JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Effect of Food Models and Low-Temperature Storage on the Adhesion of *Lactobacillus rhamnosus* GG to Caco-2 Cells

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ABSTRACT: This study evaluated the effects of fat and sugar levels on the surface properties of *Lactobacillus rhamnosus* GG during storage in food model systems, simulating yogurt and ice cream, and related them with the ability of the bacterial cells to adhere to Caco-2 cells. Freeze-dried *L. rhamnosus* GG cells were added to the model food systems and stored for 7 days. The bacterial cells were analyzed for cell viability, hydrophobicity, ζ potential, and their ability to adhere to Caco-2 cells. The results indicated that the food type and its composition affected the surface and adhesion properties of the bacterial cells during storage, with yogurt being a better delivery vehicle than ice cream in terms of bacterial adhesion to Caco-2 cells. The most important factor influencing bacterial adhesion was the storage time rather than the levels of fats and sugars, indicating that conformational changes were taking place on the surface of the bacterial cells during storage.

KEYWORDS: *Lactobacillus rhamnosus* GG, storage, survival, adhesion, hydrophobicity, Caco-2 cells

INTRODUCTION

Lactobacilli are of significant technological importance because they are involved in the manufacturing of several fermented and nonfermented foods and have been used as probiotics because of their health-promoting effects. The functional effects of lactobacilli and their behavior in the gastrointestinal tract are determined to a large extent by the surface properties of the *Lactobacillus* cell walls, such as their net hydrophobicity and surface charge,[1−4] which in turn are influenced by the composition, structure, and organization of the cell wall. The Gram-positive cell wall of lactobacilli consists of a thick peptidoglycan layer, which is decorated with various surface components, including (lipo-)teichoic acids, polysaccharides, covalently bound proteins, and S-layer proteins.[5] Alteration in any of these structures present on the surface of the bacterial cells can affect the physicochemical properties and can thus affect the ability of bacteria to adhere to the intestinal mucosa.

Yogurt is the most common food used as a delivery vehicle for probiotics,[6] while more recently, ice cream has emerged as another promising carrier. For the manufacture of such products, there are certain technological hurdles that need to be addressed to maintain the quality of the product. Among these, the effect of processing and storage on the viability of the probiotic in the food matrix and the effect of the food matrix on probiotic functionality are important ones. In terms of the former, yogurts and ice creams are stored at low temperature, which leads to cell injuries and losses in the viability of the probiotic cells.[6,7] However, little research has been performed investigating the effects of specific food components. On the other hand, there is no information regarding the effect of the food carrier and, in particular, that of specific food components on the surface and adhesion properties of probiotic lactobacilli. The aim of the study was to evaluate the effect of fat and sugar levels on the surface and adhesion properties of *Lactobacillus rhamnosus* GG, a well-established probiotic strain, during storage in food model systems simulating yogurt and ice cream.

MATERIALS AND METHODS

Bacterial Strain and Growth Conditions. *L. rhamnosus* GG [American Type Culture Collection (ATCC) 53103] was obtained from ATCC (Middlesex, U.K.), and was stored at −80 °C in 2 mL cryovials containing 20% (v/v) glycerol. To prepare preculture, 1 mL of frozen culture was used to inoculated 10 mL of MRS broth (Oxoid, Basingstoke, U.K.) and was incubated at 37 °C for overnight. Overnight grown preculture was used to inoculat fresh fermentation medium. The cells were cultivated at 200 rpm and 37 °C in 500 mL shake flasks containing 200 mL of medium. The medium consisted of 20 g/L glucose (Sigma, Poole, U.K.), 10 g/L yeast extract (Oxoid, Basingstoke, U.K.), 15 g/L vegetable peptone (Oxoid), 1% Tween 80 (Sigma, Poole, U.K.), 0.2 g/L $MgSO_4 \cdot 7H_2O$ (VWR, Lutterworth, U.K.), 0.05 g/L $MnSO_4 \cdot 4H_2O$ (VWR), and 0.1 M phosphate buffer (VWR); the pH was 7.2. The culture was grown until a stationary phase was reached (around 15 h), and then the cells were collected by centrifugation at 3000*g* for 10 min and resuspended in 5 mL of 10% (w/v) sucrose (VWR) solution to obtain an optical density (OD_{600}) value of about 4. The suspension was frozen for 24 h at −80 °C and then freeze-dried in a IEC Lyoprep-3000 freeze-dryer (Lyoprep, Dunstable, U.K.). The freeze-dried cells were stored at room temperature in desiccators for further analysis.

Experimental Design. Two food models were used, yogurt and ice cream. Table 1 shows the details of the compositions of the model foods. Three different fat concentrations of triglycerol (Sigma) and three different sucrose (Sigma) concentrations were used for both the yogurt and ice cream model systems. Phosphate-buffered saline (PBS) (10 mM, Oxoid) at pH 7 was used as the base for all of the models, whereas a bacterial suspension in PBS solution was used as the control. To prepare the model systems, appropriate volumes of components were mixed together; the emulsions were prepared by adding 1% Tween 20 and using an Ultra-Turrax homogenizer (IKA, Staufen, Germany). For the

Received: May 12, 2011
Revised: July 15, 2011
Accepted: July 15, 2011
Published: July 15, 2011

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Table 1. Composition of Yogurt and Ice Cream Model Formulations[a]

yogurt model			ice cream model		
code	fat %	sugar %	code	fat %	sugar %
0,0	0	0	0,0	0	0
0,8	0	8	0,15	0	15
0,15	0	15	0,25	0	25
3,0	3	0	10,0	10	0
3,8	3	8	10,15	10	15
3,15	3	15	10,25	10	25
5,0	5	0	15,0	15	0
5,8	5	8	15,15	15	15
5,15	5	15	15,25	15	25

[a] In the sample code, the first number represents the concentration of fat and the second number represents the concentration of sugar in the model.

yogurt model, the pH was adjusted to pH 4, and for the ice cream model, the pH was adjusted to pH 6, using 1 M HCl.

Each model system was inoculated with freeze-dried *L. rhamnosus* GG cells to obtain an OD_{600} of 1, which corresponded to approximately 10^8 cells/mL. The model yogurt formulations were stored at 4 °C, and the ice cream formulations were stored at −20 °C, all for 7 days. Samples were taken at 0, 3, and 7 days. The bacterial cells were harvested by centrifugation at 22000*g* for 20 min at 4 °C, washed with PBS, and then analyzed according to the methodologies mentioned in the following sections.

Survival during Storage. For analysis of the survival of *L. rhamnosus* GG in the model systems, the spread plate method was used. A total of 1 mL of sample was serially diluted in PBS (Oxoid) and spread (100 μL) onto a MRS agar (Oxoid) plate, in triplicate. The plates were incubated at 37 °C for 48 h aerobically, and the number of viable cells was determined as log colony forming unit (CFU)/mL.

Adhesion to Hexadecane. The microbial adhesion to hexadecane (MATH) assay was employed to evaluate the hydrophobicity of the surface of the bacterial cells stored in the food models.[3] The bacterial cells were obtained by centrifugation at 22000*g* for 20 min at 4 °C, washed with PBS, and suspended in 10 mM KH_2PO_4 (Sigma) to obtain an $OD_{600} \sim 0.8$. The pH of the suspension was then adjusted to 3 with 1 M HCl. A total of 2 mL of the bacterial cell suspension was then mixed with 2 mL of hexadecane (Sigma) in a 10 mL syringe. The mixture was vortexed for 1 min and then left undisturbed for 20 min to allow for a complete phase separation. After equilibration, the aqueous phase was removed carefully not to disturb the interfacial equilibrium, and OD_{600} was measured. The percentage of adhesion (% adhesion) was calculated using the following equation:

$$\% \text{ adhesion to hexadecane} = (1 - A_1/A_0) \times 100 \quad (1)$$

where A_0 is the initial absorbance (at 600 nm) of the bacterial suspension and A_1 is the absorbance after 20 min of incubation.

ζ Potential. The ζ potential of the bacterial cells was measured using a Zeta Master (Malvern Instruments, Malvern, U.K.) and was determined as previously described.[3] Briefly, the bacterial cells were obtained by centrifugation at 22000*g* for 20 min at 4 °C and were suspended in 10 mM KH_2PO_4 (Sigma, Poole, Dorset) to obtain an $OD_{600} \sim 0.25$. The pH was then adjusted to values ranging from 3 to 10, using 1 M HCl (VWR) and 1 M NaOH (VWR). To measure the ζ potential, 4 mL of the bacterial suspension was injected into the analyzer and the reading was taken. The measurements were carried out at 25 °C. Each sample was analyzed in triplicate.

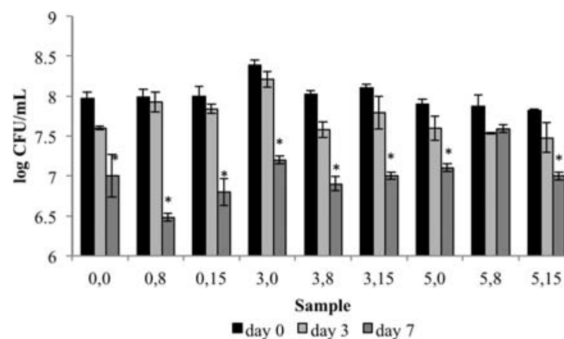

Figure 1. Survival of *L. rhamnosus* GG in the yogurt model formulations over a period of 7 days. The codes for each of the different formulations are shown in Table 1. The error bars represent standard deviation of three replicates. (∗) Stastically significant differences compared to day 0.

Adhesion to Caco-2 Cells. The Caco-2 ECACC 86010202 cell line was obtained from ECACC (Salisbury, U.K.). The cells were routinely cultured in Eagle's minimum essential medium (EMEM) (Lonza, Slough, U.K.), supplemented with 10% heat-inactivated fetal bovine serum (Lonza), 1% mixture of penicillin−streptomycin solution (Lonza), and 1% non-essential amino acid solution (Lonza), at 37 °C, in an atmosphere of 5% CO_2 and 95% air. The adhesion assay was performed as described by Deepika et al.[3] Fully differentiated cells, 21 days old, cultured in 12-well tissue culture plates (Corning, Kennebunk, ME), were used for the adhesion experiments. At 1 day before the adhesion assays, the Caco-2 cells were fed with EMEM supplemented with non-essential amino acids and fetal bovine serum, but without antibiotics. Before adhesion, the monolayer was washed twice with Dulbecco's phosphate-buffered saline (DPBS) (pH 7.2, without Ca and Mg, Lonza), to remove all traces of the medium. The cells, which were between 4×10^5 and 5×10^5 cells/mL, were counted using a Nikon microscope (Kingston Upon Thames, U.K.). The bacterial cells were obtained by centrifugation at 22000*g* for 20 min at 4 °C, washed once with DPBS, and then resuspended in DPBS to obtain an $OD_{600} \sim 1.0$, corresponding to approximately 10^7−10^8 cells/mL. Before addition to the wells, the total number of bacteria was counted by an improved Neubauer hemocytometer under a Nikon microscope (Nikon Instruments Europe B.V., Kingston, U.K.). A total of 1 mL of bacterial suspension was added to each well, and the plates were incubated for 60 min at 37 °C in 5% CO_2 and 95% air. After incubation, to calculate the number of bacteria bound to the Caco-2 cells (total and viable), DPBS containing unbound bacteria from each well was transferred into a sterile tube; the wells were further washed with 1 mL of PBS (Oxoid) to remove any non-specifically bound bacteria, and the wash was collected in another sterile tube. The bacteria from these two tubes were counted by microscope, and the counts from two fractions were pooled together to determine the number of unbound bacteria. The number of bacteria bound to the Caco-2 cells was determined by subtracting the unbound bacteria from the total number of bacteria added to the well.

Statistical Analysis. The statistical significance of the differences in the values of the various measurements was analyzed by a two-tailed *t* test using SPSS Statistics (version 17) (SPSS, Chicago, IL). A *p* value below 0.05 (presented as $p < 0.05$) was considered statistically significant.

RESULTS

Survival during Storage in the Food Models. Figure 1 depicts the survival of *L. rhamnosus* GG during storage in the yogurt model, and Figure 2 depicts the survival of *L. rhamnosus* GG during storage in the ice cream model, containing different concentrations of fat and sugar. In both the yogurt and ice cream

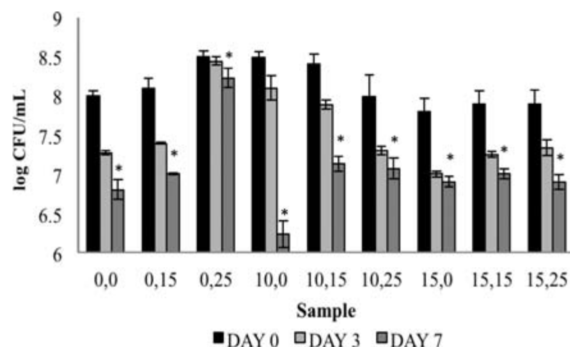

Figure 2. Survival of *L. rhamnosus* GG in the ice cream model formulations over a period of 7 days. The codes for each of the different formulations are shown in Table 1. The error bars represent standard deviation of three replicates. (*) Stastically significant differences compared to day 0.

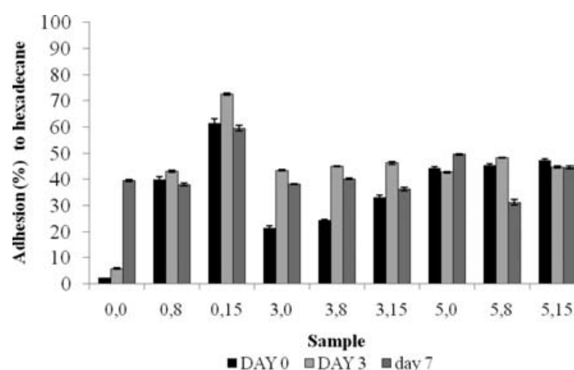

Figure 3. Adhesion (%) of bacterial cells to hexadecane. The bacterial cells were stored for up to 7 days in yogurt model formulations. The codes for each of the different formulations are shown in Table 1. The error bars represent standard deviation of three replicates.

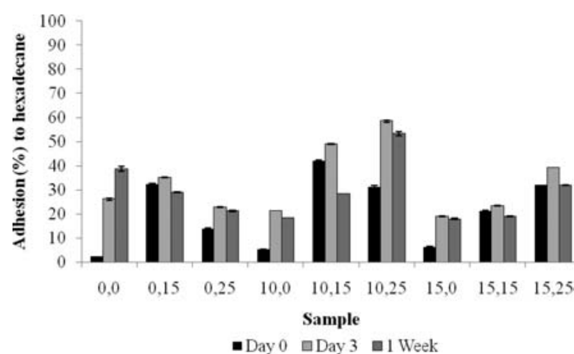

Figure 4. Adhesion (%) of bacterial cells to hexadecane. The bacterial cells were stored for up to 7 days in ice cream model formulations. The codes for each of the different formulations are shown in Table 1. The error bars represent standard deviation of three replicates.

models, a similar decrease was observed in the viable cell counts during storage of all of the samples compared to day 0. After 7 days of storage, the decrease in cell viability in both models was statistically significant ($p < 0.05$) and ranged between 1 and 2 logs, depending upon the formulation. For both models, there did not seem to be a clear relationship between the levels of fats or sugars and cell survival.

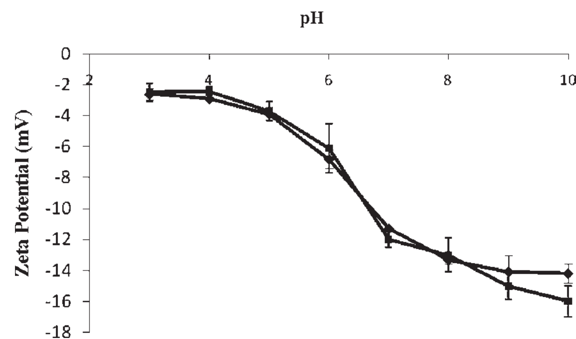

Figure 5. ζ potential measurements of representative samples, taken from (◆) 0,0 control for the yogurt model and (■) 0,0 control for the ice cream model media, as a function of pH. The bacterial cultures were suspended in 10 mM KH_2PO_4. The error bars represent standard deviations.

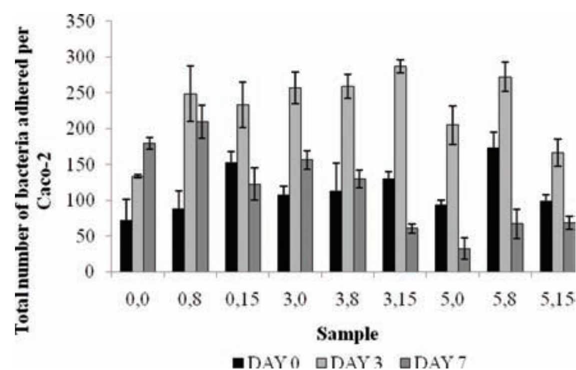

Figure 6. Total number of bacterial cells adhering to Caco-2 cells, as measured by microscopic counting. The bacterial cells were stored for up to 7 days in yogurt model formulations. The codes for each of the different formulations are shown in Table 1. The error bars represent standard deviations of three replicates.

Adhesion to Hexadecane. Figures 3 and 4 show the percentage adhesion of *L. rhamnosus* GG to hexadecane. In both models, with the obvious exception of the two control samples (0% fat and 0% sugar), an increase in the surface hydrophobicity was observed after 3 days of storage compared to day 0 for most samples; the hydrophobicity then decreased for the 7 day samples in most cases. When Figures 3 and 4 are compared, it could be deduced that the bacterial cells from the yogurt model were in general more hydrophobic than those from the ice cream model. In terms of the effects of fat and sugar, in the case of the yogurt model, the most significant differences occurred only for the day 0 samples. In particular, the hydrophobicity increased with an increasing sugar concentration for the formulations containing 0 and 3% fat. For the 5% fat formulation, the sugar did not have an effect. For the 3 and 7 day samples, it seemed that the incubation time was the main factor influencing the hydrophobicity of the cells; fat and sugar did not seem to have an effect. In the case of the ice cream model, there did not seem to be a clear relationship between the fat and sugar levels and hydrophobicity. The highest hydrophobicity values were obtained for the 10% fat and 15% sugar and the 10% fat and 25% sugar samples.

ζ Potential Measurement. The ζ potential trend of the bacterial cells over the pH range tested (pH 3 to 7) was similar

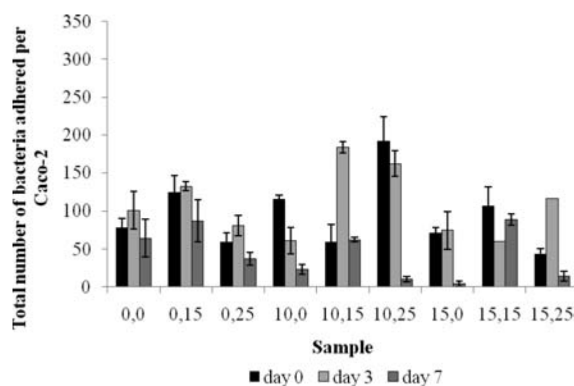

Figure 7. Total number of bacterial cells adhering to Caco-2 cells, as measured by microscopic counting. The bacterial cells were stored for up to 7 days in ice cream model formulations. The codes for each of the different formulations are shown in Table 1. The error bars represent standard deviations of three replicates.

for all of the models. Figure 5 depicts the ζ potential of the cells from the control (0,0) as a function of pH. It can be observed that the ζ potential was negative over the entire pH range, with no obvious iso-electric point observed. The ζ potential decreased slowly between pH 3 and 6 and then more steeply.

Adhesion to Caco-2 Cells. Figures 6 and 7 show the adhesion of the bacterial cells, obtained from the various model formulations, to Caco-2 cells. The bacteria that were stored in the yogurt model adhered better to Caco-2 cells than those stored in the ice cream model. In the case of the yogurt model, the adhesion levels for the samples taken after 3 days of storage were significantly ($p < 0.05$) higher than the 0 day samples; for some cases, the adhesion levels were higher than 250 bacterial cells per Caco-2 cell. The adhesion levels decreased for the 7 day samples. This trend was similar to that seen with the hydrophobicity data (Figures 3 and 4). In the case of the ice cream model, again the maximum adhesion levels were observed for the samples that were stored for 3 days. There did not seem to be a clear effect of the fat and sugar levels on bacterial adhesion. The highest adhesion levels were observed for the samples that showed the highest hydrophobicity values, i.e., the 10% fat and 15% sugar and the 10% fat and 25% sugar samples.

DISCUSSION

The present study investigated the interactions between food components and bacterial surfaces, because these are likely to affect the physiochemical properties of the bacterial surfaces and, consequently, their adhesion to the intestinal mucosa. However, despite the potential role of the food matrix, there is no information regarding its effect or specific food components on the surface and adhesion properties of bacterial cells. In this study, the surface and adhesion properties of *L. rhamonsus* GG were studied in solutions mimicking yogurt and ice cream, aiming mainly to evaluate the effect of fat and sugar on the above properties. In comparison to the complex nature of milk, the food models used were relatively simple, and as such, the possible interference from proteins and other solids was eliminated, thus focusing the study on the interactions between the bacterial cells and sugars or fats. The strain used is a well-researched probiotic strain with various therapeutic applications, established through various human studies.[8−10] This strain also shows high resistance to acid and bile[11] and high adhesion ability to Caco-2 cells.[3,12]

The reason for using model systems that simulate ice cream and yogurt was that these particular food products are commonly used for probiotic delivery and are produced with a variety of fat and sugar contents. Sugars and fats are two food components that have been suggested to affect the viability of probiotics in various foods,[11] although limited research has been conducted on the independent effects of these factors as well as their interactions on probiotic survival. In this study, it was observed that, in both the yogurt and the ice cream models, the viable cell concentration decreased with time. The survival rates were similar between the two model systems. After 7 days of storage, a significant ($p < 0.05$) decrease in cell viability was observed in both models, ranging between 1 and 2 logs, depending upon the formulations. However, there did not seem to be a clear relationship between the levels of fats and sugars and cell survival. This was also reported by Alamprese et al.,[11] who studied the survival of *L. rhamnosus* GG in ice creams of various fats and sugar concentrations, during storage at −16 °C, for up to 30 days. However, in that study, the cell viability was maintained throughout the storage period. The fact that this was not seen in the current study could be due to the additional compounds present in natural dairy foods, such as proteins (whey proteins and caseins) and salts, which are likely to contribute toward the survival of the cells. In the case of the yogurt model, the 1−2 log decrease in cell viability over a period of 7 days observed in this study was in accordance with previous studies, which used *Lactobacillus acidophilus* and *Lactobacillus casei*.[12−15] This study looked at the storage stability for 7 days, but on the basis of the previous studies, it can be speculated that, after the initial loss in viability during storage, the viability drop during long-term storage might not be that significant.[11]

According to previous studies, the surface properties of bacterial cells are determined by their cell wall makeup, which in the case of lactobacilli includes proteins, polysaccharides, and (lipo-) teichoic acid.[5,16] The interaction of these components with the surrounding environment is responsible for the attachment of bacteria to surfaces, to the intestinal mucosa, and to food components. With regard to the latter, Ly et al.[17] studied the effect of *Lactococcus lactis* cells on the emulsion stability of model foods, containing proteins and fats. That study suggested that the bacterial surfaces interacted with the hydrophobic fat droplets. In the case of the hydrophobic *L. lactis* LLD18 strain, this interaction helped to stabilize the emulsion, whereas in the case of the hydrophilic *L. lactis* LLD16 strain, there was no interaction, and this resulted in the breaking of the emulsion and phase separation. In this study, storage of the *L. rhamnosus* GG cells in the food models affected their surface properties. In both models, with the exception of the two control samples (0% fat and 0% sugar), an increase in the hydrophobicity was observed in most cases for the day 3 samples compared to the day 0 samples, which decreased for the day 7 samples. These results suggested that, during storage either at −4 °C or −20 °C, changes were taking place at the surface of the bacterial cells, affecting the hydrophobicity of the cells. Overall, when the yogurt and the ice cream models are compared, it seemed that, in the former, the bacteria were more hydrophobic than in the latter. The results suggested that the time of storage and the storage temperature were the main factors influencing the hydrophobicity of the cells and, presumably, the conformation of proteins and carbohydrates at their surface, whereas the levels of fats and sugars present in the formulation, as long as they were present even at small amounts, did not seem to have an important role.

The ζ potential of the cells was negative for the whole pH range (Figure 5) and similar to the ζ potential profiles that were previously reported for other *L. rhamnosus* strains.[2,4,18] Such profiles indicate that the surface of the cells was to a large extent dominated by anionic compounds, such as polysaccharides and teichoic acids. No significant differences were observed between models, storage, or model media composition.

The results from the adhesion of *L. rhamnosus* GG to Caco-2 cells (Figures 6 and 7) indicated that the bacterial cells when stored in the yogurt model were significantly ($p < 0.05$) more adhesive than those stored in the ice cream model. In comparison to the control, the adhesion levels (for the samples taken after 3 days of storage) were significantly higher in the case of the yogurt model system. The positive effect of fat and sugar on the adhesion of the bacterial cells, which was seen in the yogurt model in this study, is in contrast to the results by Ouwehand et al.,[19] who investigated as part of their study the effect of milk on the adhesion of various probiotic lactobacilli, including strains of *Lactobacillus brevis*, *Lactobacillus reuteri*, and *L. rhamnosus*, to human intestinal mucus glycoproteins. That study reported that the presence of milk fat reduced the adhesion of the bacterial cells. The reasons for this difference could be due to the different model of intestinal mucosa used (Caco-2 cells versus extracted mucus) and the fact that, in some cases, only the viable bacteria adhering to Caco-2 cells are counted, whereas in others, the total bacterial numbers are counted. In the case of the ice cream model, in this study, the results were inconclusive, because in some cases, the adhesion levels were higher than the control and, in some others, the adhesion levels were lower than the control. Overall, the results suggested that yogurt was a better food delivery system than ice cream, because it was likely to enhance the adhesion of the probiotic cells in the gastrointestinal tract.

Considering that the highest adhesion values were obtained for the cells that were stored for 3 days, these results correlated well with hydrophobicity results in Figures 3 and 4, which showed that the maximum hydrophobicity for all formulations was observed after 3 days of storage and decreased after 7 days. The above suggested that a possible relationship existed between the hydrophobicity of the bacteria and their ability to adhere to the intestinal mucosa. This has been shown previously for lactobacilli[20,21] and, in particular, *L. rhamnosus* GG.[3] In the case of the yogurt model, it seemed that, as long as sugar or fat was present in the formulation, even at low levels, the most important factor influencing the bacterial adhesion was the storage time rather than the actual concentrations of fat and sugar, a conclusion that was also deduced from the hydrophobicity data. It would be interesting though to study the adhesion ability of the cells during storage for longer than 7 days and evaluate whether it decreases even further.

Taking into account the fact that the different levels of fats and sugars used in the model systems did not seem to lead to significant differences in terms of bacterial adhesion and the fact that both the yogurt and ice cream model systems contained fat and sugar, it is likely that the differences between the two models were due to the pH and storage temperature. The low temperature (−20 °C) and near neutral pH (pH 6) of the ice cream model possibly resulted in preserving the original bacterial surface conformation; thus, the adhesion was relatively less affected. In contrast, the low pH of the yogurt model (pH 4) and the higher storage temperature (4 °C) meant that the cells responded more actively to these particular conditions. For example, it has been shown previously that the ζ potential of *L. rhamnosus* GG cells decreased with a decreasing pH of the solution from pH 8 to 3.[3] This suggests that, at pH 4, the cells were likely less charged compared to pH 6, and thus, the electrostatic interactions were less influential on the adhesion; in that case, the hydrophobic interactions could have played a more significant role, thus explaining our observations. Finally, an important observation in this study was that dead bacterial cells were able to adhere to Caco-2 cells. This can be deduced by the fact that the viable cell concentration for both model systems decreased considerably after 3 days of storage, whereas the adhesion levels increased. The ability of dead bacterial cells to adhere to Caco-2 cells has also been demonstrated previously[19] and is important to consider when designing food formulations for probiotic delivery.

In summary, this study indicated that the food carrier is likely to affect the surface and adhesion properties of *L. rhamnosus* GG during storage. The yogurt model enhanced the adhesion properties of the bacterial cells, suggesting that yogurt is likely to be a better delivery vehicle than ice cream in terms of bacterial adhesion. The most important factor influencing bacterial adhesion seemed to be the storage time rather than the levels of fats and sugars, indicating that conformational changes were taking place on the surface of the bacterial cells during storage.

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Funding Sources

The authors acknowledge the financial support provided by the Felix Scholarship Trust and the Society for General Microbiology (SGM) to Gurjot Deepika.

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