***Lacticaseibacillus rhamnosus* GG inhibits infection of human keratinocytes by *Staphylococcus aureus* through mechanisms involving cell surface molecules and pH reduction**

I. Spacova¹, C. O'Neill² and S. Lebeer^{1*}

¹Laboratory of Applied Microbiology and Biotechnology, ENdEMIC group, University of Antwerp, Groenenborgerlaan 171 G.V.522, Antwerp, 2020, Belgium; ²Division of Musculoskeletal & Dermatological Sciences, University of Manchester, Oxford Road, Manchester, M13 9PT, United Kingdom; sarah.lebeer@uantwerpen.be

Received: 17 April 2020 / Accepted: 2 June 2020

© 2020 Wageningen Academic Publishers

OPEN ACCESS

RESEARCH ARTICLE

Abstract

Beneficial bacteria represent an emerging tool against topical diseases, including infection caused by *Staphylococcus aureus*. Here, we investigated several anti-pathogenic mechanisms of the model probiotic *Lacticaseibacillus rhamnosus* GG against a clinical *S. aureus* isolate by implementing various mutants lacking important cell surface molecules. We analysed adhesion of *L. rhamnosus* and competitive adhesion with *S. aureus* to primary human keratinocytes, *L. rhamnosus* and *S. aureus* auto- and co-aggregation, *S. aureus* growth inhibition, keratinocyte viability increase, and monocyte Toll-like receptor (TLR) activation by *L. rhamnosus* as such, or with *S. aureus*. *L. rhamnosus* mutated in SpaCBA pili exhibited reduced adhesion to keratinocytes, reduced ability to prevent *S. aureus* adhesion to keratinocytes and reduced co-aggregation with *S. aureus*. Mutants in cell wall exopolysaccharides showed enhanced adhesion to keratinocytes and TLR activation in monocytes, suggesting involvement of additional cell surface molecules masked by exopolysaccharides. All *L. rhamnosus* strains inhibited *S. aureus* growth, likely due to acidification of the medium. Live (but not UV-inactivated) *L. rhamnosus* significantly reduced inflammatory TLR activation in monocytes by *S. aureus*. These data suggest the key role of SpaCBA pili and additional contribution of other cell surface molecules as well as secreted components of *L. rhamnosus* GG in the multifactorial inhibition of *S. aureus* adhesion and toxicity in the skin niche.

Keywords: probiotic, LGG, skin, antipathogenic, pili

1. Introduction

Topical application of live probiotic bacteria or derivatives thereof is increasingly explored as a preventive and therapeutic option against skin diseases. Several studies have demonstrated the *in vitro* effectiveness of probiotic lactobacilli and their products against skin pathogens, such as *Pseudomonas aeruginosa* (Ramos *et al.*, 2015), *Staphylococcus aureus* (Mohammedsaeed *et al.*, 2014) and *Cutibacterium acnes* (Lebeer *et al.*, 2018b). Furthermore, beneficial effects in clinical trials including patients with skin burns (Peral *et al.*, 2009) and acne (Lebeer *et al.*, 2018b) have been reported.

The model probiotic strain *Lacticaseibacillus rhamnosus* GG represents a promising live skin probiotic for application in humans (Lebeer *et al.*, 2018b). *L. rhamnosus* GG is widely studied and used in various body niches, where it demonstrates anti-pathogenic and immunomodulatory properties, as well as a favourable safety profile, and robust viability and adhesion to host cells (reviewed by Segers and Lebeer, 2014). Anti-pathogenic mechanisms of *L. rhamnosus* GG include prevention of pathogen biofilm formation (Petrova *et al.*, 2016; Spacova *et al.*, 2018) competitive adhesion to host cells (Mohammedsaeed *et al.*, 2014; Van den Broeck *et al.*, 2018), inhibition of bacterial pathogen growth (De Keersmaecker *et al.*, 2006;

Van den Broeck *et al.*, 2018) and reduction of pathogenic structure morphogenesis, such as hyphae formation of the fungal pathogen *Candida albicans* (Allonsius *et al.*, 2019). *L. rhamnosus* GG was specifically shown to reduce adhesion of the prominent pathogen *S. aureus* to human intestinal mucus (Vesterlund *et al.*, 2006) and primary skin cells (Mohammedsaeed *et al.*, 2014), however the contributing probiotic molecules have not yet been elucidated.

L. rhamnosus GG mechanisms of action have mostly been studied in the gastrointestinal niche, and several molecular structures of *L. rhamnosus* GG were previously suggested to promote its probiotic interactions. The SpaCBA pili consisting of three pilin subunits (SpaA, SpaB and SpaC) are especially relevant for probiotic-host interactions, and represent one of the first examples of pili in probiotic lactobacilli (Kankainen *et al.*, 2009; Lebeer *et al.*, 2012). Their potential role in the gastrointestinal tract has previously been described in relation to biofilm formation and efficient adhesion to human intestinal epithelial cells (Lebeer *et al.*, 2012; Spacova *et al.*, 2018) and murine macrophages (Vargas-Garcia *et al.*, 2015), as well as in immunomodulation of intestinal epithelium (Lebeer *et al.*, 2012). Other molecules of *L. rhamnosus* GG that can contribute to its probiotic action are cell wall exopolysaccharides (EPS) (Allonsius *et al.*, 2017; Lebeer *et al.*, 2009; Zhang *et al.*, 2016), and the major secreted protein 1 (Msp1), a cell wall hydrolase with anti-pathogenic activity that is also involved in daughter cell separation (Allonsius *et al.*, 2019; Claes *et al.*, 2012).

Isogenic mutants of *L. rhamnosus* GG have been extensively used to elucidate and validate the role of molecular components involved in cellular physiology and probiotic action. These mutants have played a key role in unravelling the structure and function of SpaCBA pili (Lebeer *et al.*, 2012; Vargas *et al.*, 2015). Likewise, the *L. rhamnosus* GG knockout mutant in *welE* encoding the priming glycosyltransferase for a long galactose-rich EPS, showed that the EPS protects *L. rhamnosus* GG from host factors and might also shield its surface adhesive molecules, including pili (Kiekens *et al.*, 2019; Lebeer *et al.*, 2009, 2011). Additional examples of genetically and phenotypically confirmed *L. rhamnosus* GG mutants and are listed in Table 1.

Our previous research showed that *L. rhamnosus* GG can protect primary skin cells from the prominent skin pathogen *S. aureus* through several mechanisms, including competitive exclusion and pathogen displacement (Mohammedsaeed *et al.*, 2014). Here, we used knock-out mutants of *L. rhamnosus* GG to further explore the role of probiotic surface molecules and secreted components for probiotic-host and probiotic-pathogen interactions with *S. aureus* in the skin niche.

2. Material and methods

Bacterial strains and culturing conditions

Bacterial strains used in this study are listed in Table 1. *L. rhamnosus* strains were grown in de Man-Rogosa-Sharpe medium at 37 °C in static conditions (Spacova *et al.*, 2018). *S. aureus* was grown at 37 °C in nutrient broth (Oxoid, Basingstoke, UK) in shaking conditions. Bacteria were harvested after overnight growth by centrifugation at 2,000×g for 5 min and washed twice in phosphate-buffered saline (PBS) before use in further assays. Fluorescence induction in *L. rhamnosus* was performed with nisin from *Lactococcus lactis* (Sigma-Aldrich, Diegem, Belgium, N5764) as previously described in Spacova *et al.* (2018).

Cell culturing and adhesion assay

Normal human epidermal keratinocytes (NHEK) (Promocell, Heidelberg, Germany) were cultured in supplemented Keratinocyte Growth Medium 2 at 37 °C with 5% CO₂ according to the manufacturer's instructions. For adhesion assays, NHEK cells were grown in 12-well plates until fully confluent. Live bacteria were added to NHEK cells at approximately 10⁶ cfu/well for *S. aureus* and 10⁸ cfu/well for the *L. rhamnosus* strains, as previously described (Mohammedsaeed *et al.*, 2014). After 1 h of co-incubation, NHEK cells were washed in PBS, detached using trypsin (Promocell) and serial dilutions in PBS were plated out on either MSA or Baird-Parker (BP) agar (for *S. aureus* counts) or MRS agar (for *L. rhamnosus* counts). Percentage of adherent bacteria was calculated and standardised to the *L. rhamnosus* GG (for *L. rhamnosus* counts) or *S. aureus* control well counts.

Fluorescence microscopy

Fluorescence microscopy assay was performed as previously described (Spacova *et al.*, 2018). Briefly, NHEK cells were grown on glass slides until confluent and fixed in formaldehyde/glutaraldehyde buffer. Fluorescent strains of wild-type *L. rhamnosus* GG, *L. rhamnosus* GG *spaCBA* mutant or wild-type *L. rhamnosus* GR-1 expressing mCherry were subsequently added to the cells at a concentration of 10⁸ cfu/well, incubated for 1 h and washed three times with PBS. *L. rhamnosus* binding was visualised by fluorescence microscopy (Zeiss Axio Imager Z1, Carl Zeiss AG, Oberkochen, Germany). Three repetitions have been performed for each condition.

Autoaggregation and co-aggregation of bacteria

A modified quantitative spectrophotometric assay (Collado *et al.*, 2008; Ledder *et al.*, 2008) was implemented to determine autoaggregation of *L. rhamnosus* strains and co-aggregation with *S. aureus*. Briefly, *L. rhamnosus* strains

Table 1. Bacterial strains used in this study.

	Strain name	Phenotype	References
Wild-type strains	<i>Staphylococcus aureus</i> (clinical isolate)	Clinical (skin boil) isolate of <i>S. aureus</i> from a human patient	Gift of Prof. Andrew McBain, University of Manchester, UK
	wt LGG	Wild-type (wt) <i>L. rhamnosus</i> GG ATCC 53103	Gorbach, 1996; Segers and Lebeer, 2014
	<i>Lactocaseibacillus rhamnosus</i> GG		
	LGR-1	Wild-type (wt) <i>L. rhamnosus</i> GR-1 ATCC 5582	Reid <i>et al.</i> , 2003
Fluorescent strains	<i>S. aureus</i> -GFP	<i>S. aureus</i> constitutively expressing the green fluorescent protein (GFP)	Gift of Prof. Alexander Horswill, University of Colorado (USA); Malone <i>et al.</i> , 2009
	CMPG11261	<i>L. rhamnosus</i> GG expressing the fluorescent mCherry protein using the nisin-inducible gene expression (NICE) system	Spacova <i>et al.</i> , 2018
	CMPG11265	<i>L. rhamnosus</i> GR-1 expressing the fluorescent mCherry protein using the nisin-inducible gene expression (NICE) system	Spacova <i>et al.</i> , 2018
	CMPG11270	<i>L. rhamnosus</i> GG SpaCBA pili mutant expressing the fluorescent mCherry protein using the nisin-inducible gene expression (NICE) system	Spacova <i>et al.</i> , 2018
<i>L. rhamnosus</i> mutants	LGG <i>spaCBA</i>	SpaCBA pili mutant; impaired biofilm formation and adhesion to human intestinal epithelial cells and macrophages due to lack of pili	Kiekens <i>et al.</i> , 2019; Lebeer <i>et al.</i> , 2012; Vargas Garcia <i>et al.</i> , 2015
	<i>L. rhamnosus</i> CPMG5357		
	LGG <i>welE</i>	Cell wall exopolysaccharides (EPS) mutant, diminished EPS layer; pili and potentially other cell surface adhesins are more exposed, increased adhesion to human intestinal epithelial cells and murine macrophages	Lebeer <i>et al.</i> , 2009, 2011; Kiekens <i>et al.</i> , 2019
	<i>L. rhamnosus</i> CPMG5351		
	LGG <i>welE/spaC</i>	Cell wall EPS and SpaC pili subunit mutant; deficient in adhesion to human intestinal epithelial cells and biofilm formation	Lebeer <i>et al.</i> , 2012
	<i>L. rhamnosus</i> CPMG5365		
	LGG <i>msp1</i>	Major secreted protein 1 (Msp1) mutant producing truncated version of Msp1; impaired in cell separation during cell division	Lebeer <i>et al.</i> , 2012
	<i>L. rhamnosus</i> CPMG10200		
	LGG <i>llp1</i>	Lectin-like protein 1 mutant; reduced adhesion to human intestinal epithelial cells	Petrova <i>et al.</i> , 2016
	<i>L. rhamnosus</i> CPMG10701		
	LGG <i>llp2</i>	Lectin-like protein 2 mutant; reduced adhesion to human intestinal and vaginal epithelial cells, increased biofilm formation	Petrova <i>et al.</i> , 2016
	<i>L. rhamnosus</i> CPMG10706		
<i>L. rhamnosus</i> mutants	LGG <i>llp1/2</i>	Lectin-like proteins 1 and 2 mutant; reduced adhesion to human intestinal and vaginal epithelial cells	Petrova <i>et al.</i> , 2016
	<i>L. rhamnosus</i> CPMG10707		
	LGG <i>mabA</i>	Modulator of adhesion and biofilm formation (MabA) mutant; reduced adhesion to human intestinal epithelial cells and biofilm formation	Perea Vélez <i>et al.</i> , 2010
	<i>L. rhamnosus</i> CPMG5230		
	LGG <i>adhA</i>	Putative adhesion (AdhA) exoprotein mutant	Lebeer <i>et al.</i> , 2012
	<i>L. rhamnosus</i> CPMG5355		
<i>L. rhamnosus</i> mutants	LGG <i>secX</i>	Mutant in SecX accessory transport system for glycosylated proteins	Tytgat <i>et al.</i> , 2015
	<i>L. rhamnosus</i> CPMG10816		

as such or mixed with *S. aureus* were resuspended in PBS to a concentration of 2×10^8 cfu/ml. After incubation at room temperature, optical density in cuvettes was recorded at 600 nm (OD_{600}). The following equation was used to determine the percentages of auto- or co-aggregation:

Aggregation percentage =

$$\frac{OD_{600}(\text{before incubation}) - OD_{600}(\text{after incubation})}{OD_{600}(\text{before incubation})} \times 100$$

Keratinocyte viability assay

Keratinocyte viability in co-culture with bacteria was determined as previously described (Prince *et al.*, 2012). Briefly, live bacteria were added to fully confluent NHEK cells in 12-well plates at approximately 10^6 cfu/well for *S. aureus* and 10^8 cfu/well for the *L. rhamnosus* strains, as previously described (Mohammedsaeed *et al.*, 2014). After 24 h of co-incubation at 37 °C and 5% CO₂, NHEK cells were washed in PBS and detached using trypsin. Cell viability was determined by Trypan blue exclusion assay was implemented to assess NHEK viability as described by Strober (1997).

Time-course analysis of *Staphylococcus aureus* growth with spent culture supernatants or live *Lactocaseibacillus rhamnosus*

Spent culture supernatant was obtained from overnight cultures of *L. rhamnosus* in MRS by centrifugation (15 min, 4,000×g, 4°C) with subsequent filter sterilisation (0.20 µm, VWR, Leuven, Belgium) and pH measurement (Mettler-Toledo AG, Zaventem, Belgium). *S. aureus* growth inhibition by spent culture supernatant or live *L. rhamnosus* was performed as previously described (Van den Broeck *et al.*, 2018). Briefly, overnight cultures of wt or GFP-producing *S. aureus* were diluted 1:100 in Mueller Hinton (MH) medium in a 96-well plate, and either live *L. rhamnosus* at the same dilution, or *L. rhamnosus* supernatant at 1:5 dilution were added to the wells. Growth of *S. aureus* was assessed over the course of 19 to 48 h, either by optical density (OD) at 600 nm (OD₆₀₀), or GFP fluorescence measurement at 488/510 nm in a multi-mode microplate reader (Synergy™ HTX, BioTek, Winooski, VT, USA).

NF-κB pathway activation as a result of Toll-like receptor stimulation in a THP-1 reporter cell line

THP1-Dual™ cells (InvivoGen, San Diego, CA, USA) were maintained according to manufacturer's instructions in growth medium containing RPMI 1640, 2 mM L-glutamine, 25 mM HEPES, 10% heat-inactivated foetal bovine serum, 100 µg/ml Normocin™ and Pen-Strep (100 U/ml-100 µg/ml). For assessment of nuclear factor kappa beta (NF-κB) pathway activation as a result of Toll-like receptor (TLR) stimulation, *S. aureus* was added to THP-1 at 10^6 cfu/ml, while *L. rhamnosus* strains were added at 10^7 cfu/ml, and co-incubated for 24 h at 37 °C and 5% CO₂. NF-κB-SEAP activity was measured at 405 nm according to the manufacturer's instructions after addition of a p-nitrophenyl phosphate (pNPP) solution.

Statistical analysis

Normal distribution of data was evaluated using Shapiro-Wilk and Kolmogorov-Smirnov normality tests. One-

sample t test (for parametric data) or the Wilcoxon Signed Rank Test (for non-parametric data) were performed to statistically compare data to control values corresponding to 100%. In other cases, either an unpaired t test (for parametric data) or Mann-Whitney U (for non-parametric data) test was used for pairwise comparisons, and one-way or two-way ANOVA was used for comparisons between several groups. For combining adhesion experiments data from different 12-well plates, values obtained in control wells (either *S. aureus* or wt *L. rhamnosus* GG) in each plate were set to 100%, and other tested conditions were percentage scaled to control wells values. Figure 6 was created with [BioRender.com](https://www.biorender.com).

3. Results

Presence of SpaCBA pili is crucial for *Lactocaseibacillus rhamnosus* GG adhesion to keratinocytes

First, we aimed to identify key cell surface molecules involved in efficient binding of *L. rhamnosus* GG to human epidermal keratinocytes *in vitro*. We screened a selection of previously validated *L. rhamnosus* GG knock-out mutants in probiotic and adhesion-related molecules (Table 1) and compared their adhesion to wild-type (wt) *L. rhamnosus* GG (Figure 1A). The mutants used in this study have been extensively characterised in previous studies as to their mutant phenotype and molecular and cellular interactions (Table 1). However, their interactions with *S. aureus* or effects on keratinocytes have not yet been described.

Adhesion decreased significantly in *spaCBA* and *welE/spaC* mutants (~24 times in LGG *spaCBA* and ~4 times in LGG *welE/spaC*, $P < 0.01$) and increased, albeit non-significantly, in two of the *welE* mutants (LGG *welE*, $P = 0.0534$ and LGG *welE/msp1*) compared to wt *L. rhamnosus* GG. Of note, adhesion was significantly higher ($P < 0.01$) for the *L. rhamnosus* GG *welE/spaC* mutant compared to *L. rhamnosus* GG *spaCBA* mutant and significantly lower ($P < 0.01$) compared to the *L. rhamnosus* GG *welE* mutant. Adhesion was not significantly affected in *L. rhamnosus* GG *msp1*, *mabA*, *adhA*, *llp1*, *llp2* and *llp1/2* mutants, and the *llp1*, *llp2* and *llp1/2* mutants were omitted from further analysis. A closely related urogenital probiotic strain *L. rhamnosus* GR-1 lacking SpaCBA pili was used for comparison and was found to have approximately 12 times lower adhesion levels to keratinocytes ($P < 0.0001$) compared to *L. rhamnosus* GG (Figure 1A).

To further validate these results, binding of fluorescent *L. rhamnosus* GG (strain CMPG11261), *L. rhamnosus* GG *spaCBA* mutant (strain CMPG11270) and *L. rhamnosus* GR-1 (strain CMPG11265) to keratinocytes was visualised by fluorescence microscopy (Figure 1B). The images confirmed that the fluorescent *L. rhamnosus* GG *spaCBA* knock-out mutant and *L. rhamnosus* GR-1 do not efficiently

Figure 1. (A) Adhesion of *Lactocaseibacillus rhamnosus* GG knock-out mutants and *L. rhamnosus* GR-1 to human keratinocytes compared to wt *L. rhamnosus* GG. **(B)** Adhesion of fluorescent *L. rhamnosus* GG (strain CMPG11261) to human keratinocytes compared to fluorescent *L. rhamnosus* GG *spaCBA* mutant (strain CMPG11270) and *L. rhamnosus* GR-1 (strain CMPG11265) visualised by fluorescence microscopy. Results are depicted as mean $\pm$ standard deviation ($n=3-9$). Data is expressed as percentages of the wild-type *L. rhamnosus* GG (wt LGG) adhesion percentage, which is set to 100% (dotted line). ** $P<0.01$ and **** $P<0.0001$ compared to 100% representing adhesion of wt LGG; + $P<0.01$ compared to LGG *spaCBA*; ° $P<0.01$ compared to LGG *welE*. Strains are labelled according to Table 1.

adhere to human keratinocytes, in contrast to the wt *L. rhamnosus* GG that binds to keratinocytes in high numbers.

Prevention of *Staphylococcus aureus* adhesion is decreased in *Lactocaseibacillus rhamnosus* GG *spaCBA* mutants

L. rhamnosus GG, *L. rhamnosus* GG knock-out mutants and *L. rhamnosus* GR-1 were further tested for their ability to inhibit *S. aureus* adhesion to human keratinocytes. *L. rhamnosus* GG significantly inhibited *S. aureus* adhesion, in contrast to *L. rhamnosus* GR-1 (Figure 2A). The *spaCBA* and *welE/spaC* mutants showed significantly less inhibition of *S. aureus* adhesion (Figure 2A, $P<0.01$ and $P<0.05$, respectively). Interestingly, the *L. rhamnosus* GG *msp1* knock-out mutant known to form aggregates due to a defect in daughter cell separation demonstrated a significantly stronger inhibition of *S. aureus* adhesion compared to wt *L. rhamnosus* GG ($P<0.05$). *L. rhamnosus* GG *mabA* and *adhA* knock-out mutations did not significantly affect *L.*

rhamnosus GG adhesion to keratinocytes or prevention of *S. aureus* adhesion and were omitted from further analysis.

Adhesion to keratinocytes in co-cultures of fluorescent *S. aureus* with either *L. rhamnosus* GG, *L. rhamnosus* GG *spaCBA* mutant or *L. rhamnosus* GR-1 was visualised by fluorescence microscopy (Figure 2B). *S. aureus* cells were less adherent to regions of keratinocytes densely occupied by *L. rhamnosus* GG, while no regions densely occupied with *L. rhamnosus* GG *spaCBA* knock-out mutant and *L. rhamnosus* GR-1 were observed.

Lactocaseibacillus rhamnosus GG co-aggregates with *Staphylococcus aureus*, which is less pronounced in the *spaCBA* and *welE* mutants

Next, we analysed *L. rhamnosus* GG, *L. rhamnosus* GR-1 and a selection of *L. rhamnosus* GG knock-out mutants to assess autoaggregation, and co-aggregation with *S. aureus*. Wt *L. rhamnosus* GG showed more pronounced

Figure 2. (A) Prevention of *Staphylococcus aureus* adhesion to human keratinocytes by *Lactocaseibacillus rhamnosus* GG knock-out mutants and *L. rhamnosus* GR-1 compared to *L. rhamnosus* GG; **(B)** Adhesion of fluorescent *S. aureus*-GFP (in green) in the presence of fluorescent *L. rhamnosus* GG (CMPG11261), fluorescent *L. rhamnosus* GG *spaCBA* mutant (CMPG11270) or fluorescent *L. rhamnosus* GR-1 (CMPG11265) (in red) to human keratinocytes visualised by fluorescence microscopy. Keratinocytes were incubated either with *S. aureus* alone (*S. aureus* condition) or with different *L. rhamnosus* strains (all + conditions). Results are depicted as mean $\pm$ standard deviation, and expressed as percentages of adherent *S. aureus* cfu counts in control wells (*S. aureus* with no *L. rhamnosus* added) ($n=5-13$). Dotted line indicates the result obtained for the *S. aureus* with wild-type *L. rhamnosus* GG condition. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ when compared to wt LGG condition. Strains are labelled according to Table 1.

co-aggregation with *S. aureus* compared to *L. rhamnosus* GG *spaCBA* ($P<0.001$) and *welE* mutants (not significant), and *L. rhamnosus* GR-1 ($P<0.0001$) (Figure 3A). The *L. rhamnosus* GG *msp1* ($P<0.0001$) and *L. rhamnosus* GG *welE/msp1* ($P<0.001$) mutants, and *L. rhamnosus* GR-1 ($P<0.0001$), had a significantly stronger autoaggregative phenotype compared to wt *L. rhamnosus* GG (Figure 3B).

Lactocaseibacillus rhamnosus* GG *spaCBA* and *welE/msp1* mutants are less efficient in improving keratinocyte viability in long-term co-culture with *Staphylococcus aureus

In addition to assessing probiotic-pathogen interactions over short periods of time, we further aimed to elucidate the effects of long-term co-incubation of *L. rhamnosus* GG and its knock-out mutants on *S. aureus* infection of human keratinocytes. Hereto, *S. aureus* was co-incubated with primary human keratinocytes for 24 h, alone or in combination with *L. rhamnosus* GG, a selection of

L. rhamnosus GG knock-out mutants or *L. rhamnosus* GR-1 (Figure 4A). All tested *L. rhamnosus* strains except for *L. rhamnosus* GG *spaCBA* and *welE/msp1* mutants significantly improved keratinocyte viability in a 24 h co-culture with *S. aureus* (Figure 4A).

Secreted factors of *Lactocaseibacillus rhamnosus* GG and GR-1 contributing to medium acidification inhibit long-term *Staphylococcus aureus* growth

To explore additional anti-pathogenic mechanisms of *L. rhamnosus* GG during long-term co-incubation with pathogens, we investigated the potential role of secreted probiotic molecules that inhibit *S. aureus* growth. Hereto, *S. aureus* was co-incubated for 48 h with spent culture supernatant of *L. rhamnosus* GG, *L. rhamnosus* GG knock-out mutants or *L. rhamnosus* GR-1 (Figure 4). Supernatants of *L. rhamnosus* GG and *L. rhamnosus* GR-1 drastically inhibited *S. aureus* growth (Figure 4B). However, when the supernatant pH was adjusted to 7.4, the inhibition was

Figure 3. Co-aggregation with *Staphylococcus aureus* after 1 h (A) and autoaggregation after 1h and 15h (B) for *Lactocaseibacillus rhamnosus* GG, *L. rhamnosus* GG knock-out mutants and *L. rhamnosus* GR-1. *S. aureus* was incubated with different *L. rhamnosus* strains (all + conditions) (A), or bacterial strains were incubated alone (B). Representative results are depicted as mean $\pm$ standard deviation, and expressed as percentages of co- or autoaggregation ($n=3$). Dotted line indicates the results obtained for wild-type *L. rhamnosus* GG. *** $P<0.001$ and **** $P<0.0001$ when compared to wt LGG condition. Strains are labelled according to Table 1.

Figure 4. Effects of long-term co-incubation of *Lactocaseibacillus rhamnosus* with *Staphylococcus aureus*. (A) Improvement of keratinocyte viability by wild-type *L. rhamnosus* GG mutants and *L. rhamnosus* GR-1 after 24 h co-incubation with *S. aureus*. Representative results are depicted as mean $\pm$ standard deviation, and expressed as percentages of live cells from the total number of counted cells ($n=3-4$). Dotted line indicates the results obtained for cells co-incubated with *S. aureus* and wild-type *L. rhamnosus* GG. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ and **** $P<0.0001$ when compared to *S. aureus*. *S. aureus* growth kinetics with (B-C) supernatants (SN) of *L. rhamnosus* GG mutants and *L. rhamnosus* GR-1, or (D) live *L. rhamnosus* over the course of 19-48 h depicted as means per time point ($n=3$). Strains are labelled according to Table 1.

completely abrogated. Likewise, when the MRS medium control pH was adjusted to 4 (approximately equal to the pH of *L. rhamnosus* GG and *L. rhamnosus* GR-1 supernatants, Table 2), *S. aureus* growth was inhibited, although not as strongly as with *Lactocaseibacillus* supernatants. Knock-out mutations in *L. rhamnosus* GG had no significant effects on the ability of live lactobacilli or their supernatants to inhibit *S. aureus* growth. *L. rhamnosus* GG *secX* mutant impaired in secretion of glycosylated protein compounds was additionally included in this analysis, and there was a tendency towards a less pronounced *S. aureus* growth inhibition by the supernatant of this strain (Figure 4C). Co-culturing of an alternative fluorescent *S. aureus* strain with live *L. rhamnosus* GG, *L. rhamnosus* GG knock-out mutants or *L. rhamnosus* GR-1 likewise completely inhibited *S. aureus* growth (Figure 4D).

***Lactocaseibacillus rhamnosus* reduces inflammatory cascade activation by *Staphylococcus aureus* when live and stimulates host Toll-like receptors**

Finally, we aimed to explore the degree of host TLR stimulation in co-culture with live *S. aureus*, alone or in combination with *L. rhamnosus*. Hereto, we implemented a THP-1 monocyte reporter cell line that allows estimation of NF- κ B activation in response to TLR agonists.

Co-incubation with all live *L. rhamnosus* strains except for *L. rhamnosus* GG *welE/msp1* mutant reduced NF- κ B pathway activation by *S. aureus* in these monocytic human cells (Figure 5A). Significant effects of *L. rhamnosus* GG knock-out mutations were detected: live *L. rhamnosus* GG *spaCBA*, *msp1* and *welE/spaC* mutants lowered TLR activation by *S. aureus* in these cells more efficiently, compared to wt *L. rhamnosus* GG (Figure 5A). The *L. rhamnosus* GG *welE* and *L. rhamnosus* GG *welE/msp1* mutants increased *S. aureus*-related TLR activation in the THP-1 reporter cells when compared to wt *L. rhamnosus* GG (Figure 5A). When UV-inactivated *S. aureus* and *L. rhamnosus* were combined, all *L. rhamnosus* strains rather showed a cumulative immunostimulatory effect with *S. aureus* (Figure 5B), in contrast to when live bacteria were used. This immunostimulatory effect was more pronounced for the *L. rhamnosus* GG *welE* mutants in *welE*, *welE/spaC* and *welE/msp1* (Figure 5B). Similarly, when UV-inactivated *L. rhamnosus* was co-incubated with live *S. aureus*, no reduction in TLR activation was observed (Figure 5C).

TLR activation by UV-inactivated *S. aureus* (Figure 5D) in the THP-1 reporter cells was much lower compared to live *S. aureus*. Live and UV-inactivated *L. rhamnosus* GG, *L. rhamnosus* GG mutants and *L. rhamnosus* GR-1 alone were also capable of weak TLR induction (Figure 5D), although at a significantly lower degree than *S. aureus*. The pattern observed for TLR induction by live *L. rhamnosus* (Figure 5D, 'live') was generally similar to that induced by UV-inactivated *L. rhamnosus* (Figure 5D, 'UV'). Intriguingly, only *L. rhamnosus* GG *welE/msp1* induced a much stronger TLR-facilitated response when added live. Live and UV-inactivated *L. rhamnosus* GG *spaCBA* mutant caused less TLR receptor activation compared to wt *L. rhamnosus* GG (Figure 5C) in these specific THP-1 derived reporter cells, while all other tested knock-out mutants appear to be more immunostimulatory compared to wt *L. rhamnosus* GG.

4. Discussion

Cell surface and secreted molecules of probiotic bacteria play a crucial role in their direct and indirect interaction with the surrounding environment. In particular, efficient adherence of probiotic *L. rhamnosus* GG to host cells mediated by these molecules contributes to longer host colonisation and competitive exclusion of pathogens (Lebeer *et al.*, 2018a; Segers and Lebeer, 2014). In this study, we implemented *L. rhamnosus* GG, *L. rhamnosus* GR-1 and a range of *L. rhamnosus* GG knock-out mutants in cell surface and secreted molecules to identify the *L. rhamnosus* GG molecules involved in its probiotic mechanisms of action against a clinical *S. aureus* isolate in co-culture with human skin cells.

Our data suggests an important contribution of SpaCBA pili to pathogen exclusion of *S. aureus* by *L. rhamnosus* GG. Specifically, *L. rhamnosus* GR-1 and *L. rhamnosus* GG mutants lacking SpaCBA pili or the SpaC pilin were significantly less efficient in prevention of *S. aureus* adhesion to keratinocytes. Numerous studies demonstrated inhibition of pathogen adhesion to host cells by lactobacilli in co-cultures with intestinal (Burkholder and Bhunia, 2009) or vaginal (Zárate and Nader-Macias, 2006) cells, either through steric hindrance or competition for receptors and specific adhesion sites. Interestingly, in contrast to our data, *L. rhamnosus* GR-1 was previously shown to displace and prevent the binding of *S. aureus* to female uroepithelial cells (Reid *et al.*, 1995), possibly highlighting

Table 2. pH of the corresponding *Lactocaseibacillus rhamnosus* supernatants.¹

Strain	LGG	LGG <i>spaCBA</i>	LGG <i>welE</i>	LGG <i>msp1</i>	LGG <i>secX</i>	LGR-1
pH	3.84	3.81	3.91	4.08	4.18	4.08

¹ Strains are labelled according to Table 1.

Figure 5. Toll-like (TLR) receptor activation in THP-1 reporter cells co-cultured with (A) live *Staphylococcus aureus* together with live *Lactocaseibacillus rhamnosus*; (B) UV-inactivated *S. aureus* together with UV-inactivated *L. rhamnosus*; (C) Live *S. aureus* together with UV-inactivated *L. rhamnosus*; (D) Live or UV-inactivated *S. aureus* or *L. rhamnosus* alone. Representative results are depicted as mean $\pm$ standard deviation bars, and expressed as OD₄₀₅ values representing NF- κ B-induced SEAP activity as a result of TLR activation assessed with pNPP (n=3). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ when compared to either *S. aureus* with wt LGG, or wt LGG alone conditions. Strains are labelled according to Table 1.

the body niche-dependent differences in probiotic-pathogen and host interactions. Furthermore, our results suggest that the observed probiotic-pathogen interactions are complex, and not determined solely by the presence of SpaCBA pili or the adhesion efficacy of *L. rhamnosus* GG mutants to keratinocytes.

We showed that the presence of SpaCBA pili is also crucial for efficient adhesion of *L. rhamnosus* to keratinocytes. Previous studies suggest that pili of gram-positive bacteria govern tissue tropism – for example, the SpaB and SpaC pilins of *Corynebacterium diphtheriae* facilitate its specific adherence to human pharyngeal epithelial cells (Mandlik *et al.*, 2007). The importance of SpaCBA pili was demonstrated for adhesion to Caco-2 intestinal epithelial cells (Lebeer *et al.*, 2012), murine macrophages (Vargas Garcia *et al.*, 2015) and intestinal mucus (Von Ossowski *et al.*, 2010), and our findings also suggest their key role

for skin probiotics. In addition, the *L. rhamnosus* GG *weE* mutant impaired in the EPS layer formation showed higher adhesion to keratinocytes, possibly as a result of higher exposure of pili (Burgain *et al.*, 2014; Lebeer *et al.*, 2009; Vargas-Garcia *et al.*, 2015). The results from double mutants impaired in both EPS and SpaC production suggest that cell surface molecules masked by EPS other than SpaCBA might be additionally involved. These surface adhesion molecules potentially include adhesive LPXTG- and WxL-anchored cell wall proteins of *L. rhamnosus* GG (Perea Vélez *et al.*, 2010; Petrova *et al.*, 2016; Von Ossowski *et al.*, 2010). While previous studies identified modulator of adhesion and biofilm formation (MabA) (Perea Vélez *et al.*, 2010) and Lectin-like proteins 1 and 2 (Llp1 and Llp2) (Petrova *et al.*, 2016) as *L. rhamnosus* GG adhesion factors to vaginal and/or intestinal epithelial cells, mutations in the corresponding genes did not significantly alter *L. rhamnosus* GG adhesion to keratinocytes in our experimental set-

up. Further research with knock-out mutants, blocking antibodies and cell wall fractions of *L. rhamnosus* GG is required to elucidate the additional factors contributing to *L. rhamnosus* GG adhesion to skin cells besides SpaCBA pili.

Probiotic-pathogen co-aggregation is another property of *L. rhamnosus* GG that can help capture free pathogenic bacteria and prevent their adhesion to host cells (Collado *et al.*, 2008). Additionally, probiotic autoaggregation can lead to more efficient competition with pathogens for host adhesion sites. In our experimental set-up, *L. rhamnosus* GG demonstrates significantly higher co-aggregation with *S. aureus* compared to *L. rhamnosus* GR-1, with co-aggregation percentages in line with previously reported data (Collado *et al.*, 2008). Our results with *L. rhamnosus* GG knock-out mutants imply a role of SpaCBA pili, and to a lesser degree EPS, for binding with *S. aureus*. While the effects of each of these molecules on co-aggregation might be considered relatively small in our study, in combination with other cell surface compounds they could contribute to the multifactorial action of *L. rhamnosus* GG. The involvement of EPS would not be surprising, as lectin-carbohydrate interactions have previously been suggested to play a role in co-aggregation of *L. rhamnosus* GG with oral, intestinal and vaginal pathogens (Allonsius *et al.*, 2017; Ledder *et al.*, 2008). Interestingly, in our study *L. rhamnosus* GR-1 demonstrated higher autoaggregation values compared to wt *L. rhamnosus* GG, despite its inferior ability to co-aggregate with *S. aureus*. Previous research in *L. rhamnosus* showed a correlation between auto- and co-aggregative properties of probiotics (Collado *et al.*, 2008). Nevertheless, our data demonstrates that autoaggregation is not always a predictor for co-aggregation with pathogens, which can be explained by the involvement of different surface molecules during auto- and co-aggregation with other bacterial strains.

All tested live and UV-inactivated *L. rhamnosus* strains were significantly less immunostimulatory than *S. aureus* in monocyte reporter cells, which is in line with their non-pathogenic status. Additionally, host TLR receptor activation by the pathogen *S. aureus* and probiotic lactobacilli are likely different (Kim *et al.*, 2007), and would therefore lead to differential induction of immune signalling cascades in the host. The key role of cell surface molecules in probiotic-host cell interactions was emphasised by the ability of UV-inactivated *L. rhamnosus* to stimulate TLR activation in host cells. Impairment in EPS formation, a clumping phenotype due to mutated Msp1, or a combination thereof, resulted in more pronounced TLR activation. Previous studies demonstrated that EPS can serve as a protective shield against host factors (Lebeer *et al.*, 2011) and mask bacterial surface structures of *L. rhamnosus* GG, such as lipoteichoic acid (LTA) that interacts with TLR2/6 (Claes *et al.*, 2012). This suggests that increased and closer contact of bacterial surface molecules with host cells due to highly

autoaggregative and adhesive probiotic phenotypes could be more immunostimulatory for monocytes/immune cells, although this requires further substantiation.

Other probiotic mechanisms of action in addition to adhesion prevention might become important when *S. aureus* infection of host cells is performed over a longer time period. Live but not UV-inactivated intact *L. rhamnosus* was able to downregulate the long-term inflammatory response to *S. aureus* in monocytes. Both *L. rhamnosus* GR-1 and the *L. rhamnosus* GG mutant lacking SpaCBA pili still partially increased the viability of keratinocytes in long-term co-culture with *S. aureus*, despite their inability to prevent *S. aureus* adhesion. Finally, in our experimental set-up, pro-inflammatory responses induced by *S. aureus* in monocytes were highly dependent on its viability and possibly infectivity, which might be influenced by secreted *L. rhamnosus* molecules.

In fact, our data points to the key role of secreted *L. rhamnosus* metabolites with pH-reducing properties in preventing *S. aureus* infection of keratinocytes. As live *L. rhamnosus* was shown to inhibit *S. aureus* growth, the reduction in immunostimulation of monocytes might be partly explained by *S. aureus* growth inhibition as a result of pH decrease. We indeed further demonstrated that the growth inhibition of *S. aureus* by *L. rhamnosus* was dependent on pH reduction of the culture medium. Although a minor contribution of secreted glycosylated components could be suggested, the inhibitory effect of *L. rhamnosus* GG on long-term *S. aureus* growth is likely linked to lactic acid production. This contributes to previous data on the key role of lactic acid for anti-pathogenic activity of *L. rhamnosus* GG against bacterial pathogens *Salmonella* Typhimurium (De Keersmaecker *et al.*, 2006) and *Moraxella catarrhalis* (Van den Broek *et al.*, 2018).

Healthy skin surface pH is estimated to be 4.7 on average (Lambers *et al.*, 2006) and lactic acid contributing to an acidic pH is already used for dermatological applications, for example to improve keratoses and acne (Tang *et al.*, 2018). While the capacity for lactic acid production by lactobacilli in the desiccated skin niche is yet to be validated, the knowledge that lactic acid has a strong antimicrobial effect on clinical skin isolates of *S. aureus* can contribute to the development of lactic acid-containing products based on probiotic supernatants or lysates.

The proposed multifactorial *L. rhamnosus* GG mechanism of action and the role of different *L. rhamnosus* GG properties in our experimental set-up against *S. aureus* as a skin pathogen is summarised in Figure 6. Our data suggests that different molecules of *L. rhamnosus* GG can demonstrate anti-pathogenic action at different stages of *S. aureus* skin infection. For example, the presence of SpaCBA

Figure 6. Proposed multifactorial *Lactocaseibacillus rhamnosus* GG mechanism of action against *Staphylococcus aureus* as a skin pathogen. Combined action of several molecular components produced by *L. rhamnosus* GG makes it an attractive candidate skin probiotic with potential action at different stages of *S. aureus* skin infection. Genotypes contributing to or enhancing the described properties are indicated in italics. Arrows indicate promotion, T lines indicate inhibition and dotted lines indicate putative indirect effects.

pili ensures *L. rhamnosus* GG adhesion to keratinocytes and prevents initial adhesion of *S. aureus* (likely through host adhesion site blocking and partially co-aggregation), while secreted acidic components prevent further *S. aureus* growth. Ultimately, the combination of these and other probiotic mechanisms that are yet to be explored would result in less cytotoxicity, inflammation and cell death and in the host.

5. Conclusions

In conclusion, we showed that protection of human keratinocytes from *S. aureus* by live *L. rhamnosus* GG is multifactorial and involves both surface and secreted molecules. Adhesive probiotic surface molecules, such as the SpaCBA pili, play a key role during the initial colonisation stage, while soluble secreted factors become more important during long-term interactions of *S. aureus* with the host. The positive anti-inflammatory effects of *L. rhamnosus* on keratinocytes during long-term co-incubation with *S. aureus* are likely not purely immunostimulatory, and involve inhibition of both *S. aureus* growth and adhesion to host cells. The SpaCBA pili and cell wall EPS were

identified as likely key factors in the probiotic-pathogen-host interactions, and involvement of other cell surface molecules embedded in EPS is hypothesised. Future *in vivo* studies will validate whether these mechanisms play an equally important role in the environment of the skin niche and against other clinical *S. aureus* isolates.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

We would like to thank Abigail Elias and Cécile El-Chami for their technical support and insightful discussions. We would also like to express our gratitude to Mariya Petrova, Tine Verhoeven and Camille Allonsius for providing several *Lactocaseibacillus rhamnosus* GG mutants used in this study, and the guidance with their experimental implementation. We also thank Prof. Andrew McBain and Prof. Alexander Horswill for providing the *S. aureus* isolates. IS was partially supported by a personal research stay travel grant from FWO-Vlaanderen K224618N.

References

- Allonsius, C.N., Van den Broek, M.F.L., De Boeck, I., Kiekens, S., Oerlemans, E.F.M., Kiekens, F., Foubert, K., Vandenheuveld, D., Cos, P., Delputte, P. and Lebeer, S., 2017. Interplay between *Lactobacillus rhamnosus* GG and *Candida* and the involvement of exopolysaccharides. *Microbial Biotechnology* 10: 1753-1763. <https://doi.org/10.1111/1751-7915.12799>
- Allonsius, C.N., Vandenheuveld, D., Oerlemans, E.F.M., Petrova, M.I., Donders, G.G.G., Cos, P., Delputte, P. and Lebeer, S., 2019. Inhibition of *Candida albicans* morphogenesis by chitinase from *Lactobacillus rhamnosus* GG. *Scientific Reports* 9: 2900. <https://doi.org/10.1038/s41598-019-39625-0>
- Burgain, J., Scher, J., Francius, G., Borges, F., Corgneau, M., Revol-Junelles, A.M., Cailliez-Grimal, C. and Gaiani, C., 2014. Lactic acid bacteria in dairy food: Surface characterization and interactions with food matrix components. *Advances in Colloid and Interface Science* 213: 21-35. <https://doi.org/10.1016/j.cis.2014.09.005>
- Burkholder, K.M. and Bhunia, A.K., 2009. *Salmonella enterica* serovar Typhimurium adhesion and cytotoxicity during epithelial cell stress is reduced by *Lactobacillus rhamnosus* GG. *Gut Pathogens* 1: 14. <https://doi.org/10.1186/1757-4749-1-14>
- Claes, I.J.J., Schoofs, G., Regulski, K., Courtin, P., Chapot-Chartier, M.-P., Rolain, T., Hols, P., Von Ossowski, I., Reunanen, J., De Vos, W.M., Palva, A., Vanderleyden, J., De Keersmaecker, S.C.J. and Lebeer, S., 2012. Genetic and biochemical characterization of the cell wall hydrolase activity of the major secreted protein of *Lactobacillus rhamnosus* GG. *PLoS ONE* 7: e31588-e31588. <https://doi.org/10.1371/journal.pone.0031588>
- Collado, M.C., Meriluoto, J. and Salminen, S., 2008. Adhesion and aggregation properties of probiotic and pathogen strains. *European Food Research and Technology* 226: 1065-1073. <https://doi.org/10.1007/s00217-007-0632-x>
- De Keersmaecker, S.C., Verhoeven, T.L., Desair, J., Marchal, K., Vanderleyden, J. and Nagy, I., 2006. Strong antimicrobial activity of *Lactobacillus rhamnosus* GG against *Salmonella typhimurium* is due to accumulation of lactic acid. *FEMS Microbiology Letters* 259: 89-96. <https://doi.org/10.1111/j.1574-6968.2006.00250.x>
- Gorbach, S.L., 1996. The discovery of *Lactobacillus* GG. *Nutrition Today* 31: 5S.
- Kankainen, M., Paulin, L., Tynkkynen, S., Von Ossowski, I., Reunanen, J., Partanen, P., Satokari, R., Vesterlund, S., Hendrickx, A.P.A., Lebeer, S., De Keersmaecker, S.C.J., Vanderleyden, J., Hämäläinen, T., Laukkanen, S., Salovuori, N., Ritari, J., Alatalo, E., Korpela, R., Mattila-Sandholm, T., Lassig, A., Hatakka, K., Kinnunen, K.T., Karjalainen, H., Saxelin, M., Laakso, K., Surakka, A., Palva, A., Salusjärvi, T., Auvinen, P. and De Vos, W.M., 2009. Comparative genomic analysis of *Lactobacillus rhamnosus* GG reveals pili containing a human-mucus binding protein. *Proceedings of the National Academy of Sciences of the USA* 106: 17193-17198. <https://doi.org/10.1073/pnas.0908876106>
- Kiekens, S., Vandenheuveld, D., Broeckx, G., Claes, I., Allonsius, C., De Boeck, I., Thys, S., Timmermans, J.-P., Kiekens, F. and Lebeer, S., 2019. Impact of spray-drying on the pili of *Lactobacillus rhamnosus* GG. *Microbial Biotechnology* 12: 849-855. <https://doi.org/10.1111/1751-7915>
- Kim, H.G., Gim, M.G., Kim, J.Y., Hwang, H.J., Ham, M.S., Lee, J.M., Hartung, T., Park, J.W., Han, S.H. and Chung, D.K., 2007. Lipoteichoic acid from *Lactobacillus plantarum* elicits both the production of interleukin-23p19 and suppression of pathogen-mediated interleukin-10 in THP-1 cells. *FEMS Immunology and Medical Microbiology* 49: 205-214. <https://doi.org/10.1111/j.1574-695X.2006.00175.x>
- Lambers, H., Piessens, S., Bloem, A., Pronk, H. and Finkel, P., 2006. Natural skin surface pH is on average below 5, which is beneficial for its resident flora. *International Journal of Cosmetic Science* 28: 359-370. <https://doi.org/10.1111/j.1467-2494.2006.00344.x>
- Lebeer, S., Bron, P.A., Marco, M.L., Van Pijkeren, J.P., O'Connell Motherway, M., Hill, C., Pot, B., Roos, S. and Klaenhammer, T., 2018a. Identification of probiotic effector molecules: present state and future perspectives. *Current Opinion in Biotechnology* 49: 217-223. <https://doi.org/10.1016/j.copbio.2017.10.007>
- Lebeer, S., Claes, I., Tytgat, H.L.P., Verhoeven, T.L.A., Marien, E., Von Ossowski, I., Reunanen, J., Palva, A., De Vos, W.M., De Keersmaecker, S.C.J. and Vanderleyden, J., 2012. Functional analysis of *Lactobacillus rhamnosus* GG pili in relation to adhesion and immunomodulatory interactions with intestinal epithelial cells. *Applied and Environmental Microbiology* 78: 185-193. <https://doi.org/10.1128/AEM.06192-11>
- Lebeer, S., Claes, I.J., Verhoeven, T.L., Vanderleyden, J. and De Keersmaecker, S.C., 2011. Exopolysaccharides of *Lactobacillus rhamnosus* GG form a protective shield against innate immune factors in the intestine. *Microbial Biotechnology* 4: 368-374. <https://doi.org/10.1111/j.1751-7915.2010.00199.x>
- Lebeer, S., Oerlemans, E., Claes, I., Wuys, S., Henkens, T., Spacova, I., Van den Broek, M., Tuytarts, I., Wittouck, S., De Boeck, I., Allonsius, C.N., Kiekens, F. and Lambert, J., 2018b. Topical cream with live lactobacilli modulates the skin microbiome and reduce acne symptoms. *bioRxiv* 463307. <https://doi.org/10.1101/463307>
- Lebeer, S., Verhoeven, T.L.A., Francius, G., Schoofs, G., Lambrichts, I., Dufrêne, Y., Vanderleyden, J. and De Keersmaecker, S.C.J., 2009. Identification of a gene cluster for the biosynthesis of a long, galactose-rich exopolysaccharide in *Lactobacillus rhamnosus* GG and functional analysis of the priming glycosyltransferase. *Applied and Environmental Microbiology* 75: 3554-3563. <https://doi.org/10.1128/AEM.02919-08>
- Ledder, R.G., Timperley, A.S., Friswell, M.K., Macfarlane, S. and McBain, A.J., 2008. Coaggregation between and among human intestinal and oral bacteria. *FEMS Microbiology Ecology* 66: 630-636. <https://doi.org/10.1111/j.1574-6941.2008.00525.x>
- Malone, C.L., Boles, B.R., Lauderdale, K.J., Thoendel, M., Kavanaugh, J.S. and Horswill, A.R., 2009. Fluorescent reporters for *Staphylococcus aureus*. *Journal of Microbiological Methods* 77: 251-260. <https://doi.org/10.1016/j.mimet.2009.02.011>
- Mandlik, A., Swierczynski, A., Das, A. and Ton-That, H., 2007. *Corynebacterium diphtheriae* employs specific minor pilins to target human pharyngeal epithelial cells. *Molecular Microbiology* 64: 111-124. <https://doi.org/10.1111/j.1365-2958.2007.05630.x>

- Mohammedsaeed, W., McBain, A.J., Cruickshank, S.M. and O'Neill, C.A., 2014. *Lactobacillus rhamnosus* GG inhibits the toxic effects of *Staphylococcus aureus* on epidermal keratinocytes. *Applied and Environmental Microbiology* 80: 5773-5781. <https://doi.org/10.1128/AEM.00861-14>
- Peral, M.C., Huaman Martinez, M.A. and Valdez, J.C., 2009. Bacteriotherapy with *Lactobacillus plantarum* in burns. *International Wound Journal* 6: 73-81. <https://doi.org/10.1111/j.1742-481X.2008.00577.x>
- Perea Vélez, M., Petrova, M.I., Lebeer, S., Verhoeven, T.L., Claes, I., Lambrichts, I., Tynkkynen, S., Vanderleyden, J. and De Keersmaecker, S.C., 2010. Characterization of MabA, a modulator of *Lactobacillus rhamnosus* GG adhesion and biofilm formation. *FEMS Immunology and Medical Microbiology* 59: 386-98. <https://doi.org/10.1111/j.1574-695X.2010.00680.x>
- Petrova, M.I., Imholz, N.C.E., Verhoeven, T.L.A., Balzarini, J., Van Damme, E.J.M., Schols, D., Vanderleyden, J. and Lebeer, S., 2016. Lectin-like molecules of *Lactobacillus rhamnosus* GG inhibit pathogenic *Escherichia coli* and *Salmonella* biofilm formation. *PLoS ONE* 11: e0161337-e0161337. <https://doi.org/10.1371/journal.pone.0161337>
- Prince, T., McBain, A.J. and O'Neill, C.A., 2012. *Lactobacillus reuteri* protects epidermal keratinocytes from *Staphylococcus aureus*-induced cell death by competitive exclusion. *Applied and Environmental Microbiology* 78: 5119-5126. <https://doi.org/10.1128/AEM.00595-12>
- Ramos, A.N., Sesto Cabral, M.E., Arena, M.E., Arrighi, C.F., Arroyo Aguilar, A.A. and Valdéz, J.C., 2015. Compounds from *Lactobacillus plantarum* culture supernatants with potential pro-healing and anti-pathogenic properties in skin chronic wounds. *Pharmaceutical Biology* 53: 350-358. <https://doi.org/10.3109/13880209.2014.920037>
- Reid, G., Charbonneau, D., Erb, J., Kochanowski, B., Beuerman, D., Poehner, R. and Bruce, A.W., 2003. Oral use of *Lactobacillus rhamnosus* GR-1 and *L. fermentum* RC-14 significantly alters vaginal flora: randomized, placebo-controlled trial in 64 healthy women. *FEMS Immunology and Medical Microbiology* 35: 131-134. [https://doi.org/10.1016/S0928-8244\(02\)00465-0](https://doi.org/10.1016/S0928-8244(02)00465-0)
- Reid, G., Tieszer, C. and Lam, D., 1995. Influence of lactobacilli on the adhesion of *Staphylococcus aureus* and *Candida albicans* to fibers and epithelial cells. *Journal of Industrial Microbiology* 15: 248-253. <https://doi.org/10.1007/BF01569832>
- Segers, M.E. and Lebeer, S., 2014. Towards a better understanding of *Lactobacillus rhamnosus* GG – host interactions. *Microbial Cell Factories* 13, Suppl. 1: S7. <https://doi.org/10.1186/1475-2859-13-S1-S7>
- Spacova, I., Lievens, E., Verhoeven, T., Steenackers, H., Vanderleyden, J., Lebeer, S. and Petrova, M.I., 2018. Expression of fluorescent proteins in *Lactobacillus rhamnosus* to study host-microbe and microbe-microbe interactions. *Microbial Biotechnology* 11: 317-331. <https://doi.org/10.1111/1751-7915.12872>
- Strober, W., 1997. Trypan blue exclusion test of cell viability. *Current Protocols in Immunology* 21: A.3B.1-A.3B.2. <https://doi.org/10.1002/0471142735.ima03bs21>
- Tang, S.C. and Yang, J.H., 2018. Dual effects of alpha-hydroxy acids on the skin. *Molecules* 23: 863. <https://doi.org/10.3390/molecules23040863>
- Tytgat, H., 2015. Glycoproteins in probiotic bacteria – exploring the glycosylation potential of *Lactobacillus rhamnosus* GG by genomics and glycoproteomics. Doctoral dissertation, KU Leuven, Leuven, Belgium.
- Van den Broek, M.F.L., De Boeck, I., Claes, I.J.J., Nizet, V. and Lebeer, S., 2018. Multifactorial inhibition of lactobacilli against the respiratory tract pathogen *Moraxella catarrhalis*. *Beneficial Microbes* 9: 429-439. <https://doi.org/10.3920/BM2017.0101>
- Vargas García, C.E., Petrova, M., Claes, I.J., De Boeck, I., Verhoeven, T.L., Dilissen, E., Von Ossowski, I., Palva, A., Bullens, D.M., Vanderleyden, J. and Lebeer, S., 2015. Piliation of *Lactobacillus rhamnosus* GG promotes adhesion, phagocytosis, and cytokine modulation in macrophages. *Applied and Environmental Microbiology* 81: 2050-2062. <https://doi.org/10.1128/AEM.03949-14>
- Vesterlund, S., Karp, M., Salminen, S. and Ouwehand, A.C., 2006. *Staphylococcus aureus* adheres to human intestinal mucus but can be displaced by certain lactic acid bacteria. *Microbiology* 152: 1819-1826. <https://doi.org/10.1099/mic.0.28522-0>
- Von Ossowski, I., Reunanen, J., Satokari, R., Vesterlund, S., Kankainen, M., Huhtinen, H., Tynkkynen, S., Salminen, S., De Vos, W.M. and Palva, A., 2010. Mucosal adhesion properties of the probiotic *Lactobacillus rhamnosus* GG SpaCBA and SpaFED pilin subunits. *Applied and Environmental Microbiology* 76: 2049-2057. <https://doi.org/10.1128/AEM.01958-09>
- Zárate, G. and Nader-Macias, M.E., 2006. Viability and biological properties of probiotic vaginal lactobacilli after lyophilization and refrigerated storage into gelatin capsules. *Process Biochemistry* 41: 1779-1785. <https://doi.org/10.1016/j.procbio.2006.03.024>
- Zhang, Z., Zhou, Z., Li, Y., Zhou, L., Ding, Q. and Xu, L., 2016. Isolated exopolysaccharides from *Lactobacillus rhamnosus* GG alleviated adipogenesis mediated by TLR2 in mice. *Scientific Reports* 6: 36083. <https://doi.org/10.1038/srep36083>