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

Walaa Mohammedsaeed[1], Andrew.J. McBain[2], Sheena.M. Cruickshank [3]and Catherine.A. O'Neill[1]

Schools of Medicine[1], Manchester Pharmacy School[2] and Faculty of Life Science[3], the University of Manchester, UK

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Corresponding author: catherine.oneill@manchester.ac.uk
Postal address: Room 1.704, Inflammation Sciences, Stopford Building, University of Manchester, Oxford Road, Manchester M13 9PT

ABSTRACT

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 24h 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 24h incubation. Keratinocyte survival was significantly enhanced regardless of whether the probiotic was applied in the viable form, or as cell lysates, 2h before or simultaneously (*P*=0.005) or 12h after (*P*=0.01) *S. aureus* infection. However, spent culture fluid was only protective if added before or simultaneously to *S. aureus*. With respect to mechanism, both *L. rhamnosus* GG lysate or 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.

INTRODUCTION

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 (46) including increasing epithelial barrier function (13) and modulation of the immune response (21). There is also evidence that probiotics can prevent colonisation of the gut by pathogens. This can be via mechanisms such as down regulation of virulence factors and inhibition of pathogen adherence to the epithelium (6, 9, 15). For example, *Lactobacillus* species inhibit the adhesion of *Enterobacter sakazakii* to intestinal mucus by competitive exclusion (9, 10). Other studies demonstrated that some probiotics increase the production of intestinal mucin thus inhibiting pathogen adherence to intestinal epithelial cells (34). Probiotics are also able to produce antimicrobial peptides (bacteriocins) and acids. Collectively, there are numerous probiotic mediated mechanisms that limit pathogen colonisation (39, 44, 46).

Since probiotics may have positive impacts on the gut, their potential effects on other systems, such as the mouth (36) and the urogenital tract (43) have also been investigated. For example, a study in 2002, examined the impact of oral administration of *L. plantarum* to patients who had abdominal surgery and showed that this bacterium lowered the incidence of post-surgical infection (42). 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 (8, 15, 16, 18, 50). The benefits of topical application of probiotics are still speculative and researchers are now focussing on this area to improve conditions such as excessive skin sensitivity, atopic dermatitis, psoriasis or to stimulate the wound healing process (3, 8, 12, 52). However, an important consideration will be the safety of using live bacteria especially in situations whether 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 (12). The topical application of *Lactobacillus plantarum* lysate inhibited the pathogenic activity of *Pseudomonas aeruginosa* in infected burns. (52). *In vivo*, *L.*

plantarum lysate has also been shown to improve wound healing in burned patients (53).

Staphylococcus aureus is both a transient coloniser of skin and a major opportunistic skin pathogen, causing diseases ranging from impetigo to life threatening conditions such as sepsis (58, 59). Previously, our lab demonstrated that the probiotic *L. 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 (40). In the present study, we have identified *L. 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.

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 0.004mg/ml, epidermal growth factor (recombinant-human) 0.125ng/ml, insulin (recombinant human) 5µg/ml, hydrocortisone 0.33µg/ml, epinephrine 0.39µg/ml and transferrin, holo (human) 10µg/ml) and 0.06mM $CaCl_2$ (Promocell, Heidelberg, Germany), were used as a model system. These were cultured routinely at 37^{o}C in a humid atmosphere of 5% CO_2 in T-75 culture flasks as described previously (40).

Bacterial cell culture

Lactobacillus rhamnosus Goldin and Gorbach (*L. rhamnosus* GG, ATCC 53103) *Lactobacillus reuteri* (ATCC55730) and *Lactobacillus salivarius* (UCC118) (ATCC, Middlesex, UK), were grown routinely in Wilkins-Chalgren Broth or Agar (Oxoid, Basingstoke, UK) at 37°C in incubated 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, Basingstoke, UK) as described previously (40).

Treatment of keratinocytes with bacteria

Bacteria (10^8 CFU/ml of probiotics and10^6 CFU/ml of *S. aureus*) were centrifuged at 15,000 x g, washed twice in 0.85% NaCl and re-suspended in keratinocyte basal medium. This suspension was added directly to 5 x 10^3 cells/cm^2 of NHEK growing in 24 well plates. For experiments using a probiotic lysate, 100ml of 10^8 CFU/ml of *L. rhamnosus* GG were centrifuged, washed, re-suspended in 25ml of Phosphate Buffer Saline (PBS, Invitrogen, Life Technologies Ltd, Paisley, UK) pH=7.4 and lysed using a MSE Soniprep 150. Samples were filtered using a 0.22µm pore filter (Millipore, Billerica, USA) to remove any whole bacteria remaining. Approximately 100µl of this lysate was used to treat keratinocytes (5 x 10^5 cells/cm^2). In some experiments, cells were sedimented in a centrifuge at 15,000 x g for 5 minutes and the cell-free supernatant (spent culture fluid) collected and filtered using a 0.22µm pore

filter (Millipore, Billerica, USA) to remove any whole bacteria remaining. In other experiments, keratinocyte monolayers were co-infected 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 hours 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 (40). In other experiments using heated lysates, these were heat inactivated by placing them in a boiling water bath at 100°C for 5 minutes.

Measurement of *S. aureus* viability in cell culture

To determine whether *L. rhamnosus* GG lysates or spent culture fluid were able to inhibit the growth of *S. aureus* in cell culture, keratinocytes were grown to confluence in a 24 well plate. These were exposed to100µl of 10^6 CFU/ml of *S. aureus* alone, or *S. aureus* plus 100µl *L. rhamnosus* GG lysates or 100µl spent culture fluid. In separate experiments, cells were exposed to *L. rhamnosus* GG lysates for 2, 4, 6, 8 and 12 hours post infection with *S. aureus*. The total number of viable staphylococci was determined by counting the colonies as described previously (40).

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 1hour. Cells were then washed three times in PBS, pH=7.4, to remove non adherent bacteria. The cells were trypsinised 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 *S. aureus* combined with 100µl of lysate or spent culture fluid of *L. reuteri* or *L. salivarius* UCC118. The experiment was carried out three times and results were taken as triplicates.

In separate experiments, cells were exposed to100µl of 10^8 CFU/ml of probiotic bacteria or lysates or spent culture fluid for 1hour before the addition 100µl of 10^6 CFU/ml of *S. aureus* at the same time or 2, 4, 6, 8 and 12 hours post infection with *S. aureus.*

Determination of bacterial antagonism

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

Determination of the outcome of co-culture (competition assays)

Aliquots (100µl) of *L. rhamnosus* GG lysates and 100µl of 10^6 CFU/ml *S. aureus* were inoculated into 10ml WCB broths. The pH and optical density of cultures was measured at 0 and 24h. At regular intervals (indicated in the text) 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 analysed by one way ANOVA and post hoc Tukey test using SPSS (IBM SPSS Statistics version 16.0) program. Results were considered significant if $P<0.05$. 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 24h incubation, there was no difference in the viability of keratinocytes incubated with the probiotic bacteria vs 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 (40) 24h exposure of keratinocytes to 10^6 CFU/ml *S.aureus* resulted in significant keratinocyte cell death. However, keratinocytes incubated simultaneously with pathogen and *L. rhamnosus* GG had a significantly higher percentage viability (57% P=0.01) than monolayers infected with pathogen alone (Figure 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 viability of treated keratinocytes was 65% and 55.93% respectively compared to 25% in keratinocytes infected with *S. aureus* alone (P= 0.006 and P=0.01 respectively, Figure 1B). This is in contrast to the effects observed with *L. reuteri* which we showed previously to be protective to pathogen infected keratinocytes (40). *L. reuteri* only provides protection 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* (Figure 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 pre or post infection of keratinocytes with *S. aureus*. The percentage of keratinocytes remaining viable was significantly greater in monolayers exposed to *L. rhamnosus* GG for 2h prior to infection with *S. aureus*, than in monolayers infected with *S. aureus* alone (P=0.006). Both the lysate and spent culture fluid afforded a similar levels of protection (P=0.005,

205 *P*=0.004, Figure 2A). In 'post-exposure' experiments, keratinocytes were exposed to *S. aureus* for 2h,
206 4h, 6h, 8h and 12h before addition of the live *L. rhamnosus* GG, lysate, or spent culture fluid. The
207 viability of the keratinocytes was then measured at 24h post infection with *S. aureus*. The data in Figure
208 2 (B, C) shows that both live probiotic and its lysate could protect the keratinocytes when added after *S.*
209 *aureus*. Even at 12 h post *S. aureus* infection, *L. rhamnosus* GG or lysate still afforded protection to the
210 keratinocytes such that 58% and 55% respectively of cells remained viable compared to 25% when
211 exposed to *S. aureus* alone (*P*=0.003, *P*=0.01 respectively). However, the spent culture fluid from *L.*
212 *rhamnosus* GG had no protective effect on keratinocytes when added after *S. aureus* (Figure 2 D).
213
214 ***L. rhamnosus* GG lysate, but not spent culture fluid, inhibits the growth of *S. aureus*.**
215 We investigated whether the probiotic lysate had direct effects on the growth of the pathogen by
216 growing them simultaneously in culture. Competition assays showed a significant reduction in *S.*
217 *aureus* growth over a period of 24 h in the presence of 100µl of *L. rhamnosus* GG lysate compared to
218 untreated cultures (*P*=0.02, Figure 3A). This effect was specific to the lysate because the spent culture
219 fluid from *L. rhamnosus* GG had no effect on the growth of *S. aureus* (Figure 3B). Furthermore, the
220 ability of the lysate to inhibit pathogenic growth was negated by heating the lysate to 100^{o}C for 10 min
221 (Figure 3C), Finally, this direct effect of *L. rhamnosus* GG on pathogenic growth appeared to be
222 species specific because the lysate from *L. reuteri*, made in exactly the same way had no effect on the
223 growth of *S. aureus* (Figure 3D).
224 We counted the numbers of viable staphylococci following 24h incubation with keratinocytes in the
225 presence or absence of the *L. rhamnosus* GG lysate. When *S. aureus* was added to keratinocytes at the
226 same time as the *L. rhamnosus* GG lysate, the total number of viable staphylococci was also
227 significantly reduced to 5 $\log_{10}$ cfu/ml, (compared to 8 $\log_{10}$ cfu/ml for *S. aureus* alone, *P*=0.02 Figure
228 5). Furthermore, when the *L. rhamnosus* GG lysate was added 12h post infection of the keratinocytes,
229 a reduction in number of viable *S. aureus* was observed when these were counted 24h later (Figure
230 4).These effects were not seen with either the spent culture fluid from *L. rhamnosus* GG nor a lysate

231 from *L. reuteri* (data not shown). Since *lactobacilli* can produce organic acids, we measured the pH of
232 keratinocyte media infected for 24h with *S. aureus*, *L. rhamnosus* GG lysate or both simultaneously.
233 However, there was no significant difference in the pH between treatments group (data not shown).
234 We also measured the pH of lysate alone and found it be pH= 7.2 thus suggesting that acid mediated
235 effects were not likely to be the mechanism underlying inhibition of pathogenic growth. The
236 antimicrobial properties of *L. rhamnosus* GG and lysate were evaluated using a spot-on-lawn assay.
237 This assay showed significant inhibition of *S. aureus* growth (as evidenced by the presence of zones of
238 inhibition) by anaerobic live cultures or lysates of *L. rhamnosus* GG grown anaerobically (Table 1). By
239 contrast, live *L. reuteri* or lysate did not induce zones of inhibition in this assay (Table 1).
240
241 ***L. rhamnosus* GG inhibits adhesion of *S. aureus* to keratinocytes.**
242 Another mechanism by which live bacteria, lysate or spent culture fluid of *L. rhamnosus* GG may
243 protect the keratinocytes is by inhibition of pathogenic adhesion. Previously, we showed that agents that
244 reduce adhesion of *S. aureus* to keratinocytes also reduce its toxicity (40). Hence, we considered that
245 inhibition of adhesion may also be part of the protective mechanism of *L. rhamnosus* GG, lysate or spent
246 culture fluid. Adhesion assays were performed to determine whether inhibition was due to competition,
247 exclusion or displacement of pathogen from binding sites on keratinocytes. *L. rhamnosus* GG, either as
248 viable cells or lysate, was able to inhibit pathogen adhesion if keratinocytes were co-infected
249 (competition, P= 0.03), pre-exposed (exclusion, P= 0.04) or applied 12h after infection with *S. aureus*
250 had begun (displacement, P= 0.01, (Figure 5A, B). By comparison, and as shown previously, live *L.*
251 *reuteri* or its lysate, could reduce staphylococcal adhesion if it was added same time as addition of the
252 pathogen (40, Figure 5D). However, the spent culture fluid did not reduce *S. aureus* adhesion.
253 Interestingly, the spent culture fluid from *L. rhamnosus* GG only inhibited pathogen adhesion if it was
254 added to keratinocytes either before or at the same time as the pathogen in keeping with the data on
255 viability (Figure 5C). Finally, *L. salivarius*, its lysate or spent culture fluid did not affect the adhesion of
256 *S. aureus* to keratinocytes (Figure 5D).

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, either as viable cells, in the form of a cell-free lysate or spent culture fluid enhanced keratinocyte viability on 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 co-exposed to *L. rhamnosus* GG or lysate were protected from *S. aureus* induced cell death. However, the probiotic spent culture fluid only protected keratinocytes if it was added either before or at the same time as pathogen. These data contrast with those for *L. reuteri* and *L. salivarius* since *L. reuteri* can only protect as a live organism or lysate when added before or at the same time as the pathogen and *L. salivarius* has no ability to protect keratinocytes (40).

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 (40). 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 post infection, the spent culture fluid only protects viability when added before 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 protect against the effects of *S. aureus* by exclusion and displacement whereas the spent culture fluid can only exclude pathogens. By contrast, *L. salivarius*, which cannot protect keratinocytes from *S. aureus*, does not inhibit adhesion either as 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 anti-adhesive 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 (9,10,11). For example, in an in *vitro* study, probiotics (alone or in combinations) including *L. rhamnosus* NCC4007, *L. paracasei* NCC2461, were shown to inhibit *E. sakazakii* adhesion to intestinal mucus through competitive exclusion and displacement from the binding sites (9,10). Another study by Satu and colleagues (2006) reported 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 Satu *et al* study, the authors also noted reduced *staphylococcal* viability in the presence of some of the probiotic organisms (49).

The molecules mediating the inhibitory effects of probiotics against pathogens have been investigated in a number of studies. In some cases, the molecules mediating anti-adhesive activity are largely associated with other functions i.e. the so-called "moonlighting proteins" (23, 24, 25,35). For example, enolase from *L. crispatus* can bind to laminin and collagen I, which reduces the adhesion of *S. aureus* to epithelial cell lines through these binding sites (4). Similarly, enolase from *L. plantarum* has been reported as binding to fibronectin to prevent *S. aureus* adhesion to epithelial cell lines (6,7). 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 (30, 39,41). However, thus far, the molecules mediating the anti-adhesive effects 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 this is the case: firstly, 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 are 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* (13). Other studies have reported that probiotics can improve gut health by inhibiting growth of pathogens through production of bacteriocins or lactic acid (31, 32, 49, 51). 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 (5, 14).

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 has 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 shows 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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356 **References**

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Figure Legends

558 **Figure 1** ***A)L. rhamnosus*** **GG, lysate or spent culture fluid protect keratinocytes from the toxic**
559 **effects of *S. aureus***. A combination of *S. aureus* (SA) and *L. rhamnosus* GG (LGG+SA), resulted in a
560 significantly higher (P=0.01) percentage of viable keratinocytes after 24 hours than in monolayers
561 infected with *S. aureus* alone. The data were compared to those produced by uninfected control cells
562 (control). **B)** The viability of *S. aureus* infected keratinocytes treated with *L. rhamnosus* GG lysate
563 (SA+LGGLYS) or spent culture fluid (SA+LGGCM) was significantly increased compared to
564 keratinocytes infected with *S.aureus* (SA) alone. **C)** Monolayers exposed to *S. aureus* and a lysate of *L.*
565 *reuteri* (SA+LRLYS) had a significantly higher percentage of viable keratinocytes than those infected
566 with pathogen alone but the same effect was not found with the spent culture fluid of *L. reuteri*
567 (SA+LRCM). Data are representative of three individual experiments and all values represent mean ±
568 SEM of percentage viability (n=3). *P<0.05.
569
570 **Figure 2** ***L. rhamnosus*** **GG protects and rescues keratinocytes from infection with *S. aureus*. A)**
571 The percentage viability of infected keratinocytes was significantly higher in cells that were pre-exposed
572 to *L. rhamnosus* GG (LGG+SA), lysate (LGG LYS+SA) or spent culture fluid (LGG CM+ SA)
573 compared to *S. aureus* (SA) infected cells. **B)** The viability of *S. aureus* infected keratinocytes was
574 significantly higher in cells exposed to *L. rhamnosus* GG 12h post infection with *S. aureus (*'post
575 exposed*')*. A similar effect was observed with lysate (C). However, **D)** Cells post-exposed to *L.*
576 *rhamnosus* GG spent culture fluid (CM) did not have significant protection. Data are representative of
577 three individual experiments and all values represent mean ± SEM of percentage viability (n=3). *
578 P<0.05.
579
580 **Figure 3 The effect of *L. rhamnosus* GG or *L. reuteri* lysates and spent culture fluid on *S. aureus***
581 **growth in a competition assay.** The optical densities of cultures of *S. aureus* (SA) growing in the
582 presence of **(A)** *L. rhamnosus* GG lysate (LGG LYS) or **(B)** spent culture fluid (LGGCM) or **(C)** heated
583 *L. rhamnosus* GG lysate (heated LGG LYS) or **D)** *L. reuteri* lysate (LR LYS) were determined every
584 hour to monitor the growth of the bacteria. In the presence of the *L. rhamnosus* GG lysate, the growth of
585 *S. aureus* was significantly lower than when it was grown alone (P=0.02, n=3), whereas the heated *L.*
586 *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 mean ± SEM of percentage viability (*n*=3). **P*<0.05.

Figure 4 ***L. rhamnosus*** **GG lysate, but not spent culture fluid, reduced the numbers of viable staphylococci.** The number of viable *S. aureus* (SA) was 8log CFU/ml, whereas 5 log CFU/ml of *S. aureus* (SA) were viable in the present 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 hours 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 mean ± SEM of percentage viability (*n*=3). **P*<0.05.

Figure 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 infection of cells with *S. aureus* (LGG+Post). **B)** A similar effect was also observed with the lysate. **C)** Spent culture fluid (LGG CM+SA) reduced the adhesion number 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 mean ± SEM of viability percentage (n=3). **P*<0.05.

Table 1 ***L. rhamnosus*** **GG bacteria or lysate reduce the growth of S. aureus in a spot on lawn assay.** Spot on the lawn assay demonstrating zones of inhibition produced by *L. rhamnosus* (LGG) and lysate (LGG LYS) under anaerobic condition, but not under aerobic condition**.** However, neither live *L. reuteri* (LR) nor lysate (LR LYS) inhibited *S. aureus* growth under either condition. The inhibition zone was evaluated after overnight incubation by measuring the diameter of zone sizes in mm using a ruler. Results are expressed as the mean ± SEM/mm of three individual experiments. **P*<0.05.

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	Zone of inhibition mm(Aerobic)	Zone of inhibition mm(Anaerobic)
SA+LGG	No inhibition	11±1.3
SA+LGG LYSATE	No inhibition	18.38±0.7
SA+ LR	No inhibition	No inhibition
SA+LR LYSATE	No inhibition	No inhibition

Figure 1.

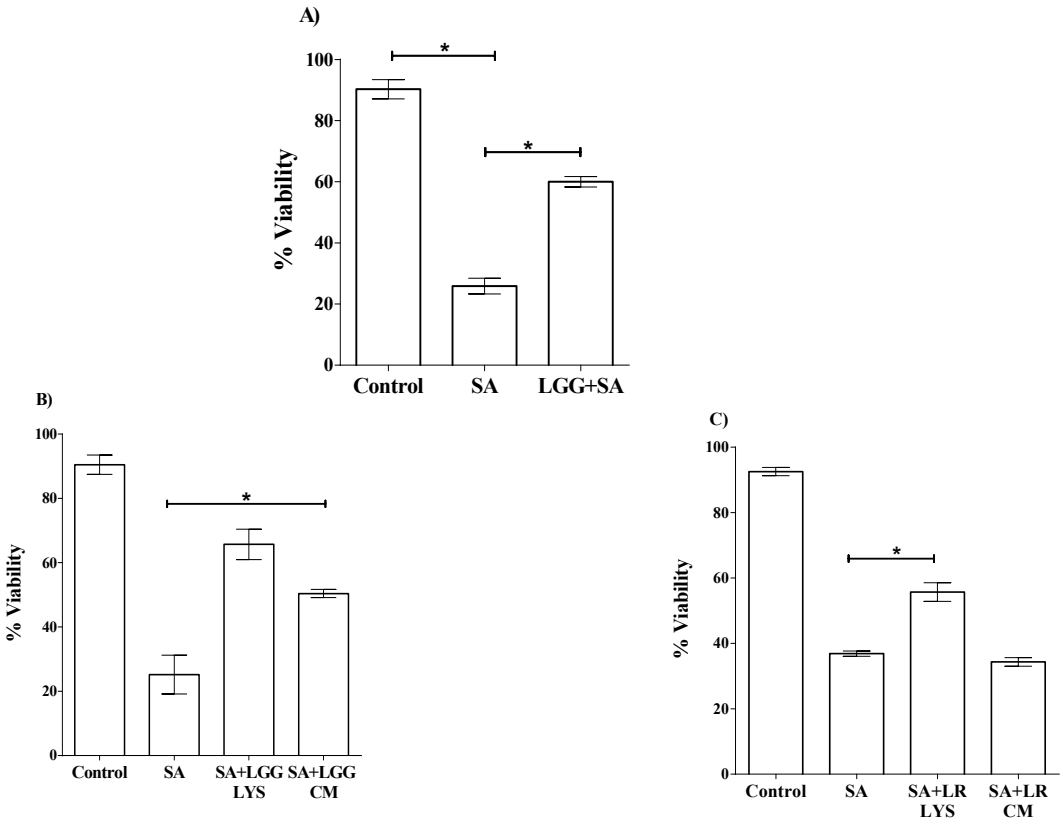

Figure 2.

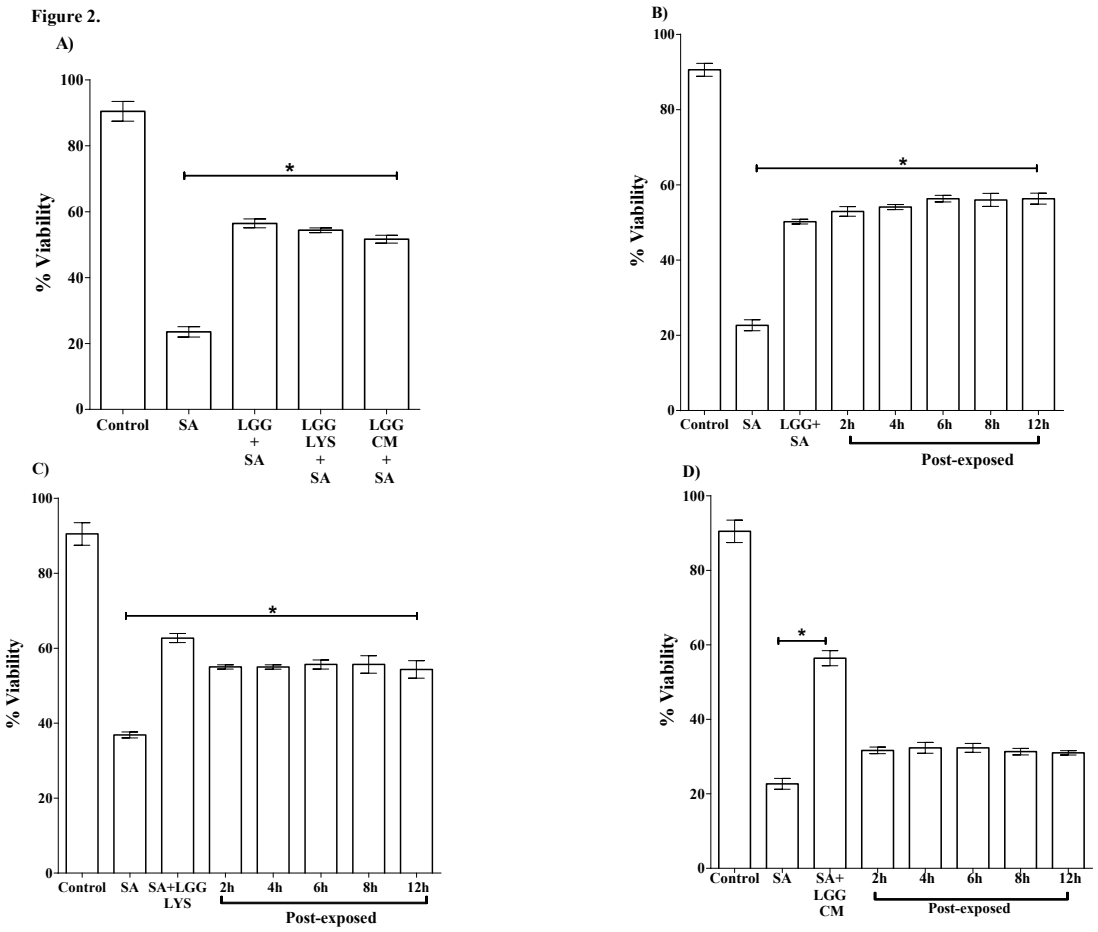

Figure 3.

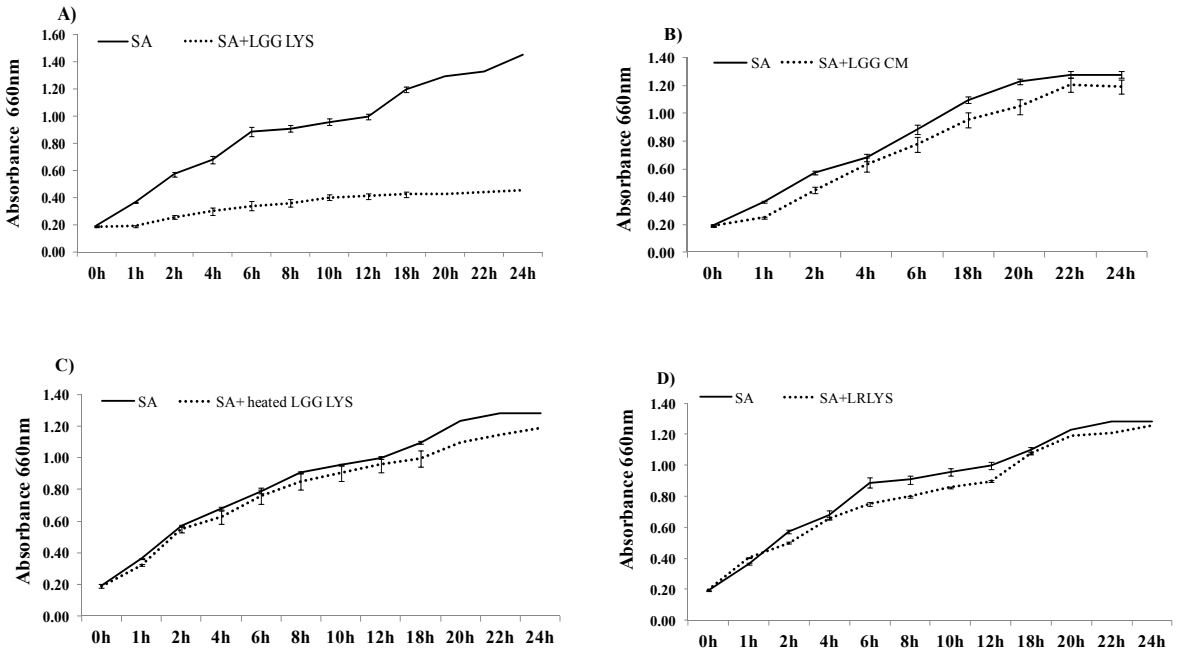

Figure 4.

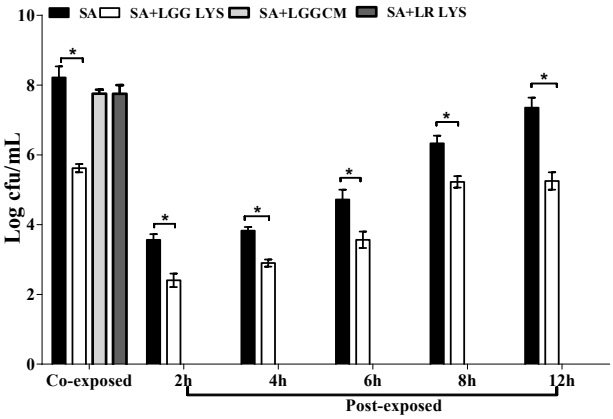

Figure 5.