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Lactobacillus rhamnosus GG Inhibits the Toxic Effects of *Staphylococcus aureus* on Epidermal Keratinocytes

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Few studies have evaluated the potential benefits of the topical application of probiotic bacteria or material derived from them. We have investigated whether a probiotic bacterium, *Lactobacillus rhamnosus* GG, can inhibit *Staphylococcus aureus* infection of human primary keratinocytes in culture. When primary human keratinocytes were exposed to *S. aureus*, only 25% of the keratinocytes remained viable following 24 h of incubation. However, in the presence of 10^8 CFU/ml of live *L. rhamnosus* GG, the viability of the infected keratinocytes increased to 57% ($P = 0.01$). *L. rhamnosus* GG lysates and spent culture fluid also provided significant protection to keratinocytes, with 65% ($P = 0.006$) and 57% ($P = 0.01$) of cells, respectively, being viable following 24 h of incubation. Keratinocyte survival was significantly enhanced regardless of whether the probiotic was applied in the viable form or as cell lysates 2 h before or simultaneously with ($P = 0.005$) or 12 h after ($P = 0.01$) *S. aureus* infection. However, spent culture fluid was protective only if added before or simultaneously with *S. aureus*. With respect to mechanism, both *L. rhamnosus* GG lysate and spent culture fluid apparently inhibited adherence of *S. aureus* to keratinocytes by competitive exclusion, but only viable bacteria or the lysate could displace *S. aureus* ($P = 0.04$ and 0.01, respectively). Furthermore, growth of *S. aureus* was inhibited by either live bacteria or lysate but not spent culture fluid. Together, these data suggest at least two separate activities involved in the protective effects of *L. rhamnosus* GG against *S. aureus*, growth inhibition and reduction of bacterial adhesion.

The concept that probiotics are beneficial to gut health has been investigated for a number of years. Studies have demonstrated that probiotics improve gut function potentially through a number of mechanisms (1, 2), including increasing epithelial barrier function (3) and modulation of the immune response (4–6). There is also evidence that probiotics can prevent colonization of the gut by pathogens. This can be via mechanisms such as downregulation of virulence factors and inhibition of pathogen adherence to the epithelium (7–9). For example, *Lactobacillus* species inhibit the adhesion of *Enterobacter sakazakii* to intestinal mucus by competitive exclusion (8, 10). Other studies demonstrated that some probiotics increase the production of intestinal mucin, thus inhibiting pathogen adherence to intestinal epithelial cells (11). Probiotics are also able to produce antimicrobial peptides (bacteriocins) and acids. Collectively, there are numerous probiotic-mediated mechanisms that limit pathogen colonization (1, 12–14).

Since probiotics may have positive impacts on the gut, their potential effects on other systems, such as the mouth (15, 16) and the urogenital tract (17), have also been investigated. For example, a study in 2002 examined the impact of oral administration of *Lactobacillus plantarum* to patients who had abdominal surgery and showed that this bacterium lowered the incidence of postsurgical infection (18). Currently, research is also investigating the topical use of probiotics to augment the skin barrier function to promote skin health or prevent or treat disease (9, 19–24). The benefits of topical application of probiotics are still speculative, and researchers are now focusing on this area to improve conditions such as excessive skin sensitivity, atopic dermatitis, and psoriasis and to stimulate the wound healing process (19, 25–29). However, an important consideration will be the safety of using live bacteria, especially in situations where the skin barrier is breached. For this reason, many investigators have used bacterial lysates in their studies. Topical application of sonicated *Streptococcus thermophilus* strains to patients suffering from atopic dermatitis resulted in improved barrier function apparently through increasing the level of ceramides in the stratum corneum (26). The topical application of *Lactobacillus plantarum* lysate inhibited the pathogenic activity of *Pseudomonas aeruginosa* in infected burns (29). *In vivo*, *L. plantarum* lysate has also been shown to improve wound healing in burn patients (30).

Staphylococcus aureus is both a transient colonizer of skin and a major opportunistic skin pathogen, causing diseases ranging from impetigo to life-threatening conditions such as sepsis (31, 32). Previously, our laboratory demonstrated that the probiotic *Lactobacillus reuteri* or its lysate could protect epidermal keratinocytes from the toxic effects of *S. aureus* via competitive exclusion of the pathogen from keratinocyte binding sites (33). In the present study, we have identified *Lactobacillus rhamnosus* GG as a second probiotic with the ability to protect skin cells from the effects of *S. aureus*. The selection of *L. rhamnosus* GG was based on the results of a screening assay testing a range of probiotics for their ability to protect human keratinocytes from the effects of *S. aureus* (data not shown). In this assay, *L. rhamnosus* GG proved to be extremely efficacious either live or as a lysate and uses multiple mechanisms to protect against infection, including inhibition of *S. aureus* growth, competitive exclusion, and displacement of the pathogen from keratinocytes.

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MATERIALS AND METHODS

Mammalian cell culture. Normal human epidermal keratinocytes (NHEK) cultured 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 holo-transferrin [human], 10 μg/ml) and 0.06 mM $CaCl_2$ (Promocell) were used as a model system. These were cultured routinely at 37°C in a humid atmosphere of 5% CO_2 in T-75 culture flasks as described previously (33).

Bacterial cell culture. *Lactobacillus rhamnosus* Goldin and Gorbach (*L. rhamnosus* GG; ATCC 53103), *Lactobacillus reuteri* (ATCC 55730), and *Lactobacillus salivarius* (UCC118) (ATCC, Middlesex, United Kingdom) were grown routinely in Wilkins-Chalgren broth or agar (Oxoid, Basingstoke, United Kingdom) at 37°C in an anaerobic cabinet (atmosphere,10:10:80 H_2-CO_2-N_2). *Staphylococcus aureus* was grown aerobically at 37°C in nutrient broth (Oxoid) as described previously (33).

Treatment of keratinocytes with bacteria. Bacteria (10^8 CFU/ml of probiotics and 10^6 CFU/ml of *S. aureus*) were centrifuged at 15,000 × *g*, washed twice in 0.85% NaCl, and resuspended in keratinocyte basal medium. This suspension was added directly to 5×10^3 cells/cm^2 of NHEK growing in 24-well plates. For experiments using a probiotic lysate, 100 ml of 10^8-CFU/ml *L. rhamnosus* GG was centrifuged, washed, resuspended in 25 ml of phosphate-buffered saline (PBS, pH 7.4; Invitrogen, Life Technologies Ltd., Paisley, United Kingdom), and lysed using an MSE Soniprep 150. Samples were filtered using a 0.22-μm-pore filter (Millipore, Billerica, MA) to remove any whole bacteria remaining. Approximately 100 μl of this lysate was used to treat keratinocytes (5×10^5 cells/cm^2). In some experiments, cells were sedimented in a centrifuge at 15,000 × *g* for 5 min, and the cell-free supernatant (spent culture fluid) was collected and filtered using a 0.22-μm-pore filter (Millipore) to remove any whole bacteria remaining. In other experiments, keratinocyte monolayers were coinfected with pathogen plus probiotics or lysates simultaneously. In separate experiments, cells were exposed to *L. rhamnosus* GG lysate for 2, 4, 6, 8, and 12 h after *S. aureus* infection had commenced. In all experiments, keratinocytes were detached and cell viability was determined using trypan blue exclusion assays as described in reference 33. In other experiments using heated lysates, these were heat inactivated by placing them in a boiling water bath at 100°C for 5 min.

Measurement of *S. aureus* viability in cell culture. To determine whether *L. rhamnosus* GG lysates or spent culture fluid was able to inhibit the growth of *S. aureus* in cell culture, keratinocytes were grown to confluence in a 24-well plate. These were exposed to 100 μl of 10^6-CFU/ml *S. aureus* alone or *S. aureus* plus 100 μl of *L. rhamnosus* GG lysates or 100 μl of spent culture fluid. In separate experiments, cells were exposed to *L. rhamnosus* GG lysates for 2, 4, 6, 8, and 12 h after infection with *S. aureus*. The total number of viable staphylococci was determined by counting the colonies as described previously (33).

Measurement of bacterial adhesion to keratinocytes. Confluent keratinocytes were exposed to 10^6 CFU/ml of *S. aureus* and 10^8 CFU/ml of *L. rhamnosus* GG for 1 h. Cells were then washed three times in PBS (pH 7.4) to remove nonadherent bacteria. The cells were trypsinized and serial dilution plate counts performed to assess the number of adherent bacteria. Selective agar was used for growth of staphylococci. Additionally, keratinocytes were exposed to 10^6 log CFU/ml of *S. aureus* combined with 100 μl of lysate or spent culture fluid of *L. reuteri* or *Lactobacillus salivarius* UCC118. The experiment was carried out three times and results were taken as triplicates.

In separate experiments, cells were exposed to 100 μl of 10^8-CFU/ml probiotic bacteria or lysates or spent culture fluid for 1 h before the addition of 100 μl of 10^6-CFU/ml *S. aureus* at the same time or 2, 4, 6, 8, and 12 h after infection with *S. aureus*.

Determination of bacterial antagonism. A 10-μl aliquot of an overnight culture of *S. aureus* was inoculated into 7 ml of soft agar medium (0.7% agar) and was added directly onto plates prepoured with agar base. A volume of 50 μl of live organisms or 50 μl of lysate extracted from 10^8 CFU/ml of *L. rhamnosus* GG or *L. reuteri* cultures was spotted onto an *S. aureus* lawn. The inhibition zone was evaluated after overnight incubation by measuring the diameter of the zone in millimeters using a ruler.

Determination of the outcome of coculture (competition assays). Aliquots (100 μl) of *L. rhamnosus* GG lysates and 100 μl of 10^6-CFU/ml *S. aureus* were inoculated into 10-ml Wilkins-Chalgren broth. The pH and optical density of cultures were measured at 0 and 24 h. At regular intervals (indicated below), bacteria were counted by serial dilution plate counts using selective agar.

Statistical analyses. All experiments were performed a minimum of three times, with three replicates within each experiment. Data generated were analyzed by one-way analysis of variance (ANOVA) and *post hoc* Tukey test using SPSS (IBM SPSS Statistics version 16.0) program. Results were considered significant if the *P* value was <0.05. Data are expressed as means ± standard errors of the means (SEM).

RESULTS

***L. rhamnosus* GG protects keratinocytes from the pathogenic effects of *S. aureus*.** Initially, we investigated whether the viability of keratinocytes was affected by incubation with *L. rhamnosus* GG. However, following 24 h of incubation, there was no difference in the viability of keratinocytes incubated with the probiotic bacteria versus the control of untreated keratinocytes (data not shown). Next, the ability of *L. rhamnosus* GG to protect keratinocytes from the effects of *S. aureus* was investigated. In agreement with our previous findings (33), 24 h of exposure of keratinocytes to 10^6 CFU/ml of *S. aureus* resulted in significant keratinocyte death. However, keratinocytes incubated simultaneously with the pathogen and *L. rhamnosus* GG had a significantly higher viability (57%; $P = 0.01$) than monolayers infected with the pathogen alone (Fig. 1A).

We investigated whether viable bacteria were essential for the protective effect of *L. rhamnosus* GG by examining the effect of probiotic lysate and spent culture fluid on *S. aureus*-infected keratinocytes. Neither lysate nor spent culture fluid significantly affected the viability of keratinocytes ($P > 0.05$) (data not shown). However, both the lysate and spent culture fluid reduced the toxicity of *S. aureus* such that the viabilities of treated keratinocytes were 65% and 55.93%, respectively, compared to 25% in keratinocytes infected with *S. aureus* alone ($P = 0.006$ and $P = 0.01$, respectively) (Fig. 1B). This is in contrast to the effects observed with *L. reuteri*, which we showed previously to be protective to pathogen-infected keratinocytes (33). *L. reuteri* provides protection only when added either live or as a lysate, but the spent culture fluid has no ability to protect keratinocytes from the effects of *S. aureus* (Fig. 1C).

***L. rhamnosus* GG lysate but not spent culture fluid rescues keratinocytes from *S. aureus* toxicity.** We next investigated the timing of the protective effect of *L. rhamnosus* GG by adding the live bacteria, the lysate, or the spent culture fluid either before or after infection of keratinocytes with *S. aureus*. The percentage of keratinocytes remaining viable was significantly greater in monolayers exposed to *L. rhamnosus* GG for 2 h prior to infection with *S. aureus* than in monolayers infected with *S. aureus* alone ($P = 0.006$). The lysate and spent culture fluid afforded similar levels of protection ($P = 0.005$ and $P = 0.004$) (Fig. 2A). In postexposure experiments, keratinocytes were exposed to *S. aureus* for 2 h, 4 h, 6 h, 8 h, and 12 h before addition of the live *L. rhamnosus* GG, lysate, or spent culture fluid. The viability of the keratinocytes was then measured at 24 h after infection with *S. aureus*. The data in

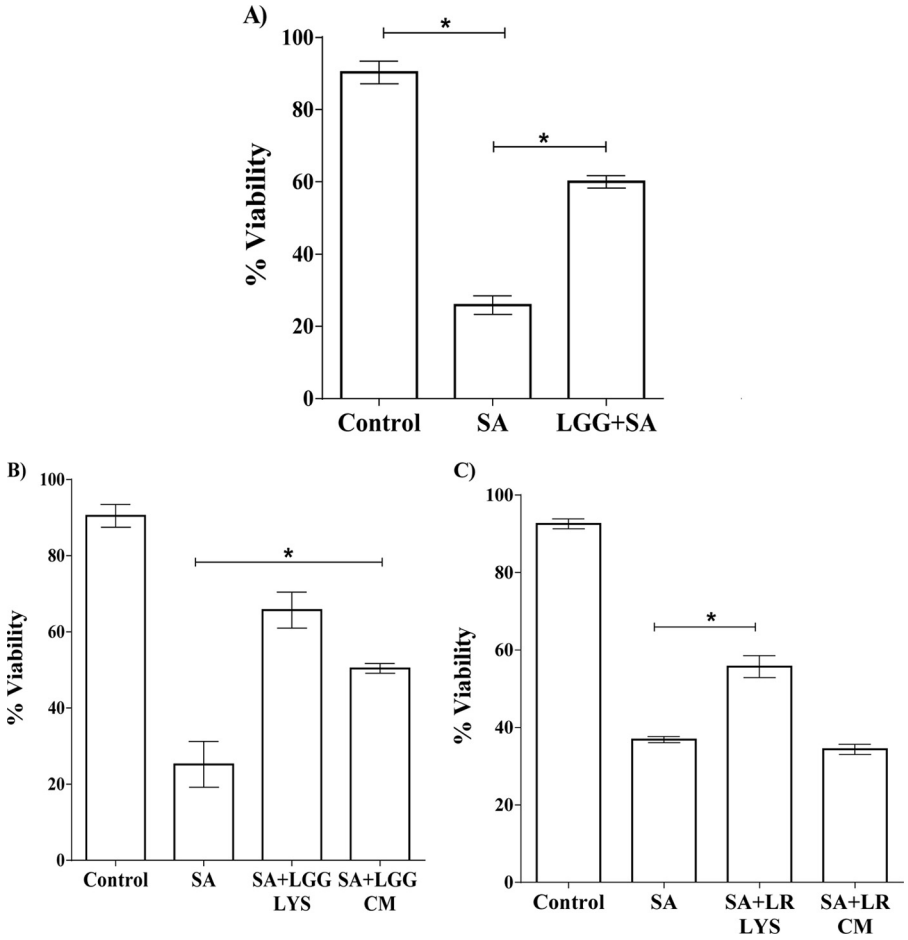

FIG 1 (A) *L. rhamnosus* GG, lysate, or spent culture fluid protects keratinocytes from the toxic effects of *S. aureus*. A combination of *S. aureus* (SA) and *L. rhamnosus* GG (LGG+SA) resulted in a significantly higher ($P = 0.01$) percentage of viable keratinocytes after 24 h than in monolayers infected with *S. aureus* alone. The data were compared to those produced by uninfected control cells (control). (B) The viability of *S. aureus*-infected keratinocytes treated with *L. rhamnosus* GG lysate (SA+LGG LYS) or spent culture fluid (SA+LGG CM) was significantly increased compared to that of keratinocytes infected with *S. aureus* (SA) alone. (C) Monolayers exposed to *S. aureus* and a lysate of *L. reuteri* (SA+LR LYS) had a significantly higher percentage of viable keratinocytes than those infected with pathogen alone, but the same effect was not found with the spent culture fluid of *L. reuteri* (SA+LR CM). Data are representative of three individual experiments, and all values represent means ± SEM. *, $P < 0.05$.

Fig. 2B and C show that both live probiotic and its lysate could protect the keratinocytes when added after *S. aureus*. Even at 12 h after *S. aureus* infection, *L. rhamnosus* GG or lysate still afforded protection to the keratinocytes such that 58% and 55%, respectively, of cells remained viable, compared to 25% when exposed to *S. aureus* alone ($P = 0.003$ and $P = 0.01$, respectively). However, the spent culture fluid from *L. rhamnosus* GG had no protective effect on keratinocytes when added after *S. aureus* (Fig. 2D).

***L. rhamnosus* GG lysate, but not spent culture fluid, inhibits the growth of *S. aureus*.** We investigated whether the probiotic lysate had direct effects on the growth of the pathogen by growing them simultaneously in culture. Competition assays showed a significant reduction in *S. aureus* growth over a period of 24 h in the presence of 100 μl of *L. rhamnosus* GG lysate compared to untreated cultures ($P = 0.02$) (Fig. 3A). This effect was specific to the lysate, because the spent culture fluid from *L. rhamnosus* GG had no effect on the growth of *S. aureus* (Fig. 3B). Furthermore, the ability of the lysate to inhibit pathogenic growth was negated by heating the lysate to 100°C for 10 min (Fig. 3C). Finally, this direct effect of *L. rhamnosus* GG on pathogenic growth appeared to be species specific, because the lysate from *L. reuteri* made in exactly the same way had no effect on the growth of *S. aureus* (Fig. 3D).

We determined the numbers of viable staphylococci following 24 h of incubation with keratinocytes in the presence or absence of the *L. rhamnosus* GG lysate. When *S. aureus* was added to keratinocytes at the same time as the *L. rhamnosus* GG lysate, the total number of viable staphylococci was also significantly reduced, to 5 $\log_{10}$ CFU/ml (Fig. 4), compared to 8 $\log_{10}$ CFU/ml for *S. aureus* alone ($P = 0.02$) (Fig. 5). Furthermore, when the *L. rhamnosus* GG lysate was added 12 h after infection of the keratinocytes, a reduction in number of viable *S. aureus* organisms was observed when these were counted 24 h later (Fig. 4). These effects were not seen with either the spent culture fluid from *L. rhamnosus* GG or a lysate from *L. reuteri* (data not shown). Since lactobacilli can produce organic acids, we measured the pH of keratinocyte media infected for 24 h with *S. aureus*, *L. rhamnosus* GG lysate, or both simultaneously. However, there was no significant difference in the pH between treatment groups (data not shown). We also measured the pH of lysate alone and found it be 7.2, thus suggesting that acid-mediated effects were not likely to be the mechanism

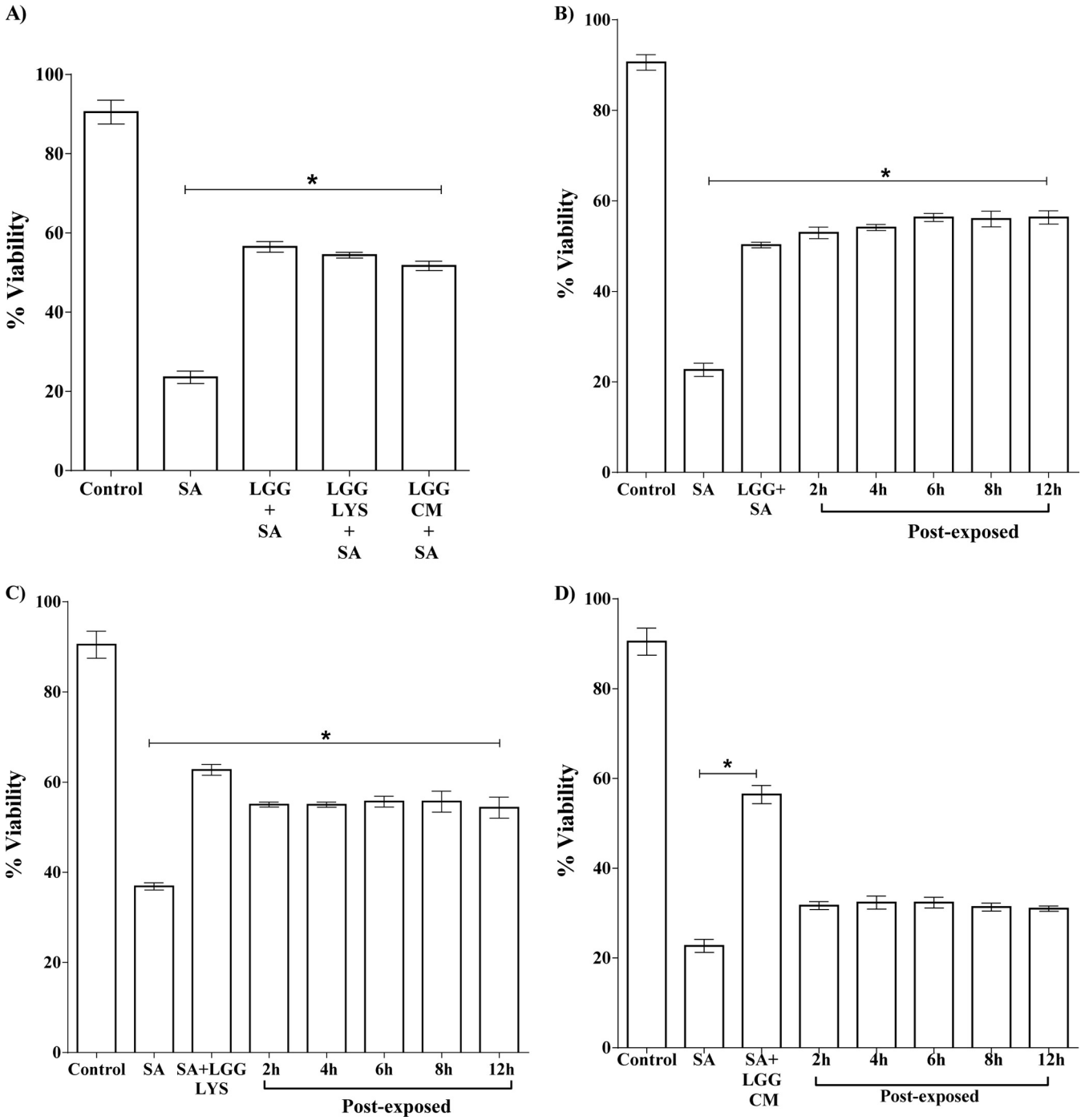

FIG 2 *L. rhamnosus* GG protects and rescues keratinocytes from infection with *S. aureus*. (A) The percent viability of infected keratinocytes was significantly higher in cells that were preexposed to *L. rhamnosus* GG (LGG+SA), lysate (LGG LYS+SA), or spent culture fluid (LGG CM+ SA) than that of *S. aureus* (SA)-infected cells. (B) The viability of *S. aureus*-infected keratinocytes was significantly higher in cells exposed to *L. rhamnosus* GG 12 h after infection with *S. aureus* ("Post-exposed"). A similar effect was observed with lysate (C). However, cells postexposed to *L. rhamnosus* GG spent culture fluid (CM) did not have significant protection (D). Data are representative of three individual experiments, and all values represent means ± SEM ($n = 3$). *, $P < 0.05$.

underlying inhibition of pathogenic growth. The antimicrobial properties of *L. rhamnosus* GG and lysate were evaluated using a spot-on-lawn assay. This assay showed significant inhibition of *S. aureus* growth (as evidenced by the presence of zones of inhibition) by anaerobic live cultures or lysates of *L. rhamnosus* GG grown anaerobically (Table 1). In contrast, live *L. reuteri* or lysate did not induce zones of inhibition in this assay (Table 1).

***L. rhamnosus* GG inhibits adhesion of *S. aureus* to keratinocytes.** Another mechanism by which live bacteria, lysate, or spent culture fluid of *L. rhamnosus* GG may protect keratinocytes is by

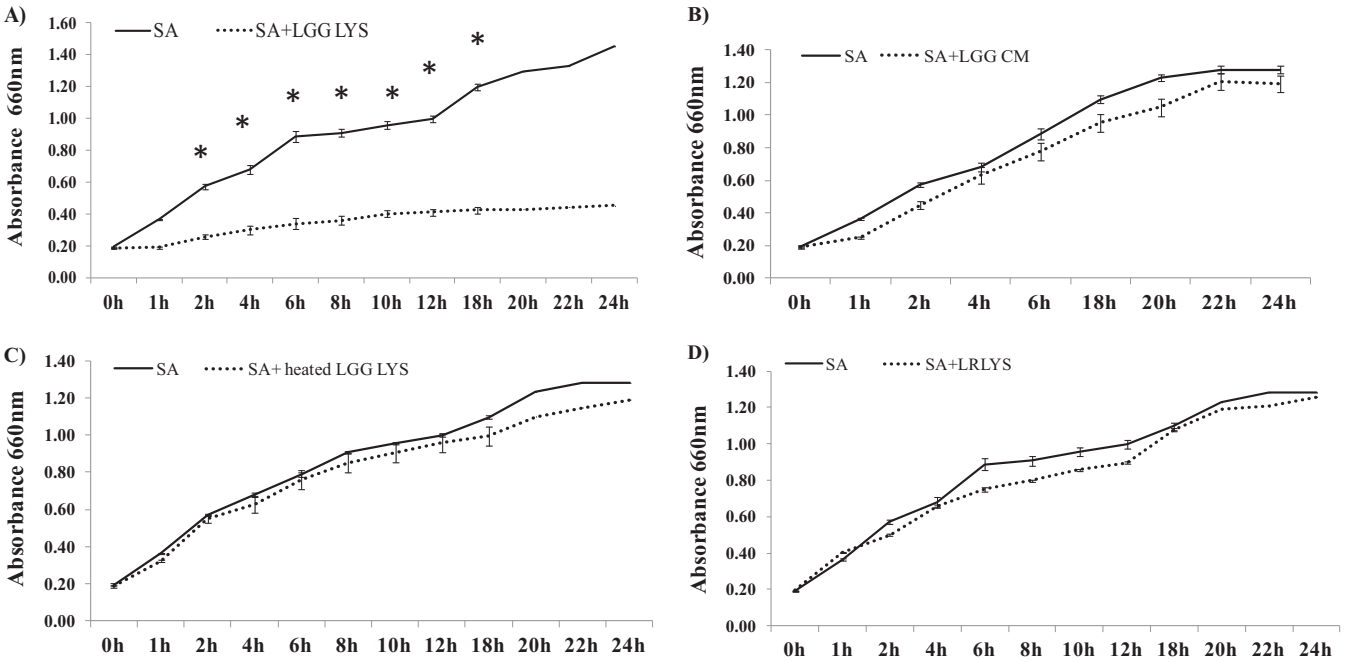

FIG 3 Effect of *L. rhamnosus* GG or *L. reuteri* lysates and spent culture fluid on *S. aureus* growth in a competition assay. The optical densities of cultures of *S. aureus* (SA) growing in the presence of *L. rhamnosus* GG lysate (LGG LYS) (A) or spent culture fluid (LGG CM) (B) or heated *L. rhamnosus* GG lysate (heated LGG LYS) (C) or *L. reuteri* lysate (LRLYS) (D) were determined every hour to monitor the growth of the bacteria. In the presence of the *L. rhamnosus* GG lysate, the growth of *S. aureus* was significantly lower than when it was grown alone ($P = 0.02$; $n = 3$), whereas the heated *L. rhamnosus* GG lysate or spent culture fluid had no significant effect ($P > 0.05$; $n = 3$). Furthermore, a lysate of *L. reuteri* had no effects on the growth of *S. aureus*. Data are representative of three individual experiments, and all values represent means ± SEM ($n = 3$). *, $P < 0.05$.

inhibition of pathogenic adhesion. Previously, we showed that agents that reduce adhesion of *S. aureus* to keratinocytes also reduce its toxicity (33). Hence, we considered that inhibition of adhesion may also be part of the protective mechanism of *L. rhamnosus* GG, lysate, or spent culture fluid. Adhesion assays were performed to determine whether inhibition was due to competition, exclusion, or displacement of pathogen from binding sites on keratinocytes. *L. rhamnosus* GG, either as viable cells or as lysate, was able to inhibit pathogen adhesion if keratinocytes were coinfected (competition; $P = 0.03$), preexposed (exclusion; $P = 0.04$), or applied 12 h after infection with *S. aureus* had begun (displacement; $P = 0.01$) (Fig. 5A and B). By comparison, and as shown previously, live *L. reuteri* or its lysate could reduce staphylococcal adhesion if it was added at the same time as the pathogen (33) (Fig. 5D). However, the spent culture fluid did not reduce *S. aureus* adhesion. Interestingly, the spent culture fluid from *L. rhamnosus* GG inhibited pathogen adhesion only if it was added to keratinocytes either before or at the same time as the pathogen, in keeping with the data on viability (Fig. 5C). Finally, *L. salivarius*, its lysate, or spent culture fluid did not affect the adhesion of *S. aureus* to keratinocytes (Fig. 5D).

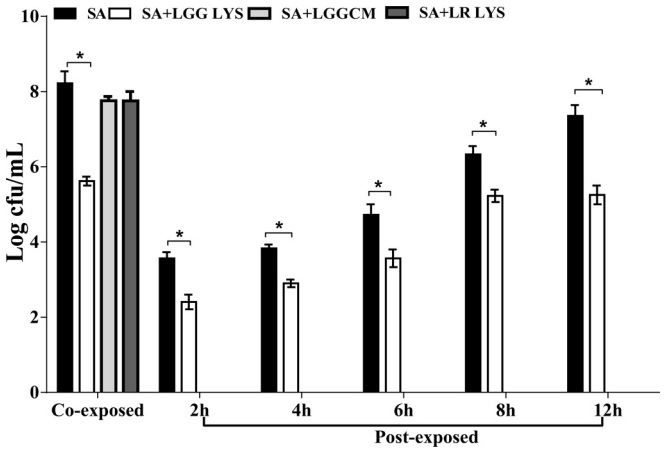

FIG 4 *L. rhamnosus* GG lysate, but not spent culture fluid, reduced the numbers of viable staphylococci. The number of viable *S. aureus* organisms (SA) was 8 log CFU/ml, whereas 5 log CFU/ml of *S. aureus* were viable in the presence of *L. rhamnosus* GG lysate ("Co-exposed"). Additionally, the total number of viable staphylococci in keratinocyte culture was reduced by the *L. rhamnosus* GG lysate when this was added 2, 4, 6, 8, and 12 h after infection of the keratinocytes with pathogen ("Post-exposed"; $P = 0.05$; $n = 3$). Data are representative of three individual experiments, and all values represent means ± SEM ($n = 3$). *, $P < 0.05$.

DISCUSSION

This study explored whether an enteric probiotic, *L. rhamnosus* GG, could protect keratinocytes from the pathogenic effects of *S. aureus*. Our data indicate that *L. rhamnosus* GG, in the form of viable cells, a cell-free lysate, or spent culture fluid, enhanced keratinocyte viability in the presence of the pathogen.

The timing of application of *L. rhamnosus* GG cells or lysate did not affect the degree of protection conferred by the probiotic or lysate because keratinocytes pre-, post-, or coexposed to *L. rhamnosus* GG or lysate were protected from *S. aureus*-induced cell death. However, the probiotic spent culture fluid protected keratinocytes only if it was added either before or at the same time as the pathogen. These data contrast with those for *L. reuteri* and *L.*

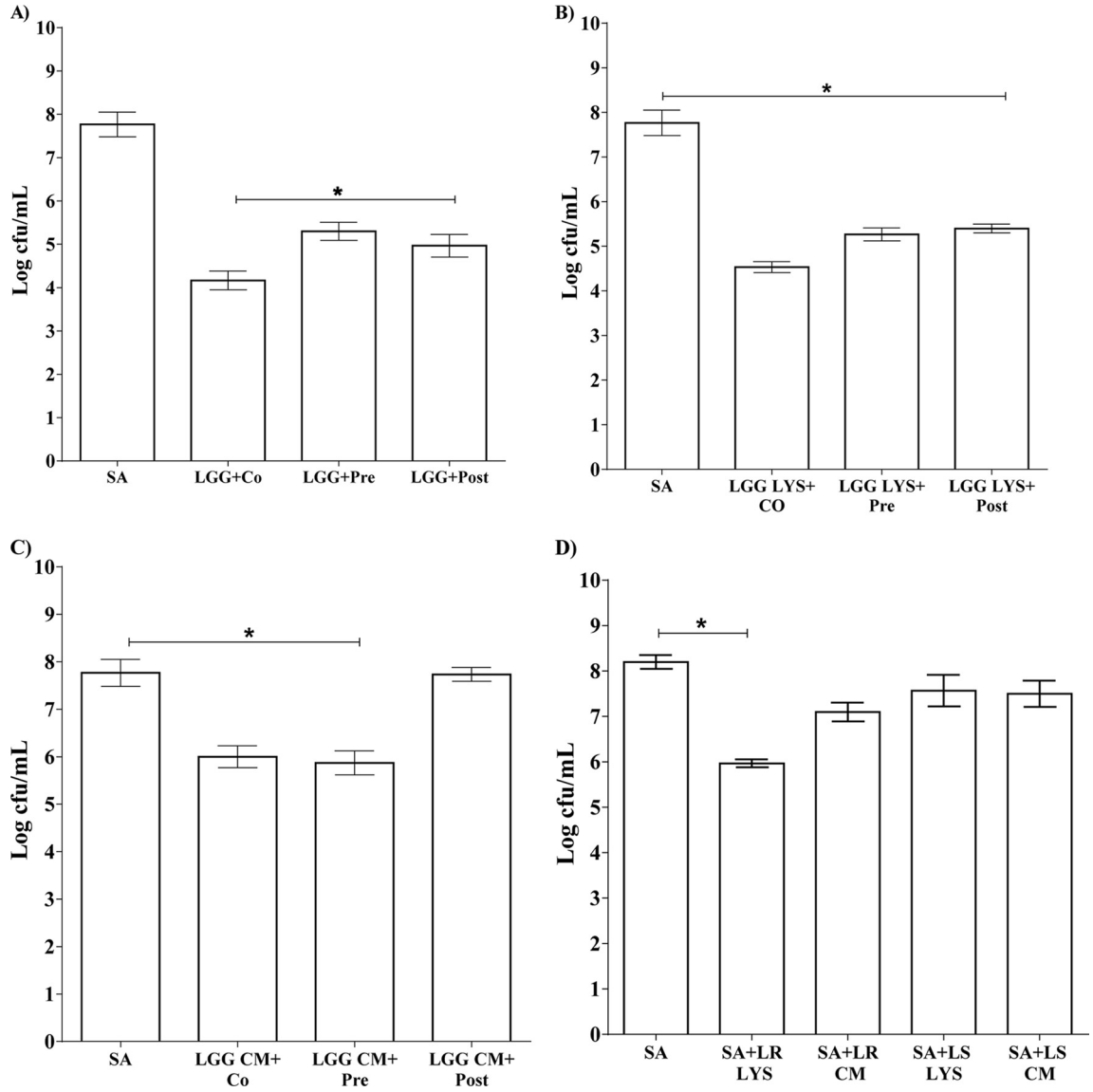

FIG 5 Live *L. rhamnosus* GG, lysate, or spent culture fluid inhibited *S. aureus* adhesion to keratinocytes. (A) Live *L. rhamnosus* GG (LGG) inhibited *S. aureus* adhesion when added at the same time (LGG+Co), before (LGG+Pre), or after (LGG+Post) infection of cells with *S. aureus*. (B) A similar effect was also observed with the lysate. (C) Spent culture fluid (LGG CM) reduced the adhesion of *S. aureus* but only when added at the same time or before infection with pathogen. (D) The *L. reuteri* lysate (SA+LR LYS) reduced the adhesion of *S. aureus* to keratinocytes when added simultaneously, but the *L. reuteri* spent culture fluid (SA+LRCM) did not. *L. salivarius* lysate (SA+LS LYS) or spent culture fluid (SA+LS CM) had no effect on the adhesion of *S. aureus* to keratinocytes. Data are representative of three individual experiments, and all values represent means ± SEM ($n = 3$). *, $P < 0.05$.

salivarius, since *L. reuteri* can protect as a live organism or lysate only when added before or at the same time as the pathogen and *L. salivarius* has no ability to protect keratinocytes (33).

The current investigation suggests that there are at least two, possibly separate, activities involved in the protective effects of *L. rhamnosus* GG. These are likely to be inhibition of pathogen adhesion and inhibition of pathogen growth. We showed previously that agents that reduce adhesion of *S. aureus* to keratinocytes also reduce its toxicity in our viability assay (33). In keeping with this, the ability of the lysate and spent culture fluid to enhance viability mirrors directly the ability of each to inhibit pathogen adhesion; i.e., while the *L. rhamnosus* GG lysate protects viability and inhibits adhesion when added pre- or postinfection, the spent culture

TABLE 1 *L. rhamnosus* GG bacteria or lysate reduces the growth of *S. aureus* in a spot-on-lawn assay[a]

Bacteria	Zone of inhibition (mm)	
	Aerobic condition	Anaerobic condition
S. aureus + *L. rhamnosus* GG	No inhibition	11 ± 1.3
S. aureus + *L. rhamnosus* GG lysate	No inhibition	18.38 ± 0.7
S. aureus + *L. reuteri*	No inhibition	No inhibition
S. aureus +*L. reuteri* lysate	No inhibition	No inhibition

[a] Spot-on-lawn assay demonstrating zones of inhibition produced by *L. rhamnosus* GG and GG lysate under the anaerobic condition but not under the aerobic condition. However, neither live *L. reuteri* nor *L. reuteri* lysate inhibited *S. aureus* growth under either condition. The inhibition zone was evaluated after overnight incubation by measuring the diameter of zone sizes using a ruler. Results are expressed as the means ± SEM of three individual experiments.

fluid protects viability only when added before the pathogen and has no ability to inhibit adhesion or protect when added after the pathogen. Thus, we suggest that the live organism or the lysate protects against the effects of *S. aureus* by exclusion and displacement, whereas the spent culture fluid can only exclude pathogens. In contrast, *L. salivarius*, which cannot protect keratinocytes from *S. aureus*, does not inhibit adhesion as either a live organism, a lysate, or spent culture fluid. Taken together, all these data point to species-specific effects in the abilities of different lactobacilli to protect keratinocytes from the toxic effects of *S. aureus*. Our data may also suggest that the antiadhesive effects contained within the *L. rhamnosus* GG lysate and spent culture fluid are mediated by different molecules. However, we cannot rule out the possibility that the same molecule(s) may be involved but that the concentration in the spent culture fluid is too low for some of the effects to be observed.

The ability of species of *Lactobacillus* species to inhibit certain pathogens from binding to epithelial cells has been demonstrated previously in models of the gut epithelium (8, 10, 34). For example, in an in *vitro* study, probiotics (alone or in combinations), including *L. rhamnosus* NCC4007 and *Lactobacillus paracasei* NCC2461, were shown to inhibit *E. sakazakii* adhesion to intestinal mucus through competitive exclusion and displacement from the binding sites (8, 10, 35). Another study by Vesterlund and colleagues (36) showed that certain lactic acid bacteria, including *L. rhamnosus* GG, were able to reduce the adhesion of *S. aureus* to intestinal cells by as much as 44%. In keeping with our study, the mechanisms involved included competition, exclusion, and displacement. Interestingly, in the study of Vesterlund et al., the authors also noted reduced staphylococcal viability in the presence of some of the probiotic organisms (36).

The molecules mediating the inhibitory effects of probiotics against pathogens have been investigated in a number of studies. In some cases, the molecules mediating antiadhesive activity are largely associated with other functions, i.e., the so-called "moonlighting proteins" (37–49). For example, enolase from *Lactobacillus crispatus* can bind to laminin and collagen I, which reduces the adhesion of *S. aureus* to epithelial cell lines through these binding sites (50). Similarly, enolase from *L. plantarum* has been reported as binding to fibronectin to prevent *S. aureus* adhesion to epithelial cell lines (7, 51). Other moonlighting proteins contributing to bacterial adhesion have been found in lactobacilli. For example, triosephosphate isomerase (TPI) from *L. plantarum* plays a role in the adhesion of lactobacilli to Caco-2 cells and has the ability to compete with pathogens such as *Clostridium sporogenes* and *Enterococcus faecalis* by excluding and displacing them from the cell-binding sites (12, 52, 53). However, thus far, the molecules mediating the effects preventing adhesion of *L. rhamnosus* GG to keratinocytes remain to be identified.

L. rhamnosus GG lysate may also protect keratinocytes is via inhibition of *S. aureus* growth. Two lines of evidence suggest that this is the case: first, a reduction in the total number of viable staphylococci in the presence of the *L. rhamnosus* GG lysate and inhibition assays demonstrating zones of inhibition when *S. aureus* was challenged with lysates from the probiotic grown anaerobically (Table 1). This could be due to the presence of a toxic molecule(s) within the probiotic that is able to directly inhibit *S. aureus* growth and/or viability. It is possible that this molecule(s) may be synthesized but not secreted because there was no effect of *L. rhamnosus* GG spent culture fluid on the viability of *S. aureus*. However, again, we cannot rule out the possibility that such molecules may be secreted but diluted once contained in the spent culture fluid. If *L. rhamnosus* GG contains bacteriostatic substances, then this may also, at least partially, explain the protective effect of the probiotic in keratinocyte survival assays. Probiotics, especially lactobacilli, have previously been shown to exert a strong inhibitory effect on *S. aureus* growth. Certain *Lactobacillus* strains have been reported to be highly antagonistic to biofilm-forming *S. aureus* (3). Other studies have reported that probiotics can improve gut health by inhibiting growth of pathogens through production of bacteriocins or lactic acid (36, 54–56). However, in the present study, we could find no evidence of the involvement of acid production as part of the protective effects of *L. rhamnosus* GG. Indeed, the lysate from this organism was neutral (pH 7.2) but was still able to inhibit *S. aureus* growth. Furthermore, neither *L. reuteri* nor *L. salivarius* showed any inhibitory activity on the growth of *S. aureus* even though both these bacteria are also able to produce acid (57, 58).

In conclusion, we have shown that *L. rhamnosus* GG uses multiple mechanisms to protect keratinocytes from *S. aureus*. These include exclusion of pathogens, inhibition of pathogen growth, and displacement of pathogen from keratinocytes. Of course, it is possible that this displacement activity may be related to the ability of *L. rhamnosus* GG to inhibit growth, and further studies will be required to clarify this point. A number of studies have suggested the utility of probiotic species of lactobacilli for use topically. In keeping with these studies, we suggest that *L. rhamnosus* GG is a potential new agent to inhibit the pathogenicity of *S. aureus* to keratinocytes. Furthermore, our data show that the utility of *L. rhamnosus* GG on skin will not be limited by whether it can grow and survive on skin, because a lysate of the organisms is just as efficacious at preventing *S. aureus* colonization as live bacteria. We suggest that the use of bacterial lysates will enhance the utility of lactobacilli since the need to produce formulations that maintain bacterial viability is negated. Furthermore, lysates potentially offer a safer option than live bacteria for treatment of damaged skin.

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