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

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Abstract

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

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

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

Keywords

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

Chemical compounds

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

1. Introduction

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

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

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

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

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

2. Materials and methods

2.1 Model system preparation

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

2.1.1 Inoculum preparation

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

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

2.1.2 Emulsion preparation

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

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

2.2 Physical characterization of emulsions and microbial cells

2.2.1 Droplet size and stability

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

2.2.2 Zeta potential measurements

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

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

2.2.3 Surface hydrophobicity

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

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

2.2.4 Microscopy

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

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

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

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

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

(assuming in this case monoexponential decay), where I_g is the attenuated signal intensity by application of gradient pulses, I_0 the optimum signal intensity without field gradient, and γ the gyromagnetic ratio). The self-diffusion coefficient (D_0) were $4.50 \times 10^{-11} \pm 7.50 \times 10^{-13} \text{ m}^2 \cdot \text{s}^{-1}$, $4.15 \times 10^{-10} \pm 1.0 \times 10^{-12} \text{ m}^2 \cdot \text{s}^{-1}$, $1.24 \times 10^{-09} \pm 3.0 \times 10^{-12} \text{ m}^2 \cdot \text{s}^{-1}$ and $5.10 \times 10^{-11} \pm 1.63 \times 10^{-12} \text{ m}^2 \cdot \text{s}^{-1}$, for pure lactic acid, hexanoic acid, acetic acid and Tween 80, respectively. Then, each weak acid and Tween 80 were diluted in water ($\phi = 0$) or prepared in two different emulsions with $\phi = 0.35$ and with $\phi = 0.50$. For each acid, the self-diffusion coefficient D in water or emulsions was compared to D_0 (pure weak acid or Tween 80) by calculating the ratio D/D_0 .

2.3 Characterization of bacterial behavior

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

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

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

(3)

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

2.3.2 Monitoring *Bacillus* spore germination by flow cytometry

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

2.3.3 Bioscreen® growth experiments

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

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

2.3.4 Individual lag times

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

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

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

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

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

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

3. Results

3.1 Physical characteristics of emulsion constituents

3.1.1 Droplet size

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

a concentration 64 % of monodisperse droplets, which represents the random-close-packing volume fraction (Leal-Calderon, Schmitt, & Bibette, 2007). The droplets were agitated by a Brownian motion. They presented a monomodal distribution of hexadecane droplets with, 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 °C, neither the creaming nor the stability of the emulsions was modified with or without organic acid and whatever the pH. As observed in the emulsions using confocal microscopy (**Figure 1**), the droplets of the dispersed phase appeared to be smaller than the spores ($\sim 1 \mu\text{m}$) (Carrera, Zandomeni, Fitzgibbon, & Sagripanti, 2007). In our systems, microbial growth seemed to occur in the aqueous phase as is commonly recognized (Brocklehurst & Wilson, 2000; Wilson et al., 2002).

3.1.2 Spore characteristics

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

3.1.3 Diffusion of emulsion constituents by NMR

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

4. Discussion

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

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

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

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

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

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

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

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

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

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

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

5. Conclusions

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

- (i) the lipophilicity of the biocide, hexanoic acid, that may have had an impact on the distribution of organic acid between hexadecane and the aqueous phases;
- (ii) the antimicrobial activity of the emulsifier Tween 80 detected at the acidic pH value.

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

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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.