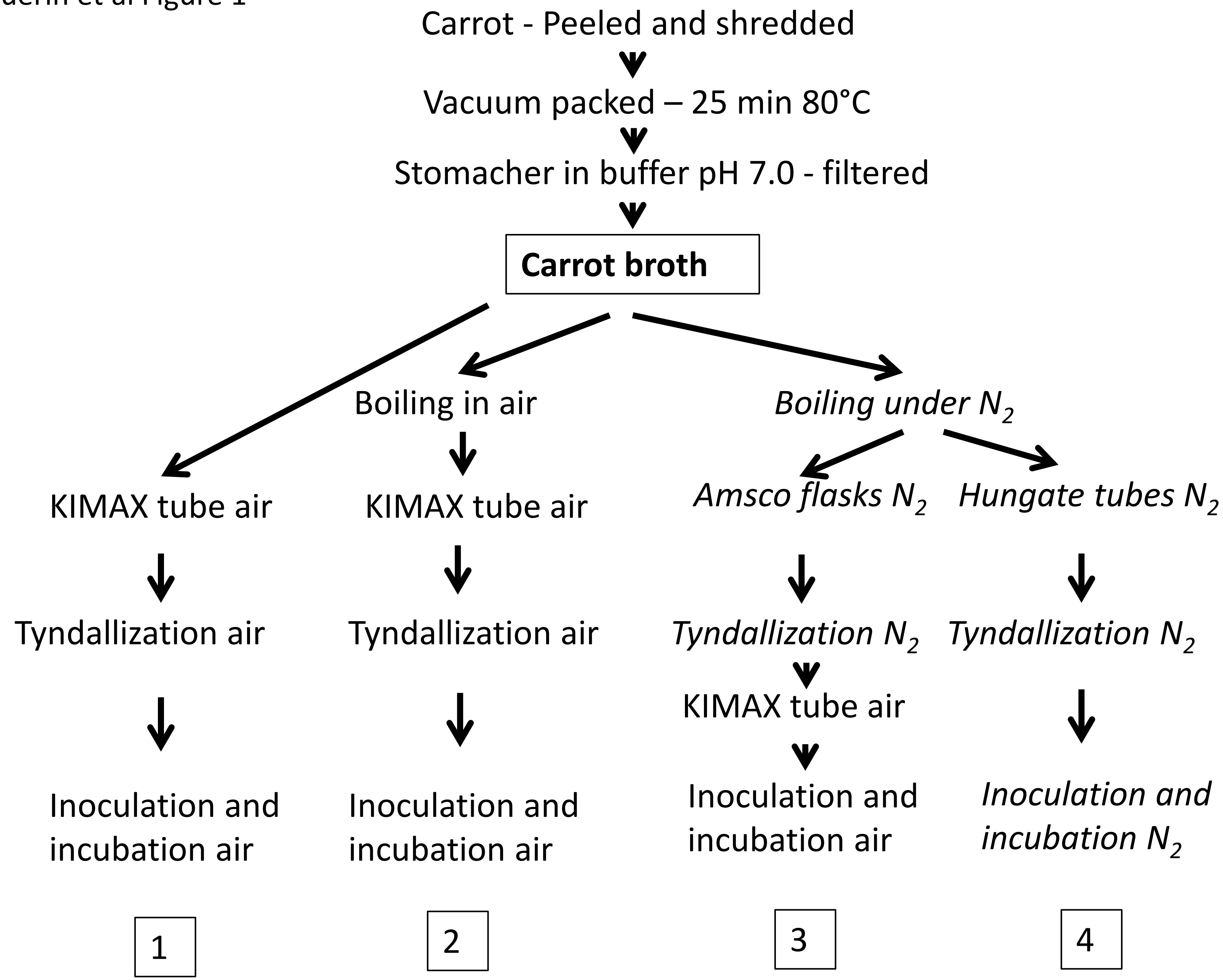
Fig. 2 - Growth of *B. weihenstephanensis* KBAB4 strain in carrot broth (winter carrots) incubated in air (panel A) (conditions 1 and 3 in Fig 1), or in anaerobic conditions (panel B) (condition 4 in Fig 1). Before inoculation, carrot broth was sterilized by tyndallization in presence (open symbols) (condition 1 in Fig 1) or in absence (closed symbols) of oxygen (conditions 3 and 4 in Fig 1). Carrot broths, inoculated with10^3 and $10^{3.5}$ CFU/ml, were incubated at 8 °C (triangles) or at 30 °C (squares).

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

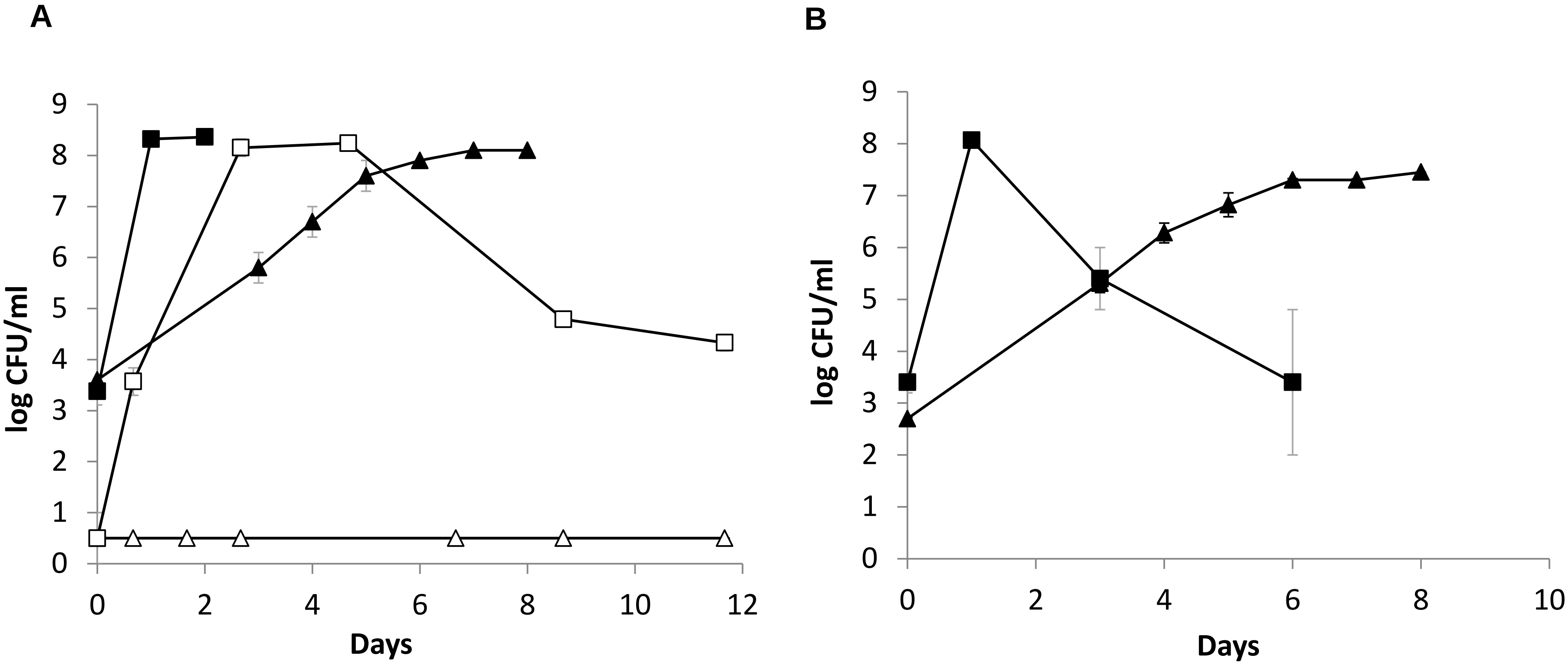
Fig. 4. Microscopic observations of *B. weihenstephanensis* KBAB4 under phase contrast (A and C) and epifluorescence (B and D) (X 1,000). KBAB4 cells were cultivated aerobically for 4 days at 30 °C in carrot broth (winter carrots) tyndallized without oxygen (condition 3 in

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.

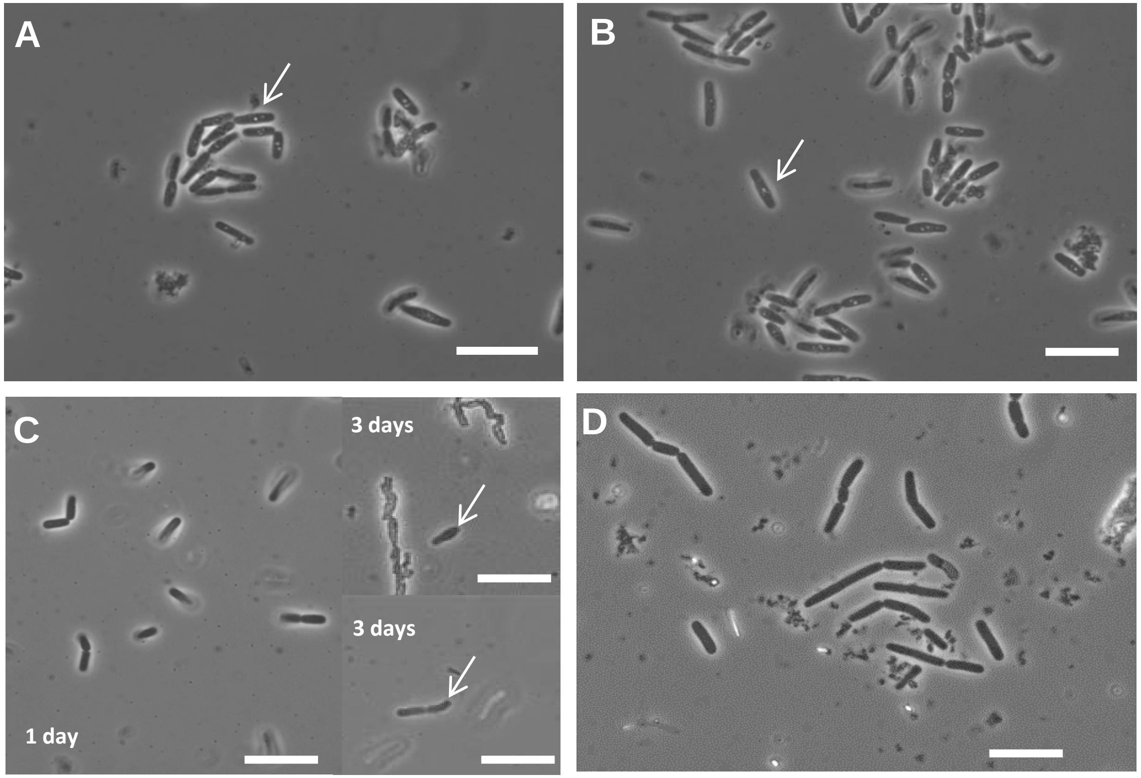
Guerin et al Figure 1



Guerin et al Figure 2



Guerin et al Figure 3



Guerin etal Figure 4