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1 Dispersed phase volume fraction, weak acids and Tween 80 in a model emulsion: Effect on
2 the germination and growth of *Bacillus weihenstephanensis* KBAB4 spores

3

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21

22

23 **Abstract**

24 In foodstuffs, physico-chemical interactions and/or physical constraints between spores,
25 inhibitors and food components may exist. Thus, the objective of this study was to investigate
26 such interactions using a model emulsion as a microbial medium in order to improve bacterial
27 spore control with better knowledge of the interactions in the formulation.

28 Emulsions were prepared with hexadecane mixed with nutrient broth using sonication and
29 were stabilized by Tween 80 and Span 80. The hexadecane ratio was either 35 % (v/v) or 50
30 % (v/v) and each emulsion was studied in the presence of organic acid (acetic, lactic or
31 hexanoic) at two pH levels (5.5 and 6). Self-diffusion coefficients of emulsion components
32 and the organic acids were measured by Pulsed Field Gradient-Nuclear Magnetic Resonance
33 (PFG-NMR). The inhibition effect on the spore germination and cell growth of *Bacillus*
34 *weihenstephanensis* KBAB4 was characterized by the measure of the probability of growth
35 using the most probable number methodology, and the measure of the time taken for the cells to
36 germinate and grow using a single cell Bioscreen® method and using flow cytometry.

37 The inhibition of spore germination and growth in the model emulsion depended on the
38 dispersed phase volume fraction and the pH value. The effect of the dispersed phase volume
39 fraction was due to a combination of (i) the lipophilicity of the biocide, hexanoic acid, that
40 may have had an impact on the distribution of organic acid between hexadecane and the
41 aqueous phases and (ii) the antimicrobial activity of the emulsifier Tween 80 detected at the
42 acidic pH value. The interface phenomena seemed to have a major influence. Future work
43 will focus on the exploration of these phenomena at the interface.

44

45 **Keywords**

46 *Bacillus*, *spoilage*, *antimicrobial effect*, *lipophilicity*, *emulsion*, *emulsifier*

47

48 **Chemical compounds**

49 Acetic acid (PubChem CID: 176); Hexadecane (PubChem CID: 11006); Hexanoic acid
50 (PubChem CID: 8892); Lactic acid (PubChem CID: 612); Tween 80 (PubChem CID:
51 5281955); Span 80 (PubChem CID: 9920342).

52 **1. Introduction**

53 Growth of microorganisms in food emulsions can lead to major economic losses for the food
54 industry through food-poisoning or spoilage due to the development of bacteria, yeasts and
55 molds in food during shelf-life (Brocklehurst & Wilson, 2000; Zalazar, Gliemmo, & Campos,
56 2016). Two forms of emulsions are mostly encountered in food: oil-in-water (such as milk,
57 dairy cream or mayonnaise) and water-in-oil (such as butter or low-fat spread) (Brocklehurst
58 & Wilson, 2000). Emulsions are metastable colloidal systems formed from two immiscible
59 liquids (Wilson et al., 2002). Due to the large interfacial area between the two immiscible
60 phases, emulsions are thermodynamically unstable systems and flocculation, coalescence and
61 phase separation (e.g. creaming) are frequently observed during processing or storage (Mao,
62 Wang, Liu, & Gao, 2016). They can be stabilized kinetically because of the repulsion forces
63 between neighboring droplets in the systems thanks to various components such as proteins,
64 lipids and emulsifiers (Mao et al., 2016; Wilson et al., 2002). In such complex food
65 emulsions, many factors may have effects on the behavior of microorganisms.

66 In emulsions, the aqueous phase is commonly recognized as a microbial growth location
67 (Brocklehurst & Wilson, 2000; Wilson et al., 2002), and the structural features of this phase
68 are relevant to the length scale of the microorganisms that influence microbial growth (Wilson
69 et al., 2002). Indeed, the food structure may influence both the growth rate and the conditions
70 in which growth is initiated (Brocklehurst & Wilson, 2000). The structure of complex
71 matrices could also involve the formation of a local chemical specific micro-environment
72 around microbial cells, for instance local pH (Robins & Wilson, 1994). Thus, the surface of
73 bacteria can interact physico-chemically with food matrix components such as proteins, lipids
74 and emulsifiers. Electrostatic, Van Der Waals and Lewis acid-base interactions must also be
75 considered between microbial surface properties and the environment (Ly et al., 2006).
76 Bacterial cells are micrometer-scale particles bearing at their surface many macromolecules
77 such as proteins, mannoproteins, peptidoglycan, teichoic acid and polysaccharides (Ly et al.,
78 2008). Their surface composition can be modified by environmental chemical characteristics,
79 as well as the adsorption of the oil phase on the bacterial outer surface (Naïtali, Dubois-
80 Brissonnet, Cuvelier, & Bellon-Fontaine, 2009).

81 The structural complexity of food emulsions has a substantial impact on the activity of
82 antimicrobial compounds (Terjung et al., 2014). Antimicrobial efficacy has been shown to
83 depend on various product characteristics such as concentrations of fats, proteins,
84 carbohydrates, and salt, as well as the pH (Castro, Rojas, Campos, & Gerschenson, 2009;
85 Jung, Bodyfelt, & Daeschel, 1992; Terjung et al., 2014). However, difficulties in compiling

[Texte]

86 the information and discrepancies in the literature remain since emulsion is a complex matrix
87 exhibiting a wide diversity of compositions and physico-chemical characteristics.

88 The objective of this study was to investigate interactions between microorganisms,
89 antimicrobial compounds and matrices. The model emulsion was composed of nutrient broth,
90 allowing bacterial growth, as the aqueous phase, hexadecane as the dispersed phase and a
91 Tween 80/Span 80 mixture as the emulsifier. These emulsifiers are non-ionic, frequently
92 encountered in food systems (McClements & Weiss, 2005) and listed as authorized additives
93 under European regulations (EU 1129/2011 and EC 1333/2008 (<https://eur-lex.europa.eu/>)).
94 According to the scientific literature, Tween 80 is particularly commonly used in food
95 emulsions containing microorganisms and its concentration ranges from 0.1 to 10 % (w/w)
96 (Buranasuksombat, Kwon, Turner, & Bhandari, 2011; Celiktas et al., 2007; Hilbig, Ma,
97 Davidson, Weiss, & Zhong, 2016; Krogsgard-Nielsen et al., 2017; Landry et al., 2016;
98 Murray, 2016; Neves et al., 2016; Ribes, Fuentes, Talens, & Barat, 2017). Both emulsifiers
99 were chosen to stabilize the emulsion because of this non-ionic characteristic to limit
100 artefacts. The use of hexadecane prevented any trace of polyunsaturated fatty acid which
101 could affect microorganism behavior (Mvou Lekogo, Coroller, Mathot, Mafart, & Leguérinel,
102 2010). The emulsion characteristics (pH, weak acid and dispersed phase volume ratio) were
103 modified and the impacts were assessed on several scales, namely *Bacillus*
104 *weihenstephanensis* germination and outgrowth, and the interface phenomenon with the
105 emulsifier. *Bacillus weihenstephanensis* was used in this study because of its capacity i) to
106 contaminate chilled and minimally processed food, ii) to constitute a potential danger for
107 customers' health, and iii) to limit products' shelf life leading to economic losses (Antolinos
108 et al., 2012; Desriac et al., 2013).

109

110 **2. Materials and methods**

111 **2.1 Model system preparation**

112 The model emulsion was composed of nutrient broth as the aqueous phase, hexadecane as the
113 dispersed phase and a Tween 80/Span 80 mixture as the emulsifier. This emulsion was
114 inoculated with *Bacillus weihenstephanensis* spores or vegetative cells.

115 **2.1.1 Inoculum preparation**

116 *Bacillus weihenstephanensis* strain KBAB4 (INRA, Avignon, France) was used in this study.
117 The strain (in the form of vegetative cells) was stored at -80 °C in Brain Heart Infusion (BHI)
118 (Biokar, Diagnostics, Beauvais, France) mixed with 50 % glycerol (v/v). A 100-mL volume
119 of BHI was inoculated with 1 mL of the stock suspension and incubated for 8 h at 30 °C, the

[Texte]

120 optimal growth temperature; then, a 1-mL volume was transferred into 100 mL of BHI for
121 16 h at 30 °C. Finally, 0.1 mL of the *Bacillus weihenstephanensis* suspension was added to
122 100 mL of BHI and was incubated for 6 h. Spores were produced through a two-step
123 sporulation process as described in Baril et al. (2011). The final concentration of the bacterial
124 spore suspension was 10^7 spores·mL⁻¹. The number of spores was estimated as the number of
125 cells surviving a 5-min, 70 °C heat treatment.

126 **2.1.2 Emulsion preparation**

127 Hexadecane (H6703, Sigma Aldrich, St-Louis, MO, USA) was mixed with nutrient broth
128 (Biokar, Diagnostics, Beauvais, France) using sonication and stabilized by Tween 80 (P4780,
129 Sigma Aldrich, St-Louis, MO, USA) and Span 80 (S6760, Sigma Aldrich, St-Louis, MO,
130 USA). Emulsions with either 35% (v/v) (dispersed phase volume ratio (ϕ) = 0.35) or 50%
131 (v/v) (ϕ = 0.50) hexadecane content were studied as a function of pH (5.5 and 6.0), adjusted
132 with HCl, with or without weak acid. Three weak acids were studied as antimicrobial
133 compounds: acetic acid ($pK_a=4.75$; $\log P=0.17$) (A6283, Sigma Aldrich, St-Louis, MO,
134 USA), hexanoic acid ($pK_a=4.88$; $\log P=1.92$) (153745, Sigma Aldrich, St-Louis, MO, USA)
135 and lactic acid ($pK_a=3.90$; $\log P=-0.72$) (69775, Sigma Aldrich, St-Louis, MO, USA). The
136 $\log P$ values were assessed by Hansch and Leo (1995). Acetic acid and lactic acid were
137 studied as food additives. Hexanoic acid was studied because of its higher hydrophobicity
138 ($\log P=1.92$) compared to acetic acid and lactic acid ($\log P=0.17$ and $\log P=-0.72$,
139 respectively).

140 The aqueous phases were composed of 0.005% iron citrate, 0.1% esculin, 2% nutrient broth,
141 3% Tween 80 for emulsions with $\phi = 0.50$ and 2.3% Tween 80 for emulsions with $\phi = 0.35$
142 (% w/w of the aqueous phase). A weak acid was mixed with the aqueous phase during the
143 preparation process to obtain final concentrations of 14 mM of acetic acid, 1.8 and 4 mM of
144 hexanoic acid, and 82 and 255 mM of lactic acid. Hexadecane and the aqueous phases were
145 sterilized by filtration (0.22 μ m, Stericup® Millipore) prior to spore inoculation. Heat-treated
146 spore suspension (5 min.; 70 °C) was added to the aqueous phase at three different
147 concentrations: 100, 20 and 3.5 spores per 100 μ L of aqueous phase. The mixtures were
148 sonicated for 1 min at 60 % amplitude using a Branson 450 W-20 kHz digital sonifier
149 equipped with a temperature probe. Thus, for each sample the temperature variation was
150 controlled and rose 40 °C in 1 min of mixing.

151

152 **2.2 Physical characterization of emulsions and microbial cells**

153

2.2.1 Droplet size and stability

[Texte]

154 The emulsion particle size distributions were measured by laser granulometry with a Malvern
155 MS3000, in 0.1 % SDS dispersant at 2000 rpm. The mean volume diameters $D_{[4,3]}$ and the
156 particle size dispersions were monitored for 10 days with storage at 30 °C which was the
157 temperature used for the microbial assay. The emulsion stability (flocculation, creaming,
158 clarification) was monitored by multiple light scattering with a TurbiScan (Formulation,
159 France). The destabilization phenomena were monitored for 10 days at 30 °C.

160 **2.2.2 Zeta potential measurements**

161 According to Bundeleva et al. (2011), zeta-potential (ξ -potential) can be used to perform the
162 change in the cell surface potential (mV) of microorganisms as a function of pH. ξ -potential is
163 an important parameter to analyze the stability of particles (MubarakAli et al., 2018).

164 The ξ -potential of vegetative cells and spores was measured using a Zetasizer Nano-ZS
165 (Malvern Instruments, United Kingdom). The analysis was performed at 25 °C between pH 4
166 and 9; a range covering those used for the microbial assay. Vegetative cells were recovered
167 from a culture in the exponential phase with an optical density of 0.5 at 600 nm and washed
168 twice in tryptone salt medium. The vegetative cell suspension and spore stock suspension
169 were diluted 10- and 1000-fold respectively in 5 mmol·L⁻¹ of imidazole/acetate buffer
170 adjusted to the appropriate pH using NaOH or HCl solutions (1 mol·L⁻¹) prior to
171 measurements. Samples were introduced into capillary cells and measured in automatic mode.
172 The ξ -potential measurements are reported as the average and standard deviation of the
173 measurements for three freshly prepared samples.

174 **2.2.3 Surface hydrophobicity**

175 The hydrophobicity of vegetative cells and spores was evaluated by Microbial Adhesion to
176 Hexadecane (MATH) (Bellon-Fontaine, Rault, & van Oss, 1996). Vegetative cells in the
177 exponential phase were harvested by centrifugation at 5000 g for 10 min at 4 °C, washed
178 twice in tryptone salt medium and resuspended to $A_{600nm} = 0.5$ (A_0) in physiological water.
179 0.5 mL of hexadecane was added to 3 mL of cell or spore suspension with optical density A_0 .
180 The two-phase system was mixed by 10 s shaking by hand, then by vortexing 1 min, and
181 finally by 10 s shaking by hand. The system was allowed to separate for 15 min. The
182 absorbance of the aqueous phase at 600 nm (A_1) was measured. The percentage of microbial
183 adhesion (Ad%) to solvent was calculated as follows (1):

$$184 \quad Ad\% = \left(1 - \frac{A_1}{A_0}\right) \times 100 \quad (1)$$

185 **2.2.4 Microscopy**

[Texte]

186 Microstructures of the model emulsion containing spores were observed with a Confocal
187 Laser Scanning Microscope (CLSM) Zeiss LSM780 (Carl Zeiss, Oberkochen, Germany). The
188 hexadecane was stained with Nile Red (72485, Sigma Aldrich, St-Louis, MO, USA)
189 (emission/excitation wave length: 488-515/>590 nm) at a final concentration of 16 mg·mL⁻¹.
190 Spores were labeled with Thioflavin T (T3516, Sigma Aldrich, St-Louis, MO, USA)
191 (emission/excitation wave length: 405/485 nm) at a final concentration of 100 μmol·L⁻¹ of
192 spore suspension. Two light sources were used: a diode laser at 405 nm and an Argon laser at
193 514 nm.

194 **2.2.5 Pulsed Field Gradient-Nuclear Magnetic Resonance (PFG-NMR)**

195 NMR measurements were performed at 30 °C with a 500 MHz Bruker spectrometer, equipped
196 with a field gradient probe (0 to 12 T/m) and using the LED-BP-PFGSTE sequence in which
197 Δ is the diffusion delay (5.64 to 100 ms) and δ the gradient pulse duration (1 ms). Within a
198 measurement, sequence parameters δ, Δ and τ (time between the end of each gradient and the
199 next radiofrequency pulse) (1 ms) remained constant and successive and increasing values of
200 the gradient amplitude (g) were applied.

201 The data was processed using the Dynamics Center (DOSY reconstruction) and the T1/T2
202 relaxation module (1D processing) developed by Bruker under Topspin 3.2. For 1D
203 processing, the self-diffusion coefficient (D) was determined on the basis of the following
204 equation (2):

$$205 \frac{I_g}{I_0} = \exp\left[-\gamma^2 \times g^2 \times \delta^2 \times \left(\Delta - \frac{\delta}{3} - \frac{\tau}{4}\right) \times D\right] \quad (2)$$

206 (assuming in this case monoexponential decay), where I_g is the attenuated signal intensity by
207 application of gradient pulses, I_0 the optimum signal intensity without field gradient, and γ the
208 gyromagnetic ratio). The self-diffusion coefficient (D0) were $4.50 \times 10^{-11} \pm 7.50 \times 10^{-13} \text{ m}^2 \cdot \text{s}^{-1}$,
209 $4.15 \times 10^{-10} \pm 1.0 \times 10^{-12} \text{ m}^2 \cdot \text{s}^{-1}$, $1.24 \times 10^{-09} \pm 3.0 \times 10^{-12} \text{ m}^2 \cdot \text{s}^{-1}$ and $5.10 \times 10^{-11} \pm 1.63 \times 10^{-12}$
210 $\text{m}^2 \cdot \text{s}^{-1}$, for pure lactic acid, hexanoic acid, acetic acid and Tween 80, respectively. Then,
211 each weak acid and Tween 80 were diluted in water ($\phi = 0$) or prepared in two different
212 emulsions with $\phi = 0.35$ and with $\phi = 0.50$. For each acid, the self-diffusion coefficient D in
213 water or emulsions was compared to D0 (pure weak acid or Tween 80) by calculating the
214 ratio D/D0.

215

216 **2.3 Characterization of bacterial behavior**

[Texte]

217 **2.3.1 Assessment of the number of spores able to germinate and to grow in**
218 **emulsion**

219 The inoculated emulsions were distributed in sterile 96-well microplates. Spores of *Bacillus*
220 *weihenstephanensis* KBAB4 able to germinate and to outgrow were detected by the loss of
221 fluorescence of wells due to the esculin metabolism. These spores were then enumerated by
222 the most probable number method (MPN). An estimation of the percentage of spores able to
223 germinate and to give active cells in the emulsion was calculated as follows (3):

224
$$OutG \% = \left(\frac{MPN}{N_0} \right) \times 100$$

225 (3)

226 with N_0 (Colony Forming Unit, CFU), the initial population of spores determined after a 5-
227 min, 70 °C heat treatment and dilution in tryptone salt broth (Biokar, Diagnostics, Beauvais,
228 France) by plating in nutrient agar (Biokar, Diagnostics, Beauvais, France).

229 **2.3.2 Monitoring *Bacillus* spore germination by flow cytometry**

230 Spore germination and outgrowth in nutrient broth in the presence of Tween 80 were
231 monitored by flow cytometry (FCM) analysis using the fluorescent dye Syto®9 (Invitrogen,
232 Carlsbad, California, United States) to investigate the effect of the emulsifier at different pH
233 values on spores. Syto®9 fluoresces only when bound to either double-stranded DNA or
234 RNA with an emission maximum of 520 nm (green). Dormant refractive spores show only
235 peripheral staining with Syto®9, however, intact germinating spores stain brightly (van Melis,
236 Groot, Tempelaars, Moezelaar, & Abee, 2011). For 1 mL of culture sample, 0.5 µL of Syto®9
237 was added (van Melis et al., 2011). Flow cytometric analyses were carried out using a
238 CyFlow®Space (Partec, France). Forward (FSC) and side (SSC) scatter signals were collected
239 from 488 nm excitation. Analysis was carried out in true volumetric absolute counting mode
240 (analysis in 200 µL). Samples were diluted with tryptone salt broth prior to analysis in order
241 to respect a sampling rate ranging from 500 to 5000 particles·s⁻¹. Analysis and gating of data
242 were performed using FloMax® software (Partec, France).

243 **2.3.3 Bioscreen® growth experiments**

244 The growth kinetics of *Bacillus weihenstephanensis* KBAB4 were evaluated in nutrient broth
245 at 30 °C with different concentrations of Tween 80 (0, 1, 5 and 10% (w/w)) to investigate the
246 effect of the emulsifier at different pH values on vegetative cells. Turbidity growth curves
247 were generated with an automatic Bioscreen C reader (Oy Growth Curves Ab Ltd., Helsinki,
248 Finland). From a pre-culture in the end exponential phase, the first line of a plate was
249 inoculated with a 10⁵ CFU·mL⁻¹ suspension and, to complete the plate, seven serial two-fold

[Texte]

250 dilutions were performed in the tested medium. Then the plate was placed in the Bioscreen C
251 reader at 30 °C. The increase in turbidity was monitored at 600 nm. Measurements were taken
252 every 10 min, and the plate was shaken at low intensity for 20 s prior to absorbance
253 measurements. The maximum specific growth rate (μ_{max}) values corresponding to each Tween
254 80 concentration were determined by linear regression as previously described (Membré et
255 al., 2005). Paired Student's *t*-tests were performed to compare the maximum specific growth
256 with and without Tween 80. P-values strictly lower than 0.05 were considered significant.

257 **2.3.4 Individual lag times**

258 The distribution of individual lag times was investigated according to a method adapted from
259 Guillier et al. (2005). From end exponential phase cells, serial dilutions were performed in the
260 tested medium in order to obtain a final bacterial suspension at 1.4 CFU·mL⁻¹. Assuming
261 thereafter that the distribution of the cells in the microplate followed a Poisson distribution,
262 the maximum concentration of 0.42 CFU·well⁻¹ will correspond to a maximum of 35 % of
263 wells showing growth and 20 % of them, i.e. 7 %, will only have one cell.

264 Then the plate was placed in the Bioscreen C reader at an incubation temperature of 30 °C.
265 The increase in turbidity was monitored at 600 nm. Measurements were taken every 10 min,
266 and the plate was shaken at low intensity for 20 s prior to absorbance measurements.

267 On the assumption that the maximum specific growth rate was constant from one well to
268 another, the detection time (T_d) is related to the lag time (lag) by the following formula
269 (Baranyi & Pin, 1999) (4):

$$270 \quad T_d = lag + \frac{\ln(N_d) - \ln(N_0)}{\mu_{max}} \quad (4)$$

271 with N_d , the bacterial number at T_d ; N_0 , the number of cells initiating growth in the considered
272 well, and μ_{max} , the maximum specific growth rate.

273 The Kolmogorov-Smirnov statistical test was applied to compare the distributions of latency
274 times with and without Tween 80 at one pH value (XLSTAT software, 2017 version).

275

276

277 **3. Results**

278 **3.1 Physical characteristics of emulsion constituents**

279 **3.1.1 Droplet size**

280 The dispersion was studied to characterize the spatial organization and locate the different
281 components. Measurements by laser granulometry showed that emulsions with $\phi = 0.35$ and
282 $\phi = 0.50$ were monodispersed. Indeed, an emulsion is considered concentrated when it reaches

[Texte]

283 a concentration 64 % of monodisperse droplets, which represents the random-close-packing
284 volume fraction (Leal-Calderon, Schmitt, & Bibette, 2007). The droplets were agitated by a
285 Brownian motion. They presented a monomodal distribution of hexadecane droplets with,
286 respectively, a $D_{[4,3]}$ of $0.484 \pm 0.005 \mu\text{m}$ and $0.437 \pm 0.006 \mu\text{m}$. During a 10-day period at 30
287 °C, neither the creaming nor the stability of the emulsions was modified with or without
288 organic acid and whatever the pH. As observed in the emulsions using confocal microscopy
289 (**Figure 1**), the droplets of the dispersed phase appeared to be smaller than the spores ($\sim 1 \mu\text{m}$)
290 (Carrera, Zandomeni, Fitzgibbon, & Sagripanti, 2007). In our systems, microbial growth
291 seemed to occur in the aqueous phase as is commonly recognized (Brocklehurst & Wilson,
292 2000; Wilson et al., 2002).

293 **3.1.2 Spore characteristics**

294 The surface of the spores and vegetative cells were characterized by MATH (Bellon-
295 Fontaine et al., 1996). We found that the spores had a hydrophobic surface contrary to
296 vegetative cells. The ξ -potential values of spores and vegetative cells, *i.e.* net charges on the
297 surface, were also determined in phosphate buffer ranging from pH 4 to 9. The electrophoretic
298 motilities of the spores and vegetative cells indicated an electronegative character with ξ -
299 potential ranging, respectively, from -9.8 to -41.0 mV and from -11.2 to -18.7 mV.

300 **3.1.3 Diffusion of emulsion constituents by NMR**

301 In order to highlight the effect of emulsion structure on the diffusion coefficients of weak
302 acids, the D/D_0 ratio was reported as a function of pH for each hexadecane/water ratio
303 (**Figure 2**). The D/D_0 ratio variations were significant as showed by the calculated standard
304 error for each ratio. Figure 2 shows that for each weak acid, whatever the pH, D/D_0 tended to
305 decrease when the hexadecane/water ratio increased. This indicates that a fraction of each
306 weak acid may be trapped in the hexadecane, and/or the emulsion structure impacts on its
307 diffusion coefficient. Discrepancies of D/D_0 values among acids were likely due to two
308 effects: $D/D_0 > 1$ may be due to a lower viscosity in water or in emulsion in comparison to
309 bulk acid, and $D/D_0 < 1$ may be due to a structure effect in emulsion either by molecule
310 trapping or interactions between weak acids and droplets. These results were obtained by
311 assuming that the total amount of weak acid was diffused in the emulsion in a unique way
312 (mono-exponential decrease of the NMR signal). In contrast to lactic acid and acetic acid,
313 D/D_0 for hexanoic acid tends to decrease with pH (**Figure 2.a**). This discrepancy could
314 account for both the higher pK_a and the higher $\log P$ for hexanoic acid, favoring its affinity for
315 the dispersed hexadecane phase.

[Texte]

316 For Tween 80, the self-diffusion coefficient was around $10^{-11} \text{ m}^2 \cdot \text{s}^{-1}$ in water and around
317 $10^{-10} \text{ m}^2 \cdot \text{s}^{-1}$ in emulsions, whatever the pH value and hexadecane volume fraction. These two
318 different values might be due to the spatial arrangement of Tween 80 in the form of micelles
319 in water and at the emulsion interfaces (Henry, Fryer, Frith, & Norton, 2009).

320

321 **3.2 Comparison of the percentage of outgrown *B. weihenstephanensis* spores in** 322 **aqueous phases and model emulsions**

323 An estimation of the percentage of outgrown *B. weihenstephanensis* KBAB4 spores (i) in
324 the nutrient broth, (ii) in the aqueous phase (nutrient broth with 3 % (w/w) Tween 80 ($\phi = 0$)),
325 (iii) in $\phi = 0.35$ emulsions and (iv) in $\phi = 0.50$ emulsions was calculated from an enumeration
326 by a most probable number (MPN) assay.

327 First of all, the percentage of germination and outgrowth in the nutrient broth with HCl at pH
328 5.5 was 1.5-fold lower compared to pH 6 (**Table 1**). The percentages of germination and
329 outgrowth obtained with the weak acids was 0 to 1.2-fold lower than with HCl at pH 6.0 and
330 1.3 to 2.8-fold lower at pH 5.5.

331 Secondly, the percentages of germination and outgrowth in the aqueous phase (nutrient broth
332 with 3 % (w/w) Tween 80 ($\phi = 0$)) used for the emulsion with HCl at pH 5.5 were lower than
333 those at pH 6, also reflecting the influence of pH on *B. weihenstephanensis* KBAB4 (**Table**
334 **1**). Yet, we noticed that the aqueous phase ($\phi = 0$) in the presence of the weak acids and HCl
335 at pH 5.5 revealed that less than 0.03 % of spores outgrew compared to 22 to 61 % in the
336 nutrient broth. This highlighted an antimicrobial synergistic effect between Tween 80 and
337 weak acid depending on the pH value.

338 Moreover, the percentages of outgrowth observed in all $\phi = 0.35$ emulsions as well as those
339 observed in the $\phi = 0.50$ emulsions with lactic acid and acetic acid at pH 5.5 and pH 6 did not
340 differ from the aqueous phase (**Table 1**). Surprisingly, data in Table 1 show that the $\phi = 0.50$
341 emulsions with HCl at pH 5.5 revealed a higher percentage of outgrowth compared to the
342 aqueous phase or the $\phi = 0.35$ emulsions with HCl at the same pH value. In the same way,
343 these data also show that the $\phi = 0.50$ emulsions with hexanoic acid at pH 6 revealed a higher
344 percentage of outgrowth compared to the aqueous phase or the $\phi = 0.35$ emulsions with
345 hexanoic acid at the same pH value.

346 These results confirm the influence of pH and of the weak acids as hurdles preventing the
347 germination and outgrowth of *B. weihenstephanensis* KBAB4 in the studied systems. Yet,
348 Tween 80, used as the emulsifier in the emulsions, also acted as an additional hurdle.
349 Furthermore, the ability of spores to recover and grow was modulated by the interactions

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350 among Tween 80, hexanoic acid and their environment. This illustrated the complexity of
351 controlling the bacterial behavior through physico-chemical factors in complex matrices.

352

353 **3.3 Effect of Tween 80 on the growth of vegetative cells (μ_{max} , lag times)**

354 In order to characterize the antimicrobial effect of Tween 80 on the growth of KBAB4 cells,
355 Bioscreen® growth experiments were carried out to determine growth parameters in the
356 presence or absence of Tween 80 at pH 5.5 and pH 6.0 (**Figure 3**). At pH 6.0 with a Tween
357 80 concentration of 5 % (w/w) and more, μ_{max} was significantly lower than the control
358 without Tween 80. At pH 5.5 with a Tween 80 concentration of 2% and more, μ_{max} was
359 significantly lower than without Tween 80 (Student's *t*-test, $p < 0.05$). Tween 80 seemed to
360 have an antimicrobial effect on the growth of vegetative cells depending on the pH value.
361 Minimum inhibitory concentrations were determined by a linear regression: at pH 6.0, MIC
362 was 64.8 % (w/w) and at pH 5.5, MIC was 19.9 % (w/w).

363 The distribution of individual lag times of vegetative cells with and without Tween 80 at pH
364 6.0 and 5.5 were assessed (**Figure 4**). The presence of Tween 80 in the growth medium
365 increased the individual lag times. At pH 6, the frequency distribution was maximal for the
366 10-11 h lag time interval without Tween 80, and for the 10-12 h interval with Tween 80. At
367 pH 5.5, the frequency distribution was maximal for the 12-13 h lag time interval without
368 Tween 80, and for the 16-18 h interval with Tween 80. The effect of Tween 80 on the growth
369 delay was more marked at acidic pH (higher D statistic at pH 5.5 (Kolmogorov-Smirnov
370 statistical test)). The frequency distributions without Tween 80 and with Tween 80 were
371 significantly different at both pH values (Kolmogorov-Smirnov statistical test). As shown by
372 these results, at pH 5.5 and with Tween 80, the variability of lag times was higher than for any
373 other combination tested, which indicates that, at single cell level, the adaptation period, and
374 therefore the lag times, had a much greater variability than in the other conditions tested. This
375 indicates that the combination has an important inhibitory effect on cells.

376

377 **3.4 Effect of Tween 80 on spore germination (kinetics determined by flow 378 cytometry)**

379 To determine whether Tween 80 induced a delay in germination, spores were exposed to
380 different concentrations of Tween 80 in nutrient broth at pH 5.5 and their germination was
381 monitored by flow cytometry (**Figure 5**). According to van Melis et al. (2011), dormant
382 refractive spores are slightly stained (**Figure 5, quadrant I**). Upon germination, spore
383 permeability to Syto®9 and the accessibility of DNA increased, leading to an increase in the

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384 fluorescence level (**Figure 5, quadrant II**). The size increase of the outgrowing spores could
385 then be monitored by an increase in the forward-scatter signal (**Figure 5, quadrant III**).

386 After 4 h, for all the conditions, the spores were still dormant. Within the following four
387 hours, the spore populations became permeable to Syto®9 (quadrant II, germinated spores).
388 Under unstressed conditions (without Tween 80), the majority of the cell population was able
389 to outgrow (located in quadrant III), while for stress conditions, a substantial part of the spore
390 population remained dormant (quadrant I) until 10 h of incubation. Without Tween 80, 4.59 %
391 of spores were still refractive spores (quadrant I) after 10 h of incubation, while with 5 % and
392 10 % of Tween 80 in nutrient broth, the percentage of dormant spores was respectively 12.04
393 % and 54.59 %. It can also be seen from Figure 5C that after 8h and 10h germinated spores
394 and growth (quadrant II and quadrant III, respectively) decreased more sharply than for other
395 conditions. Thus, increasing the concentration of Tween 80 drastically decreased germination
396 and growth.

397

398 **4. Discussion**

399 First of all, H^+ ion concentrations had an effect on spore germination and outgrowth as
400 expected. H^+ ions will move into the cytoplasm decreasing internal pH. Drastic variations in
401 cytoplasmic pH can harm bacteria by disrupting the plasma membrane or inhibiting enzyme
402 activities and membrane transport proteins. Changes in the external pH might also alter the
403 ionization of nutrient molecules and thus reduce their availability to the organism. In our
404 model emulsion, the effect of pH was detected at pH 5.5 regardless of the dispersed phase
405 volume fraction. Nevertheless, at pH 5.5 less than 0.03 % germination and outgrowth may
406 occur. The minimum growth pH value is 4.35 and the estimated optimal recovery pH is 7.80
407 for *B. weihenstephanensis* KBAB4 (Trunet et al., 2015). This result suggested a synergistic
408 effect between pH value and matrix composition.

409

410 **The role of undissociated weak acid molecules was also important.** Weak acids are the
411 most commonly used chemical preservatives in the food industry to control both pH and
412 microbial growth (van Melis et al., 2011). It is generally accepted that that bacterial growth
413 inhibition by weak acids is based on the rapid diffusion of undissociated molecules through
414 the plasma membrane, followed by immediate dissociation and acidification of the cytoplasm.
415 Concerning their effects on bacterial spores, van Melis et al. (2012) formulated two
416 possibilities. Inhibition could be due, on one hand, to the accumulation of acids in the spore

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417 inner membrane or, on the other hand, to the accumulation of foreign compounds in the inner
418 membrane. As we have shown, such effects may occur in emulsions and may be affected by
419 the dispersed phase volume fraction and the presence of Tween 80. These relevant effects
420 should be highlighted and characterized, as they may have important safety implications in
421 foods based on emulsions.

422 There was no influence of the dispersed phase volume fraction in the presence of acetic acid
423 and lactic acid, whereas hexanoic acid was less effective in $\phi = 0.50$ emulsions. However,
424 results contrary to ours are described in the literature. Prachaiyo and McLandsborough (2003)
425 showed an increase in the generation time of bacteria as the hexadecane concentration
426 increased. This is likely to be due to space limitation, nutrient limitation and/or the
427 concentration of metabolic products. In the same way, Zalazar et al. (2016) described how an
428 increase in the oil level promoted a decrease in the specific growth rate. On the other hand,
429 Dong et al. (2016) reported a comparable inactivation of *Bacillus amyloliquefaciens* spores by
430 thermal and high hydrostatic pressure treatments in an oil/water emulsion with 20 % and 10 %
431 soybean oil. As the dispersed phase droplets in our study were smaller than the *B.*
432 *weihenstephanensis* KBAB4 spores, the cells seemed to evolve in the aqueous phase
433 following a Brownian motion and the increase in hexadecane concentration did not have an
434 effect on the germination and growth percentage.

435 In addition, our results showed a decline in efficiency of hexanoic acid with the increase in
436 hexadecane concentration. This could be related to the lipophilic character of this weak acid.
437 Indeed, according to the NMR results, the impact of hexadecane/water ratio impacted the
438 hexanoic acid diffusion coefficient more, which decreased with the pH. This could mean that
439 a fraction of hexanoic acid may be trapped in the dispersed phase limiting its access to cells
440 that were in the aqueous phase. Similarly, Brocklehurst and Wilson (2000) wrote that the
441 weak acid components interact with the lipid phase, and the effect was a decrease in the
442 preservation of the products. For example, acetic acid had a low partition coefficient: just a
443 small proportion of this acid was dissolved in the lipid phase and therefore predominantly
444 available in the water phase as a preservative. This was not the case for sorbic acid or benzoic
445 acid which had respectively a $\log P$ -value of 1.33 and 1.87. Furthermore, in most emulsified
446 food, emulsifiers are above their critical micelle concentration at which aggregates known as
447 micelles are formed. The non-polar inner regions of micelles present an environment closely
448 similar to that created by an organic solvent, where solutes such as weak acid will tend to
449 partition. For this reason, the preservative effectiveness of a weak acid will be associated with
450 its distribution in the different phases of the food emulsion, considering the interior of the

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451 micelles as a third phase (Castro et al., 2009). In our system, the lipophilicity of the biocide,
452 hexanoic acid, seemed to have had an impact on the distribution of the weak acid between the
453 dispersed phase and the aqueous phase and thus, on the antimicrobial activity.

454

455 **Moreover, the structure and composition of the model emulsion seemed to affect**
456 **antimicrobial activity.** The loss of microbial inhibitory activity when the dispersed phase
457 volume fraction increased could be due to phenomena at the interfaces. Factors such as
458 hydrophobicity, surface charge, and cell density influence the attachment of microorganisms
459 to surfaces and the behavior at interfaces (Seale, Flint, McQuillan, & Bremer, 2008). The
460 main result that emerged from the work of Naïtali et al. (2009) was that the cell surface
461 hydrophobicity of *Listeria monocytogenes* increased after growth in emulsion. This may have
462 been due to modifications to the cell surface composition, as well as to the adsorption of the
463 oil phase on the outer surface of the bacteria (Naïtali et al., 2009). The hydrophobicity of
464 various species of the *B. cereus* group spores varies from moderate to high (Faille et al.,
465 2010). This hydrophobic character it thought to be linked to the presence of an exosporium on
466 the outer spore membrane composed of proteins, polysaccharides, lipids and ash (Faille et al.,
467 2010; Sagong et al., 2013). Spores are more hydrophobic than vegetative cells. It has been
468 suggested that the increased hydrophobicity of the spores may be due to a relative abundance
469 of protein in the outer coats and exosporium compared with the peptidoglycan on vegetative
470 cell surfaces (Parkar, Flint, Palmer, & Brooks, 2001). Previous works have shown that a lower
471 ξ -potential, *i.e.* net charge on the spore surface, may be correlated with increased surface
472 attachment (Husmark & Rönner, 1990), while others did not identify a correlation (Parkar et
473 al., 2001). These different observations reflect the fact that the surface attachment or the
474 location of these bacteria in a complex system is a multifactorial process, combining physical
475 factors such as surface appendages, chemical signaling between cells and chemical
476 interactions.

477

478 **Furthermore, the component linked to interfaces, namely the emulsifier, could also**
479 **interact with cells and affect germination and/or growth.** Tween 80 is commonly used as
480 an emulsion stabilizer in a wide range of food and personal care products (Henry et al., 2009).
481 Tween-type emulsifiers possess a branched hydrophilic region of three polyoxyethylene
482 chains substituted to a sorbitan ring. The results of the present study revealed an antimicrobial
483 effect of Tween 80 on *B. weihenstephanensis* KBAB4 spores and on vegetative cells which
484 depended on pH. An effect of Tween 80 on the action of antimicrobial compounds has

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485 previously been mentioned in the context of interactions in food systems (Castro et al., 2009;
486 Jung et al., 1992; Krogsgard-Nielsen et al., 2017). Tween 80 may have hampered the
487 antimicrobial properties of isoeugenol in an emulsion (Krogsgard-Nielsen et al., 2017). The
488 solubilization capacity of the emulsifier on isoeugenol was so strong that it prevented the
489 antimicrobial compound from penetrating the cell membrane. Alternatively, Tween 80 might
490 affect the biofilm or individual cells in a way that makes them less susceptible to isoeugenol,
491 by interacting with membrane proteins which made them more hydrophilic and therefore less
492 prone to interaction with the essential oil (Krogsgard-Nielsen et al., 2017). This remark might
493 also have implications for the use of other hydrophobic substances: Krsta et al. (2014) showed
494 that Tween 80 reduced the effects of the antibiotic triclosan on *Streptococcus agalactiae* and
495 *S. aureus*. In a model salad dressing, the Tween effect was entirely dependent on oil content
496 when nisin was present: (i) at 23 % oil, Tween had an antagonistic effect with nisin; (ii) at 11
497 % oil, the presence of Tween increased the preservative effect; (iii) at 46 %, Tween had no
498 effect (Castro et al., 2009). In the same way, Jung et al. (1992) demonstrated interactions
499 between nisin, milk and Tween: the addition of Tween 80 has been shown to significantly
500 increase the activity of nisin on *L. monocytogenes* in milk regardless of fat content. The
501 authors suggested that nisin may adsorb to milk fat globules, which may render it unavailable
502 to destroy microbial cells. Tween, which has the ability to displace proteins from the milk fat
503 globule, may increase nisin availability. These results highlighted the importance of
504 considering ingredient interactions when evaluating the microbial stability of food systems.
505 Nevertheless, only a few studies have mentioned the testing of the antimicrobial effect of an
506 emulsifier as a function of pH and its concentrations. In our case, Tween 80 seemed to stress
507 spores and vegetative cells, slowing down germination and growth. In previous works, Tween
508 80 appears to have affected cell permeability in certain microorganisms to promote both
509 uptake and exit of compounds from the cell through modifications of plasma membrane
510 permeability (Brown & Winsley, 1969; Noh, Kim, & Gilliland, 1997; Sattler & Youmans,
511 1948). Noh et al. (1997) showed that the influence of Tween 80 was related to alterations in
512 the cellular membrane of the organism on *Lactobacillus acidophilus*. On *Pseudomonas*
513 *aeruginosa*, 0.125 % polysorbate led to the greatest uptake of a dye showing cell membrane
514 permeability (Brown & Winsley, 1969). Non-ionics had little denaturing action on proteins,
515 the uptake of polysorbate was presumably via hydrogen bonding. Flow cytometry on
516 vegetative cells in the presence of Tween 80 in the medium did not determine whether Tween
517 80 permeabilized the membrane of *B. weihenstephanensis* KBAB4 cells. Thus, the reduction
518 in growth rate could be due to a modification of the cellular membrane and its properties

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519 (permeability, hydrophobicity/hydrophilicity). Moreover, the synergistic effect between
520 Tween 80/pH and Tween 80/organic acid shows that Tween 80 could destabilize the
521 membrane, facilitating the action of pH or organic acid. Concerning this effect on
522 germination, spore surfaces were hydrophobic, so interactions could have been possible on
523 the surface of the spores with Tween 80, inhibiting germination mechanisms. Tween 80 is a
524 non-ionic emulsifier and would therefore not be expected to have any charge, although
525 previous studies have shown that droplets stabilized by non-ionic emulsifiers may be charged
526 due to the presence of ionizable surface active impurities (such as free fatty acids) or due to
527 preferential adsorption of hydroxyl ions at high pH or hydrogen ions at low pH to the droplet
528 surfaces (Yang, Leser, Sher, & McClements, 2013).

529 These interactions between the surface of the spores and Tween 80 could explain in some
530 cases the fact that germination and outgrowth were lower in $\phi = 0.35$ emulsions than in $\phi =$
531 0.50 emulsions. The interfacial region plays a crucial role in controlling the distribution of
532 molecules in the emulsified system (Pastoriza-Gallego, Losada-Barreiro, & Bravo-Diaz,
533 2012). Amphiphilic molecules are primarily located in the interfacial region; meanwhile non-
534 polar molecules tend to be mainly distributed between the oil and interfacial regions and polar
535 molecules are distributed between the aqueous and interfacial regions (Pastoriza-Gallego et
536 al., 2012). It seems that the effect of the dispersed phase volume fraction on antimicrobial
537 activity observed in our system was due to the distribution of the emulsifier between the
538 aqueous phase and the interface. According to Henry et al. (2009), Tween 80 had a head
539 group size of 0.78 nm^2 (average of 3 values in the literature). With the dispersed phase
540 volume fraction, the dispersed phase droplet volume and the concentration of emulsifier in the
541 emulsion, it was possible to calculate the proportion of each emulsifier required to give the
542 surface coverage of dispersed phase droplets for the emulsions produced. In $\phi = 0.50$
543 emulsions, with the concentration of Tween 80 used (1.5 % (w/w) in final emulsions), all
544 molecules were at the interfaces: there were no free Tween 80 molecules. They were not in
545 contact with the cells, hence the antimicrobial activity due to Tween 80 was limited. On the
546 contrary, in $\phi = 0.35$ emulsions, Tween 80 molecules were in excess to cover all droplets, so a
547 share of the molecules remained free. They were therefore in contact with the cells in the
548 aqueous phase and the antimicrobial activity of Tween 80 was higher.

549

550

551 **5. Conclusions**

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552 The present study shows that the inhibition of spore germination and growth in the model
553 emulsion depended on several factors: pH value and the dispersed phase volume fraction.
554 Antimicrobial activity resulted in a reduction in the number of cells capable of germination
555 and outgrowth, a reduction in maximal specific growth rate and an increase in lag times. The
556 effect of the dispersed phase volume fraction was due to a combination of:

557 (i) the lipophilicity of the biocide, hexanoic acid, that may have had an impact on the
558 distribution of organic acid between hexadecane and the aqueous phases;

559 (ii) the antimicrobial activity of the emulsifier Tween 80 detected at the acidic pH value.

560 The effect of Tween 80 has rarely been described in the literature and this study highlights the
561 caution with which this emulsifier must be used. Furthermore, the addition of Tween 80 in a
562 formulation may improve the antimicrobial activity of preservatives and this model emulsion
563 could be used as a surface treatment for example. The interface phenomena seemed to have a
564 great influence. Future work will focus on the exploration of these phenomena at the interface
565 by the use of biocides with other physical-chemical characteristics (lipophilicity,
566 antimicrobial mechanism of action, hydrophobicity, electrical charge) and of bacterial cells
567 with other surface characteristics (hydrophobicity, electrical charge).

568

569

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Fig. 1. Confocal microscopy observations of inoculated $\phi = 0.50$ emulsions with labeling of Bacillus spores with Thioflavin T (green) and the dispersed phase with Nile Red (red). (a, b, c: 2D observations; d: 3D observations). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

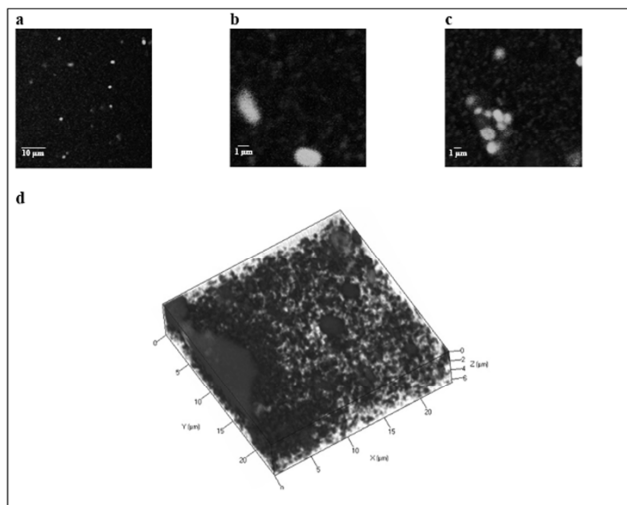


Fig. 2. D/D_0 for weak acids in distilled water or in emulsions as a function of pH at different hexadecane ratio (ϕ) (assuming a unique diffusion mechanism) (D/D_0 : ratio of self-diffusion coefficient in emulsion to self-diffusion coefficient of bulk component; ϕ , dispersed phase volume ratio).

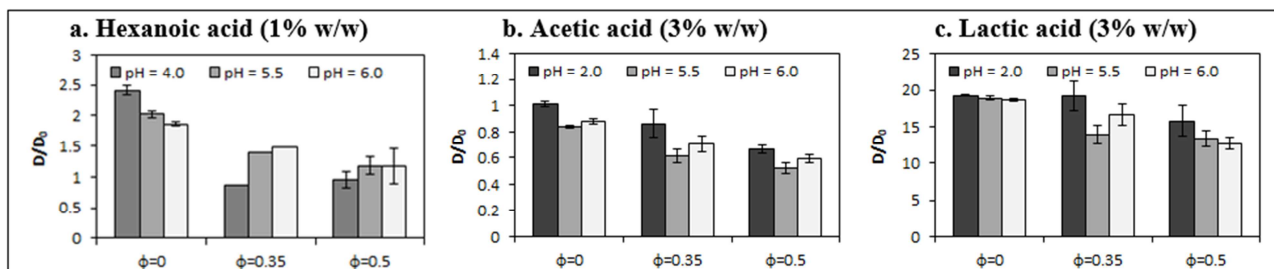


Fig. 3. Maximal growth rate (μ_{max}) at pH 6.0 and 5.5 in nutrient broth in the presence of Tween at different concentrations from 0% to 10% (*, **: μ_{max} significantly different from μ_{max} obtained without Tween 80 following a Student t-test ($P < 0.05$)).

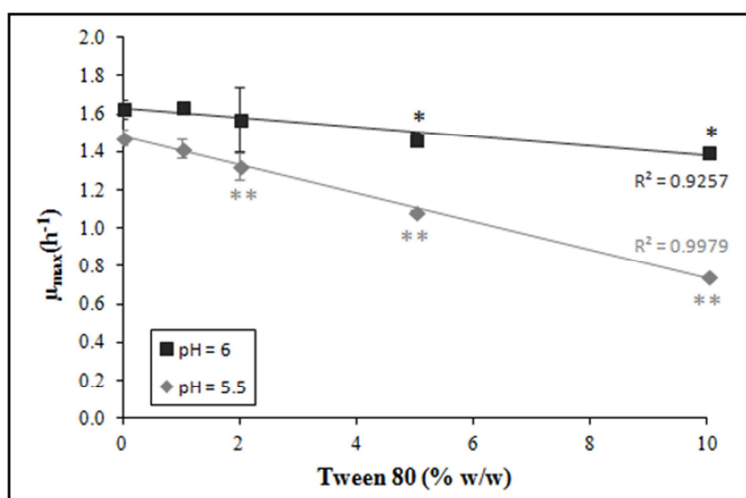


Fig. 4. Frequency distribution of individual lag times during growth of *B. weihenstephanensis* KBAB4 in nutrient broth without Tween 80 (black histogram) and with 5% (w/w) of Tween 80 (gray shaped histogram): a- at a pH 6.0; b- at a pH 5.5.

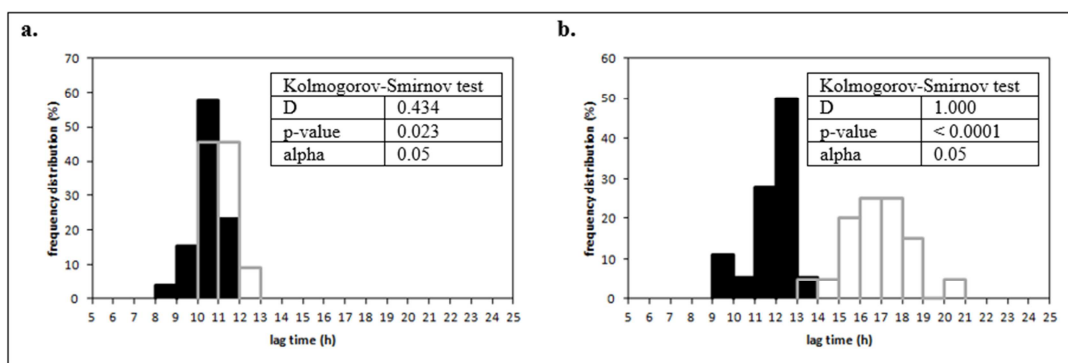


Fig. 5. Flow cytometry-derived scatterplots of green fluorescence (FL1) versus forward-scatter intensities (FSC) of Syto[®]9-stained *B. weihenstephanensis* KBAB4 spores during germination and growth. Events in different quadrants correspond to dormant refractive spores (I), germinated spores (II), and outgrowing spores (III).

- a. at pH 5.5 in nutrient broth without Tween 80
- b. at pH 5.5 in nutrient broth with 5% (w/w) Tween 80
- c. at pH 5.5 in nutrient broth with 10% (w/w) Tween 80.

