

# *Lactobacillus reuteri* Protects Epidermal Keratinocytes from *Staphylococcus aureus*-Induced Cell Death by Competitive Exclusion

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Recent studies have suggested that the topical application of probiotic bacteria can improve skin health or combat disease. We have utilized a primary human keratinocyte culture model to investigate whether probiotic bacteria can inhibit *Staphylococcus aureus* infection. Evaluation of the candidate probiotics *Lactobacillus reuteri* ATCC 55730, *Lactobacillus rhamnosus* AC413, and *Lactobacillus salivarius* UCC118 demonstrated that both *L. reuteri* and *L. rhamnosus*, but not *L. salivarius*, reduced *S. aureus*-induced keratinocyte cell death in both undifferentiated and differentiated keratinocytes. Keratinocyte survival was significantly higher if the probiotic was applied prior to ( $P < 0.01$ ) or simultaneously with ( $P < 0.01$ ) infection with *S. aureus* but not when added after infection had commenced ( $P > 0.05$ ). The protective effect of *L. reuteri* was not dependent on the elaboration of inhibitory substances such as lactic acid. *L. reuteri* inhibited adherence of *S. aureus* to keratinocytes by competitive exclusion ( $P = 0.026$ ). *L. salivarius* UCC118, however, did not inhibit *S. aureus* from adhering to keratinocytes ( $P > 0.05$ ) and did not protect keratinocyte viability. *S. aureus* utilizes the  $\alpha 5\beta 1$  integrin to adhere to keratinocytes, and blocking of this integrin resulted in a protective effect similar to that observed with probiotics ( $P = 0.03$ ). This suggests that the protective mechanism for *L. reuteri*-mediated protection of keratinocytes was by competitive exclusion of the pathogen from its binding sites on the cells. Our results suggest that use of a topical probiotic prophylactically could inhibit the colonization of skin by *S. aureus* and thus aid in the prevention of infection.

Humans live in constant contact with a multitude of microorganisms. The gut is by far the most heavily colonized environment of the human body, and the gut microbiota play multiple roles in normal physiology, including nutrient sequestration (2) and development of normal immune responses (20).

Among the normal gut microbiota are the so-called probiotic bacteria. Ingestion of these has been claimed to prevent or treat gastrointestinal disorders such as antibiotic-associated diarrhea (45) and inflammatory bowel disease (40). The mechanisms underlying these effects are largely unknown. However, studies have suggested that probiotics can inhibit colonization of the gut by pathogens. *In vitro* work has suggested that probiotics use various mechanisms to inhibit pathogens, including direct competition for binding sites on epithelial cells (10) and competition with pathogenic bacteria for nutrients. Probiotic organisms are also able to produce inhibitory substances such as bacteriocins (11) and organic acids (23) that can kill or limit the growth of pathogens. Selected probiotics, such as *Lactobacillus plantarum* 299v, have been shown to upregulate mucin production by epithelial cells, thereby preventing pathogen attachment (31). Probiotics may also produce biosurfactants that allow attachment of the probiotic while inhibiting attachment of pathogenic bacteria to cells (41).

In contrast to the gut, very little is known about the normal interactions between the skin microflora and the epidermis. Recent work suggests that skin commensals may also be able to limit the colonization of the skin by pathogenic bacteria (22, 46). Studies also suggest that certain skin diseases (such as *acne vulgaris* and atopic dermatitis) may be associated with disruptions to the normal microflora (3, 5, 7). Therefore, the idea that the skin microflora may be modulated using specific skin commensals to promote health or inhibit disease has received some attention (28, 29). However, the skin commensal bacteria can also be pathogenic

under certain circumstances (14). By contrast, probiotics are generally regarded as safe (GRAS) and therefore could potentially be used topically if they have therapeutic value (15). So far, the limited amount of research in this area suggests that conventional probiotic bacteria may be of significant value when used on the skin. For example, topical application of a *Bifidobacterium longum reuter* lysate has been shown to induce clinical improvement of “reactive skin.” This is skin that is more sensitive to physical changes such as atmospheric temperature and to chemical changes such as seen with topically applied products (18). Application of the *B. longum* lysate to the skin of volunteers decreased sensitivity and transepidermal water loss (TEWL) after tape stripping. Additionally, application of the lysate to skin *ex vivo* reportedly decreased signs of inflammation such as vasodilation, edema, and tumor necrosis factor alpha (TNF- $\alpha$ ) release (18). Topical application of *Lactobacillus plantarum* has also been demonstrated to improve tissue repair in a burned mouse model and to prevent infection in chronic leg ulcers and burns in humans (38, 39, 44). However, in general, the mechanisms underlying these effects are unknown.

*Staphylococcus aureus* is a transient colonizer of skin predominantly in the moist, warm regions of the body such as the groin, axilla, and anterior nares (26). Up to 60% of people are intermit-

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tent carriers, while 20% of people may be stably colonized (26). While normal carriage is asymptomatic, *S. aureus* may invade tissues (e.g., through broken skin) where it causes diseases ranging from the relatively minor impetigo and scalded skin syndrome to life-threatening conditions such as septicemia (27). Furthermore, *S. aureus* infection is often a secondary phenomenon in skin with underlying conditions such as atopic dermatitis (25).

Since probiotics can inhibit pathogen attachment to epithelial cells in the gut, we considered that, if the same is true in skin, conventional probiotics such as lactobacilli may have utility in the prevention of infection. In this study, we have investigated the relative ability of three probiotic strains, *Lactobacillus reuteri* ATCC 55730, *Lactobacillus rhamnosus* AC413, and *Lactobacillus salivarius* UCC118, to inhibit the effects of *S. aureus* upon normal primary human epidermal keratinocytes (NHEK). We have shown that *L. reuteri* can protect NHEK from the effects of *S. aureus*. Furthermore, the mechanism appears to be through the ability of the probiotic to inhibit pathogen attachment to NHEK.

## MATERIALS AND METHODS

**Growth of bacteria.** Probiotic bacteria were grown routinely to stationary phase in Wilkins-Chalgren broth (WCB) (Oxoid) at 37°C in a Mark 3 anaerobic work station (Don Whitley Scientific, Shipley, United Kingdom). *S. aureus* was grown aerobically at 37°C in nutrient broth (Oxoid). Culture densities were adjusted spectrophotometrically with medium to contain the number of bacteria required. For experiments utilizing keratinocytes, bacteria were washed twice in 0.85% NaCl solution, centrifuged, and reconstituted in keratinocyte medium. Selective agar was used in some experiments. This was mannitol salt agar (MSA; Oxoid) or de Man-Rogosa-Sharpe agar (MRS) for *S. aureus* or lactobacilli, respectively. For experiments utilizing heat-killed bacteria, *L. reuteri* was centrifuged and resuspended in 0.4% glucose and heat inactivated by placing in a water bath at 85°C for 45 min. Samples were Gram stained to ensure that lysis of bacterial cells had not occurred and plated onto growth medium to ensure that they had been killed. For experiments using probiotic lysates, 10 ml of 10<sup>8</sup> CFU/ml *L. reuteri* was centrifuged, washed, concentrated in 1 ml keratinocyte medium, and lysed using a bead beater (FastPrep FP120; Thermo Electron Corporation). Samples were filter sterilized to remove any whole bacteria remaining. Approximately 100 µl of this lysate was used to treat keratinocytes.

**Mammalian cell culture.** Normal human epidermal keratinocytes (Promocell, Heidelberg, Germany) were maintained in keratinocyte basal medium (Promocell, Heidelberg, Germany) containing a supplement mix (bovine pituitary extract 0.004 mg/ml, epidermal growth factor [recombinant human] 0.125 ng/ml, insulin [recombinant human] 5 µg/ml, hydrocortisone 0.33 µg/ml, epinephrine 0.39 µg/ml, and holotransferrin [human] 10 µg/ml) and 0.06 mM CaCl<sub>2</sub> (Promocell, Heidelberg, Germany) without antibiotics. Medium was substituted twice weekly and cells were cultured at 37°C in a humid atmosphere of 5% CO<sub>2</sub>. Cells were detached once grown to 80% confluence and reseeded at approximately 5 × 10<sup>3</sup> cells/cm<sup>2</sup>. For experiments using differentiated cells, calcium chloride in the medium was increased to 1.8 mM, and the cells were grown in this for 24 h prior to experimentation.

**Bacterial competition assay.** Aliquots (100 µl) of overnight cultures of *L. reuteri* and *S. aureus* were inoculated into fresh 10-ml broths (both as axenic cultures and as cocultures). The pH and optical density of cultures was measured at 0 and 48 h. At regular intervals (3, 6, 24, 30, and 48 h), bacteria were counted by serial dilution plate counts using selective and nonselective agars. At 48 h, BacLite Live/Dead stains were performed according to the manufacturer's instructions (Invitrogen).

**Production of inhibitory substances by *L. reuteri*.** Inhibition of the growth of *S. aureus* by *L. reuteri* was determined using well diffusion assays, as described by Holder and Boyce (21). For inhibition studies, *L. reuteri* was grown in either MRS medium (Oxoid) or WCB (Oxoid) an-

aerobically at 37°C to stationary phase. Whole-cell (WC) cultures of *L. reuteri* grown in WCB (1 g/liter glucose) or MRS (20 g/liter glucose) were used. In other experiments, cells were sedimented in a microcentrifuge at 15,000 × g for 5 min, and the cell-free supernatant (CFS) was extracted for use. Well diffusion plates were incubated at 37°C for 48 h, and zones of inhibition were measured using a ruler. To measure the production of organic acids by *L. reuteri*, the pH of WC and CFS was measured. In a separate experiment to determine whether *L. reuteri* produced any other inhibitory substances, cell-free supernatants were neutralized using 1 M NaOH prior to use in the well diffusion assay. To determine if *L. reuteri* produced organic acids in cell culture, the pH of infected cell cultures was measured.

**Measurement of keratinocyte viability.** NHEK cells were grown in 24-well tissue culture plates to confluence. Cell culture medium was replaced with medium containing the indicated concentration of bacteria. For preexposure and postexposure experiments, NHEK were treated with *L. reuteri* for periods of between 1 and 4 h prior to the addition/after the addition of *S. aureus*. In all experiments, cells were incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> for 24 h. The medium was then removed and cells were washed with sterile phosphate-buffered saline (PBS). Cells were detached using trypsin (0.4%)-EDTA (0.3%) (Promocell), and cell viability was determined by trypan blue exclusion assay (43).

**Measurement of *S. aureus* viability.** To determine if *L. reuteri* or NHEK were able to inhibit the growth of *S. aureus* in cell culture, cells were grown to confluence in a 24-well plate. These were infected with either *S. aureus* or *S. aureus* and *L. reuteri* together. In parallel, wells without any cells also had these combinations added to determine the effect of the keratinocytes themselves on staphylococcal viability. After 24 h of exposure, the medium was removed and centrifuged to pellet extracellular bacteria. The cells were then trypsinized, and 500 µl of 0.25% Triton X-100 (Sigma-Aldrich) in PBS was added for approximately 30 min to lyse the keratinocytes. The well contents were then combined with the cell pellet and serial dilution plate counts using MSA performed to determine the total number of viable staphylococci.

**Adhesion assays for *L. reuteri*, *L. salivarius*, and *S. aureus*.** Confluent differentiating NHEK were exposed to either the probiotic or *S. aureus* for 1 h. After incubation, cells were washed three times in PBS to remove nonadherent bacteria. The cells were trypsinized and serial dilution plate counts were performed to assess the number of adherent bacteria. Selective agar was used for growth of staphylococci. In separate experiments, cells were exposed to *L. reuteri* 1 h before the addition of *S. aureus* (exclusion), at the same time (competition), or 30 min after *S. aureus* infection had begun (displacement) to determine whether *L. reuteri* could inhibit *S. aureus* adhesion to keratinocytes. To determine whether *L. reuteri* could inhibit invasion of keratinocytes by staphylococci, cells were exposed to either *S. aureus* or *S. aureus* and *L. reuteri* for 1 h. Cells were washed three times in sterile PBS to remove nonadherent bacteria, and the growth medium was replaced with medium containing 100 µg/ml gentamicin (Sigma-Aldrich) for 2 h. Cells were then washed three times in sterile PBS and trypsinized and lysed in 0.25% Triton X-100 for 30 min to release internalized bacteria. Bacteria in lysates were counted using serial dilutions. In experiments utilizing conditioned medium (CM), NHEK were exposed to *L. reuteri* and incubated for 1 h at 37°C in 5% CO<sub>2</sub>. The CM from the exposed cells was removed and centrifuged at 15,000 × g for 3 min to pellet the bacterial cells, and the CM filtered through a 0.22-µm-pore-size filter (Millipore). In experiments exploring the role of the α5β1 integrin in *S. aureus* adhesion, NHEK were preexposed to 60 µg/ml of an anti-α5β1 integrin antibody (Millipore) for 1 h prior to infection of cells. An alternative method for assessing the number of adherent bacteria was utilized for dead preparations of *L. reuteri*. Cells were grown on LabTek chamber slides (Thermo-Scientific). Adhesion assays were performed as usual, using adjusted amounts of bacteria. After the cells were exposed to bacteria, cells were washed twice in PBS and fixed in methanol for 20 min before performing a Gram stain. The number of adherent bacteria per 100

cells was then assessed using a Keyence All-in-One fluorescence microscope.

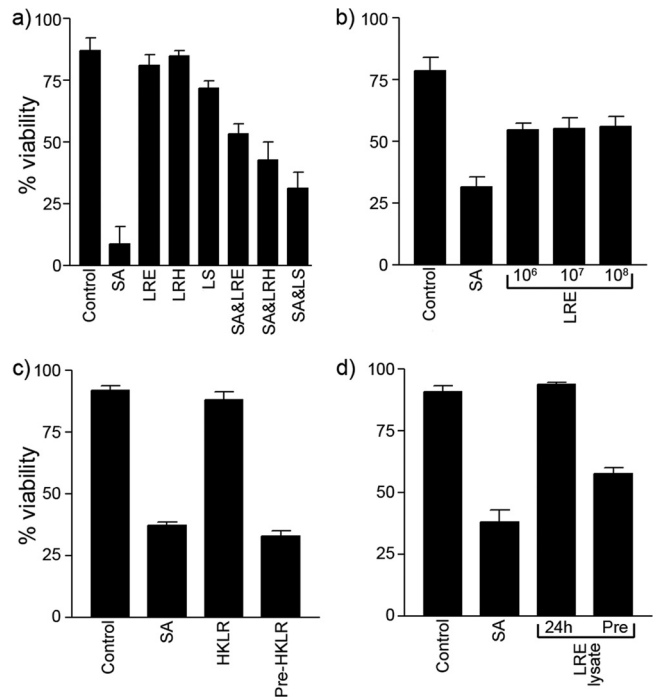
**Statistical analyses.** All experiments were performed a minimum of three times, with three replicates each time. For experiments comparing two treatments, Student's *t* test was used. For experiments comparing two or more treatments, a one-way analysis of variance (ANOVA) with blocking by experiment and *post hoc* Tukey's test was utilized. Dose-response and competition assays were analyzed using linear regression and 2-way ANOVA, respectively. Results were considered significant if *P* values were <0.05.

**RESULTS**

***S. aureus*-induced cell death in primary human keratinocytes.** We initially characterized the effects of *S. aureus* on keratinocyte viability by performing trypan blue exclusion assays on undifferentiated NHEK incubated with *S. aureus* at different doses. At a concentration of 10<sup>5</sup> CFU/ml *S. aureus*, only 49.4% of keratinocytes were viable after 24 h of infection (see Fig. S1 in the supplemental material). This was reduced in a dose-dependent manner such that only 3.3% of keratinocytes were viable at concentrations of 10<sup>8</sup> CFU/ml *S. aureus* (see Fig. S1). Approximately 10<sup>6</sup> organisms is a physiologically relevant concentration that might be found, e.g., in a skin wound infection (6). At this concentration, 30.5% of keratinocytes were viable after 24 h of infection. This concentration was therefore used in further assays.

***L. reuteri* protects keratinocytes from *S. aureus*-induced cell death.** The ability of three strains of lactobacilli (*L. reuteri* ATCC 55730, *L. rhamnosus* AC413, and *L. salivarius* UCC118) to protect keratinocytes from the effects of *S. aureus* was investigated (Fig. 1a). Infection of NHEK with 10<sup>8</sup> *S. aureus* cells reduced viability to 8.8%. In contrast to *S. aureus*, incubation of NHEK with 10<sup>8</sup> lactobacilli did not result in significant reduction in cell viability (Fig. 1a; also see Fig. S2 in the supplemental material). Coinfection with *S. aureus* and either *L. rhamnosus* or *L. reuteri* resulted in increased keratinocyte viability at 24 h of either 42.7% or 53.1% (*P* = 0.0012 and *P* < 0.0001), respectively. However, coinfection of keratinocytes with *S. aureus* and *L. salivarius* did not result in a significant elevation in viability compared to NHEK infected with *S. aureus* (*P* = 0.052) (Fig. 1a). Since *L. reuteri* was more protective than the other two strains, we further investigated the effects of *L. reuteri* by determining whether the protective effect of *L. reuteri* was dose dependent. However, there was no further significant increase in keratinocyte viability using *L. reuteri* at 10<sup>5</sup>, 10<sup>6</sup>, 10<sup>7</sup>, or 10<sup>8</sup> CFU/ml (*P* > 0.05) (Fig. 1b). Furthermore, the same level of protection was observed in cells that were completely undifferentiated (i.e., grown in low-calcium medium) or differentiated by growing them for 24 h in medium containing 1.8 mM calcium (see Fig. S3 in the supplemental material). We also investigated whether the protective effects of the probiotic were dependent on live bacteria. Although heat-killed *L. reuteri* did not protect NHEK from *S. aureus*, an *L. reuteri* lysate afforded a significant protective effect (*P* = 0.01) (Fig. 1c and d, respectively). Further experiments utilized 10<sup>8</sup> CFU/ml of live *L. reuteri* cells (multiplicity of infection [MOI] of 1,000).

**Protective effects of *L. reuteri* are time dependent.** We next investigated whether the timing of *L. reuteri* application relative to *S. aureus* infection was critical to the protective effect of the probiotic. We assessed the viability of differentiated keratinocytes exposed to *L. reuteri* 1, 2, or 4 h prior to or following the commencement of infection with *S. aureus* for 24 h. Preexposed keratinocytes had viabilities similar to those of coinfecting cells (*P* > 0.05) (Fig.

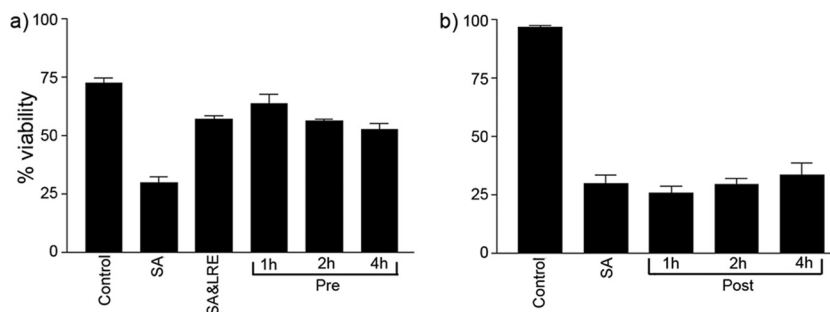


**FIG 1** Lactobacilli protect undifferentiated keratinocytes from the cytotoxic effects of *S. aureus*. (a) Percentage of viability for uninfected control NHEK (86.9% ± 5.1%) and NHEK infected with 10<sup>8</sup> CFU/ml of *S. aureus* (SA) (8.8% ± 7.1%), *L. reuteri* (LRE) (80.8% ± 4.5%), *L. rhamnosus* (LRH) (84.8% ± 2.1%), *L. salivarius* (LS) (71.7% ± 2.9%), *S. aureus* and *L. reuteri* (SA&LRE) (53.1% ± 4.2%), *S. aureus* and *L. rhamnosus* (SA&LRH) (42.7% ± 7.4%), and *S. aureus* and *L. salivarius* (SA&LS) (31.1% ± 6.5%). (b) Viability of cells infected with either 10<sup>6</sup> CFU/ml of *S. aureus* (SA) (31.6% ± 4.1%), *S. aureus* and 10<sup>6</sup> CFU/ml of *L. reuteri* (54.8% ± 2.7%), *S. aureus* and 10<sup>7</sup> CFU/ml of *L. reuteri* (55.4% ± 4.3%), and *S. aureus* and 10<sup>8</sup> CFU/ml of *L. reuteri* (56.2% ± 4.1%). (c) Keratinocytes exposed to *S. aureus* (SA) had a reduced viability of 37.4% ± 1.3% compared to controls (92% ± 1.9%), while keratinocytes exposed to heat-killed *L. reuteri* (HKLR) had little change to their viability (88.3% ± 3.3%). Keratinocytes preexposed to heat-killed *L. reuteri* (Pre-HKLR) had viabilities similar to those of *S. aureus*-exposed keratinocytes (33% ± 2.1%). (d) *L. reuteri* lysate (LRE lysate) protected keratinocytes, *P* = 0.01. Cells exposed to *S. aureus* (SA) alone had significantly lower viability (38.3% ± 4.7%) than cells preexposed to a lysate of *L. reuteri* (Pre) (57.7% ± 2.4%). Results are expressed as mean ± standard error of the mean (SEM).

2a). However, keratinocytes exposed to *L. reuteri* 1, 2, or 4 h after *S. aureus* infection had commenced had viabilities similar to those of cells exposed to *S. aureus* (*P* > 0.05) (Fig. 2b).

***L. reuteri* does not inhibit growth of *S. aureus*.** We hypothesized that the mechanism(s) underlying the protective effects of *S. aureus* might be due to direct effects of the probiotic on the pathogen. However, competition assays showed no significant reduction in either *L. reuteri* or *S. aureus* viability during coculture for 48 h (see Fig. S4 in the supplemental material) compared to axenic cultures (*P* > 0.05). Additionally, in keratinocyte culture assays, the total number of viable staphylococci was also unaffected by the presence of the probiotic *L. reuteri*. *S. aureus* grown in the absence of cells had a viable count of 8.0 (log CFU). *S. aureus* grown in the presence of differentiating keratinocytes had a viable count of 8.6 ± 0.2 (mean ± standard error of the mean [SEM]). *S. aureus* coincubated with *L. reuteri* in the absence of cells had a viable count of 8.4 ± 0.4, while *S. aureus* coincubated with *L. reuteri* in the presence of cells had a viable count of 8.0 ± 0.2. There was no

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**FIG 2** *L. reuteri* protects keratinocytes only if added prior to infection with *S. aureus*. (a) Percentage of viability was significantly higher in cells that were coinfecting (SA&LRE) ( $57.1 \pm 2\%$ ,  $P = 0.0016$ ) and preexposed (Pre) for 1 h ( $63.6 \pm 5.6\%$ ,  $P = 0.0003$ ), 2 h ( $56.1 \pm 1.2\%$ ,  $P = 0.0022$ ), and 4 h ( $52.4 \pm 4.1\%$ ,  $P = 0.0066$ ) with *L. reuteri* compared to *S. aureus* (SA)-infected cells ( $29.8 \pm 3.8\%$ ). (b) There was no significant difference between the viability of cells treated with *L. reuteri* 1 h ( $25.8 \pm 0.6\%$ ), 2 h ( $29.3 \pm 2.6\%$ ), or 4 h ( $33.6 \pm 4.9\%$ ) after infection had begun (Post) and cells that had been infected with *S. aureus* (SA) alone ( $29.9 \pm 3.4\%$ ) ( $P > 0.05$ ). Both experiments utilized differentiating NHEK. Results are expressed as the mean  $\pm$  SEM.

significant difference found between groups ( $P = 0.34$ ). A live-dead count of the coculture at 48 h also demonstrated no significant reduction in bacterial cell viability (data not shown). Furthermore, although we established that *L. reuteri* could produce organic acid that inhibited pathogen growth on agar plates (data not shown), when we measured the pH of keratinocyte media infected with *S. aureus*, *L. reuteri*, and both simultaneously for 24 h, there was no significant difference in the pH between treatments (see Fig. S5 in the supplemental material). However, since keratinocyte medium is buffered, acid could be produced but not in quantities sufficient to detect by measuring pH changes. Therefore, to eliminate the possibility of acid-induced *S. aureus* death, we compared the numbers of *S. aureus* in keratinocyte medium grown with or without *L. reuteri* (see above). There was no significant difference in numbers of *S. aureus* under these conditions.

***L. reuteri* inhibits adhesion and invasion of staphylococci to keratinocytes.** One of the mechanisms by which probiotics may protect gut epithelia is by inhibition of pathogenic adhesion to epithelial cells. Therefore, we explored whether *L. reuteri* could inhibit the adhesion of *S. aureus* to NHEK.

The graph in Fig. 3a demonstrates that both *L. reuteri* and *S. aureus* adhered to NHEK. Next we established whether *L. reuteri* could exclude or compete with *S. aureus* for binding sites by adding the probiotic either before or at the same time as the pathogen. The graphs in Fig. 3b and c demonstrate a reduction in adhesion of *S. aureus* to NHEK when *L. reuteri* was added before or at the same time as the addition of the pathogen ( $P = 0.026$  or  $P = 0.0078$ , respectively). However, if *L. reuteri* was added after *S. aureus*, the probiotic did not prevent the pathogen from adhering to NHEK ( $P > 0.05$ ) (Fig. 3d). Furthermore, gentamicin protection assays demonstrated that the probiotic inhibited *S. aureus* invasion of NHEK ( $P = 0.009$ ) (Fig. 3e). Our results showed that heat-killed *L. reuteri* did not significantly inhibit staphylococcal adhesion ( $P > 0.05$ ) (Fig. 4a). However, probiotic lysates were able to inhibit staphylococcal adhesion ( $P = 0.032$ ), though not to the same extent as live *L. reuteri* ( $P = 0.0002$ ). NHEK had approximately  $5.6 \pm 0.2$  log CFU staphylococci adhered when exposed to live *L. reuteri* compared to  $6.0 \pm 0.2$  log CFU when exposed to lysates (Fig. 4b).

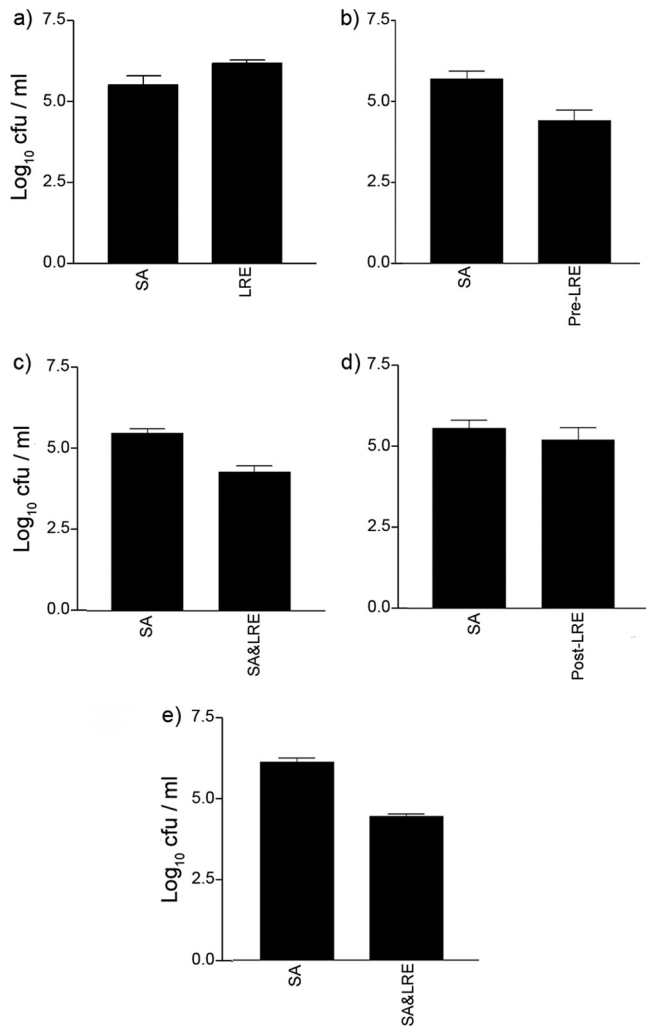
***L. salivarius* UCC118 does not prevent *S. aureus* from adhering to keratinocytes.** We considered that the protective effects of *L. reuteri* might be connected to its ability to inhibit pathogen adhesion to NHEK. To further explore this, we assessed whether

inhibition of *S. aureus* binding to NHEK resulted in a decrease in its toxicity. *S. aureus* can adhere to cells by means of a fibronectin-binding protein (FnBP) which interacts with extracellular fibronectin (24, 34). Fibronectin itself binds to the keratinocyte receptor, the  $\alpha 5\beta 1$  integrin. As shown in previous studies (25), pretreatment of cells with a blocking antibody to  $\alpha 5\beta 1$  integrin resulted in decreased *S. aureus* adhesion. Therefore, we treated NHEK with the  $\alpha 5\beta 1$  blocking antibody and investigated its ability to inhibit adherence and toxicity to NHEK (Fig. 5a and b). The blocking antibody significantly inhibited *S. aureus* adhesion to NHEK ( $P = 0.007$ ), while it also appeared to inhibit the ability of *S. aureus* to induce cell death in NHEK ( $P = 0.03$ ). Next, we investigated the ability of *L. salivarius*, which did not protect NHEK (Fig. 1a), to inhibit *S. aureus* adhesion. Keratinocytes exposed to *L. salivarius* and *S. aureus* were found to have similar numbers of adherent staphylococci ( $4.5 \pm 0.2$  log CFU) as keratinocytes exposed to *S. aureus* only ( $4.9 \pm 0.2$  log CFU) ( $P > 0.05$ ) (Fig. 6a). Furthermore, *L. salivarius* did not adhere to NHEK as efficiently ( $5.6 \pm 0.1$  log CFU) as *L. reuteri* ( $6.7 \pm 0.1$  log CFU) ( $P = 0.005$ ) (Fig. 6b).

## DISCUSSION

In this study, we have made a preliminary step toward characterizing the utility of probiotics for topical use. We have investigated the ability of three strains of lactobacilli with previously reported probiotic potential in the gut and mouth (8, 36, 42) to protect keratinocytes from the effects of the common skin pathogen *S. aureus*.

The application of *S. aureus* at physiologically relevant concentrations killed 69.5% of NHEK within 24 h. In contrast, none of the strains of lactobacilli significantly affected the viability of NHEK under the same conditions, suggesting that probiotics were well tolerated by keratinocytes. When NHEK were exposed to both pathogen and *L. reuteri*, the probiotic protected the cells from the effects of the pathogen, as indicated by the significantly higher percentage of viability of *S. aureus*-infected NHEK in the presence versus absence of *L. reuteri* (Fig. 1a; also see Fig. S2 in the supplemental material). The protective effect was also observed with lysates of *L. reuteri* but not heat-killed *L. reuteri* cells (Fig. 1d and e). Furthermore, the protective effects of the probiotic were variable between different species because the protection afforded by *L. reuteri* was greater than that provided by *L. rhamnosus*. In



**FIG 3** *L. reuteri* inhibits staphylococcal adhesion and invasion of keratinocytes. (a) *S. aureus* (SA) adhered at approximately  $5.5 \pm 0.3 \log_{10}$  CFU/ml and *L. reuteri* (LRE) adhered at approximately  $6.2 \pm 0.1 \log_{10}$  CFU/ml. (b) Exclusion. Cells preexposed to *L. reuteri* before *S. aureus* (Pre-LRE) infection had significantly fewer staphylococci adhered to them ( $4.4 \pm 0.4 \log_{10}$  CFU) than cells infected with *S. aureus* (SA) alone ( $5.7 \pm 0.2 \log_{10}$  CFU). (c) Competition. Cells coinfecting with *L. reuteri* (SA&LRE) had significantly fewer staphylococci adhered to them ( $4.4 \pm 0.4 \log_{10}$  CFU) than cells infected with *S. aureus* (SA) alone ( $5.6 \pm 0.1 \log_{10}$  CFU). (d) Displacement. There was no significant difference in the number of adherent staphylococci on cells exposed to *L. reuteri* after *S. aureus* infection had begun (Post-LRE) ( $5.3 \pm 0.5 \log_{10}$  CFU), compared to cells infected with *S. aureus* (SA) alone ( $5.7 \pm 0.3 \log_{10}$  CFU,  $P = 0.47$ ). (e) Cells coinfecting with *L. reuteri* had significantly fewer internalized staphylococci ( $4.5 \pm 0.1 \log_{10}$  CFU) than cells infected with *S. aureus* alone ( $6.1 \pm 0.1 \log_{10}$  CFU). All experiments utilized differentiating NHEK. Results are expressed as the mean  $\pm$  SEM.

contrast, *L. salivarius* did not provide keratinocytes with any significant protection from *S. aureus* (Fig. 1a).

While to our knowledge, this is the first evidence showing the ability of a probiotic to protect keratinocytes from the pathogenic effects of *S. aureus in vitro*, *L. fermentum* reportedly may inhibit *S. aureus* infection of surgical implants and abscess formation in rats (16). Probiotics have previously been shown to exert protective effects against pathogen-induced cell death in other epithelia. For example, *L. rhamnosus*, *L. reuteri*, and *L. oris* were shown to inhibit

*Streptococcus pyogenes*-induced cell death of pharyngeal epithelial cells (32). Probiotic cell-free supernatants have also been shown to be protective, since *L. delbrueckii* supernatants protected enterocytes from *Clostridium difficile* pathogenicity (4).

The protective effect of *L. reuteri* against *S. aureus*-induced cell death of keratinocytes could result from several different mechanisms. Lactobacilli produce organic acids and bacteriocins that can have direct antimicrobial effects on pathogens. Although we could demonstrate in diffusion assays that *L. reuteri* could produce acid, this does not appear to be responsible for the protective effects observed because the pH of the keratinocyte culture medium did not change in the presence of lactobacilli (see Fig. S5 in the supplemental material). Similarly, we did not detect any other antimicrobial activity since, in a competition assay, the growth rate and productivity of *S. aureus* in the presence of *L. reuteri* were identical to those of axenic cultures (see Fig. S4 in the supplemental material).

At least part of the mechanism by which *L. reuteri* protects keratinocytes appears to be linked to the ability of *L. reuteri* to prevent adhesion of *S. aureus*. Several lines of evidence point to this. First, *S. aureus* was less toxic to the cells when adhesion was inhibited using an integrin-blocking antibody (Fig. 5a and b). Second, lysates of *L. reuteri* that inhibit adhesion were also protective (Fig. 1d and 4b). On the other hand, heat-killed *L. reuteri* did not protect keratinocytes (Fig. 1c). Third, strains of lactobacilli that did not inhibit *S. aureus* adhesion (*L. salivarius*) (Fig. 6a) did not protect keratinocytes (Fig. 1a).

The timing of *L. reuteri* addition to the cells appears to be critical to its protective effects. When *L. reuteri* was added before or at the same time as *S. aureus*, NHEK were protected from the effects of the pathogen (Fig. 2a). However, if the probiotic was added after the pathogen, the levels of keratinocyte cell death were similar to that seen in cultures not treated with probiotic (Fig. 2b). Taken together, our data point to a mechanism of protection by *L. reuteri* involving competitive exclusion of *S. aureus* from keratinocyte binding sites.

How *S. aureus* induces cell death in keratinocytes is a complex process. In general, the first step in *S. aureus* pathogenesis is adherence to keratinocytes prior to invasion (25). Staphylococcal adhesins known to be involved in adhesion to keratinocytes include fibronectin-binding proteins (FnBPs) (25, 34), protein A, clumping factors, and coagulase (33). Teichoic acid mediates binding of *S. aureus* to nasal epithelial cells, though whether this is true for keratinocytes is not known (1). However, integrin blocking did not wholly inhibit binding of *S. aureus* to NHEK, suggesting that more than one adhesin is used (Fig. 5a).

Our study and others (25) have shown that blocking fibronectin receptors on the keratinocytes inhibits the adhesion of *S. aureus* (Fig. 5a). Interestingly, in separate experiments (results not shown), we could not show the involvement of this integrin in the binding of *L. reuteri* to NHEK. Indeed, neither protease nor glycosidase treatment of keratinocytes was able to prevent *L. reuteri* from binding to cells (data not shown). However, whatever the mechanism is by which the probiotic binds to cells, it involves a heat-labile molecule, as heat-killed *L. reuteri* do not adhere to keratinocytes as efficiently (see Fig. S6 in the supplemental material) or protect them from *S. aureus* (Fig. 1c).

To date, there are no published reports on the ability of probiotics to adhere to epidermal keratinocytes. Adhesion of probiotics to epithelia elsewhere has been suggested to occur via collagen

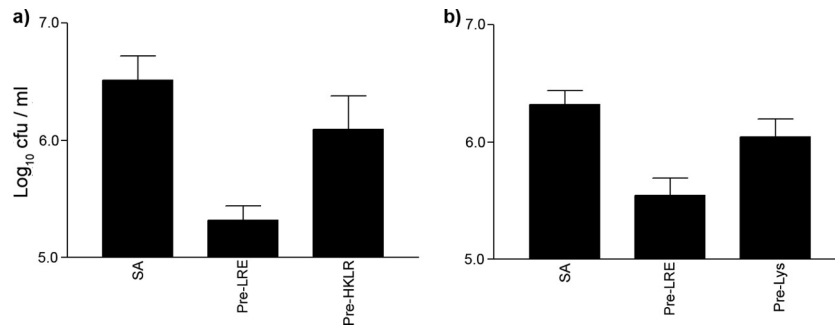


FIG 4 Heat-killed *L. reuteri* was not able to inhibit staphylococcal adhesion to keratinocytes, but lysates could. (a) The number of staphylococci adherent to NHEK when applied alone (SA) ( $6.5 \pm 0.2 \log_{10}$  CFU), when added after NHEK were preexposed to live *L. reuteri* (Pre-LRE) ( $5.3 \pm 0.1 \log_{10}$  CFU), and when preexposed to heat-killed *L. reuteri* (Pre-HKLR) ( $6.1 \pm 0.3 \log_{10}$  CFU). Cells exposed to untreated *L. reuteri* had significantly fewer staphylococci bound to them ( $P = 0.003$ ), but cells exposed to heat-killed *L. reuteri* had no significant difference in adherent staphylococci ( $P = 0.09$ ). (b) The number of staphylococci adherent to NHEK when applied alone (SA) ( $6.3 \pm 0.1 \log_{10}$  CFU) was significantly more than when they were added after NHEK were preexposed to live *L. reuteri* (Pre-LRE) ( $5.6 \pm 0.2 \log_{10}$  CFU,  $P = 0.0002$ ) and when preexposed to lysates of *L. reuteri* (Pre-Lys) ( $6.0 \pm 0.2 \log_{10}$  CFU,  $P = 0.032$ ). All experiments utilized differentiating NHEK. Results are expressed as the mean  $\pm$  SEM.

binding proteins (19), mucus binding proteins (35), lipoteichoic acids (17), and S-layer proteins (9, 12). The relevant adhesins may be proteinacious, carbohydrate, or a combination of the two, and more than one adhesin may be in use. *L. reuteri* inhibited staphylococcal adhesion to keratinocytes when cells were preexposed to the probiotic (exclusion of binding sites) (Fig. 3b) and coinfecting with *S. aureus* (competition for binding sites) (Fig. 3c) but not when applied after staphylococcal infection had begun (displacement from binding sites) (Fig. 3d). Others have shown that competitive inhibition/exclusion is often strain dependent, while displacement is generally not as effective or may require longer to occur (30, 47). Our results suggest that *L. reuteri* does not displace existing *S. aureus* from keratinocytes and support the finding that *L. reuteri* cannot protect keratinocytes from *S. aureus* cytotoxicity if added after infection has commenced. Some evidence has emerged to suggest that probiotics may have a synergistic effect on each others' adhesion, suggesting that an investigation into the effect of combinations of probiotics may be of interest in the future (37).

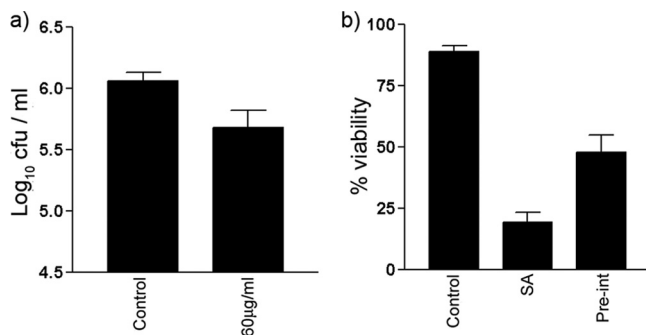


FIG 5 *S. aureus* utilizes  $\alpha 5\beta 1$  integrin to bind to keratinocytes, and blocking of this integrin is sufficient to protect keratinocytes from the effects of *S. aureus*. (a) Significantly fewer staphylococci adhered to cells treated with 60  $\mu\text{g/ml}$  blocking antibody ( $5.7 \pm 0.1 \log_{10}$  CFU) than to untreated control keratinocytes ( $6.1 \pm 0.1 \log_{10}$  CFU,  $P = 0.007$ ). (b) Significantly more keratinocytes were viable after infection with *S. aureus* for 24 h if preexposed to an anti- $\alpha 5\beta 1$  integrin (Pre-int) antibody prior to infection ( $47.9\% \pm 6.0\%$ ) compared to keratinocytes infected with *S. aureus* alone (SA) ( $19.3\% \pm 2.0\%$ ,  $P = 0.03$ ). All experiments utilized differentiating NHEK. Results are expressed as the mean  $\pm$  SEM.

It seems likely that *L. reuteri* employs multiple mechanisms to attach to host epithelia and may use several strategies to prevent *S. aureus* from adhering. It is possible that *L. reuteri* may influence the expression of adhesive structures on the surfaces of keratinocytes, an area which has yet to be explored. Previous work has shown that probiotic strains *L. rhamnosus* GG and *L. plantarum* 299v can induce upregulation of genes involved in mucin expression in the intestine, which in turn inhibits the adhesion of enteropathogenic *Escherichia coli* (31). In a study in 1999, application of the lactic acid bacterium *Streptococcus thermophilus* to keratinocytes *in vitro* and skin *in vivo* resulted in increased ceramide production (13).

We have provided evidence that a probiotic has a protective effect against the pathogenic effects of *S. aureus* on keratinocyte viability *in vitro*. These results, in agreement with those of others, suggest that a topical probiotic for skin could exclude pathogenic organisms and therefore prevent infections. The topical administration of *L. plantarum* to burns in mice has been shown to inhibit *Pseudomonas aeruginosa* colonization of burns, promote healing, and modify the inflammatory response (44). This organism has also been compared to and found to be as effective as traditional silver sulphadiazine treat-

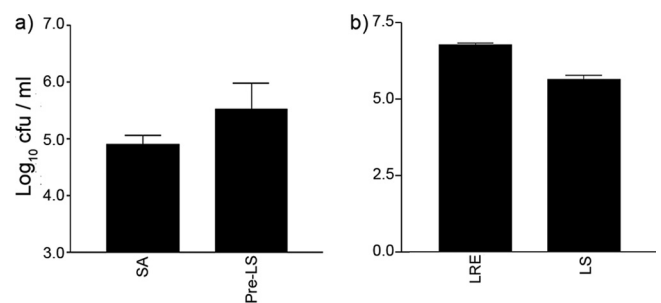


FIG 6 *L. salivarius* was not able to inhibit staphylococcal adhesion to keratinocytes. (a) Keratinocytes preexposed to *L. salivarius* (Pre-LS) had a similar number of adherent staphylococci ( $5.5 \pm 0.5 \log_{10}$  CFU) as cells exposed to *S. aureus* alone (SA) ( $4.9 \pm 0.2 \log_{10}$  CFU). (b) *L. reuteri* (LRE) adhered significantly better ( $6.7 \pm 0.1 \log_{10}$  CFU) than *L. salivarius* (LS) ( $5.6 \pm 0.1 \log_{10}$  CFU,  $P = 0.005$ ). All experiments utilized differentiating NHEK. Results are expressed as mean  $\pm$  SEM.

ments of burn wounds for limiting the bacterial load of wounds and healing *in vivo* (39). While further investigations are required to determine the full effect probiotics have on the skin, our findings show that *L. reuteri* can protect keratinocytes from the pathogenic effects of *S. aureus* through exclusion and competition for binding sites. We suggest that the prophylactic use of probiotics or their lysates in barrier creams and soaps may aid in the fight against staphylococcal skin colonization and subsequent infections.

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