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ARTICLE

Characterization of *Lactobacillus reuteri* Interaction with Milk Fat Globule Membrane Components in Dairy Products

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A set of methods has been developed to study the adhesion between four *Lactobacillus reuteri* strains and the milk fat globule membrane (MFGM) components in dairy products. By combining sucrose density gradient (SDG) centrifugation and bacterial DNA quantification it was found which strains of *L. reuteri* were more strongly associated with the dairy products, and the results were corroborated by direct binding rate and force measurements made with optical tweezers. It was determined that strong binding was associated with hydrophobicity of the bacteria and that this hydrophobicity is correlated with the presence of LiCl-extractable protein on the surface of the bacteria. Confocal laser scanning microscopy (CLSM) allowed for the visualization of interactions between bacteria and MFGM. This study demonstrates that these methods can be used in combination to characterize, both qualitatively and quantitatively, the adhesion of lactic acid bacteria strains in dairy products.

KEYWORDS: *Lactobacillus reuteri*; MFGM; adhesion; optical tweezers

INTRODUCTION

Lactic acid bacteria (LAB), including *Lactobacillus* spp., are generally used as probiotics for their beneficial impact on human health (*1*). Probiotic bacteria function to maintain a healthy balance of the beneficial bacteria in the gastrointestinal tract (GIT) and to enhance the immune system (*1*). These bacteria can play a critical role in the competitive exclusion of pathogens from attachment to the intestine (*1*). The success of this probiotic functional property is related to their ability to both survive and persist in the GIT (*1*, *2*). The selection criterion for potential probiotic strains is focused on their capability to resist GIT secretions (i.e., stomach acid and bile) and to adhere to intestinal epithelial cells (*2*). This probiotic activity, although generally accepted, is very variable in its effectiveness, and many questions remain as to the major factors contributing to this variability. It is well understood that species and strain differences in probiotic functionality are common (*1*), but the potential impact of variation within the dairy product used as a delivery vehicle, which results from different food processing technologies, needs to be evaluated.

Dairy products, such as yogurt and fermented milk, are viewed as an ideal delivery system for probiotic bacteria (*3*), and recent studies have revealed the influence of milk components on gene expression (*4*−*6*). Some specific genes associated with LAB adhesion to mucins and intestinal epithelial cells have been identified (*7*, *8*). Furthermore, there is evidence that the growth medium and environmental conditions can have a major impact on surface and adhesion properties of LAB (*9*−*11*). In dairy products, the direct adhesion of LAB to milk fat droplets in cream has been reported (*12*, *13*). LAB have been shown to be preferentially associated with the fat/protein interface in the cheese matrix (*14*) and in emulsions stabilized by various milk proteins (*15*), whereas milk has also demonstrated a protective effect on LAB to gastric acidity (*2*). These studies suggest a potential role of bacterial interaction with milk components in defining their function and in the observed protective property of dairy foods on LAB. However, the manner and the mechanisms by which the bacteria interact with dairy components has not been fully elucidated, particularly with the milk fat globule membrane (MFGM).

In milk, the fat is secreted in small spherical globules ranging from 0.2 to 15 μm in diameter, which are surrounded by a complex membrane, the MFGM (*16*). The MFGM consists of an inner monolayer of polar lipids and proteins surrounding the core fat droplet as well as an electron dense proteinaceous layer located on the inner face of the outer membrane, which consists of a true bilayer membrane of phospholipids and proteins. Several MFGM components have displayed some unique physicochemical and nutritional functionality (*16*−*18*). In addition, the MFGM contains specific components such as mucins (*9*), phospholipids (*19*), proteins (*20*), glycophospholipids, and gangliosides (*21*) that are known to have affinity to the bacteria cell surface. It has previously been shown that after pre-exposure to mucin the mucus-binding activity of *L. reuteri* was increased (*9*). To date, little is known about the interaction between LAB and MFGM components in complex dairy products. Therefore, more research and technical tools are needed to evaluate the extent of

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Table 1. Composition of Dairy Products (Wet Basis)

	proportion of each component in total product (% wet weight)			
dairy product[a]	total solids	protein	ash	fat
RC	7.1	0.3	1.1	2.9
BM	11.2	2.9	0.6	3.3
BMP	95.9	54.8	4.0	35.2

[a] RC, raw cream; BM, buttermilk; BMP, buttermilk powder.

interaction between the MFGM and LAB to better understand this relationship.

In this work we focused on the characterization of the interactions between a set of *Lactobacillus reuteri* strains and dairy products. The dairy products contained MFGM components both in the unprocessed native form and after different stages of processing. We used several tools to explore the adhesion characteristics of different *L. reuteri* strains to MFGM in the dairy products. The *L. reuteri* strains in this study were selected for their commercial relevance in the field of probiotics as well as for their differences in cell surface properties. The dairy products were chosen to exemplify the impact of varying stages of processing and MFGM composition on bacteria binding.

MATERIALS AND METHODS

Dairy Products Preparation. Three different dairy products containing milk fat globule membrane components at different processing stages were prepared: raw cream (RC), buttermilk (BM), and buttermilk powder (BMP). The raw cream was prepared before each experiment from fresh milk (Cal Poly Dairy, San Luis Obispo, CA) by centrifugation at 3200*g* for 5 min at 4 °C. The top cream layer was removed and then reconstituted at 10% (w/w) with PBS (pH 7.2). The BM was obtained from butter making performed at the Dairy Product Technology Center (DPTC) pilot plant facility (Cal Poly). In this process, pasteurized sweet cream (Producers Dairy, Fresno, CA) was churned to butter using a continuous pilot-scale butter churn (Egli AG, Bütschwil, Switzerland). The resulting BM was collected in milk cans and filtered through cheesecloth to remove small butter granules. A portion (1 L) of the BM was supplemented with sodium azide (0.02% w/v) and kept at 4 °C for the duration of the experiments. The other BM portion was used to prepare the BMP dried ingredients. Briefly, the fresh BM was processed by ultrafiltration and diafiltration (UF/DF) using a UF membrane fitted on a DDS UF Plant unit (Nakskov, Denmark) with a 10000 Da molecular weight cutoff. The BM was first concentrated by UF to a 4× volume concentration factor before being diafiltered with 2 volumes of water. The UF/DF concentrated buttermilk (12% total solids) was then spray-dried using a Niro Filterlab spray-dryer (Hudson, WI) to produce the BMP utilized in this experiment.

Chemical Analysis. For each dairy product, total solids (TS) were determined with the direct-drying method using a forced air drying oven set at 102 °C, and the ash content was determined by incineration at 550 °C (*22*). The percent of protein was determined according to the Kjeldhal method using a nitrogen to protein conversion factor of 6.38 for milk proteins (*23*). Total lipids were obtained by using the Mojonnier solvent extraction procedure (*22*). The final composition for the different dairy products is presented in **Table 1**.

Bacterial Strains and Growth Conditions. The bacteria evaluated in this study were four different strains of *L. reuteri*. The *L. reuteri* strains 1063-S, SD2112, and T-1 were obtained from Biogaia Biologics (Stockholm, Sweden), whereas the fourth strain, *L. reuteri* 23272, was obtained from the American Type Culture Collection (ATCC). All bacterial strains were grown in MRS broth (Difco, Detroit, MI) at 37 °C. For all experiments, cultures were inoculated 1:100 (v/v) in prewarmed MRS medium (10 mL) and allowed to grow for 24 h prior to being harvested (3200*g* for 5 min) at stationary phase. The bacteria were washed three times (3200*g* for 5 min) with PBS buffer before being normalized to an absorbance at 600 nm (A_{600}) of 2.0 (±0.01).

Bacterial Surface Hydrophobicity Determination. Bacterial surface hydrophobicity was determined by measuring the percent of adhesion to hexadecane according to the microbial adhesion to hydrocarbons (MATH) test (*24*). Bacteria were grown and harvested as previously described. The harvested bacteria were then adjusted to an A_{600} of 0.4 with PBS buffer (pH 7.2). An aliquot of hexadecane (0.4 mL) was then added to the adjusted bacterial suspension (2.4 mL), and both phases were mixed thoroughly with a vortex for 30 s. The mixture was then incubated at room temperature for 20 min to allow for the separation of the aqueous phase. The aqueous phase was then recovered, and the absorbance at 600 nm was measured. The percent of hydrophobicity was then reported according to the equation

$$\% \text{ hydrophobicity} = (1 - A_1/A_0) \times 100$$

where A_0 represents the absorbance value at 600 nm of the bacteria adjusted at 0.4 and A_1 is the absorbance at 600 nm of the aqueous phase recovered after the hexadecane extraction.

Bacterial Surface Protein Characterization. Cell surface proteins were extracted from the four *L. reuteri* strains using a method based on the S-layer protein LiCl extraction procedure of Mozes and Lortal (*25*). Briefly, 30 mL of the bacteria previously adjusted to an A_{600} of 2.0 was washed with an equivalent volume of 0.15 M NaCl. The bacteria were then harvested before being treated with 3.0 mL of a 5 M LiCl solution at 0 °C for 15 min. The bacteria were again harvested, and the supernatant fractions were recovered and dialyzed (3500 Da) against deionized H_2O at 4 °C for 24 h. The dialyzed fractions were then concentrated to dryness using a vacuum centrifugal concentrator (Eppendorf, model 5301, Westbury, NY). The bacterial surface protein profiles were determined by SDS-PAGE according to the method of Laemmli (*26*). Staining was performed using Coomassie Brilliant Blue R-250 (Bio-Rad), and the gel was imaged using the ChemiDoc XRS imager system and analyzed with Quantity One 1-D analysis software (Bio-Rad).

Sucrose Density Gradient (SDG) Separation. SDG separation was used to determine the relative binding frequency of each *L. reuteri* strain with the different dairy products. The RC samples and the BM were prepared as previously described. The BMP was reconstituted at 3% TS with PBS (pH 7.2). The powder was mixed on a rotary axel at 4 °C for 1 h prior to each experiment. All dairy products were prewarmed at 37 °C for 10 min before being put in contact with the bacteria. A 2 mL volume of the bacteria adjusted to an A_{600} of 2.0 was harvested and then resuspended in an equal volume of the prewarmed dairy products. The bacteria and the dairy product were then allowed to incubate at 37 °C for 5 min before being loaded (750 μL) on top of the SDG. The SDG consisted of two equal volumes (2.15 mL) of sucrose solution with 60% (w/w) sucrose at the bottom and 20% (w/w) sucrose at the top, successively layered in a 5 mL Ultraclear centrifuge tube (Beckman Coulter, Fullerton, CA). The samples were centrifuged at 54000*g* for 60 min at 4 °C in an L7-35 ultracentrifuge (SW50.1 rotor, Beckman Coulter). Samples were recovered immediately after the SDG separation. The 20% sucrose layer was removed with a syringe inserted just underneath the interface between the two layers. The bacteria were then recovered after removal of most of the 60% sucrose layer with the syringe without disturbing the bacterial pellet. A 500 μL volume of PBS was added, and the bacteria pellets were cresuspended before being transferred into a 2.0 mL microcentrifuge tube. This washing step was repeated once to recover all residual bacteria from the ultracentrifuge tube. The recovered bacteria were finally harvested (3200*g* for 5 min) and, after the supernatant had been discarded, the tube was kept at −15 °C until further analysis by DNA quantification. The same SDG separation and recovery procedures were performed on the different dairy products in the absence of bacteria to serve as product controls.

Bacterial Binding Determination by DNA Quantification. Quantitative measurement of unbound bacteria was accomplished by measuring the bacterial DNA at the bottom of the SDG tube. The cell pellets were first treated with lysozyme buffer (0.9 mg of lysozyme/mL, 0.015 M $MgCl_2$, 0.06 M potassium phosphate, pH 7.0) containing Proteinase K (20 mg/mL) at 37 °C for 1 h. DNA was extracted from each sample using the FastDNA kit (MP Biomedicals, Irvine, CA) according to the manufacturer's instructions. The DNA quantification was carried out on the undiluted extracts loaded on a SPECTRAplate UV transparent quartz microplate (Molecular Devices, Sunnyvale, CA) by measuring the absorbance at 260 nm (A_{260}) with a SpectraMAX Plus microplate spectrophotometer (Molecular Devices). Data were analyzed with Molecular Devices SoftMax Pro software (version 2.6.1.). The total DNA amount recovered was

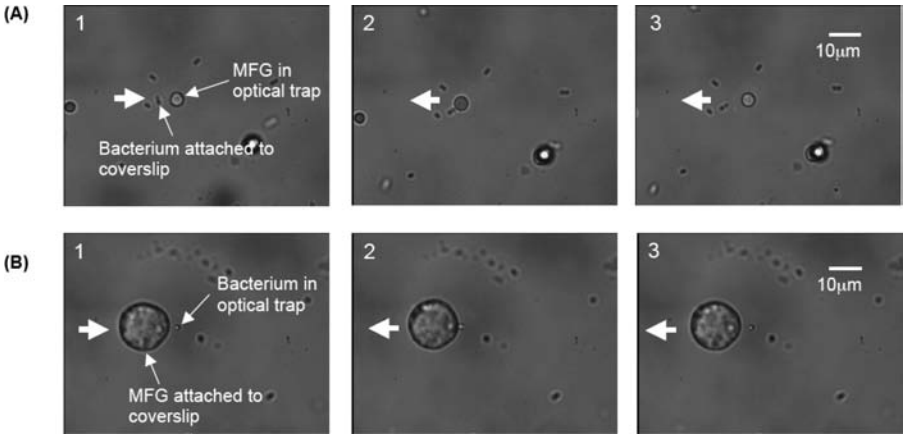

Figure 1. Sequential step images showing the optical tweezer force measurements procedure for the two trapping geometries used. (**A**) When bacteria are adhered to the coverslip, (1) a milk fat globule (MFG) is held in the optical trap while the bacterium (*Lactobacillus reuteri* SD2112), which is attached to the slide coverslip, is moved toward the MFG by moving the microscope stage, (2) attachment has occurred and the bacterium is being pulled from the MFG, and (3) the bacterium has detached. (**B**) When MFG is adhered to the coverslip, (1) the bacterium (*L. reuteri* SD2112) is held in the trap while the MFG approaches (note that the rod-like-shaped bacterium aligns upright in the trap and appears as a point), (2) binding has occurred, and the MFG is being withdrawn (elongation of the bacterium can now be seen), and (3) the MFG has detached. The thick white arrow shows the direction of the microscope stage travel.

calculated by assuming that 1 absorbance unit at 260 nm of double-strand DNA is equal to 50 μg/mL and using the equation

$$\text{DNA}\ (\mu\text{g}) = (A_{260}) \times (50\ \mu\text{g/mL}) \times V \times \text{DF}$$

where V is the DNA extract volume (0.1 mL) and DF is the dilution factor (undiluted). The DNA corresponding to the unbound *L. reuteri* bacteria (DNA_{UB}) was then estimated according to the equation

$$\text{DNA}_{\text{UB}} = \text{DNA}_{\text{B+DP}} - \text{DNA}_{\text{DP}}$$

where $\text{DNA}_{\text{B+DP}}$ is the DNA amount in the SDG pellet for the *L. reuteri* strain after contact with the dairy product and DNA_{DP} is the DNA amount collected in the SDG pellet of the corresponding dairy product control.

Confocal Laser Scanning Microscopy (CLSM). We used CLSM to visualize the binding location of the different strains to the various MFGM and fat structures found in the dairy products. The dairy products and bacteria were stained separately. Each dairy product was labeled with the phosphatidylethanolamine−lissamine rhodamine B (RH-PE) probe (Avanti, Alabaster, AL). This probe is fluorescently labeled at its polar head with rhodamine B. Briefly, 2 μL of the RH-PE probe in $CHCl_3$ was first evaporated in a microcentrifuge tube (1.5 mL) for 5 min. The evaporated probe was resuspended in 225 μL of PBS. The dairy product (25 μL) was then added and allowed to contact with the probe for 15 min at room temperature protected from light. To stain the bacteria, a sample (500 μL) of each culture was adjusted to an A_{600} of 2.0 with PBS and mixed with an acridine orange (AO) hydrochloride solution (10 mg/mL in water, Sigma, St. Louis, MO) at a ratio of 1:1000. The samples were allowed to incubate for 5 min at room temperature protected from light before being washed twice with PBS. The harvested bacteria were resuspended to the initial volume with PBS. Equal volumes of the labeled bacteria and the dairy products were then mixed and allowed to incubate for 15 min. Then, the bacteria in dairy product suspension were mixed 1:2 with agarose (0.5% w/v), previously melted and kept at 37 °C. After transfer to coverslipped slides, the samples were imaged with a CLSM Fluoview FV1000 system (Olympus America Inc., Center Valley, PA). The observations were made with a Plan Apo N 60x 1.42NA immersion oil objective lens (Olympus). Laser excitation parameters were set at 559 and 488 nm for the RH-PE and the AO probes, respectively. The emission spectra were recorded using a fluorescence detector (405/488/559) with a confocal aperture of 110 μm. The images were analyzed with Fluoview FV1000 software (Olympus, version 1.7.2.2.).

Optical Tweezers Binding Force Measurements. Optical tweezers were used to evaluate the interaction forces between the different *L. reuteri* strains and MFG in raw cream. Trapping was done at 1064 nm and the trap strength estimated by the Stokes−drag method (*27*). All of the experiments reported were carried out at room temperature (21 ± 1 °C). Samples were made up from the RC samples (10% w/w in PBS, pH 7.2) diluted 1:25 with PBS. To this was added 2 μL of bacteria A_{600} at 2.0, and after mixing, the suspension was introduced by capillarity into a coverslipped microscope slide. The slides were left for 5 min with the coverslip facing up, allowing the fat to rise toward the top. Then, when placed in an inverted microscope, there were fat globules attached to and floating near the coverslip.

In this work we used two different trapping geometries. With the bacteria attached to the coverslip (**Figure 1A**) it is possible to trap a MFG and push it against a bacterium to see if binding occurs. If so, then the trap strength is slowly increased and the microscope stage moved to pull the MFG from the bacterium. If detachment occurs, then the stage is moved so that the bacterium is about 10 μm from the trapped MFG. The piezo-positioning stage of the microscope is then set in sinusoidal motion at a frequency (f) of 40 Hz using the signal from an attached function generator. The amplitude of the motion is gradually increased until the MFG can be seen escaping from the trap. The amplitude of the motion (A) required for the MFG to be pulled from the trap can be accurately measured from the microscope image, and the maximum velocity of the fluid with respect to the trap is calculated from

$$v = 2\pi f A$$

Once the velocity is known, then the strength of the trapping force can be estimated using Stokes law for the drag force due to a moving fluid which, when applied to spherical particles, reads

$$F = 6\pi\varpi\ \ \eta r$$

where ϖ is the viscosity of the fluid (10^{-3} Ns/m^2 for water at 20 °C) and r is the radius of the MFG (which can be measured in the microscope image).

It was also possible to estimate the binding force when the bacteria are held in the optical trap (**Figure 1B**). This is especially useful when the bacteria themselves do not adhere to the coverslip. In this case the trapped bacteria are pushed against the MFG attached to the coverslip. If binding occurs, then the trap strength is adjusted until the bacterium is pulled from the MFG, as explained previously. Because the bacterial cell is not a spherical particle as for the MFG, the trap strength is estimated in the following way. First, we note that when the fluid is stationary, the bacteria tend to align in the trap with their long axis perpendicular to the coverslip. However, when the fluid moves, the bacteria reorient and become trapped at one end by the tweezers and aligned with their long axes parallel to the coverslip. This is the same geometry the bacterium assumes when it is being pulled from the MFG (**Figure 1B**). The bacteria retain this configuration until the fluid velocity is sufficiently large to pull them from the trap. The drag force is estimated by assuming the bacterium is a straight, slender

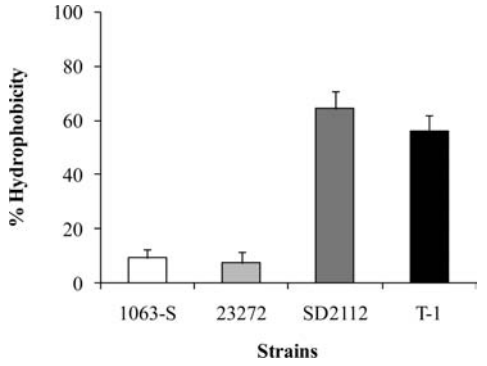

Figure 2. Bacterial cell surface hydrophobicity expressed as the percent of adhesion to hexadecane for the different *Lactobacillus reuteri* strains as determined by the microbial adhesion to hydrocarbons (MATH) test. Values are means ± SD of three independent determinations.

cylindrical rod with a much greater length than radius. The drag force in this case is given by eq 7.10 of Cox (*28*):

$$F = \frac{2\pi\eta l v}{\ln(1/r) - 0.81}$$

where l is the rod length and r is the radius of the rod.

To make a force measurement, a MFG (or bacterium) is pushed against a bacterium (or MFG) for a few seconds and then the stage is moved to see if attachment has taken place. If there is no attachment, then this is repeated with the MFG and bacterium touching at different points on their surfaces. This is repeated four times, and if no attachment is seen, then different pairs of MFG/bacterium are selected. Once attachment is observed, then the force-measuring procedure outlined above is carried out and the final forces calculated. More than 25 repeats were performed for each strain studied.

Statistical Analysis. Statistical analysis of bacteria binding data was performed using the mixed procedure of SAS (SAS/STAT version 9.1, SAS Institute Inc., Cary, NC). The fixed effects of the strains, the dairy products, and their interaction were tested by ANOVA, using a factorial design (4×3). When the F value of the ANOVA was significant ($P < 0.05$), the means were compared using adjusted Tukey tests.

RESULTS

Bacteria Surface Hydrophobicity Determination. The evaluation of the *L. reuteri* strains' surface hydrophobicity revealed two distinct cell surface hydrophobicity patterns (**Figure 2**). The *L. reuteri* 1063-S and the 23272 strains showed a more hydrophilic surface character with a rather poor affinity for the hexadecane phase at approximately 10% adhesion. In contrast, the percent of adhesion to hexadecane for *L. reuteri* strains SD2112 and T-1 were 65 and 55%, respectively. The greater percent adhesion demonstrates a more hydrophobic cell surface.

Bacterial Surface Protein Characterization. The SDS-PAGE cell surface protein profiles of the different *L. reuteri* strains were determined after LiCl extraction (**Figure 3**). The protein profiles for *L. reuteri* 1063-S and 23272 strains showed only very faint protein bands in a range between 40 and 50 kDa. In contrast, the protein profiles for both the SD2112 and T-1 strains demonstrated increased band intensity when compared to the other two strains. Furthermore, the band intensity of the surface proteins for *L. reuteri* strain SD2112 was overall greater than for *L. reuteri* strain T-1.

Sucrose Density Gradient Separation. The SDG procedure allowed for the separation of any unbound bacteria as a result of their higher density from the lower density components in the dairy product. For each strain, SDG control runs of the bacteria in the absence of dairy products confirmed that the bacteria were

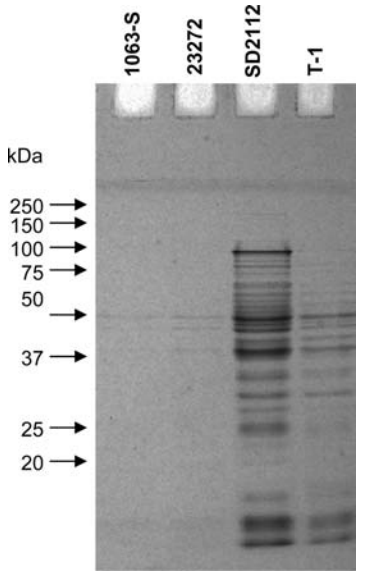

Figure 3. SDS-PAGE image of the *Lactobacillus reuteri* surface proteins extracted with 5 M LiCl. Each lane represented sample extracts from 30 mL of bacterial suspension adjusted at an absorbance at 600 nm of 2.0.

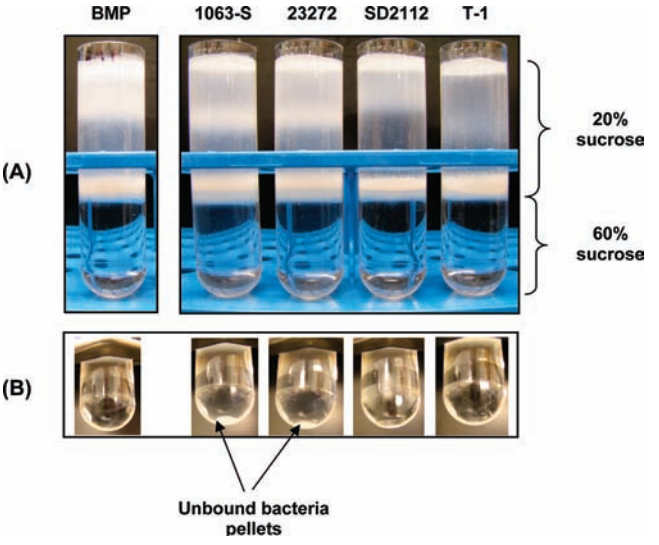

Figure 4. (**A**) Frontal view of the ultracentrifuge tubes after sucrose density gradient (SDG) separation for the buttermilk powder (BMP) control and for the different *Lactobacillus reuteri* strains that have been previously incubated with BMP. (**B**) Views of the corresponding bottom pellet fractions after SDG separation. The black arrow shows unbound bacteria that have migrated to the bottom of the tube during the SDG separation.

dense enough to migrate through both sucrose layers to form a pellet in the bottom of the tube after centrifugation (results not shown). Therefore, any bacteria that bound with the lower density components of the dairy product were subsequently retained in the sucrose layers after centrifugation. An example of the centrifuge tubes recovered after SDG separation of the *L. reuteri* strains incubated with the BMP is given in **Figure 4**.

Bacteria Binding Determination by DNA Quantification. The extent of the *L. reuteri* strains' association with the dairy products was confirmed with bacterial DNA quantification of pellets obtained after SDG separation. Using this methodology the quantity of unbound bacterial DNA was measured. The results revealed that significantly ($p < 0.05$) more bacterial DNA was recovered for *L. reuteri* 1063-S and 23272 strains than for the

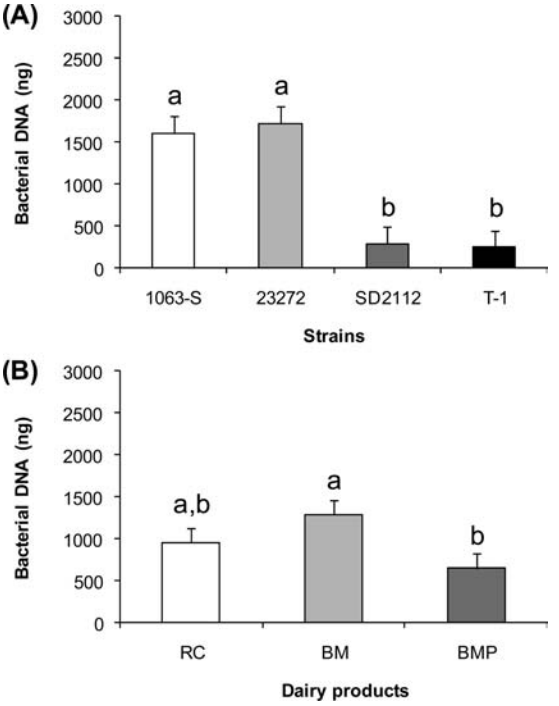

Figure 5. Amount of bacterial DNA recovered in the pellet fraction after sucrose density gradient separation corresponding to the unbound bacteria from the statistically significant ($p < 0.05$) two main effects: (**A**) strain; (**B**) dairy product. Error bars represents standard error of the mean (±SEM) of three independent observations ($n = 3$). All ratings are based on readings observed with the corresponding dairy product control sample.

SD2112 and T-1 strains for each of the dairy products studied (**Figure 5A**). The mean amounts of DNA measured in the SDG bottom fractions for *L. reuteri* 1063-S and 23272 ranged between 1500 and 2000 ng. In contrast, *L. reuteri* strains SD2112 and T-1 yielded lower amounts of DNA, with an average lower than 250 ng, for each dairy product tested. These results indicated that *L. reuteri* SD2112 and T-1 have higher binding affinities toward the different dairy products, whereas *L. reuteri* 1063-S and 23272 were weaker binding strains. The effect of the different processing steps that the fat has undergone during the manufacture of the dairy products is shown in **Figure 5B**. The results showed that significantly less unbound bacterial DNA ($p < 0.05$) was recovered from the BMP than from the BM (**Figure 5B**). However, the amounts of DNA recovered from the RC samples were not different from the BM and the BMP. No difference in the interaction (strain × dairy product) was observed ($p = 0.60$).

Confocal Laser Scanning Microscopy. These microscopic observations were carried out to determine the nature and binding localization site of the bacteria. Only the CLSM images of strains SD2112 and T-1, which demonstrated greater binding to the dairy products, are presented (**Figure 6**). Both *L. reuteri* strains (in green) bound to the surface of the intact MFG (in red) obtained from raw cream. The *L. reuteri* strains also interacted with the surface of small spherical droplets labeled with the RH-PE in the BM samples. MFGM materials likely stabilize these small droplets. Binding to the surface of particles from the BMP was also observed (**Figure 6**). This latest result showed that the hydrophobic *L. reuteri* bound preferentially to the surface of buttermilk particles that are covered with fat material as revealed by the RH-PE probe.

Optical Tweezers Binding Force Measurements. Optical tweezers were used to measure the interaction force between the four different *L. reuteri* strains and MFG in a raw cream sample. This

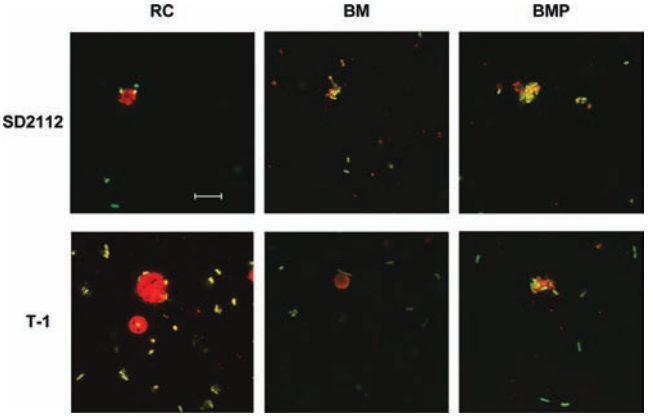

Figure 6. Confocal laser scanning images showing interactions between *Lactobacillus reuteri* SD2112 and T-1 strains with milk fat globule membrane components in raw cream (RC), buttermilk (BM), and buttermilk powder (BMP). The dairy products were stained with phosphatidylethanolamine−lissamine rhodamine B (in red) and the bacteria with acridine orange (in green). (Scale bar = 10 μm).

Table 2. Binding Rates and Forces between the Different *Lactobacillus reuteri* Strains and Milk Fat Globules As Determined Using Optical Tweezers

bacteria	binding rate (%)	binding force (pN)
L. reuteri 1063-S	8	10−15
L. reuteri 23272	11	10−15
L. reuteri SD2112	33	15−180
L. reuteri T-1	57	30−200

direct method allowed for single-cell measurements to determine the rate of binding as well as estimate the binding force to MFG present in raw cream. The optical tweezers technique confirmed the adhesion of the bacteria to MFG, which was indirectly measured using the SDG method and visualized with CLSM. The higher binding rates for *L. reuteri* strains SD2122 and T-1 were 33 and 57%, respectively (**Table 2**). The binding forces measured ranged from 15 to 180 pN for *L. reuteri* SD2122, whereas *L. reuteri* T-1 showed a higher range of force from 30 to >200 pN (no observed detachment). For *L. reuteri* strains 1063-S and 23272 the incidence of successful binding events was only 8 and 11%, respectively, with measured forces ranging from a low of 10 to 15 pN. The successful binding event rates were in agreement with the observations made with the SDG technique with the RC, which showed higher binding rates recorded for the SD2112 and T-1 strains (**Table 2**).

DISCUSSION

The MATH test allowed for the correlation of the preferential binding of more hydrophobic strains to the lipid fraction and hydrophobic compounds of the MFGM. The MATH technique has also been successfully used to demonstrate the preferential binding of LAB strains, with hydrophobic surface characteristics, toward the lipid fraction of complex food matrices (*13*, *29*). Specifically, these authors reported the preferential adhesion of several *Lactoccocus lactis* strains, with hydrophobic cell surface properties, toward hydrophobic compounds such as milk fat globules. Therefore, surface hydrophobicity as determined with the MATH method could be a useful criterion to predict the adhesion properties of probiotic strains toward MFGM components in dairy products.

The *L. reuteri* cell surface protein profiles revealed a greater concentration of proteins extracted from the surface of the SD2112 and T-1 strains compared to the 1063-S and 23272

strains (**Figure 3**). These results in conjunction with the hydrophobicity results suggest that the surface proteins are related to the hydrophobicity index of these bacteria. Specifically, a high level of LiCl extractable cell surface proteins was strongly associated with high cell surface hydrophobicity. Furthermore, the band intensity of the surface proteins for *L. reuteri* strain SD2112 was overall greater than for strain T-1, which might explain its slightly higher hydrophobicity. Several surface proteins have been identified in various *L. reuteri* strains (*30*−*35*) including the 1063-S (*32*) and the SD2112 (*36*) strains used in this study. For instance, Bath et al. (*36*) identified 126 extracellular proteins from the SD2112 (ATCC 55730) genome sequence. This could explain why numerous protein bands of high intensity were observed in the SDS-PAGE profile of the SD2112 surface protein extract (**Figure 3**). Moreover, several *L. reuteri* surface proteins have been related to functionally characterized adherence factors such as the mucus-binding (Mub) protein in strain 1063-S (*32*), the collagen-binding (CnBP) protein (*31*), the epithelium-adhering (LspA) protein (*34*), and the mucus adhesion-promoting (MapA) protein (*30*). The potential presence of these proteins that mediate adhesion to extracellular matrices could also be responsible for adhesion of the different *L. reuteri* strains to MFGM compounds in our dairy products. However, in this study we did not observe higher adhesion of the *L. reuteri* 1063-S strain to MFGM material despite the known presence of the Mub protein at the cell surface. Nevertheless, binding of the bacteria to the different dairy products is strongly associated with the surface hydrophobicity of the strain. Thus, the hydrophobic character is probably due to the large presence of proteins at the surface of the SD2112 and T-1 bacteria. However, the LAB Gram-positive cell wall structure is rather complex. It consists mainly of peptidoglycans, polysaccharides, (lipo)teichoic acids, and proteins. All of these compounds are likely to contribute to the bacterial surface properties such as hydrophobicity/hydrophilicity, Lewis acid/base character, and net charge. Therefore, other hydrophobic molecules, namely, lipoteichoic acids and lipoglycans, are also expected to contribute to the cell surface hydrophobicity. In the present study, the potential contribution of these other hydrophobic components to the overall cell surface hydrophobicity has not been determined.

The SDG separation method was used to assess bacterial binding in the different dairy products, and a decrease in bacteria density was expected upon binding to lower density material in the dairy products. Indeed, interaction with the different dairy products allowed the bacteria to be recovered in the top 20% sucrose layer, whereas the higher density nonbinding bacteria were recovered at the bottom of the tubes (**Figure 4B**). The SDG method was particularly powerful in determining the binding affinity of the various *L. reuteri* strains among the different dairy products. This method was used in conjunction with DNA analysis to quantify the relative interaction of each strain with the different dairy products. The DNA quantification method is rapid and independent of the bacterial species or the growth conditions, conferring a major advantage of this technique over the conventional enumeration methods. The results from the bacterial DNA analysis (**Figure 5**) were in agreement with SDG visual observations (**Figure 4**) and allowed for a quantitative comparison of the interaction between different strains and the dairy products. *L. reuteri* strains SD2112 and T-1 had the highest binding toward the lower density material in each of the dairy products studied, whereas minimal interaction was observed for the 1063-S and 23272 strains (**Figure 5A**). As seen in **Figure 5B**, the effect of processing the RC into BM or BMP did not alter the binding properties of the strains under study. However, more bacterial interactions were observed with the dried ingredient (BMP) than for the BM. The difference could be due to the high presence of fat material at the surface of the BMP particles. In fact, the surface of spray-dried dairy powder particles is known to be over-represented by fat, especially in high fat containing dairy powders, which are usually almost totally covered by surface fat (*37*−*40*).

The CLSM images showed that the RH-PE probe stained the periphery of the following: the MFG in raw cream, small droplets in BM, likely MFGM fragments as mentioned above, and fat material located at the surface of the BMP particles (**Figure 6**). The CLSM observations allowed us to confirm that the presence of MFGM material and milk fat in different dairy products was responsible for the adhesion of the more hydrophobic SD2112 and T-1 strains.

The use of the independent optical tweezers method to directly measure adhesion forces and to characterize binding events between bacteria and MFG was in agreement with the indirect methods utilized in this research. Recent studies have shown the ability of optical tweezers to precisely measure adhesion forces, in the piconewton range, for various bacteria (*41*−*45*). In this study, the technique allowed us to record binding events and to measure the corresponding range of detachment forces for each strain and the MFG in raw cream (**Table 2**). The results obtained were in line with the previous SDG observations with strains SD2112 and T-1 showing more successful binding events than strains 1063-S and 23272. The corresponding detachment forces measured were generally also higher for SD2112 and T-1 compared to the other two strains. The lower range of adhesion forces reported (10−15 pN) is characteristic of single bond rupture forces observed in protein−protein interaction studies with bacteria (*44*, *46*, *47*). This might explain the weaker adherence of strains 1063-S and 23272. Conversely, for the better binding strains the range of forces was higher, suggesting multiple binding sites for attachment of the bacteria to the MFG. Within the resolution of these measurements we were unable to see contributions from individual binding sites. It must be noted that because of the unknown distribution of refractive index within the bacterium and the variable geometry of trapping (and hence the grasp of the tweezers), our measurements at this stage are probably accurate only to about 10 pN. More refined measurements are possible, but would require "handles" attached to the bacteria (*27*). Despite the need for refinement, the optical tweezers approach enabled us to measure objectively the differences in both the adherence forces and percent of binding events between the different *L. reuteri* strains under study.

In conclusion, in this study we developed a set of methods to characterize the interaction occurring between *L. reuteri* strains and MFGM components in a complex dairy product. It was found that the cell surface characteristics of the bacteria influence the binding properties of the different strains under study, with the more hydrophobic strains showing greater adhesion properties toward MFGM. A strong association was found between the strain hydrophobicity and the presence of extractable cell surface proteins, suggesting that they might play an important role in the binding of these bacteria. The binding properties of the different strains of *L. reuteri* toward MFGM components in dairy products representing different stages in processing were investigated. The results obtained showed that the binding of the *L. reuteri* toward raw cream was not affected by churning and spray-drying of the buttermilk. However, differences were observed between the BM and the BMP processed samples. The results demonstrated that methods such as SDG separation combined with bacterial DNA quantification, CLSM microscopic observation, and optical tweezers are very powerful tools to characterize, both qualitatively and quantitatively, the

binding of LAB strains to MFGM in dairy products. These methods will ultimately add to our understanding of the relationship between MFGM components and LAB in dairy products. However, further work is needed to assess if this interaction has an impact on LAB probiotic activity and their delivery in the GIT.

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