

Interactions between bacterial surfaces and milk proteins, impact on food emulsions stability

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Abstract

Bacteria possess physicochemical surface properties such as hydrophobicity, Lewis acid/base and charge which are involved in physicochemical interactions between cells and interfaces. Moreover, food matrices are complex and heterogeneous media, with a microstructure depending on interactions between the components in media (van der Waals, electrostatic or structural forces, etc.). Despite the presence of bacteria in fermented products, few works have investigated how bacteria interact with other food components. The objective of the present study was to determine the effects of the surface properties of lactic acid bacteria on the stability of model food emulsions. The bacteria were added to oil/water emulsions stabilized by milk proteins (sodium caseinate, whey proteins concentrate or whey proteins isolate) at different pH (from 3 to 7.5). The effect of bacteria on the emulsions stability depended on the surface properties of strains and also on the characteristics of emulsions. Flocculation and aggregation phenomena were observed in emulsion at pHs for which the bacterial surface charge was opposed to the one of the proteins. The effects of bacteria on the stability of emulsion depended also on the concentration of cations present in media such as Ca^{2+} . These results show that the bacteria through their surface properties could interact with other compounds in matrices, consequently affecting the stability of emulsions. The knowledge and choice of bacteria depending on their surface properties could be one of the important factors to control the stability of matrices such as fermentation media or fermented products.

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1. Introduction

Food matrices are complex media containing very diverse compounds. The interactions between them through physicochemical forces may modify the microstructure of food products, leading to changes in the texture and retention of aroma compounds (Dickinson, 2003; Gupta & Muralidhara, 2001). Moreover, most food products, such as desserts and salad dressings, are oil-in-water emulsions. The stability of emulsions is dependent on

many factors: oil/water ratio, nature of the dispersed oil phase and of the interfacial adsorbed layer (oil–water interface) (Dickinson, 2003). The latter factor depends on characteristics such as hydrophobicity, surface charge, the concentration of emulsifier and also the competition between the various surface active compounds present in emulsion. For this reason, many studies have investigated the interactions of emulsifiers with other compounds for the stability of emulsions (Dickinson, 2003; Tcholakova, Denkov, Ivanov, & Campbell, 2006), particularly in the case of milk proteins that are known for their capacity of emulsifying and their nutritional properties (Cayot & Lorient, 1998). Interactions between milk proteins and other components such as non-ionic surfactant, polysaccharides

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(pectin, xanthan, carrageenan, chitosan, etc.) and salt have been extensively studied (Hemar, Tamehana, Munro, & Singh, 2001; Ramkumar, Singh, Munro, & Singh, 2000; van Aken, 2003; Ye, Hemar, & Singh, 2004; Ye & Singh, 2000). In general, these interactions of physicochemical nature are weak but they may become important when they occur between macromolecules, leading to possible changes in the stability of emulsions. However, they depend on the physicochemical properties of both proteins and other compounds and on the conditions of media: pH, ionic strength (Dickinson, 2003; van Aken, 2003).

In fermented food, bacteria are present in important concentrations (more than 10^9 cultivable cells/ml at the end of fermentations, amount that is maintained over 10^8 cells/ml for some probiotic products to which many biologically inactive cells can be added) (Yoon, Woodams, & Hang, 2006). Moreover, bacterial cells are micrometer-scale particles bearing at their surface many macromolecules such as proteins, mannoproteins, peptidoglycan, teichoic acid and polysaccharides (Boonaert & Rouxhet, 2000; van der Mei, de Vries, & Busscher, 2000). These compounds bring to cells their physicochemical properties that enable bacteria to interact with other compounds through Lewis acid/base, electrostatic and hydrophobic interactions (Hermansson, 1999; Schar-Zammaretti & Ubbink, 2003). The microbial surface properties have been widely studied in order to understand the interactions between bacteria and interfaces resulting in the formation of biofilms, phenomenon important in many fields such as biomedical and food safety, corrosion and environment (Bellon-Fontaine, Rault, & Van Oss, 1996; Briandet, Herry, & Bellon-Fontaine, 2001; Strevett & Chen, 2003; van der Mei, Busscher et al., 2000). Up to now, the behavior of bacteria in food matrices through physicochemical interactions has not received such attention. Recently, we have studied the physicochemical properties of bacteria in order to understand the interactions between them and food components (Ly, Vo, Le, Belin, & Waché, 2006). The bacteria with more hydrophobic surfaces had a bigger affinity for milk fat and aroma compounds. On the other hand, the diversity of bacterial surface charge results in different electrostatic interactions between bacteria and droplets modifying the emulsion stability (Ly, Naïtali et al., 2006). In the present study, we present the impact of the surface of bacteria on the stability of emulsions stabilized by different milk proteins: sodium caseinate, whey protein isolate (WPI) and whey protein concentrate (WPC). After the preliminary evaluation of the properties of more than 20 strains from the bacterial collection of the laboratory (Ly, Vo et al., 2006), two strains of the same species *Lactococcus lactis* subsp. *lactis* biovar. diacetylactis exhibiting different surface properties were selected for the experiments. These strains of lactic acid bacteria are among the most important microorganisms used in the elaboration of fermented products. They have a key role in the production of yoghurts, cheese, sausages, sauerkraut (McKay & Baldwin, 1990; Ross, Morgan, & Hill, 2002).

2. Material and methods

2.1. Chemicals

The WPC powder (Nollibel) was obtained from Bel Industries (France). WPC consists of a serum mixture of proteins, minerals and lactose. The mineral and lactose concentrations are close to the concentrations in milk. The protein fraction (about 35% of the total content) contains 90% serum proteins and about 10% caseins. WPC was obtained from serum by evaporation and nanofiltration.

The WPI is also composed of serum proteins but which have been isolated and concentrated. The WPI, containing 95% proteins (60% β -lactoglobuline; 40% α -lactalbumin); 0.4% fat and 5% moisture (moist weight basis), was from Davisco International (France). WPI was obtained by separation of proteins from the serum, ion exchange, ultrafiltration and heat treatment.

The other chemicals, including sodium caseinate, were of the highest purity and have been purchased from Sigma-Aldrich (France) except the sunflower oil, which was purchased from the local market.

2.2. Determination of protein zeta potential (ζ)

The zeta potential of the proteins at each pH was measured by the Zetasizer system ZS (Malvern, Worcs), which is a combined static, dynamic, and electrophoretic scattering instrument for the characterization of protein samples (sample size, molecular weight and zeta potential). The powder of proteins (5%, m/v) was dissolved in distilled water for 1 h at 30 °C under agitation at 140 rpm. This protein suspension was diluted at pH 3 and 4.5 in citrate buffer (100 mM) and at pH 7 in phosphate buffer (100 mM) to an adequate concentration prior to analysis. One milliliter of the sample was slowly injected into the cell of a Zetasizer Nano ZS that consists of electrodes and a folded capillary, checking that all air bubbles were removed. The cells were moved in the measurement chamber. Depending on the electric field, the ζ was determined by measuring the direction and velocity of particles. All measurements were performed at a temperature of 25 °C.

2.3. Bacteria and characterization of cell surfaces

2.3.1. Bacterial strains and growth conditions

Two *L. lactis* subsp. *lactis* biovar. diacetylactis strains: LLD16, LLD18 (formerly SD16 and SD18) were selected from the *L. lactis* laboratory collection. They are technological strains isolated from dairy products and reflecting the natural diversity of lactococci. Bacteria conserved at –70 °C in MRS media (De Man, Rogosa, & Sharpe, 1960) (without Tween 80 and for which glucose was replaced by lactose) containing 25% (v/v) glycerol were thawed, subcultured overnight, and grown in liquid MRS media. Cultures were performed at 27 °C and bacterial growth was

evaluated by the measurement of the absorbance at 600 nm and of the pH of the medium. For the experiments, cells were harvested in early stationary phase.

The relationship between cellular concentration and absorbance was tested by the Malassez cell counting. 10^9 cells/ml of bacteria correspond to an absorbance at 600 nm of $A_{600\text{nm}} = 1$. The cells harvested in early stationary phase were washed twice with the same buffer used for experiments by centrifugation at 7000g for 5 min.

2.3.2. Hydrophobicity of cell surfaces

The hydrophobicity of cell surface was evaluated as a function of pH by microbial adhesion to hydrocarbons (MATH) according to the method proposed by Rosenberg (1991). Bacterial cells were harvested by centrifugation at 7000g for 5 min and resuspended in citrate buffer 100 mM at pH 3; 4.5 and in potassium phosphate buffer 100 mM at pH 6.5. The cell suspension has an absorbance (A) at 600 nm of 0.4 (A_0). A 0.4 ml of hexadecane was added to 2.4 ml of cell suspension. The two-phase system was mixed by vortexing for 30 s and allowed to separate for 20 min. The aqueous phase was removed with a Pasteur pipette and its absorbance at 600 nm (A_1) was measured. The percentage of microbial adhesion to solvent was expressed by the difference of the absorbance of cell suspension before (A_0) and after (A_1) mixing with the solvent: $(1 - A_1/A_0) \times 100$.

2.3.3. Zeta potential (ζ) of cell surfaces

The electrical properties of the cell surfaces were assessed by microelectrophoresis. The electrophoretic mobility (EM) was determined in the pH 2–8 range. Cells in the stationary phase were harvested by centrifugation at 7000g for 5 min, and resuspended twice in physiological water (NaCl 9 g/l) at a concentration of about 10^7 cells/ml. pHs were adjusted by addition of KOH and HNO₃ (100 mM). EMs were evaluated at room temperature on a ZM 77 Zetameter model (Zetameter Inc., New York, NY). The EM, expressed in $10^{-8} \text{ m}^2 \text{ V}^{-1} \text{ s}^{-1}$, was derived from the velocity of the bacteria in suspension under an applied electric field of 100 mV.

2.4. Characterization of emulsions stability

2.4.1. Preparation of the model food emulsions

Stock emulsions were prepared by mixing 70% of a 5% (w/v) aqueous solution of protein and 30% of sunflower oil with an ultra turrax (Kika T25 basic, Germany) at 16,000 rpm. Stock emulsions (at 25 °C) were diluted 10-fold in citrate (100 mM, pH 3 or 4.5) or phosphate buffer (100 mM, pH 6, 6.5 and 7) before use. A bacterial concentration of approximately 10^9 cells/ml was added to the emulsions by vortexing for 5 s. The 10^9 cells/ml concentration corresponds to an absorbance at 600 nm of $A_{600\text{nm}} = 1$. An adequate volume of a concentrated suspension of bacteria was added in emulsion to reach the wanted concentration. The pH was checked after

addition of bacteria. The CaCl₂ (20 mM) was added to the WPI emulsions at pH 7. Two orders of addition of CaCl₂ were realized with an addition before or after the addition of the bacteria.

The stability of emulsions was evaluated for 24 h of storage at 25 °C by the observation of the phase separation, by the measurement of the emulsion turbidity and by microscopic observations of fluorescently stained emulsions.

2.4.2. Observation of phase separation

Four milliliters of emulsion were put into a tube of 10 cm height and 0.8 cm radius. The apparition of a cream layer was observed.

2.4.3. Determination of emulsion turbidity

One milliliter of emulsion was placed into a 1-cm path length plastic spectrophotometer microcuvette. The change in the emulsion turbidity was measured at 600 nm. The oil droplets of the emulsion moved upwards due to gravity, which led to the formation of a relatively clear serum layer at the bottom of the cuvette. The light beam passed through the emulsions at a height of about 10 mm from cuvette bottom and always 30% inferior to the emulsion's phase height. The turbidity of the emulsion in the bottom part of the cuvette indicates the stability of emulsion: the turbidity was reduced for unstable emulsions. Diminution of the absorbance was calculated by: $(A_{\text{after}}/A_{\text{before}}) \times 100$.

2.4.4. Staining and microscopic observation

The emulsions with or without bacteria were observed in fluorescence microscopy (Zeiss Axioplan 2 imaging). The emulsions were stained with Nile Red (8 µg/ml, stock solution: 4 mg/ml in acetone) and the bacteria were stained with DAPI (4',6-diamidino-2-phenylindole, a DNA-specific fluorescent probe) (10 µg/ml, stock solution: 5 mg/ml in distilled water) for 15 min at 27 °C under a 140 rpm agitation. The emulsion and the cells were stained separately and then cells were added to the emulsion under agitation at room temperature. The images from the Axiocam MRm camera were treated with the software AxioVision 4 (Zeiss).

3. Results

The growth and acidification kinetics of the two strains were evaluated in MRS medium. They were very similar (data not shown). The stationary phase was reached after 6 h of culture when the pH of media was about 4.3.

3.1. Hydrophobicity of cell surfaces

The partitioning of cells between water and hexadecane depends on hydrophobic interactions between microorganisms and the hydrocarbon. In buffer at concentration of 100 mM, the adhesion of LLD18 to *n*-hexadecane was similar at pH 3, 4.5 and 7 with more than 40% adhesion. Contrasting with LLD18, LLD16 did not adhere to hexadecane at any pH (Fig. 1).

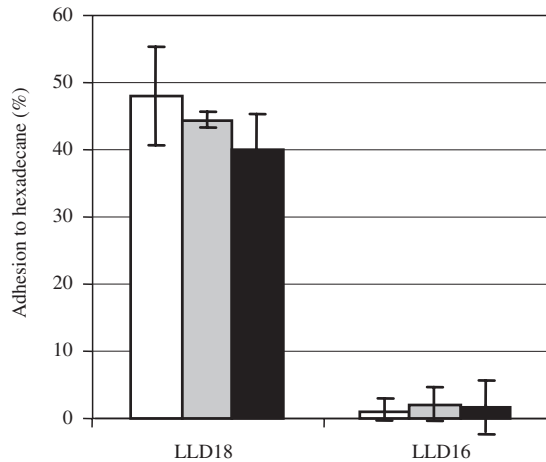


Fig. 1. Adhesion of two strains LLD18 and LLD16 to hexadecane in phosphate buffer 100 mM at pH 3 (□), 4.5 (▣) and 7 (■).

3.2. Zeta potential (ζ) of proteins and bacteria

The ζ of the two strains and of milk proteins at different pHs are presented in Fig. 2. For the bacteria, the ζ depended upon strains and pH. The ζ of both strains was negative at near-neutral pH values and became more positive with decreasing pHs (Fig. 2A). The LLD18 strain had a net negative charge at pH 7.5 (−32 mV) and a positive one (7 mV) at pH 2. The isoelectric point (pI) of LLD18 was around pH 3.5. Contrasting with these results, the LLD16 strain was negatively charged at all pH values of the test (−30 mV at pH 2 and −40 mV at pH 7.5). In all cases, LLD16 was more negatively charged than LLD18. The ζ of LLD16 was almost constant from pH 7.5 to 4.5.

The profile of ζ of proteins WPI, WPC and sodium caseinate, was rather similar (Fig. 2B). The value of ζ decreased from +30 to −30 mV when pH increased from 3 to 7. The isoelectric point (pI) of WPI and WPC was around 4.5 and the one of sodium caseinate was close to 4.

3.3. Emulsion stability characteristics

The emulsion stability evolution was first evaluated through the macroscopic observation of the apparition of a thick cream layer (phase separation). Then, the emulsions were examined more in detail by the measurement of the turbidity of the serum phase and by the microscopic observation of the microstructure.

3.3.1. Phase separation in tubes

Observations of the phase separation phenomena for the emulsions at different pHs are presented in Fig. 3. The emulsions made with sodium caseinate were stable at pH above 6 but unstable at pH 3 and 4.5 (Fig. 3A). A visible thick cream layer and a clear lower serum phase were observed at pH 3 and 4.5. The stability of emulsions was similar with or without bacteria at pH superior to 6

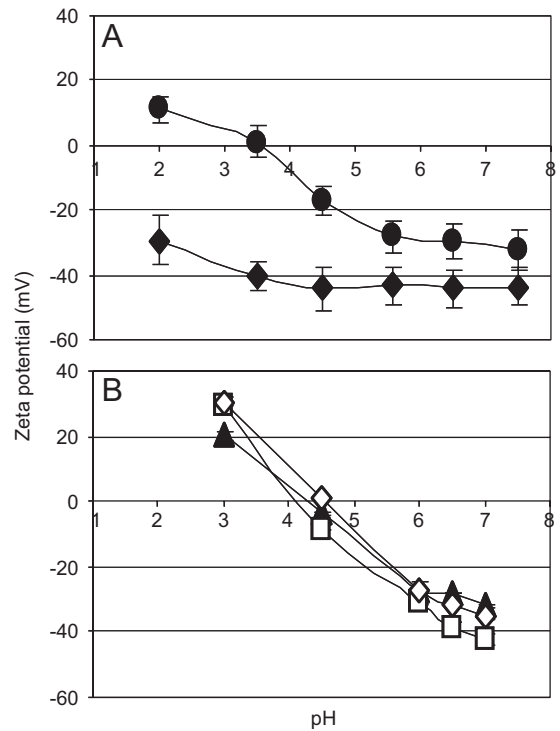


Fig. 2. Zeta potential of bacteria (A) and milk proteins (B) as a function of pH: (◆) LLD16; (●) LLD18; (▲) WPC; (□) sodium caseinate; (◇) WPI.

showing that the presence of the bacteria in the emulsions did not affect it.

In the case of emulsions made with WPI, the stability was similar to that of sodium caseinate emulsions, except at pH 3 for which the WPI emulsion without bacteria was stable (Fig. 3B). When bacteria were added, the emulsion was destabilized immediately in presence of strain LLD16 but not in presence of LLD18. The emulsions in the presence of LLD18 were stable and similar to emulsions without bacteria. At pH 4.5, close to the isoelectric point of whey proteins, emulsions were unstable with and even without bacteria. No effect of bacteria was observed at pH 6, 6.5 or 7.

The stability of emulsions made with WPC was similar to that of emulsions made with WPI at pH 3 and 4.5 but different at pH superior to 6 (Fig. 3C). At pH 3, the emulsion was not stable in presence of LLD16 whereas the emulsions made without bacteria or in the presence of LLD18 were stable. Without bacteria the emulsion made with WPC was stable at pH 6 and 6.5 but not at pH 7. An effect of bacteria was observed at pH 6 and 6.5: the emulsions without bacteria were stable but became unstable, with a visible creaming and phase separation, after the addition of bacteria.

3.3.2. Turbidity of emulsions

The instability of an emulsion can be deduced from the evolution of its turbidity (Fig. 4). When the emulsions were unstable, the turbidity decreased immediately. This decrease

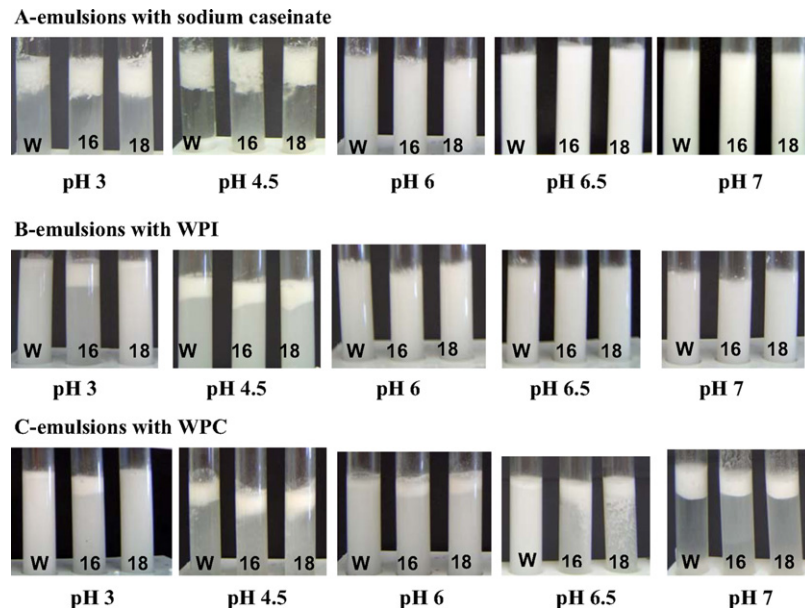


Fig. 3. Phase separation of emulsions as a function of pH: emulsions stabilized by sodium caseinate (A); WPI (B) and WPC (C). W—without bacteria, 18—with LLD18 and 16—with LLD16, 24 h after cell addition.

may be due to the rising up of the light absorbing lipid droplets. The measurement of the decrease in turbidity was correlated with the observation of phase separation.

3.3.3. Microscopic observation

To examine the microstructure and the relationship between the structure and the stability of emulsions, a microscopic observation was carried out. The positions of bacteria and emulsion droplets were evaluated by fluorescent staining.

The observation of emulsions made with WPI at pH 3 and 7 are shown in Fig. 5. The microscopic observation confirmed the instability of emulsions in presence of the strain LLD16 at pH 3. At this pH (Fig. 5, up), the oil droplets of emulsions without bacteria (Fig. 5, up-left) or with LLD 18 (Fig. 5, up-right) were uniformly small. When LLD16 was added to the emulsion, the droplets coalesced and became bigger (Fig. 5, up-center). The fluorescence images showed that the LLD16 were located on the surface of the droplets, acting as bridges between them. This phenomenon was not observed for LLD18. Similar observations were done for the emulsion made with WPC at pH 3 (data not shown).

At pH 7, the emulsion droplets with WPI were rather similar with or without bacteria but no adhesion to droplets was observed for LLD16 whereas some cells of LLD18 adsorbed (Fig. 5, down). This adhesion may be driven by hydrophobic interactions. At pH 6 and 6.5, the aspect of emulsions was similar to observation at pH 7 (data not shown).

3.3.4. Effect of calcium chloride addition on emulsion stabilized by WPI

The emulsions made with WPI without bacteria at pH 6, 6.5, 7 were stable and became unstable when calcium was

added. When the emulsions contained both bacteria and calcium cations, the stability of emulsions depended on the order of addition of calcium before or after bacteria. In Fig. 6, tube A presents the creaming of the emulsion (A). When calcium was added before the bacteria, the emulsion was broken dramatically. Tube B presents the creaming of the emulsion for which calcium was added after bacteria. The emulsion was also unstable but less than the emulsion (A) when calcium was added before bacteria. In tube C, the calcium was added in the buffer containing the bacteria and the stock emulsion was added after. The emulsion was more stable.

4. Discussion

The MATH result is considered as a measure of the hydrophobicity of microbial cell surface, which is evaluated by the partition of cells between water and hexadecane (Rosenberg, 1991). According to some authors (Bellon-Fontaine et al., 1996; Busscher, van de Belt-Gritter, & van der Mei, 1995), this partition depends on hydrophobic interactions but also on electrostatic ones because the hexadecane droplets have a charge depending on pH. The hydrophobic interactions could be dominant in two conditions: (1) at pH which are close to the *pI* of bacteria or *pI* of hexadecane droplets, (2) in suspensions with ionic strength sufficiently high to neglect the electrostatic interactions. In this study, the adhesion of bacteria was evaluated in ionic conditions similar to the ones used to study the emulsion stability (buffer at 100 mM; pH 3, 4.5 and 7). Although the charge of the two bacteria and of hexadecane droplets varied from pH 2 to pH 7, the percentages of adhesion of LLD16 and LLD18 to hexadecane were similar at all pHs indicating that the

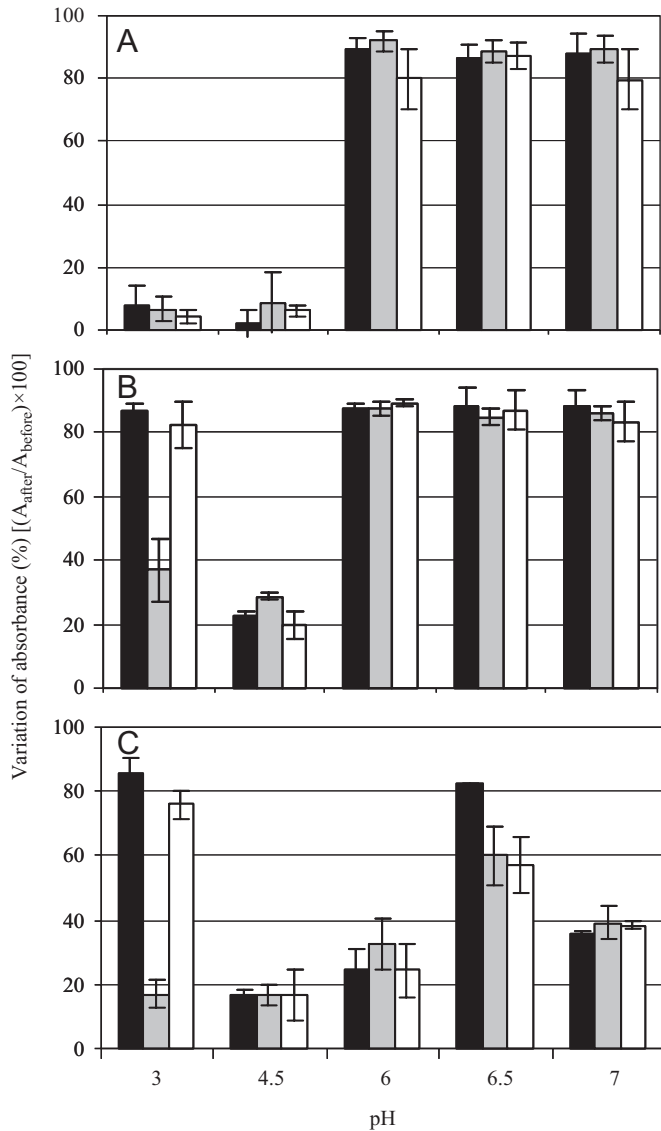


Fig. 4. Variation of absorbance $[(A_{\text{after}}/A_{\text{before}}) \times 100]$ of emulsions stabilized by sodium caseinate (A); WPI (B) and WPC (C). Without bacteria (■); in the presence of LLD18 (□) and in the presence of LLD16 (▒) 24 h after cell addition.

electrostatic interactions did not affect adhesion. The LLD18 strain was more hydrophobic than LLD16. The surface of lactic acid bacteria strains studied in literature is rather hydrophilic (Boonaert & Rouxhet, 2000; Pelletier et al., 1997). According to these authors, the chemical groups of proteins, polysaccharides, peptidoglycans and (lipo)teichoic acids at the cell surface are responsible for its physicochemical properties.

The bacterial surface charge results from the dissociation or protonation of three main ionizable groups, the phosphate-group of (lipo)teichoic acids and the carboxyl- and amino-groups of proteins, which depend on pH. At physiological pHs between 5 and 7, the number of ionized carboxyl- and phosphate-groups exceeds the number of amino-groups and most bacterial strains are negatively

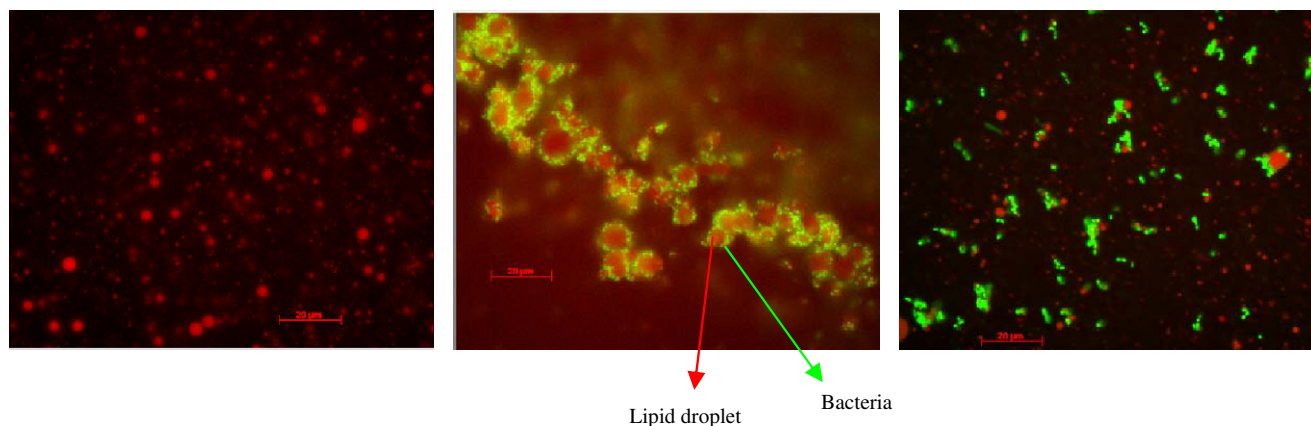
charged (Boonaert & Rouxhet, 2000). Recently, cellular surfaces of some microorganisms have been analyzed by the X-ray photoelectron spectroscopy (XPS) technique which enables to determine the ratios of atoms N/C, O/C, P/C at the cell surface (van der Mei, Busscher et al., 2000; van der Mei, de Vries et al., 2000). These authors found a correlation between the isoelectric point, hydrophobicity and the N/C ratio. Similar results were reported by Latrache et al. (2002) who found a correlation between atom ratios and the hydrophobicity of cells. Both water contact angles and MATS tests correlated with N/C ratios ($r = 0.67$ and 0.88 , respectively) and with O/C ratios ($r = 0.91$ and 0.46 , respectively).

The cell surface properties including the cell surface charge and hydrophobicity are involved in the interactions between bacteria and solid surfaces or interfaces. These properties have been studied to understand the first step of the adhesion of bacteria which is the cause of biofilms formation (Donlan, 2002; Strevett & Chen, 2003). In this paper, the cell surface properties were studied to understand the interactions of bacteria with other food components such as proteins surrounding lipids, as these interactions may affect the stability of emulsions and the localization of bacteria in the different regions of the matrix. In this objective, the bacteria with different surface properties LLD18 and LLD16 were added in emulsions made with the various milk proteins: WPI, WPC and sodium caseinate.

The surface charge of emulsion droplets is dependent on the charge of proteins coating the droplet surface (van der Mei, Meijer, & Busscher, 1998). The droplets have a positive charge when pH is far lower than protein's pI , a neutral charge when pH is close to protein's pI and a negative one when pH is superior to protein's pI (Kulmyrzaev & Schubert, 2004). This suggests that the emulsions stabilized by these proteins possess positively charged droplets at pH 3, negatively charged droplets at pH 7 and non-charged ones at protein's pI .

In all the cases, the emulsions were not stable at pH 4.5 which was close to the pI of proteins. The droplets flocculated and an important creaming occurred. This phenomenon may be explained by the fact that at pH close to the pI of proteins, the electrostatic repulsion between the droplets is reduced, leading to coalescence and flocculation (Kulmyrzaev, Chanamai, & McClements, 2000). According to these authors, to produce a stable emulsion, it is important to adjust pH to a value inferior to 4 or superior to 6. The emulsions made with serum proteins were stable at pH 3 but not sodium caseinate. It may be explained by the pI of sodium caseinate which was lower than the one of WPC and WPI. Similar droplets aggregation have been observed in the literature for sodium caseinate stabilized oil-in-water emulsions (Surh, Decker, & McClements, 2006). It was attributed to the fact that the pH was close to the protein's pI and the electrostatic repulsion was insufficient to prevent aggregation. Moreover, the structure and properties of whey proteins and caseinate are different

pH 3



pH 7

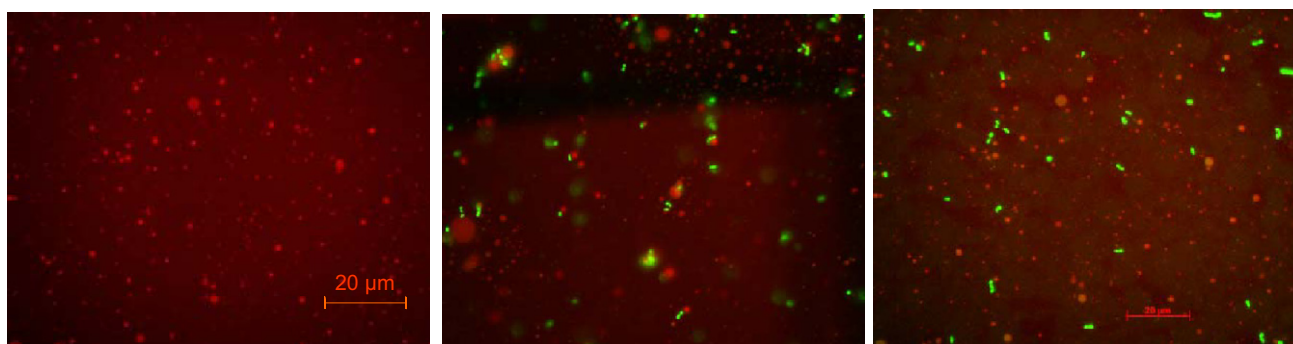


Fig. 5. Fluorescence microscopy observations: emulsion made with WPI at pH 3 (up) and at pH 7 (down), without bacteria (left), with LLD16 (center) and with LLD18 (right) 24 h after cell addition.

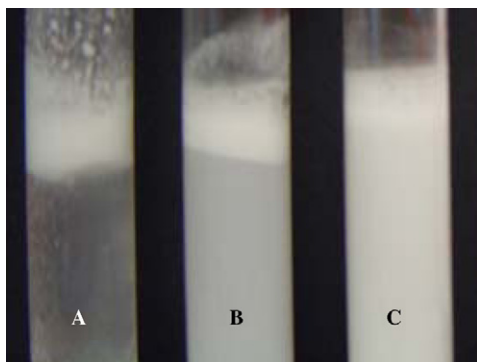


Fig. 6. Variation of emulsion stability depending on the order of addition of bacteria and calcium. (A) ((emulsion + Ca^{2+}) + bacteria). (B) ((emulsion + bacteria) + Ca^{2+}). (C) ((bacteria + Ca^{2+}) + emulsion).

in terms of hydrophobicity and nature of amino acids residues exposed to the surface. It has been shown already that WPI emulsions are destabilized only in a narrow range around the pI (Kulmyrzaev et al., 2000) whereas caseinate emulsions are destabilized in a larger range and particularly, below the pI (Surh et al., 2006). This could be the result of non-electrostatic interactions as it has been shown that the hydrophobicity of caseinate increases dramatically below the pI , due to changes in the protein conformation in

which more aromatic and aliphatic amino acids residues are exposed to the surface (Jahaniaval, Kakuda, Abraham, & Marcone, 2000).

Without bacteria, most emulsions were stable in the pI range but when cells were added to emulsions, these latter could induce instability even at pHs relatively distant from pI .

The instability was firstly observed for the emulsions made with WPC and WPI at pH 3 after the addition of LLD16. At this pH, the emulsions contained positively charged droplets and the LLD16 had a negative charge. We suggest that, due to the adsorption by electrostatic interactions of bacteria to the proteins coating the oil droplets, the repulsion between the droplets was reduced, leading to aggregation phenomena. Thus, the emergence of big droplets may be a result of the contacts between small ones. On another hand, the bacteria with a negative charge on the surface were able to form bridges between droplets, promoting their aggregation (Fig. 5C, up). The aggregation of emulsion droplets observed by microscopy for LLD 16 at pH 3 could be the cause of the coalescence and creaming phenomenon resulting in phase separation in tubes. These results suggest that if the bacteria interacted with the emulsion droplets, they adsorbed on the droplet surface, raised consequently to the top with the droplets and were found in the creaming phase. These results could be

compared to the destabilization of emulsions with whey proteins at pHs for which emulsions contained negatively charged droplets and when calcium ions (Ca^{2+}) were added: a flocculation and an increase in the size of the droplets were observed (Gu, Decker, & McClements, 2005; Kulmyrzaev et al., 2000; Ramkumar et al., 2000; Ye & Singh, 2000). In these cases, Ca^{2+} could also be involved in the formation of bridges. According to these authors, the addition of Ca^{2+} in the emulsion decreased the electrostatic repulsion between the droplets and increased the potential associations and aggregations. Van Aken (2003) also suggested that the calcium adsorbed on the proteins coating the droplets can decrease the charge of the adsorption layer of the droplets, thus enabling connections between them. Similar results were obtained for emulsions stabilized by whey proteins in presence of pectin at pHs for which the emulsion droplets were positively charged and the pectin negatively charged (Gancz, Alexander, & Corredig, 2006). In our results, the emulsion in presence of LLD18 was stable at pH 3. In this condition, both the strain and proteins had a net positive charge, and an electrostatic repulsion between them was likely to occur. However, the adsorption of LLD18 cells to the droplets at pH 7 suggests that the bacteria can also adsorb to proteins by hydrophobic interactions. The hydrophobicity of bacteria can explain and enable to predict the affinity of microorganism for apolar compounds (Bouchez-Naitali, Blanchet, Bardin, & Vandecasteele, 2001; Bruinsma, van der Mei, & Busscher, 2001; Ly, Vo et al., 2006; Pascual, De Cal, Magan, & Melgarejo, 2000). A previous study (Dickinson, 2003) reported, for a mixture of proteins and another surfactant (i.e. polysaccharide or Tween 80), the flocculation of an emulsion by hydrophobic interactions. In our study, the hydrophobic interaction between bacteria and proteins led to the adsorption of bacteria on lipid droplets but did not result in a destabilization of the emulsion.

The difference in stability of emulsions made with WPC and WPI at pH 7 may be due to the composition of whey proteins preparations. The WPI contains β -lactoglobulin and α -lactalbumin which possess an emulsifier capacity at neutral pH. These proteins are also contained in WPC, but in lower concentrations. In addition to these two proteins, WPC contains lactose and minerals, especially calcium. The presence of salt in emulsion stabilized by whey proteins may lead to coalescence (Gu et al., 2005; Kulmyrzaev et al., 2000; Ramkumar et al., 2000; Ye & Singh, 2000). We suggest that the instability of WPC at pH 7 can be due to the presence of minerals. This hypothesis was verified by the addition of calcium in emulsion made with WPI in the later part. The stability of WPI emulsion depends on the pH and also the concentration of CaCl_2 presence in media. The difference of creaming was observed for WPI emulsion at pH 7, 6.5 and 6 when the concentration of CaCl_2 added in emulsion between 0 and 5 mM (Kulmyrzaev et al., 2000).

The emulsion made with WPC became unstable at pH 6 and 6.5 when the bacteria were added. These results were

not observed for emulsions made with WPI. These results showed that the interactions of bacteria with other compounds in emulsion depend on the composition of media. The pH dependence of the surface charge of proteins may influence the flocculation stability. With the decrease in pH, the number of negatively charged groups decreases and the one of positively charged groups increases. As a result, the droplets acquired a net negative charge. At pH 7, if the cations are present in the emulsion, they can adsorb strongly on negatively charged groups of proteins coating the droplets and facilitate their aggregation and reduce the surface charge of droplets (Cayot & Lorient, 1998; Kulmyrzaev et al., 2000). In that case, the electrostatic repulsion between the droplets is not sufficient to prevent destabilization. At pH 6 and 6.5, the emulsions with WPC may be more stable because the droplets had less negatively charged groups than those at pH 7 and the adsorption of minerals to droplets is likely to be lower. The electrostatic repulsion is sufficient to prevent droplets from aggregation. But in these cases when negative bacteria were added in emulsions with WPC at pH 6 and 6.5, the bacteria may adsorb to emulsion droplets by positively charged groups of proteins and also the cations coating the droplets. The bacteria could make bridges between the emulsion droplets and the emulsion became unstable.

In the case of emulsions which contained bacteria and cations, the order of addition of the elements was important, if the bacteria and cations were added in solution before adding the emulsion stock, the final emulsion was stable. This phenomenon can be explained by the negative charges of bacteria which may neutralize Ca^{2+} (Poortinga, Bos, Norde, & Busscher, 2002) decreasing thus the effect of calcium.

5. Conclusion

The interactions between proteins and other components have been much studied to understand the stability of emulsions. In this paper, the impact of the surface of bacteria on the stability of emulsions made with milk proteins was investigated. The results show that the bacteria can be involved in interactions with other compounds changing the emulsion stability. The effect is notably due to the surface charges of bacteria. If negatively charged bacteria are added to an emulsion containing positively charged droplets, the bacteria can adsorb directly to the emulsion droplets by electrostatic interactions decreasing the repulsion between the emulsion droplets and resulting in an aggregation of the oil globules. The different bacterial effect, which was observed for LLD18 and LLD16 at pH 3, shows that the choice of a bacterium according to its surface properties can have an important impact on the stability and characteristics of an emulsion. The negatively charged bacteria could also interact with the negatively charged droplets by the intermediate of cations. The design of new food products with specific surfactants

and functional ingredients have also to take into account the presence of these properties on the surface of bacteria. Properties such as ζ , isoelectric point and hydrophobicity are of great interest in many food processes. In the case of fermented products or in fermentation media containing lipids, proteins or minerals, the presence of bacteria could affect the organization of matrices and also the localization of bacteria.

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