Effects of lactoferrin and milk on adherence of *Streptococcus uberis* to bovine mammary epithelial cells

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Objective—To determine whether lactoferrin (LF) or milk influenced adherence of *Streptococcus uberis* to bovine mammary epithelial cells.

Sample Population—Three strains of *S uberis* from cows with mastitis, pooled milk samples from 3 clinically healthy Jersey cows early in the lactation period, and bovine mammary epithelial cells from a clonal cell line.

Procedures—Adherence of *S uberis* to bovine mammary epithelial cells in the presence of various concentrations of LF or milk and after pretreatment of bacteria with LF or milk was tested. Bacteria were cultured with mammary epithelial cell monolayers for 1 hour. The culture supernatant was removed, and the epithelial cells were lysed. Adherence index was calculated as number of colony-forming units (CFU) in the cell lysate divided by number of CFU in the supernatant times 10,000.

Results—All 3 strains of *S uberis* were found to bind to purified LF and LF in milk. Addition of LF to the culture medium enhanced adherence of all 3 strains to mammary epithelial cells, whereas addition of milk enhanced adherence of 2 strains and decreased adherence of the third. Pretreatment of bacteria with LF or milk increased adherence of 1 of the strains but decreased adherence of the other 2. Increased adherence was antagonized by rabbit antibovine LF antibody.

Conclusions—Results suggest that LF may function as a bridging molecule between *S uberis* and bovine mammary epithelial cells, facilitating adherence of the bacteria to the cells. (*Am J Vet Res* 2000;61:275–279)

Infection with *Streptococcus uberis* is one of the major causes of mastitis in cattle, and control measures currently in use against contagious mastitis pathogens are not effective for controlling intramammary infection with *S uberis*. This is possibly attributable to a lack of full understanding of the epidemiologic and pathogenic aspects of *S uberis* mastitis, even though encapsulation,[1,2] adherence to and invasion into mammary epithelial cells, binding to extracellular matrix proteins,[3,4] and possession of a plasminogen activator[5] have been identified as potential virulence factors of *S uberis*.

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Bacteria are mostly planktonic in milk during intramammary infections; however, some may adhere to mammary epithelial cells. Because milk is a protein-rich exosecretion, it is important to determine what roles the interactions between milk proteins and invading bacteria have in the pathogenesis of intramammary infection. Previous research revealed that **lactoferrin (LF)** is the major milk protein to which *S uberis* binds.[6] Lactoferrin is an iron-binding glycoprotein that is present in bovine milk in various concentrations, depending on the physiologic and pathophysiologic conditions of the mammary gland. Concentration of LF is markedly increased during involution of the mammary gland and during intramammary infections.[7-9]

Traditionally, LF has been considered one of the nonspecific antibacterial factors in bovine milk,[10,11] and it has been suggested that LF exerts its antibacterial effect through sequestration of iron from the environment in which bacteria reside, so that the bacteria are deprived of the iron needed for their growth.[12,13] However, streptococci, including *S uberis*, are more resistant to the antibacterial effects of LF than gram-negative bacteria,[10,11,14] probably because of their low requirement for iron.[15]

Lactoferrin has been found to bind to several types of host cells, including human and bovine mammary epithelial cells.[16,17] It also affects surface properties of human leukocytes.[18] Because *S uberis* binds to LF in milk and purified LF,[6] we hypothesize that LF may act as a bridging molecule between bacteria and mammary epithelial cells or phagocytic cells in the pathogenesis of *S uberis* mastitis. The purpose of the study reported here was to determine whether LF and milk influenced adherence of *S uberis* to bovine mammary epithelial cells and what role anti-LF antibodies have on adherence of *S uberis* pretreated with LF and milk.

Materials and Methods

Bacteria—Three strains of *S uberis* (UT102, UT366, and UT888) originally isolated from dairy cows with mastitis were used. *Streptococcus uberis* UT888 is an encapsulated strain isolated from a cow with chronic mastitis; this strain has been used extensively in our laboratory to induce mild clinical mastitis during experimental challenge exposure studies. *Streptococcus uberis* UT366 is an old stock strain that was first used in an experimental mastitis model in 1987.[19] It appeared more virulent than *S uberis* UT888, and in recent experimental challenge exposure studies, could cause clinical mastitis with associated systemic signs. *Streptococcus uberis* UT102 is a nonencapsulated strain from a cow with subclinical mastitis.[20] Bacteria were stored at –80 C in **Todd-Hewitt broth (THB)**[a] containing 20% glycerol and refreshed, when needed, on blood agar plates before being subcultured in THB for assays involved in the present study.

Milk samples—Milk samples were collected from 3 Jersey cows in the early part of the lactation period. Cows were considered free from intramammary infections on the basis of results of bacteriologic culture of milk samples obtained monthly for 3 months. Milk samples were defatted by means of centrifugation at 1,400 × g for 30 minutes. Skim milk samples were centrifuged at 22,000 × g at 4 C for 2 hours, filter sterilized (pore size 0.45 µm),[b] and stored at –80 C. Prior to use in assays of bacterial adherence to bovine mammary epithelial cells and of bacterial binding to LF, milk samples from the 3 cows were thawed and pooled.

Bacterial adherence to bovine mammary epithelial cells—A bovine mammary epithelial cell line (MAC-T)[c] was grown to confluence in 24-well plates[d] in **Dulbecco's modified Eagle's medium (DMEM)**[e] containing 10% **fetal bovine serum (FBS)**[e] at 37 C and 5% CO_2. Epithelial cell monolayers were washed twice with **phosphate-buffered saline solution (PBSS)** prior to use in adherence assays.

To determine adherence of *S uberis* to mammary epithelial cells, strains of *S uberis* were subcultured in THB at 37 C for 5 hours, washed once with PBSS, and resuspended in DMEM containing 10% FBS. Bacterial density was calibrated to about 1 to 2 × 10^8 **colony-forming units (CFU)** per ml. Aliquots (0.5 ml) of the bacterial suspensions were added to wells containing MAC-T–cell monolayers, along with 0.5 ml of filter-sterilized LF solution (in DMEM containing 10% FBS) or 0.5 ml of milk. Final concentrations of LF were 0, 0.01, 0.1, and 1 mg/ml. Final concentrations of milk were 0, 12.5, 25, and 50%. Each concentration was tested in triplicate. Bacteria were incubated with MAC-T cells for 1 hour at 37 C and 5% CO_2. At the end of the incubation period, supernatants were aspirated and diluted for bacterial counting. Wells were washed 3 times with PBSS, each followed by shaking, and mammary epithelial cells were lysed with a solution containing 0.1% trypsin[e] and 0.03% Triton-X 100.[f] Cell lysates were diluted 10-fold for bacterial counting. Adherence was expressed as number of CFU in the cell lysate divided by number of CFU in the supernatant times 10,000. Each experiment was conducted at least twice.

In separate assays, adherence of *S uberis* pretreated with LF was tested. Bacterial strains that had been grown in THB at 37 C for 5 hours were washed with PBSS and pretreated with LF (final concentration, 1 mg/ml of PBSS) or milk (100%) for 1 hour at room temperature (approx 20 C). Bacteria were washed twice with PBSS, centrifuged at 1,400 × g for 10 minutes, resuspended in PBSS, calibrated to about 1 to 2 × 10^8 CFU/ml, and used in adherence assays. Bacterial suspensions in PBSS (without LF or milk) were included as parallel controls.

To test the effect of antibovine LF antibody on adherence, *S uberis* UT366 was pretreated with LF or milk and examined for adherence to MAC-T–cell monolayers in the presence of various dilutions (fivefold dilutions from 0 to 1:12,500) of rabbit antibovine LF antibody, produced as described.[7,8] The remainder of the procedure was the same as the adherence assay described previously. Each dilution was tested in 4 replicates.

Microscopic examination of bacterial adherence—Strains of *S uberis* were grown in THB for 5 hours at 37 C, washed once with PBSS, and resuspended in sterile sodium bicarbonate buffer (pH, 9.6) containing 0.5 mg of **fluorescein isothiocyanate (FITC)**/ml.[g] After 1 hour of labeling at room temperature, bacteria were washed twice with PBSS to remove free FITC, and FITC-labeled bacteria were resuspended in DMEM containing 10% FBS at a concentration of about 1 to 2 × 10^8 CFU/ml. Aliquots (0.15 ml) of sterile LF solution (1 mg/ml) in DMEM containing 10% FBS and 0.15 ml of calibrated bacterial suspensions were added to wells of 8-well chamber slides[h] containing confluent MAC-T cell monolayers. After incubation at 37 C in 5% CO_2 for 1 hour, bacterial supernatants were removed. Slides were washed 3 times with PBSS and examined under a microscope, using a mercury arc lamp as the UV light source.[i]

Hexadecane-based hydrophobicity test—Adhesion of *S uberis* to hydrophobic substrata *n*-hexadecane was tested by the method of Doyle and Rosenberg,[21] with some modifications to suit the microtitration plate-based photometric format. Briefly, strains of *S uberis* were grown in THB at 37 C for 10 hours. Bacteria were washed twice with 50 m*M* phosphate buffer containing 150 m*M* sodium chloride (pH, 7.2) and centrifuged at 1,400 × g for 10 minutes at room temperature. Bacterial density was adjusted to an absorbance of 0.8 to 0.9 at 450 nm with a 1 cm lightpath. A volume of 0.95 ml of each calibrated bacterial suspension was transferred to a 6-ml glass tube and overlaid with 0.05 ml of *n*-hexadecane.[f] Each mixture was vortexed uniformly for 2 minutes and left to stand for 20 minutes to allow the phases to separate. The aqueous phase was removed carefully and transferred in triplicate into wells (200 µl/well) on a 96-well microtitration plate. Absorbance was measured at 450 nm, using an automated reader.[j] Percentage reduction in absorbance of the aqueous phase was determined by considering initial absorbance of the suspensions prior to mixing as 100%. Each strain was tested in 4 replicates.

Binding of bacteria to purified LF and LF in milk—Strains of *S uberis* were grown in THB at 37 C for 10 hours. Bacterial cultures were centrifuged at 1,400 × g for 15 minutes, resuspended in PBSS, and split into 3 equal portions. After further centrifugation at 1,400 × g for 10 minutes, bacterial pellets were resuspended in 3 ml of LF (1 mg/ml of PBSS; portion 1), 3 ml of pooled sterile milk (portion 2), or 3 ml of PBSS (control; portion 3). Binding was allowed to proceed for 1 hour at room temperature, and samples were centrifuged at 1,400 × g for 15 minutes at 4 C. Bacterial pellets were washed 3 times with sterile PBSS and centrifuged at 14,000 × g for 10 minutes to minimize water content in the pellets before detergent extraction. Bacterial surface proteins from pellets were extracted using 0.2% **sodium dodecyl sulfate (SDS)** in PBSS (30 mg wet weight of bacteria per 100 µl of 0.2% SDS) at room temperature for 1 hour. Extraction mixtures were centrifuged at 14,000 × g for 10 minutes, and supernatant protein samples were stored at –20 C until use.

Bacterial proteins were electrophoresed on 10% polyacrylamide gels in the presence of SDS as described.[22] Separated proteins on gels were silver-stained, using the ammoniacal silver procedure,[23] or transferred to nitrocellulose membrane sheets for immunoblotting.[24,k] After being blocked with 3% casitone[a] in PBSS containing 0.05% Tween 20, blots were probed overnight at 4 C with rabbit antibovine LF antibody produced as described[7,8] (1:2,000 dilution in PBSS containing 0.05% Tween 20 and 0.1% casitone). Blots were washed 4 times with PBSS containing 0.05% Tween 20 and probed at room temperature for 1 hour with peroxidase-conjugated donkey antirabbit IgG antibody[l] (1:2,000 dilution in PBSS containing 0.05% Tween 20 and 0.1% of casitone). After another washing cycle, horseradish peroxidase activity on blots was visualized with 4-chloro-1-naphthol[m] as the substrate.

Statistical analyses—The Student *t*-test was used to compare adherence indices for control and test conditions.

Results

All 3 strains of *S uberis* were bound to purified LF and to LF in milk as revealed by probing immunoblots

with rabbit antibovine LF antibody (**Fig 1**). At concentrations > 0.1 mg/ml, LF significantly enhanced adherence of all 3 strains of *S uberis* to MAC-T cells (**Table 1**), whereas for *S uberis* UT366, addition of LF, even at a concentration of 0.01 mg/ml enhanced adherence to MAC-T cells. Addition of milk at concentrations > 12.5% to the medium enhanced adherence of *S uberis* UT366 and *S uberis* UT888 but significantly decreased adherence of *S uberis* UT102 (**Table 2**). Addition of LF

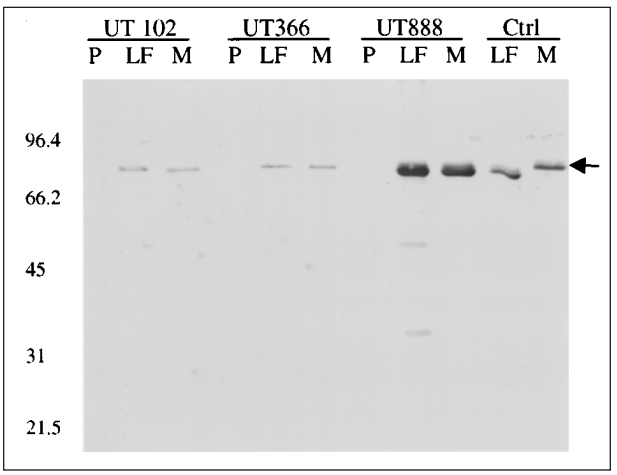

Figure 1—Binding of 3 strains of *Streptococcus uberis* (UT102, UT366, and UT888) to purified lactoferrin and lactoferrin in milk as detected by western blot. P = Bacteria incubated in phosphate-buffered saline solution as controls. LF = Bacteria incubated in 1 mg/ml of purified lactoferrin/ml. M = Bacteria incubated in 100% milk. Ctrl LF = Positive control consisting of 1 mg of purified lactoferrin/ml. Ctrl M = Positive control consisting of 100% milk. Rabbit antibovine lactoferrin antibody was used to identify lactoferrin bands in each lane (arrow).

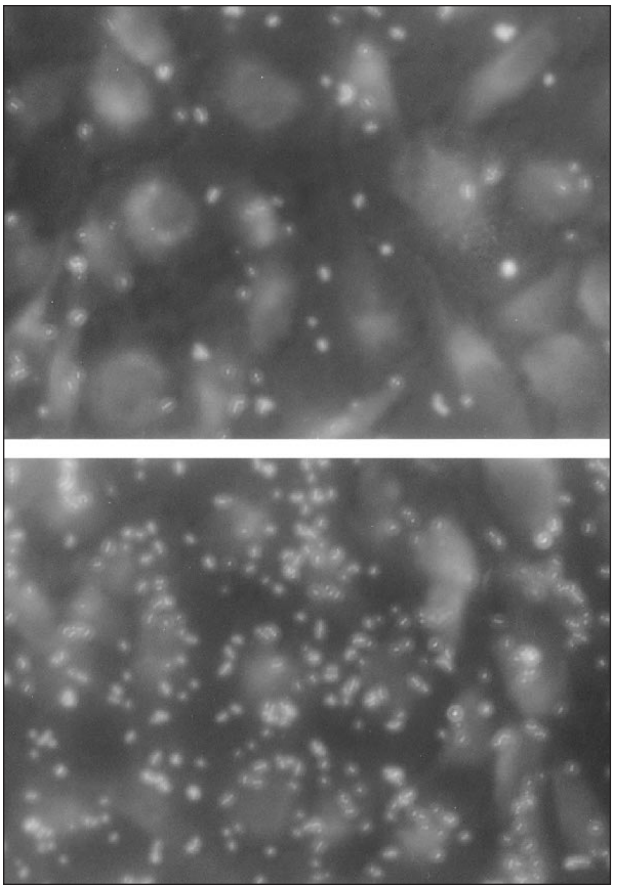

Figure 2—Adherence of fluorescein isothiocyanate-labeled *S uberis* UT102 to mammary epithelial cell monolayers without (top) or with (bottom) addition of lactoferrin to the culture medium.

Table 1—Effect of lactoferrin on adherence of 3 strains of *Streptococcus uberis* (UT102, UT366, and UT888) to bovine mammary epithelial cells

	Adherence index*					
	UT102		UT366		UT888	
Lactoferrin (mg/ml)	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2
0	15.7 ± 1.38	6.5 ± 1.85	6.6 ± 2.15	0.8 ± 0.05	23.3 ± 4.45	7.6 ± 1.4
0.01	23.9 ± 1.47†	8.4 ± 1.43	22.8 ± 4.12†	3.0 ± 1.01†	28.7 ± 1.64	9.7 ± 0.62
0.1	26.3 ± 3.94†	18.3 ± 2.6†	20.2 ± 3.2†	2.9 ± 0.37†	38.4 ± 1.74†	22.9 ± 2.76†
1.0	27.2 ± 2.41†	17.5 ± 2.69†	17.0 ± 0.58†	2.1 ± 0.45†	38.9 ± 2.56†	32.8 ± 1.98†

*Bacteria were cultured with mammary epithelial cell monolayers for 1 h. The culture supernatant was removed, and epithelial cells were lysed. Adherence index was calculated as number of colony-forming units (CFU) in the cell lysate divided by number of CFU in the supernatant times 10,000. †Significantly ($P \leq 0.01$) different from value obtained when samples were incubated without lactoferrin.

Data are given as mean ± SD for samples tested in triplicate; each experiment (Exp) was conducted twice.

Table 2—Effect of milk on adherence of 3 strains of *S uberis* to bovine mammary epithelial cells

	Adherence index*					
	UT102		UT366		UT888	
Milk (%)	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2
0	26.9 ± 1.6	28.3 ± 3.73	5.8 ± 0.12	6.8 ± 0.69	36.7 ± 1.69	94.6 ± 9.11
12.5	14.0 ± 1.05†	5.0 ± 1.01†	16.5 ± 0.78†	19.6 ± 2.06†	73.1 ± 6.46†	122.3 ± 15.8
25	15.9 ± 2.66‡	9.7 ± 1.02†	39.6 ± 3.29†	32.9 ± 6.91†	72.6 ± 6.72†	104.6 ± 8.36
50	19.7 ± 2.94‡	13.1 ± 0.40†	43.7 ± 5.68†	45.7 ± 3.60†	47.7 ± 5.53‡	90.3 ± 5.92

†Significantly ($P \leq 0.001$) different from value obtained when samples were incubated without milk. ‡Significantly ($P \leq 0.02$) different from value obtained when samples were incubated without milk.

Data are given as mean ± SD for samples tested in triplicate; each experiment (Exp) was conducted twice.

See Table 1 for remainder of key.

Table 3—Effect of pretreatment of 3 strains of *S uberis* with lactoferrin on adherence to bovine mammary epithelial cells

	Adherence index*					
	UT102		UT366		UT888	
Treatment	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2
PBSS	12.1 ± 5.53	10.4 ± 3.23	6.8 ± 1.48	3.6 ± 1.45	28.8 ± 3.63	48.1 ± 6.16
Lactoferrin	17.6 ± 3.13	10.5 ± 2.64	16.3 ± 5.29†	11.7 ± 2.37†	26.8 ± 4.44	47.4 ± 5.4

†Significantly ($P \leq 0.003$) different from value obtained when samples were not pretreated with lactoferrin.
Data are given as mean ± SD for samples tested in triplicate; each experiment (Exp) was conducted twice.
PBSS = Phosphate-buffered saline solution.
See Table 1 for remainder of key.

Table 4—Effect of pretreatment of 3 strains of *S uberis* with milk on adherence to bovine mammary epithelial cells

	Adherence index*					
	UT102		UT366		UT888	
Treatment	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2
PBSS	68.5 ± 6.13	55.3 ± 3.72	8.9 ± 1.17	15.6 ± 1.69	60.2 ± 5.57	82.5 ± 6.51
Milk	49.1 ± 8.61†	37.9 ± 5.36‡	22.3 ± 4.03‡	25.4 ± 4.4‡	42.6 ± 4.3‡	62.8 ± 4.36‡

†Significantly ($P \leq 0.01$) different from value obtained when samples were not pretreated with milk. ‡Significantly ($P \leq 0.004$) different from value obtained when samples were not pretreated with milk.
Data are given as mean ± SD for samples tested in triplicate; each experiment (Exp) was conducted twice.
PBSS = Phosphate-buffered saline solution.
See Table 1 for remainder of key.

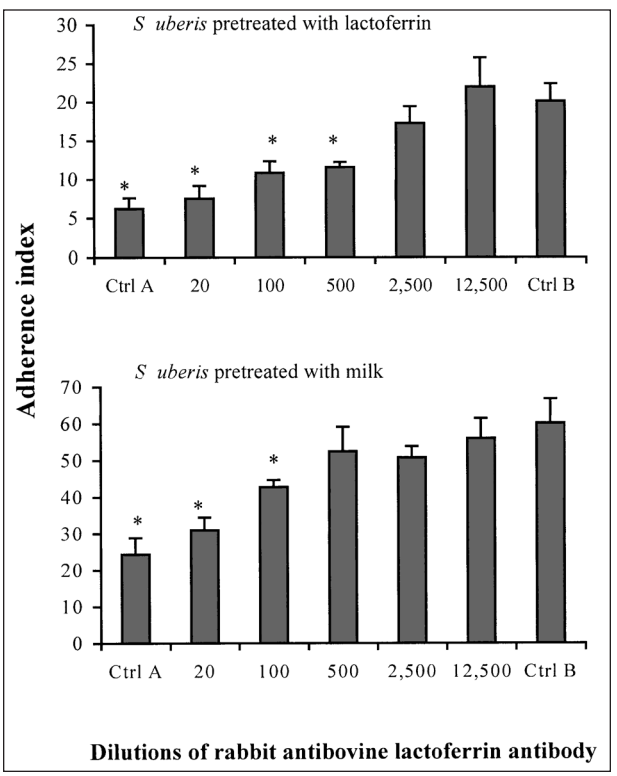

Figure 3—Adherence of *S uberis* UT366 that had been pretreated with purified lactoferrin (top) or milk (bottom) to mammary epithelial cells (MAC-T) in the presence of rabbit antibovine lactoferrin antibody. Ctrl A = Adherence of *S uberis* that had not been pretreated with lactoferrin or milk. Ctrl B = Adherence of *S uberis* that had been pretreated with lactoferrin or milk but without addition of rabbit antibovine lactoferrin antibody. Each bar represents mean ± SD for 4 replicates of each antibody dilution. * = Significantly ($P < 0.01$) less than Ctrl B value.

to the culture medium enhanced adherence of FITC-labeled *S uberis* to mammary epithelial cells (**Fig 2**).

Pretreatment of bacteria with purified LF enhanced adherence of *S uberis* UT366 to MAC-T cells but had no effect on *S uberis* UT102 and *S uberis* UT888 (**Table 3**). Pretreatment of bacteria with milk markedly increased adherence of *S uberis* UT366 to MAC-T cells but significantly decreased adherence of *S uberis* UT102 and *S uberis* UT888 (**Table 4**). Rabbit antibovine LF antibody was found to antagonize the LF-mediated increase in bacterial adherence to mammary epithelial cells. An antibody dilution of 1:500 had a significant antagonistic effect on adherence when bacteria were pretreated with LF (**Fig 3**), and a dilution of 1:100 had a significant antagonistic effect on adherence when bacteria were pretreated with milk. *Streptococcus uberis* UT888 had the highest adhesion capacity to hydrophobic hexadecane (87.7 ± 3.9%), followed by *S uberis* UT102 (29.6 ± 2.1%) and *S uberis* UT366 (15.1 ± 2.9%).

Discussion

As in another study,[6] *S uberis* was found to bind to purified LF and LF in milk. Addition of LF to the culture medium enhanced adherence of all 3 *S uberis* strains to mammary epithelial cells, but addition of milk enhanced adherence of *S uberis* strains UT366 and UT888 but decreased adherence of strain UT102. On the other hand, pretreatment of bacteria with LF or milk increased adherence of *S uberis* UT366 but decreased the adherence of *S uberis* UT102 and *S uberis* UT888.

Because LF also binds to mammary epithelial cells,[16] addition of LF to the culture medium may enhance the potential of LF to act as a bridging molecule between bacteria and MAC-T cells, thus increas-

ing adherence. Differences in intrinsic surface properties among strains of *S uberis* likely account for differences in adherence to MAC-T cells and interactions with LF, although it is not known whether LF could alter surface properties of the bacteria as it does with leukocytes.[18] We did find that these 3 *S uberis* strains varied in degree of hydrophobicity, as determined by adhesion to hexadecane. Differences in adherence among replicates were likely attributable to variations in the degree of confluence of MAC-T cell monolayers used or differences in number of bacteria inoculated.

The role that milk plays in adherence of *S uberis* to MAC-T cells may be more complicated than that of purified LF because of other milk components that also play a role in the interactions between bacteria and epithelial cells. The concentration of LF in the pooled milk sample, determined by use of an ELISA using purified LF as the reference standard, was 106 µg/ml, which should be considered normal for milk from lactating cows.[7] To confirm the specific role purified LF and LF in milk may play in adherence, we used *S uberis* UT366 as the model strain for use in adherence inhibition assays involving antibovine LF antibody, because this strain exhibited enhanced adherence after pretreatment with LF or milk. Rabbit antibovine LF antibody significantly decreased adherence of LF- or milk-pretreated bacteria to MAC-T cells, indicating that LF was specifically involved in adherence.

We have learned from our experimental challenge exposure studies that *S uberis* UT888 is a strain of moderate virulence that causes mild clinical mastitis. Conversely, *S uberis* UT366 is much more virulent and capable of inducing clinical mastitis with systemic signs, including fever.[19] However, *S uberis* UT102, although originally isolated from a cow with subclinical mastitis, appears to no longer be pathogenic after repeated subculturing. In this context, the negative impact of milk on adherence of *S uberis* UT102 to MAC-T cells is supporting evidence of its lack of pathogenicity. Bacteria of higher hydrophobicity seem to be more adherent to mammary epithelial cells, as seen with *S uberis* UT888. This is in agreement with findings by Calvinho et al[25] that a hydrophobic strain of *S dysgalactiae* was more adherent to bovine mammary epithelial cells than a hydrophilic one. In the present study, the most virulent strain, *S uberis* UT366, had the lowest hydrophobicity and was also less adherent to MAC-T cells than the other 2 strains of *S uberis*. We postulate that binding to LF could be one of the mechanisms that *S uberis* UT366 exploits to increase its pathogenicity in intramammary infections, because its adherence to mammary epithelial cells was greatly increased by addition of LF or milk to the culture medium and by pretreatment with LF or milk.

[a]Difco Laboratories Inc, Detroit, Mich.
[b]AcroDisc, Gelman Sciences, Ann Arbor, Mich.
[c]Provided by Dr. J. D. Turner, McGill University, Montreal, QC, Canada.
[d]Becton Dickinson Labware, Becton, Dickson & Co, Lincoln Park, NJ.
[e]Gibco BRL, Grand Island, NY.
[f]Sigma Chemical Co, St Louis, Mo.
[g]Polyscience Inc, Warrinton, Pa.
[h]Nalge Nunc International Corp, Naperville, Ill.
[i]BH-2, Olympus Optical Co Ltd, Tokyo, Japan.
[j]Microplate Autoreader EL 311, Bio-Tek Instruments Inc, Winooski, Vt.
[k]Semi-dry transfer cell, Bio-Rad Laboratories, Hercules, Calif.
[l]Jackson ImmunoResearch Laboratories Inc, West Grove, Pa.
[m]Bio-Rad Laboratories, Hercules, Calif.

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