

Influence of Preadsorbed Milk Proteins on Adhesion of *Listeria monocytogenes* to Hydrophobic and Hydrophilic Silica Surfaces

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The adsorption of β -lactoglobulin, bovine serum albumin, α -lactalbumin, and β -casein for 8 h and β -lactoglobulin and bovine serum albumin for 1 h at silanized silica surfaces of low and high hydrophobicity, followed by incubation in buffer and contact with *Listeria monocytogenes*, resulted in different numbers of cells adhered per unit of surface area. Adhesion to both surfaces was greatest when β -lactoglobulin was present and was lowest when bovine serum albumin was present. Preadsorption of α -lactalbumin and β -casein showed an intermediate effect on cell adhesion. Adsorption of β -lactoglobulin for 1 h resulted in a generally lower number of cells adhered compared with the 8-h adsorption time, while the opposite result was observed with respect to bovine serum albumin. The adhesion data were explainable in terms of the relative rates of arrival to the surface and postadsorptive conformational change among the proteins, in addition to the extent of surface coverage in each case.

Listeria monocytogenes is a pathogenic bacterium that can adhere to the types of contact surfaces used in food processing (22, 23). Colonization and growth of this microorganism on food-processing surfaces would represent a serious obstacle to consistently providing healthful, high-quality products. The fact that *L. monocytogenes* can attach to such surfaces at refrigeration temperatures makes this problem of even greater concern, particularly to the dairy industry (22). Better understanding and hence optimal control of this problem seem best accomplished by focusing attention on the early events that take place at the food contact interface which lead to bacterial attachment and biofilm formation.

Microbial adhesion to surfaces is, to some extent, dependent on the presence, composition, and conformational state of a preadsorbed protein film. Characteristics of this film are dependent on molecular properties of the individual protein as well as those of the bare contact surface. Contact surface hydrophobicity or hydrophilicity, and its influence on protein adsorption as well as cell adhesion, has received much attention for several decades (2, 9, 12, 19, 25).

Adsorbed protein may change its conformation over time, and the rate of conformational change would then vary among different proteins and from one surface to another (3, 9, 16, 29). Such surface-induced conformational changes should be expected to influence protein and surface reactivities with bacterial cells and spores just as the conformation of blood serum proteins affects platelet and whole cell adhesion to implanted biomedical materials. It is the purpose of this research to begin to evaluate the influence of adsorbed milk proteins (α -lactalbumin [α -Lac], β -lactoglobulin [β -Lg], β -casein, and bovine serum albumin [BSA]) on the extent of bacterial adhesion measured at hydrophilic and hydrophobic silica surfaces.

MATERIALS AND METHODS

Monocrystalline and polished silicon plates (hyperpure, type N, phosphorus doped, 1-0-0 orientation, resistivity = 0.8 to 2 Ω /cm) were obtained from Wacker-Siltronic Corp., Portland, Oreg. Xylene and sodium phosphate (mono- and dibasic) were of analytical grade. The bovine milk proteins β -Lg, which contained the genetic variants A and B ($3\times$ crystallized and lyophilized, lot 98F8080), α -Lac (type III, lot 128F8140), BSA (lot 15F-0112), and β -casein (lyophilized, essentially salt free, lot 40H9510) were obtained from Sigma Chemical Co. (St. Louis, Mo.). Dichlorodimethylsilane (DDS) (lot 11905cx) was obtained from the Aldrich Chemical Company, Inc. (Milwaukee, Wis.).

Surface modification. Silicon wafers were cut into plates of approximately 1 cm² with a tungsten pen. The silanizations were slightly modified from the method described by Jönsson et al. (14). Surfaces were silanized by reaction with 0.01 or 0.1% DDS in xylene as described previously (15). These surface derivatizations are stable and exhibit a low or high hydrophobic character, respectively, as shown by contact angle analysis (15). Surfaces silanized with 0.01% DDS exhibit a partial negative charge in addition to low hydrophobicity, and we will refer to them as hydrophilic surfaces.

Protein adsorption. Equimolar solutions of protein (equivalent to 1 mg of β -Lg per ml) were prepared by first dissolving each in 0.01 M phosphate buffer (pH 7.0) while stirring for 20 min. Each protein solution was filtered through a preassembled, presterilized Nalgene bottle top filter (pore size, 0.2 μ m; Nalge Company, Rochester, N.Y.). From this solution, 10 ml was pipetted into small petri dishes (60 by 15 mm, disposable polystyrene, Falcon 1007; Becton Dickinson and Company, Lincoln Park, N.J.), and surfaces were then introduced to the protein solution. Surfaces were allowed to contact protein solution for 8 or 1 h, after which they were rinsed sequentially in three 300-ml volumes of distilled, deionized water. Each individual surface was rinsed for about 15 s in each step. Protein films formed on surfaces were then aged in 20 ml of buffer for 0, 5, 10, or 15 h, and then surfaces were rinsed again

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as described above and introduced to the medium with bacterial cells. Great care was taken not to allow drying of the adsorbed protein films. For the purpose of quantifying the amount of protein desorbed during incubation in buffer, the same tests were performed for β -Lg and BSA adsorption for 1 h but in the absence of any cell contact. Protein films in this case were dried after the final rinse by blowing the surfaces with nitrogen gas and then were kept in a dust-free desiccator for 24 h. The amount of protein remaining on the surface was then determined by ellipsometry (7).

Culture and adhesion. *L. monocytogenes* Scott A was grown in 50 ml of protein-free defined medium and incubated for 24 h at 37°C to reach the stationary phase. The defined medium was made up of 20.8 g of RPMI 1640 medium (lot 119F-4645-1; Sigma) per liter. The medium was modified by the addition of 1% glucose and 2% Casamino Acids (Difco Laboratories, Detroit, Mich.). Before use, this medium was filter sterilized with a preassembled, presterilized Nalgene bottle top filter (pore size, 0.2 μ m). Culture was diluted (in Butterfield's buffer [pH 7.0; 0.00426% potassium phosphate]) to a cell density of 10^7 to 10^9 CFU/ml. Ten milliliters of this diluted culture was transferred to small petri dishes. Hydrophilic or hydrophobic silica surfaces, either bare or coated with a protein film, were introduced to the petri dishes and allowed to contact the culture for 3 h.

Rinsing. After contact with cells, surfaces were affixed to a microscope glass slide with Elmer's Stix All glue (Borden Inc., Columbus, Ohio) without allowing the biofilm to dry. Rinsing to remove the loosely adhered cells was done by passing 1,200 ml of distilled water through a flow cell into which the glass slides with the affixed surfaces had been inserted. The flow cell (volume, 87.5 ml; a generous gift from The Swedish Institute for Food Research) is shown in Fig. 1.

Criteria used for rinsing unattached bacterial cells. Unmodified silica surfaces were allowed to contact cells for 3 h. Contact with cells was terminated, and the surfaces were rinsed with distilled water at different flow rates for different periods. The flow rate and rinse time chosen for all subsequent experiments were those for which little change in the number of attached cells was seen with increasing time and/or flow rate.

Image analysis. Following rinsing, surfaces were quickly covered by a coverslip, and images of bacterial cells adhered to surfaces were taken with an incident light microscopy image analysis system (IAS). The incident light microscopy image analysis system is composed of a 486 IBM compatible computer interfaced to a Sony monitor and a Reichert Epistar incident light microscope to which a video camera (solid state camera model 4815-2000/0000; Cohu Inc., San Diego, Calif.) is attached. Into the computer, two programs were installed. Image-Pro Plus version 2.0 (Media Cybernetics; Silver Spring, Md.) for recording images and Visionplus-AT (Imaging Technology Inc., Bedford, Mass.) for image processing. Images were recorded under the 100/1.25 oil objective. Eight to 25 images from each surface were digitized and saved for later analysis. The number of cells in a 2,500- μ m² field in each image was determined.

Statistical analysis. Data of the completely randomized design with two replications were evaluated by analysis of variance and least significant difference at the 95% significance level by using Statistical Analysis System programs PROC ANOVA and PROC GLM (26).

RESULTS AND DISCUSSION

Rinsing of unattached bacterial cells. The procedure for rinsing unattached cells was established by contacting unmod-

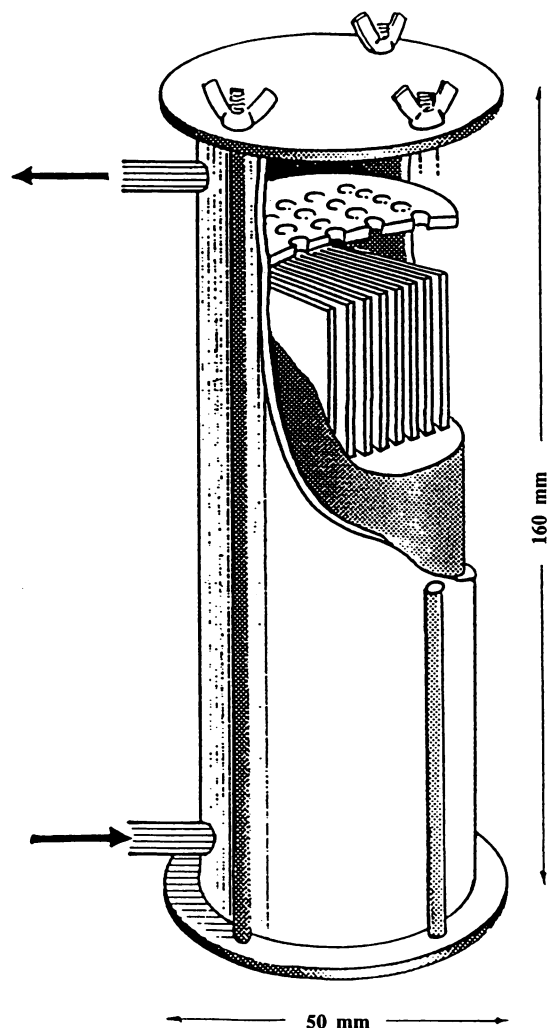


FIG. 1. Schematic of the flow cell used to rinse loosely adhered *L. monocytogenes* from the surfaces.

ified silica surfaces with *L. monocytogenes* and then rinsing at different flow rates for different periods of time. In Fig. 2, the number of cells that remained on the surface after rinsing is plotted against rinsing time at flow rates of 100, 300, and 550 ml/min. For subsequent experiments, rinsing was performed with a flow rate of 300 ml/min for 4 min. A one-way analysis of variance (least significant difference; 95% confidence level) indicated that there is no significant difference ($P > 0.05$) between rinsing at a flow rate of 300 ml/min for 4 min and rinsing at 550 ml/min for 4 or 10 min. Although the rinsing protocol was established with reference only to adhesion to bare surfaces, the aim in these experiments was simply to assist selection of a method that could be consistently applied in each adhesion experiment.

Adhesion following protein contact for 8 h. (i) Hydrophobic surfaces. Figure 3 illustrates the effect of film age on the number of cells adhered to hydrophobic silica surfaces following contact with protein for 8 h. It also shows the extent of adhesion measured on bare hydrophobic and hydrophilic silica surfaces. Figure 3 indicates that the β -Lg film encouraged adhesion more than did the other protein films ($P < 0.05$), although the extent of adhesion to preadsorbed β -Lg remained

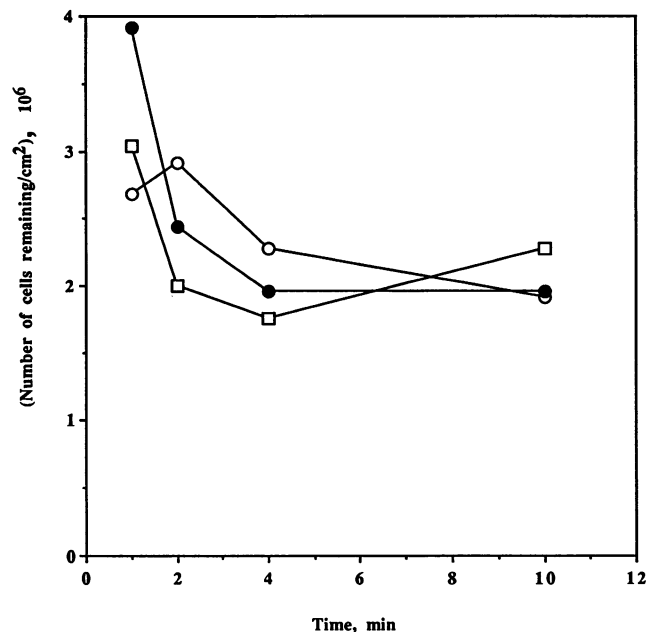


FIG. 2. Effects of flow rates on residual adherent cells of *L. monocytogenes*. ○, 100 ml/min; ●, 300 ml/min; □, 550 ml/min.

lower than that measured on bare hydrophobic surfaces. While β -Lg had the greatest effect on adhesion in terms of the number of cells adhered per unit area, preadsorbed BSA reduced the number of cells adhered to values well below that of the bare hydrophilic surfaces ($P < 0.05$). The other two proteins, β -casein and α -Lac, showed an intermediate effect.

The effects of protein preadsorbed to these hydrophobic surfaces are consistent with previous observations. Proteins

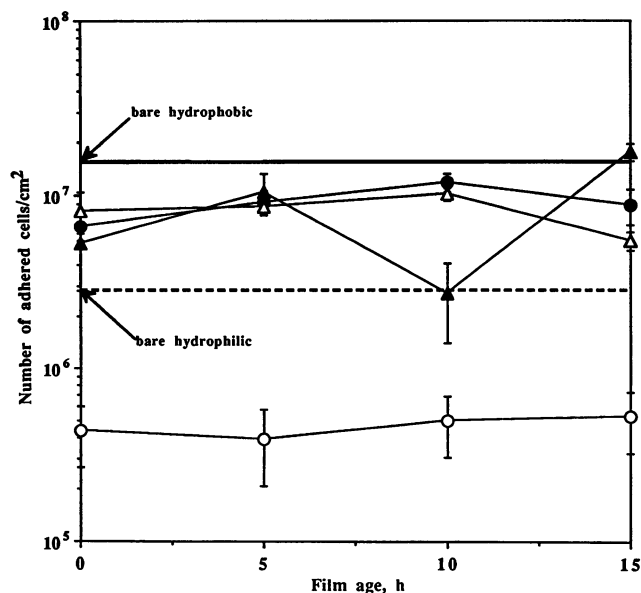


FIG. 3. Effects of preadsorbed protein type and film age on adhesion of *L. monocytogenes* to hydrophobic silica surfaces. Bars indicate standard deviations of the means. ○, BSA; ●, β -Lg; ▲, β -casein; △, α -Lac.

adsorbed to solid surfaces alter the original interfacial properties (6, 27, 31, 33). Yang et al. (33) used contact angle methods to measure the change in hydrophilic-hydrophobic balance exhibited by a number of different materials following adsorption of β -Lg. They found that adsorption of β -Lg rendered hydrophilic surfaces more hydrophobic and hydrophobic surfaces more hydrophilic. Adsorption of each of the four proteins studied to hydrophobic surfaces is entropically driven (17, 18) and probably accompanied by orientation of negatively charged, hydrophilic regions to solution, yielding a less hydrophobic interface. Considering only the general observation that *Listeria* adhesion is greater on hydrophobic as opposed to hydrophilic surfaces (1), it is thus expected that adhesion of *Listeria* spp. to such film-covered surfaces would result in lower numbers of adhered cells relative to those measured at a bare hydrophobic surface.

The previous argument, however, cannot explain the differences in adhesion responses evoked by β -Lg and BSA. BSA has a net charge of -18 at pH 7.0, while that of β -Lg is -5 (32), and would be expected to take part in a more repulsive electrostatic interaction with a bacterium. β -Casein, on the other hand, is a linear amphiphile, with essentially all of the protein's net charge (-12 at pH 7.0) found on the first 43 residues of the N-terminal domain (10). Upon adsorption at a hydrophobic surface, the N-terminal domain is oriented toward the solution. This mechanism has been used to explain the observation that a preadsorbed layer of β -casein prevents a sequential adsorption of β -Lg (24). Nevertheless, the difference between the effect of a preadsorbed film of β -casein and that of α -Lac (net charge, -3 at pH 7.0) on *Listeria* adhesion was found to be statistically insignificant.

A more comprehensive treatment of these results would account for relative rates at which each of the four proteins is able to adopt a passivating conformation at the hydrophobic surface.

One such treatment begins with reference to a model for interactions between adsorbed protein and protein in solution. Lundström and Elwing (21) considered an experimental situation in which adsorption to a solid surface is allowed to occur from a single-component protein solution and is followed by incubation in buffer, after which a second, dissimilar protein is added. They showed that the fraction of originally adsorbed protein that is not exchangeable with dissolved protein is related to rate constants governing conversion of the originally adsorbed protein to a nonremovable form and exchange of adsorbed protein by dissimilar protein introduced to the solution. Krisdhasima et al. (16) adapted this concept to the sodium dodecyl sulfate-mediated removal of α -Lac, β -Lg, BSA, and β -casein from silica surfaces silanized by the procedure used here for hydrophobic surfaces. They were able to rank the rate constants governing arrival of each protein to the surface (k_1) and surface-induced conversion to a nonremovable state (S_1). Results of that ranking showed the following: $S_{1,BSA} > S_{1,\beta\text{-casein}} > S_{1,\alpha\text{-lac}} > S_{1,\beta\text{-Lg}}$. This finding is consistent with the adhesion results of Fig. 3, as it indicates that once adsorbed, BSA would offer greater resistance to removal by an incoming adsorbate than would β -Lg. So assuming that each protein would have some capacity to passivate the surface, β -Lg would be more likely to leave the interface upon exposure to surface active species (in this case, *Listeria* spp.) than would BSA, exposing a greater area of bare, hydrophobic silica to adhering bacteria.

(ii) **Hydrophilic surfaces.** Figure 4 depicts the effects of preadsorption of each protein on adhesion to hydrophilic surfaces. It also indicates the extent of adhesion expected on bare hydrophilic and hydrophobic surfaces. β -Lg and BSA

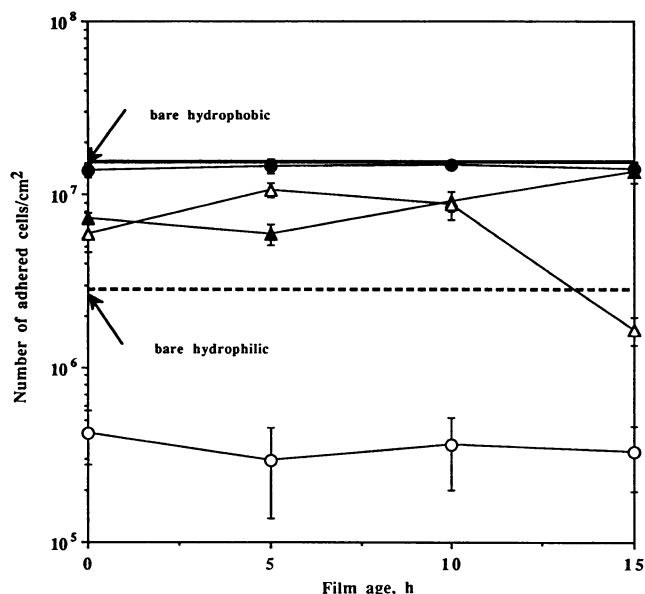


FIG. 4. Effects of preadsorbed protein type and film age on adhesion of *L. monocytogenes* to hydrophilic silica surfaces. Bars indicate standard deviations of the means. ○, BSA; ●, β -Lg; ▲, β -casein; △, α -Lac.

again behaved most dissimilarly, with β -Lg evoking much greater adhesion than BSA, while β -casein and α -Lac exhibited an intermediate effect. However, adhesion mediated by β -Lg preadsorbed to hydrophilic silica was greater than that exhibited on hydrophobic silica ($P < 0.05$). Adsorption of β -Lg in this case would involve interaction between regions of negative charge on the surface and positive charge on the molecule, in addition to the entropically driven attraction described earlier, as these surfaces were silanized as well. Adsorption occurring via favorable electrostatic attraction would orient hydrophobic regions of the molecule away from the surface. Indeed, the presence of these hydrophobic domains leads to a higher water contact angle than that measured on bare hydrophilic surfaces (33). Moreover, Arnebrant et al. (5) reported that adsorption of β -Lg to hydrophilic chromium oxide surfaces results in a protein bilayer in which the second layer, bound via hydrophobic association to the partially unfolded first layer, can be partially removed by rinsing. This was not observed for β -Lg adsorption to hydrophobic surfaces. Concerning the results of Fig. 4, any amount of a second layer removed by rinsing would leave a more hydrophobic interface for bacterial adhesion.

The BSA-mediated inhibition of bacterial adhesion on hydrophilic surfaces was similar in order of magnitude but more extensive than that observed on hydrophobic surfaces ($P < 0.05$). Slightly less adhesion measured for BSA-coated hydrophilic surfaces may simply be due to any removal of adhered molecules being accompanied by exposure of hydrophilic, as opposed to hydrophobic, interface. That the passivating effect of preadsorbed BSA was similar on each type of surface, and more pronounced in each case than that of the other preadsorbed proteins, is not surprising. BSA adsorption to both hydrophobic and negatively charged hydrophilic interfaces has been postulated to take place according to a similar mechanism (4). In particular, BSA consists of three similarly sized globular domains arranged in series. One end domain is neutral at pH 7.0, while the remaining domains carry a high net

negative charge. Adsorption with the neutral domain oriented toward the surface in each case accompanied by structural rearrangement of that domain to minimize free energy yields a more or less stable, hydrated, and negatively charged interface that is probably not favorable for bacterial adhesion. This thinking is consistent with the facts that unlike the other proteins, both BSA adsorption kinetic (16) and adsorption equilibrium behavior (30) have been observed to be similar at hydrophobic and hydrophilic silica surfaces. Moreover, human serum albumin shows great homology with BSA and is known to passivate biomedical material surfaces to fibrinogen adsorption and platelet adhesion (8). In fact, retention of albumin on blood-contacting implants to reduce thrombogenesis remains a major focus in biomedical materials research (13).

Other studies have directly identified BSA as capable of passivating surfaces against cell adhesion. Tamada and Ikada (31) investigated the effect of preadsorbed BSA, bovine gamma globulin, and plasma fibronectin on L-cell adhesion to 14 polymer surfaces of various wettabilities. They found that BSA preadsorption entirely inhibited L-cell adhesion, independent of substrate wettability. Fletcher (11) investigated the effect of BSA and several other proteins on adhesion of a marine pseudomonad to polystyrene petri dishes. She found that BSA, gelatin, fibrinogen, and pepsin impaired cell attachment through adsorption to the dish surface. Protamine and histone were found not to markedly inhibit adhesion.

Figures 3 and 4 would suggest a real decrease in the number of cells adhered with film age for α -Lac, while Fig. 4 shows an increase in adhesion with film age for β -casein. For α -Lac, the decrease was observed most clearly at hydrophilic surfaces. Krisdhasima et al. (16) found α -Lac to exhibit a very low affinity for hydrophilic silica and α -Lac desorption occurring with longer incubation time might be a contributing factor to the low adhesion observed in that case. At hydrophobic surfaces, the decrease is consistent with slow conformational adaptations exposing a more hydrophilic interface to incoming bacteria.

The increased number of cells adhered to β -casein-covered hydrophilic surfaces with film age could be related to the flexibility of β -casein. β -Casein is more flexible than the globular proteins, and this might contribute to conformational adaptations continuing to occur over the time scale of these experiments such that the hydrophilic surface is rendered more hydrophobic with time.

Adhesion following protein contact for 1 h. Since β -Lg and BSA exhibited the most dissimilar effects on adhesion, with little dependence on film age, adhesion following a shorter preadsorption period was monitored. In this way, the possible impact of time-dependent conformational changes on adhesion could be better resolved.

Preadsorption of β -Lg. Figure 5 shows the effect of preadsorbed β -Lg on adhesion at hydrophilic and hydrophobic surfaces, where preadsorption was allowed to occur for 1 h. Relevant data from Fig. 3 and 4 are included for comparison. At hydrophobic surfaces, cell adhesion was lower than that observed for 8-h preadsorption, for film ages of 0 and 5 h. It is important to note that after 1 h (and in the absence of rinsing), the surface coverage of β -Lg is only about 79% of that measured after 8 h of contact and well below that expected for a monolayer (15, 16). The greater amount of interfacial area per molecule may allow β -Lg to adopt either a more energetically favorable orientation at the interface or more rapidly adopt a more passivating conformation as described earlier. As film age is increased, cell numbers increase, probably because of desorption of β -Lg. To verify whether β -Lg desorbs from hydrophobic silica after preadsorption for 1 h, similar tests

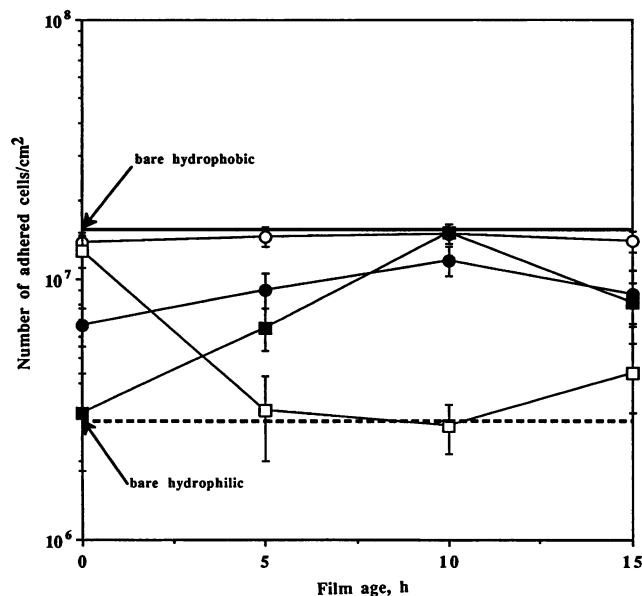


FIG. 5. Effects of β -Lg adsorption time and film age on adhesion of *L. monocytogenes* to hydrophobic and hydrophilic silica surfaces. ●, adhesion following adsorption to hydrophobic surfaces for 8 h; ■, adhesion following adsorption to hydrophobic surfaces for 1 h; ○, adhesion following adsorption to hydrophilic surfaces for 8 h; □, adhesion following adsorption to hydrophilic surfaces for 1 h.

were run over the 15-h period but in the absence of cell contact. Between film ages of 0 and 15 h, the adsorbed mass of β -Lg decreased about 20% (Table 1). The increased exposure of bare hydrophobic silica to incoming cells would facilitate adhesion.

At hydrophilic surfaces, the opposite behavior was observed in the sense that initially, cell numbers were comparable to those observed after the 8-h preadsorption and then decreased to values consistent with those expected for a bare hydrophilic surface. Thus, the same argument would serve to explain the results in this case. After 1 h (and in the absence of rinsing), the surface coverage of β -Lg is only about 74% of that measured after 8 h of contact, again well below that expected for a monolayer (15, 16). The increased area per molecule at the interface may have facilitated an energetically favorable orientation with concomitant exposure of hydrophobic regions to the solution. Decreasing cell numbers with increasing film age are consistent with β -Lg desorption which, between film ages of 0 and 15 h, was observed to decrease the initial value of adsorbed mass by about 25%. The importance of available interfacial area, i.e., area not occupied by protein, has been

TABLE 1. Masses of proteins remaining on surfaces following 1 h of protein contact and incubation in buffer for different times

Film age (h)	Adsorbed mass ($\mu\text{g}/\text{cm}^2$)			
	BSA		β -Lg	
	Hydrophobic surface	Hydrophilic surface	Hydrophobic surface	Hydrophilic surface
0	0.375	0.356	0.297	0.279
5	0.356	0.311	0.301	0.227
10	0.373	0.315	0.250	0.197
15	0.365	0.324	0.256	0.217

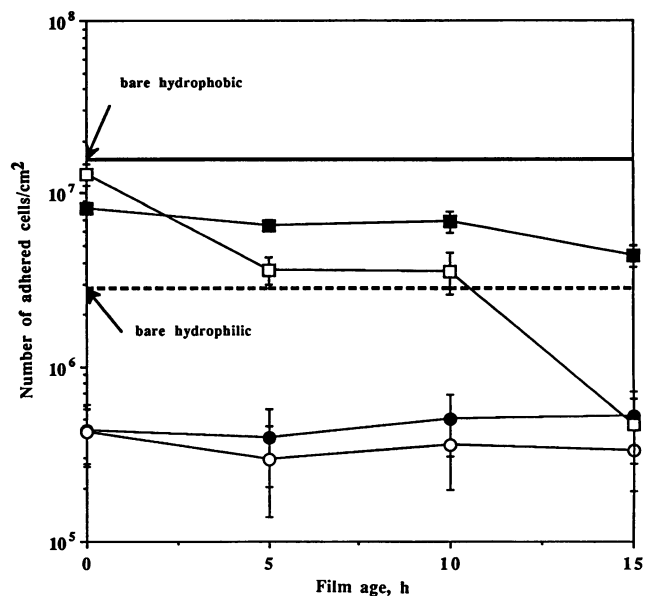


FIG. 6. Effects of BSA adsorption time and film age on adhesion of *L. monocytogenes* to hydrophobic and hydrophilic silica surfaces. ●, adhesion following adsorption to hydrophobic surfaces for 8 h; ■, adhesion following adsorption to hydrophobic surfaces for 1 h; ○, adhesion following adsorption to hydrophilic surfaces for 8 h; □, adhesion following adsorption to hydrophilic surfaces for 1 h.

well documented with reference to models (20) and experimental interpretation (28).

Preadsorption of BSA. The adhesion response evoked on BSA-coated surfaces following a 1-h preadsorption is shown in Fig. 6, along with relevant data from Fig. 3 and 4. Krisdhasima et al. (16) showed that a rate constant describing initial arrival of BSA to a hydrophobic silica surface, involving end-on orientation with its neutral domain adjacent to the surface, was lower than the arrival rate constants of the other three milk proteins used in this study. Based on that, it is not surprising that the amount of cell adhesion was higher than that observed following an 8-h preadsorption. At hydrophobic surfaces, although the adsorbed mass of BSA after 1 h is about 86% of that attained after 8 h (16), kinetic analysis would indicate that the population of BSA molecules tightly adsorbed in the passivating orientation may be relatively low. Little desorption was observed for BSA between 0 and 15 h, which suggests that the passivating orientation characteristic of the 8-h data was not attained, even after 15 h. The relatively small decrease in cell numbers with increasing film age suggests that breaking intermolecular and protein-surface associations to reorient in the passivating orientation was a rather slow process.

At hydrophilic surfaces, the same argument holds for explanation of the response evoked immediately after protein contact (i.e., film age = 0 h), but the decrease in adhesion with increasing film age is substantial in comparison to that observed at the hydrophobic surface. In this case, the high negative charge of the molecule may have served to facilitate molecular reorientation and conformational adaptation on the surface with increasing age of the film.

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