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Research Article

RESEARCH ARTICLE

Antimicrobial activity of *Lactobacillus salivarius* and *Lactobacillus fermentum* against *Staphylococcus aureus*

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One sentence summary: *L. salivarius* kills planktonic cells and biofilms of *S. aureus*.
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ABSTRACT

The increasing prevalence of methicillin-resistant *Staphylococcus aureus* has become a major public health threat. While lactobacilli were recently found useful in combating various pathogens, limited data exist on their therapeutic potential for *S. aureus* infections. The aim of this study was to determine whether *Lactobacillus salivarius* was able to produce bactericidal activities against *S. aureus* and to determine whether the inhibition was due to a generalized reduction in pH or due to secreted *Lactobacillus* product(s). We found an 8.6-$\log_{10}$ reduction of planktonic and a 6.3-$\log_{10}$ reduction of biofilm *S. aureus*. In contrast, the previously described anti-staphylococcal effects of *L. fermentum* only caused a 4.0-$\log_{10}$ reduction in planktonic *S. aureus* cells, with no effect on biofilm *S. aureus* cells. Killing of *S. aureus* was partially pH dependent, but independent of nutrient depletion. Cell-free supernatant that was pH neutralized and heat inactivated or proteinase K treated had significantly reduced killing of *L. salivarius* than with pH-neutralized supernatant alone. Proteomic analysis of the *L. salivarius* secretome identified a total of five secreted proteins including a LysM-containing peptidoglycan binding

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protein and a protein peptidase M23B. These proteins may represent potential novel anti-staphylococcal agents that could be effective against *S. aureus* biofilms.

Keywords: antibacterial activity; biofilm; *Lactobacillus fermentum*; *Lactobacillus salivarius*; LysM; *Staphylococcus aureus*

INTRODUCTION

Staphylococcus aureus is a Gram-positive coccal bacterial species that persistently colonizes the skin, nares or pharyngeal surfaces in 25%–30% humans (Wertheim *et al.* 2004), but also causes invasive infections (Montoiro Allué, Moreno Loshuertos and Sánchez Marteles 2010). Morbidity and mortality of *S. aureus* infections are high, specifically with the increasing occurrence of methicillin-resistant staphylococcus aureus (MRSA), and hence leading to extensive health care costs (Lowy 1998). Therefore, the development of a successful treatment strategy is warranted.

Lactobacilli are Gram-positive, non-spore-forming bacilli that produce antibacterial peptides and small proteins called bacteriocins, which have beneficial effect on the host when administered as live organisms in adequate amounts (Alvarez-Olmos and Oberhelman 2001; Reid and WHO 2005; Messaoudi *et al.* 2013). It has been postulated that their probiotic activity may be due to (i) direct inhibition of microbial growth (Alvarez-Olmos and Oberhelman 2001; Karska-Wysocki, Bazo and Smoragiewicz 2010), (ii) competition for space or nutrients, (iii) immune-modulatory activity and/or (iv) modulation of the intestinal barrier (Drago *et al.* 2015).

While many strains of lactobacilli are known to have probiotic effects against *S. aureus*, oral strains such as *Lactobacillus salivarius* have largely been ignored (Varma *et al.* 2011; Messaoudi *et al.* 2013; Drago *et al.* 2015). It has been found that *L. salivarius* was able to directly inhibit non-staphylococcal intestinal bacterial strains through a peptide bacteriocin (Silva *et al.* 1987; Pridmore *et al.* 2008; Dobson *et al.* 2012). However, it is unknown if this inhibitory effect also occurs against *S. aureus*, and whether any noted bactericidal properties are due to pH modification, nutrient deprivation or secretome components.

Therefore, the aim of this study was to demonstrate and determine the anti-staphylococcal properties of *L. salivarius* on planktonic and biofilm *S. aureus* cells compared to a well-studied *Lactobacillus* species, *L. fermentans*. Furthermore, the mechanism of inhibition of *S. aureus* growth was investigated, with a focus on identifying potential secretome proteins that may be responsible for the antimicrobial activity, using 2D gel electrophoresis and matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry (MALDI-ToF/ToF MS) analysis.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Methicillin-resistant *Staphylococcus aureus* (MRSA) (M2 (Harro *et al.* 2013), USA300 JE2 (Diep *et al.* 2006) and USA300 SAP149 (Plaut *et al.* 2013)), and methicillin-susceptible *S. aureus* strains (ATCC 25923 (Treangen *et al.* 2014), RN6390 (Cassat *et al.* 2006) and NCTC 8325-4 (Baek *et al.* 2013)) were used. For initial experiments, two commercially available laboratory lactobacilli strains were used for proof of principle tests (*Lactobacillus salivarius* KCTC3156 (Li *et al.* 2006), *L. fermentum* ATCC 14931). Thereafter, two *Lactobacillus* strains were isolated from the oral mucosa of healthy children (4–7 years). Isolates were identified as *L. salivarius* and *L. fermentans* by standard biochemical testing (API CH50 system, BioMérieux, Marcy l'Etoile, France) and further characterized as described below.

Molecular identification of two oral isolated *Lactobacillus* strains

For molecular identification of the two *Lactobacillus* strains, 16S rDNA sequence analysis was performed. Chromosomal DNA was isolated as previously described with slight modifications (Wilson and Carson 2001) and the 16S rDNA gene was amplified by PCR, using the universal primers 27F [5′-AGAGTTTGATCCTGGCTCAG; positions 8–27 (*Escherichia coli* numbering)] and 1522R (5′-AAGGAGGTGATCCAGCCGCA; positions 1541-1522) (Weisburg *et al.* 1991). The PCR products were then purified with a Wizard PCR Preps DNA Purification System (Promega, Madison, WI, USA) according to the manufacturer's protocol, and sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) on an automatic sequencer (model 310, Applied Biosystems). The sequences of known strains closely related to the two newly identified strains were retrieved (GenBank, Ribosomal Database Project databases) and nucleotide sequence similarities determined (PHYDIT).

Antibacterial activity of *Lactobacillus salivarius* and *Lactobacillus fermentum* in co-culture with planktonic *Staphylococcus aureus* cells

Lactobacilli strains and *S. aureus* were separately grown in De Man, Rogosa, Sharpe (MRS) broth (Difco Laboratories, Detroit, MI, USA) at 37°C for 16 h and in Tryptic Soy broth (TSB) (Sigma, St. Louis, MO, USA) at 37°C overnight, respectively. Then, both lactobacilli and *S. aureus* were equally inoculated (1:1) using a starting inoculum of 5×10^6 CFUs in amounts adjusted to OD_{600} (optical density at 600 nm). Growth (CFU/ml) of *S. aureus* at 37°C was determined after 4, 8 and 24 h using serial fold dilutions on MRS agar for *Lactobacillus* strains and CHROM agar (CHROMagar Microbiology, Paris, France) for *S. aureus* strains (in triplicates). Acidification of the culture medium by bacterial byproducts and acid production was furthermore detected (Accumet, model AP61, Fisher Scientific).

Antibacterial activity of *Lactobacillus salivarius* and *Lactobacillus fermentum* against biofilm formation in *Staphylococcus aureus*

The effect of *L. salivarius* and *L. fermentans* on *S. aureus* biofilms was studied using a colony biofilm assay as described by Anderl, Franklin and Stewart (2000) with slight modifications. Briefly, sterile polycarbonate semipermeable membrane filters

(diameter, 25 mm; pore size, 0.2 μm; GE Water & Process Technologies, Trevose, PA, USA) were placed on Tryptic Soy agar (TSA) to allow easy transfer of the surface-grown biofilm from one media to another. Liquid overnight cultures of *S. aureus* strain M2 were diluted to an OD_{600} of 0.1 in TSB, spotted onto the center of individual membrane filters (100 μl; 1×10^7 CFU/ml) and incubated at 37°C. After 24 h, the membrane-supported *S. aureus* biofilm was transferred to fresh culture TS agar plates mixed 1:1 with cell-free supernatants (CFS) of *Lactobacillus* (described below) and incubated at 37°C. As a negative control, MRS without CFS of *Lactobacillus* was used. Finally, the antibacterial activity of *L. salivarius* and *L. fermentum* was detected by two methods.

Staphylococcus aureus biofilm was harvested after 0, 8 and 24 h, homogenized at high speed for 1 min in 5 ml of PBS (Polytron PT1200E, Kinematica AG, Lucerne, Switzerland), serially diluted in PBS and plated in triplicates for viable CFU counts.

Staphylococcus aureus biofilm membranes were stained with BacLight LIVE/DEAD viability kit (Invitrogen, Carlsbad, CA, USA) to visualize viable (in green, Syto9) and dead cells (in red propidium iodide). Membrane-supported biofilms were mounted onto glass slides with Vectashield (Vector Laboratories, Burlingame, CA, USA) and processed for confocal laser scanning microscope. The biofilm was determined by analysis of confocal z-axis image slices using the LSMIX software package (Carl Zeiss, Thornwood, NY, USA).

Evaluating the effects of lactobacilli-dependent nutrient deprivation, pH or antimicrobial proteins on *Staphylococcus aureus*

Depletion of metabolic substances through medium adjustment

To test whether the underlying mechanism of action is related to depletion of metabolic substances, the antimicrobial activity on *S. aureus* was tested after changing media. Growth medium was changed after 8 h of incubation by centrifugation at 4000 $\times$ *g* for 15 min and resuspension of the pellet in fresh medium. Cultures were incubated for another 16 h at 37°C and serial dilutions were performed in PBS (triplicates) on either MRS or CHROM agar plates to enumerate the *Lactobacillus* species or *S. aureus* CFUs, respectively. CFUs/ml of samples with medium change were compared to samples without change.

Acidification of the environment by medium adjustment

A similar approach as described above was used, but by use of pH-buffered medium (pH 6.5, 0.1 M MES (2-[N-Morpholino] ethanesulfonic acid monohydrate). All other steps were identical. Furthermore, a cell-free culture (CFS) supernatant assay was conducted that allows determining whether the inhibitory factor on *S. aureus* is an organic acid (Kang *et al.* 2012). Briefly, CFS of lactobacilli obtained from liquid culture by centrifugation (6000 $\times$ *g*, 20 min, 4°C) was sterile-filtered (0.22 μm pore size; Millipore, Billerica, MA, USA) after 24, 48 and 72 h of growth and neutralized with NaOH (5 M, 37°C, 2 h). Treated and untreated CFSs (100 μl) were inoculated with *S. aureus* (100 μl, 5×10^5 CFU/ml) in TSB growth medium. After 4, 8 and 24 h incubation, the level of microbial growth was measured at OD_{600} using a DTX880 multimode detector (Beckman Coulter, Brea, CA, USA). The reduction of optimal density in percentage (in correlation to microbial growth) was calculated as 100% –[(OD Test group ÷ OD *S. aureus*) $\times$ 100%].

Depletion of proteins and secretome analysis

The CFS assay described above was adjusted to specifically investigate protein involvement. Briefly, CFS of lactobacilli were NaOH-neutralized heated at 95°C for 10 min or treated with proteinase K (1 mg/ml; Sigma) at 37°C for 2 h followed by enzymatic heat inactivation at 95°C for 1 min. All other steps were identical to acidification experiments.

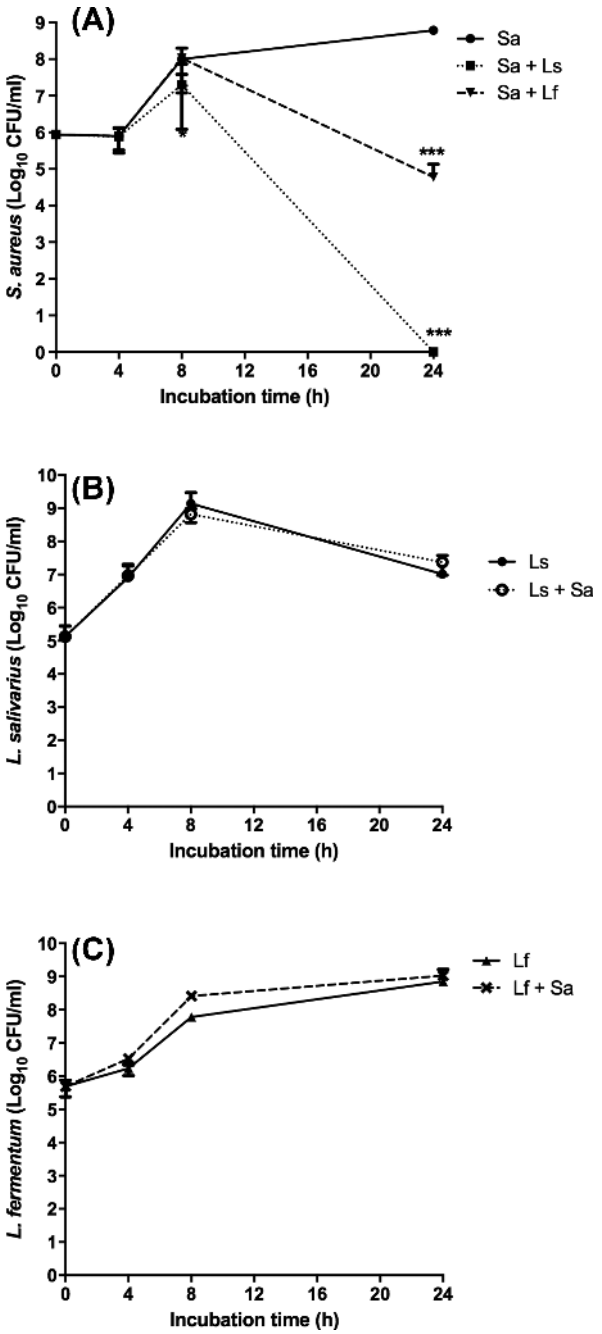

Figure 1. Killing curve of *S. aureus* M2 (*Sa*) in co-culture with *L. salivarius* (*Ls*) and *L. fermentum* (*Lf*) using two laboratory standard and two orally isolated clinical strains (*Lf* CNU1334, KCTC3156 and *Lf* CNU1969, ATCC14931) over 24 h. Viable cells of *S. aureus* (**a**), *L. salivarius* (**b**) and *L. fermentum* (**c**) of mono- and co-cultures were determined and expressed as the mean ± SEM performed in triplicate. In (a), significant differences to *Sa* culture alone at 24 h determined as $^*P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

To identify possible proteins within the secretome of *Lactobacillus* isolates that were responsible for the anti-staphylococcal effects, total secreted proteins in the stationary phase of lactobacilli growth were precipitated as previously described with minor modifications(Sánchez *et al.* 2009). Sodium deoxycholate (Sigma) was added at a final concentration of 0.2% (v/v) to CFS of either *L. salivarius* or *S. fermentum*, mixed and incubated on ice for 30 min. Thereafter, chilled trichloroacetic acid (Sigma) was added at a final concentration of 6% (v/v), vortexed for 30 s and allowed to precipitate for overnight at 4°C. Proteins were recovered by centrifugation (9300 × *g*, 10 min, 4°C). Pellets were washed twice with 2 ml of chilled acetone (Sigma), harvested by centrifugation (15000 × *g*, 10 min, 4°C), dried at room temperature and proteins were re-solubilized in 1 ml of 0.02 M Tris (pH 8.8) by ultrasonication for 3 min (Laboratory Supplies Co, Hicksville, NY, USA). Crude secreted protein extracts were precipitated and purified with Perfect-Focus reagent (G-Biosciences, Maryland Heights, MO) according to the manufacturer's directions. The minimal bactericidal concentration of the lactobacilli-secreted proteins was identified, using a broth microdilution method (Kang *et al.* 2013). A total of 200 μg proteins and successive 2-fold dilutions of proteins were resuspended in 100 μl PBS, and 100 μl each of bacteria was added to prepare 96-well plates. The final inoculum concentration of bacterium was 5×10^5 CFU/ml. The controls consisted of cells grown in the medium only. After 24 h incubation, the level of microbial growth was measured as described above.

Isolated secreted proteins (200 μg) were separated on 2D gel electrophoresis (Brady *et al.* 2006; Achermann *et al.* 2015), proteins were stained with Sypro Ruby (Lonza, Rockland, ME, USA) and gel images were captured using the FluorChem 8900 (Alpha Innotech, San Leandro, CA, USA). Protein spots were visually selected and excised for MALDI-ToF/ToF MS analysis as described previously (Brady *et al.* 2006). Protein spots were equilibrated in 50 mM Ammonium bicarbonate (Sigma) and washed twice with ultrapure water, followed by an extraction of pure acetonitrile (Thermo Scientific, Rockford, IL, USA). To remove any excess liquids from the gel spots, samples were kept in a speedvac for several hours. Thereafter, activated Trypsin (Trypsin Gold, Promega) was added to each sample for digestion overnight at 37°C. Finally, 1 μl of the peptide solution and 10 μg of the Matrix (Alpha-cyano-4-hydroxycinnamic acid (Thermo Scientific) in 1 μl volume was directly spotted onto the MALDI Target plate (MTP 384, ground steel T F Bruker, Nr. 209519) and allowed to dry. The MALDI-Tof MS instrument was operated in positive ion reflector mode, mass range 700–3500 Da. An external calibration was performed using a peptide mixture (Bruker Peptide Calibration Mixture II, Thermo Scientific). The data were analyzed with the MASCOT software.

Statistical analyses

Statistical analysis was carried out using SPSS (Version 19.0; SPSS Inc., Chicago, IL, USA). To compare categorical variables, Student's *t*-tests (two-sided) or Fisher's exact test (as appropriate) were used. Results were considered significant if *P* values were < 0.05.

RESULTS

Isolation and identification of two *Lactobacillus* isolates

16S rDNA sequencing revealed that one of the oral isolate was *Lactobacillus salivarius* subsp. *salicinius* (CNU1334) with high similarity to the published reference strain *L. salivarius* subsp. *salicinius* JCM 1230 and *L. salivarius* subsp. *salivarius* ATCC 11741T. An according phylogenetic tree is shown in Fig. S1 (Supporting Information). The other oral isolate was identified as *L. fermentum* (CNU1969) with 99.04% similarity to *L. fermentum* ATCC 14931T (accession no. M58819) (nt D/C: 6/622), *L. thermotolerans* DSM 14792T (accession no. AF317702) (93.45%, nt D/C: 41/626) and *L. ingluviei* LMG 20380T (accession no. AF333975) (93.45%, nt D/C: 41/626).

Killing of planktonic *Staphylococcus aureus* cells in co-culture with *Lactobacillus salvarius* and *Lactobacillus fermentum*

Both tested laboratory and oral strains of *L. salivarius* (CNU1334, KCTC 3156) and *L. fermentum* (CNU1969, ATCC 14931) led to significant reductions in log10 CFUs of *S. aureus* strain M2 over 24 h (8.6 and 4.0-log10 reduction, respectively, $P < 0.05$), with

Table 1. Antimicrobial activity of *L. salivarius* CNU1334 (*Ls*) and *L. fermentum* CNU1969 (*Lf*) against different *S. aureus* (*Sa*) strains (1a), and different *Sa* strains against *Ls* and *Lf* (Log_{10} CFU/ml) (1b).

1a	Growth of *S. aureus* (Log10 CFU/ml) [a]					
	Sa M2	*Sa* USA 300 JE2	*Sa* USA 300 SAP149	*Sa* ATCC 25923	*Sa* RN6390	*Sa* 8325-4
Sa	8.97 ± 0.03	8.10 ± 0.04	8.42 ± 0.09	7.67 ± 0.19	7.79 ± 0.10	7.51 ± 0.09
SA + *Ls*	ND	ND	ND	ND	ND	ND
Sa + *Lf*	3.00 ± 2.61	2.10 ± 1.83	1.00 ± 1.73	ND	3.52 ± 0.07	1.10 ± 1.90

1b	Growth of *Lactobacillus* (Log_{10} CFU/ml)[a]	
	Ls	*Lf*
Lactobacillus alone	6.52 ± 0.07	7.87 ± 0.15
S. aureus M2 in co-culture	7.60 ± 0.00	9.39 ± 0.01
S. aureus USA300 JE2 in co-culture	7.85 ± 0.20	9.10 ± 0.11
S. aureus USA300 SAP149 in co-culture	7.54 ± 0.28	8.93 ± 0.10
S. aureus ATCC25923 in co-culture	7.40 ± 0.35	9.14 ± 0.17
S. aureus RN6390 in co-culture	7.67 ± 0.06	9.08 ± 0.07
S. aureus 8325-4 in co-culture	8.51 ± 0.11	9.18 ± 0.06

[a]Viable cell counts of *S. aureus* and *Lactobacillus* in co-cultures were determined after 24 h incubation. The data are expressed as the mean ± SD of a representative experiment performed in triplicate.
ND, non-detectable.

L. salivarius leading to complete killing of *S. aureus* cells (Fig. 1a). The killing effect of both *L. salivarius* CNU1334 and *L. fermentum* CNU1969 in co-culture with *S. aureus* was independent of the *S. aureus* strains and their antimicrobial resistance (Table 1). The effect of killing against different *S. aureus* strains was generally enhanced using *L. salivarius* compared to *L. fermentum* except against the *S. aureus* ATCC 25923 strain with complete killing by *L. fermentum*. Growth of *Lactobacillus* strains was not affected by *S. aureus* in co-culture over 24 h (Fig. 1b and c, Table 1). The starting pH of 6.5 decreased over 24 h to pH 4.4 in *S. aureus* alone and to pH 3.7 and pH 4.1 in co-cultures with *L. salivarius* and *L. fermentum*, respectively.

Impact of *Lactobacillus salivarius* and *Lactobacillus fermentum* on preformed *Staphylococcus aureus* biofilm

Growth of preformed biofilm with *S. aureus* M2 strain in co-culture with *L. salivarius* CNU1334 in a static biofilm assay over

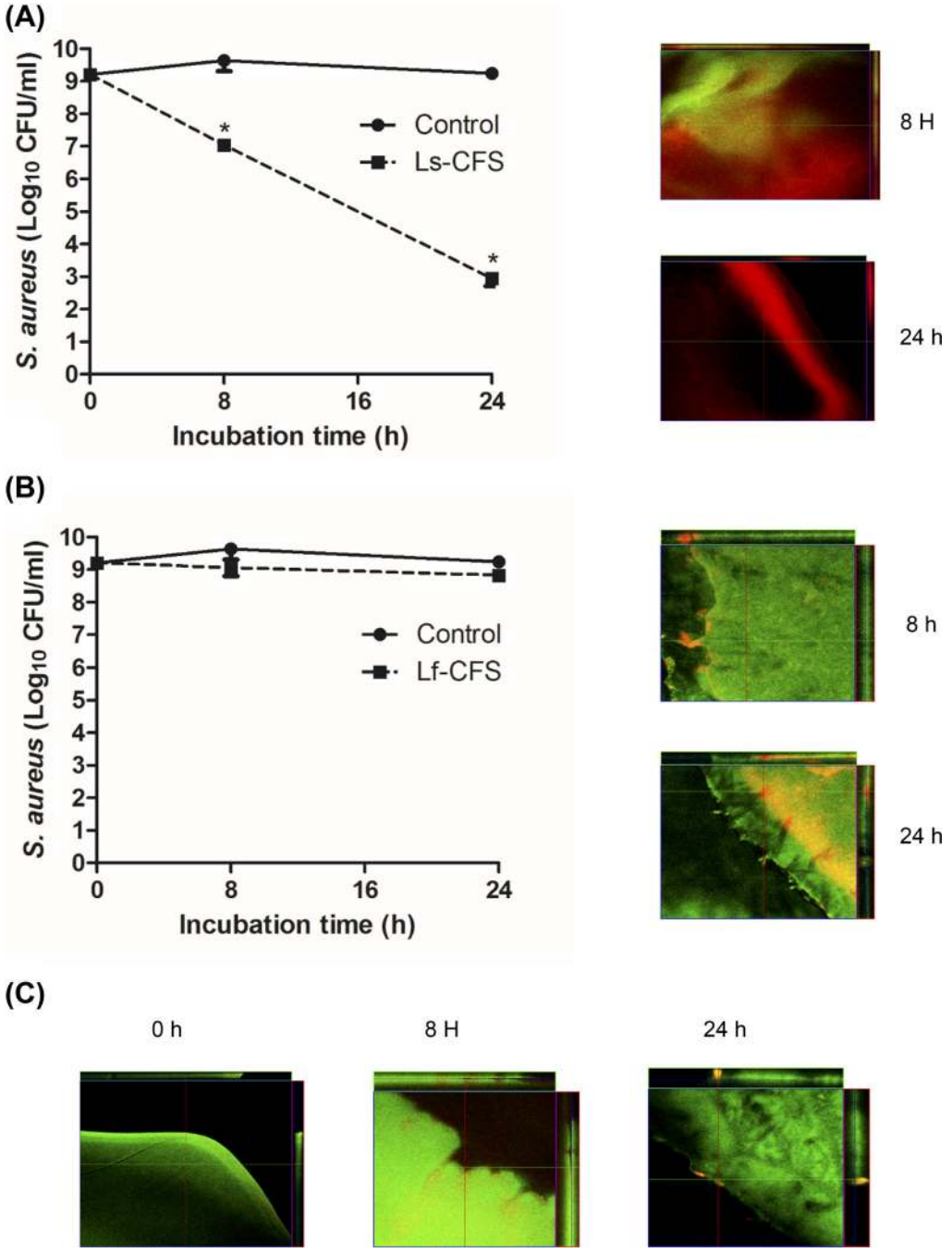

Figure 2. Effect of *L. salivarius* CNU1334 (**a**) and *L. fermentum* CNU1969 (**b**) on *S. aureus* M2 biofilm in co-culture at 8 and 24 h using a colony biofilm assay for CFU counting and CLSM for visualizing live/dead bacteria. Panel **c** shows an *S. aureus* M2 biofilm as a monobacterial culture 0, 8 and 24 h as the control without any *Lactobacillus* treatment. All images are shown as representative confocal z-stack images.

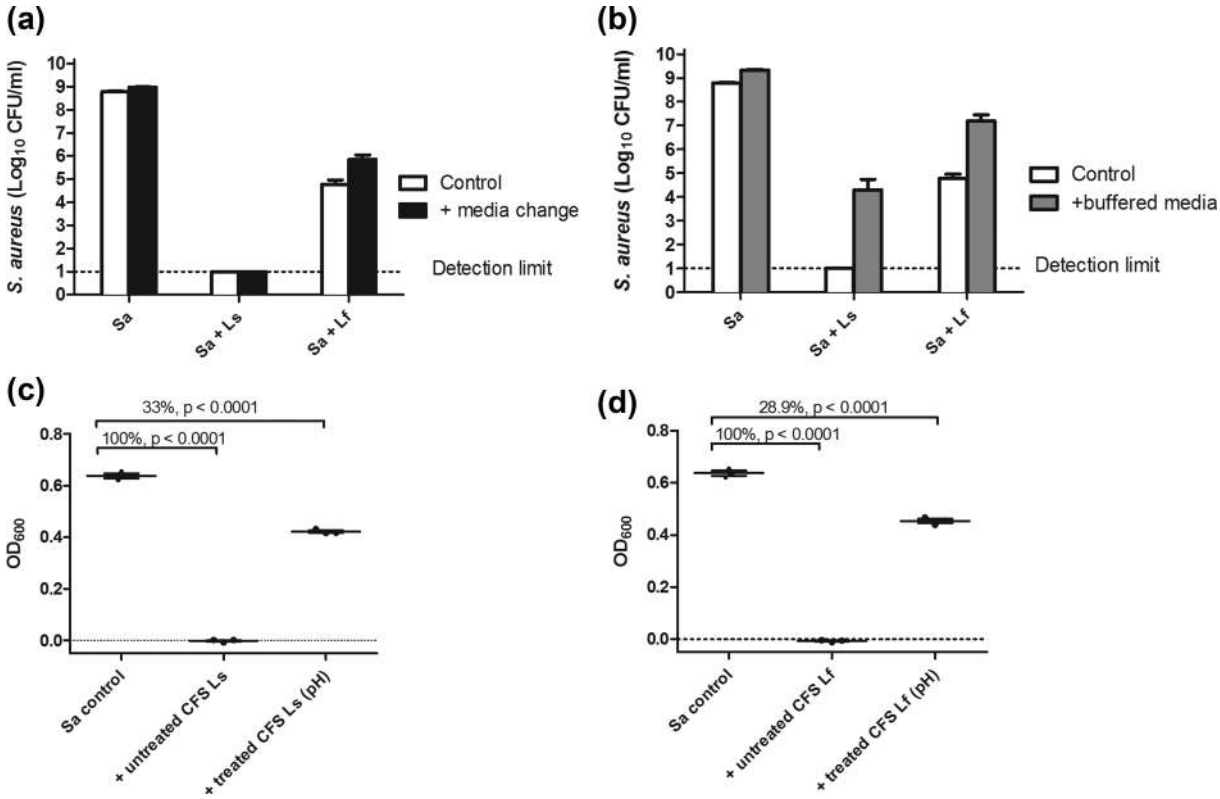

Figure 3. Effect of nutrient depletion (**a**) and acidification (**b–d**) on *S. aureus* M2 (*Sa*) growth in co-culture with the oral isolates *L. salivarius* CNU1334 (*Ls*) and *L. fermentum* CNU1969 (*Lf*). (**a**) Media change neither change the antibacterial activity of *Ls* nor *Lf* after 24 h. (b–d) However, pH-buffered media (0.1 M MES) strongly reduced killing effect of both *Ls* and *Lf* on *Sa* in a co-culture (b) and in a NaOH-neutralized CFS assay (**c**, d). Data are expressed as the mean ± SD of a representative experiment performed in triplicate.

24 h lead to a 6.3-$\log_{10}$ reduction ($P = 0.007$) (Fig. 2a), whereas *L. fermentum* CNU1969 only slightly decreased the number of viable *S. aureus* cells over time (Fig. 2b). Confocal microscopy and LIVE/DEAD staining confirmed these results.

Effect of lactobacilli-dependent nutrient depletion on anti-staphylococcal activity

Changing media neither changed the antibacterial activity of *L. salivarius* nor of *L. fermentum* against *S. aureus* (Fig. 3a), indicating that nutrient depletion does not contribute to their antimicrobial activity.

Effect of lactobacilli-dependent media acidification on anti-staphylococcal activity

As low pH may play a role in the observed effects, pH adjustment experiments were conducted and demonstrated that fresh pH-buffered media showed a strong effect on *S. aureus* killing with reduction of 2.6-$\log_{10}$ in *L. salivarius*, and 1.8-$\log_{10}$ reduction in *L. fermentum* (Fig. 3b). The crucial role of acidification could be confirmed by use of CFS assays. As the most effective antibacterial activity of CFS of *L. salivarius* CNLU1334 and *L. fermentum* CNU1969 on *S. aureus* was observed with supernatants collected after 24 h of growth with a 24-h incubation time (Fig. S2, Supporting Information), this experimental design was further used for all CFS assays. When CFS of *L. salivarius* and *L. fermentum* was pH neutralized, the optimal density was significantly decreased to 33.9% and 29.9%, respectively (100%, $P < 0.0001$) (Fig. 3c and d).

Effect of lactobacilli secretome on anti-staphylococcal activity

CFS assays were furthermore used to test whether the release of antimicrobial proteins is one of the underlying mechanisms of action. Proteinase K treatment, which was used to inactivate secreted proteins in the CFS, reduced the optimal density of *L. salivarius* to 22.8% (Fig. 4a) and of *L. fermentum* to 30.1% (Fig. 4b). These results indicate that secreted proteins are important for the antimicrobial activity of these strains against *S. aureus*. Importantly, relatively low concentrations of secreted proteins of *L. salivarius* (25 μg, Fig. 4c) and *L. fermentum* (100 μg, Fig. 4d) were able to effectively eradicate *S. aureus*.

Identification of secreted proteins of *Lactobacillus*

In order to identify proteins with antibacterial activity against *S. aureus*, secreted proteins of the oral strains *L. salivarius* CNU1334 and *L. fermemtum* CNU1969 were separated by 2D gel electrophoresis (Fig. S3, Supporting Information). Using MALDI-TOF, a total of 21 secreted proteins were identified in the two *Lactobacillus* strains (Table 2), while no homologs were found for eight spots. Amongst the identified proteins, the following candidates may be of specific relevance: a LysM domain protein in both a

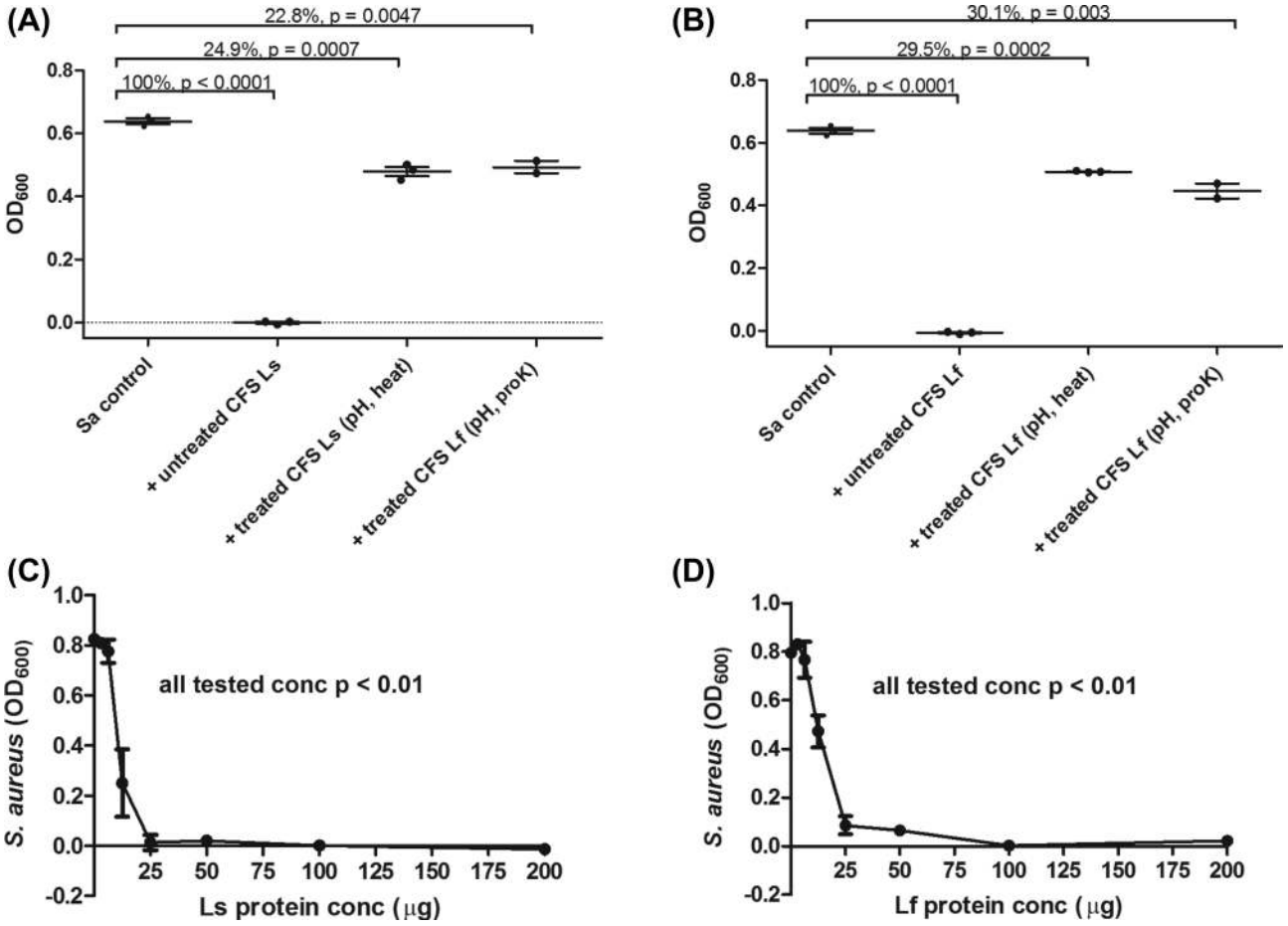

Figure 4. Effect of antimicrobial peptides of the oral isolates *L. salivarius* CNU1334 (*Ls*) and *L. fermentum* CNU1969 (*Lf*) on *S. aureus* (*Sa*) growth. Heat and proteinase K (proK) treatment on NaOH-neutralized CFS of Ls (**a**) or Lf (**b**) reduced the killing effect on *Sa* after 24 h presented as the reduction of the optimal density (OD600). Optimal density reduction compared to growth of *Sa* alone (control) was calculated using the following formula: Reduction (%) = 100% – [(Test group ÷ *Sa* alone) × 100%]. Concentration-dependent killing assay of isolated secreted proteins of *Ls* (c) and *Lf* (c) against *Sa* after incubation for 24 h. Bacterial growth was determined by measuring the optical density of the cultures at 600 nm using different concentrations (conc) of proteins. The data are expressed as the mean ± SEM of a representative experiment performed in triplicate.

protein peptidase M23B in *L. salivarius* and an APF-like surface protein in *L. fermentum*.

DISCUSSION

Staphylococcus aureus is the major pathogen responsible for community- and nosocomial-acquired infections worldwide (Simor *et al.* 2001; Klein, Smith and Laxminarayan 2007; Klevens *et al.* 2007). Successful treatment not only requires target-oriented antibiotic therapy, but also surgical intervention in many cases. Antibiotic treatment options are limited due to an increasing rates of antimicrobial agent resistance development and the antibiotic tolerance due to biofilm formation (Ceri, Olson and Turner 2010). The use of probiotics has been recently proposed as a viable option for the prevention or treatment of *S. aureus* infectious diseases (Sikorska and Smoragiewicz 2013). It has been reported that some lactobacilli species such as *Lactobacillus acidophilus* and *L. casei* (Karska-Wysocki, Bazo and Smoragiewicz 2010) have an inhibitory effect on *S. aureus*, possibly though nutritional competition, secretion of antibacterial peptides/proteins or immunomodulation (Dennis *et al.* 2009; Karska-Wysocki, Bazo and Smoragiewicz 2010). Little data exist on the antibacterial activity of oral *L. salivarius* against planktonic and biofilm *S. aureus* and the underlying mode of action.

In this study, we showed that both *L. salivarius* and *L. fermentum* effectively inhibited six *S. aureus* strains including three MRSA strains. We were able to demonstrate that *L. salivarius*, which has previously been shown to kill different pathogenic bacteria such as *Salmonella* (Olivares *et al.* 2006), also had a strong bactericidal effect against planktonic and biofilm *S. aureus*. In contrast, *L. fermentum* had no effect on *S. aureus* biofilm cells, suggesting that the mechanism of action of the two *Lactobacillus* species differs. Importantly, the antimicrobial effects of *Lactobacillus spp.* are not limited to *S. aureus*, but also spans other pathogens (Chen *et al.* 2012). However, in contrast to this study, stronger bactericidal effects for other pathogens were observed by *L. fermentum* than *L. salivarius* (Chen *et al.* 2012).

The anti-staphylococcal properties of lactobacilli have been noted in a number of *in vitro* and *in vivo* studies in *Lactobacillus* spp. other than *L salivarius*. The *L. salivarius*-dependent anti-staphylococcal activity seen in this study may also occur *in vivo* and have important clinical relevance. In one study, oral administration of *L. salivarius* PS2 during late pregnancy was shown to reduce the prevalence of staphylococcal mastitis in the first three months after delivery (Fernández *et al.* 2016). Other

Table 2. Secreted proteins identified in the supernatant of the oral isolated strains *L. salivarius* CNU1334 (*Ls*) and *L. fermentum* CNU1969 (*Lf*).

Spot number	Protein description[a]	Accession number[b]	MW (kDa)[a]	PI[c]	Protein score CI (%)
Proteins secreted by *Ls*					
1A	Peptidoglycan binding protein, LysM domain protein *L. salivarius* (strain CECT 5713)	WP_014568494	28.062	9.748	99.96
2A	Hypothetical secreted protein *L. salivarius* (strain CECT 5713)	YP_005863218	52.805	8.654	100
3A	Cobalamin (Vitamin B12) biosynthesis CbiM protein *Aminobacterium colombiense* (strain DSM 12261 / ALA-1)	YP_003552938	20.692	8.327	99.84
4A	Uncharacterized protein, aggregation promoting *Lactobacillus ultunensis* DSM 16047	WP_007125060	24.800	9.794	100
5A	Peptidoglycan binding protein, peptidase M23B *L. salivarius*	WP_004564200	20.110	5.826	100
Proteins secreted by *Lf*					
1,2B	Dextran sucrase *L. reuteri* (strain DSM 20016)	YP_001842264	154.301	5.082	100
3B	Cobalamin (Vitamin B12) biosynthesis CbiM protein *Aminobacterium colombiense* (strain DSM 12261 / ALA-1)	WP_023465553	20.692	8.327	99.84
4,5B	LysM domain protein, mannosyl-glycoprotein endo-beta-N-acetylglucosamidase *L. fermentum* ATCC 14931	WP_023465553	49.651	6.737	100
6B	Phosphoketolase *L. fermentum* MTCC 8711	WP_021816608	90.658	4.873	100
7,12B	Uncharacterized protein *L. fermentum* MTCC 8711	WP_021816398	49.800	5.192	96.85
8B	6-phosphogluconate dehydrogenase (decarboxylating) *L. fermentum* ATCC 14931	WP_003681101	52.523	4.709	100
9B	Putative muramidase *L. fermentum* (strain CECT 5716)	YP_005848973	27.448	5.603	100
10B	Uncharacterized protein (fragment) *L. fermentum* 3872	WP_021349642	46.019	5.866	99.97
11B	NlpC/P60 family protein *Lactobacillus gasseri* JV-V03	WP_003649984	42.593	9.62	100
13-15B	Peptidoglycan binding protein, LysM domain protein *L. fermentum* 28-3-CHN	WP_004563255	21.194	9.495	100
16B	Aggregation promoting factor-like surface protein *L. gasseri* K7	WP_020807431	27.946	9.633	100

[a]Protein description derived from UniProt database (www.uniprot.org)
[b]Information obtained from NCBI Protein Database (www.ncbi.nih.gov)
[c]Values derived from Isoelectric Point Calcultor (http://isoelectric.ovh.org)
CI, confidence interval

clinical studies are presently in progress to evaluate the ability of lactobacilli to reduce *S. aureus* carriage (Eggers *et al.* 2016). The pronounced anti-staphylococcal properties of *L. salivarius* seen in this study warrant further study of this particular species in colonization reduction studies (Bessesen *et al.* 2015).

Our results suggest that there are at least two different mechanisms by which lactobacilli kill *S. aureus*: an acidic pH shift and the secretion of specific proteins with antimicrobial activity. In contrast, nutrient depletion does not seem to be an essential factor. Although the *in vitro* production of antibacterial substances called bacteriocins in *L. salivarius* have been identified previously (Messaoudi *et al.* 2013), no specific substance is known with antimicrobial activity against *S. aureus* planktonic and biofilm modes of growth. Flynn *et al.* (2002) described a small heat-stable bacteriocin, called ABP-118, which is able to inhibit a number of microbial pathogens such as *Bacillus, Listeria, Enterococcus and Staphylococcus* species. However, the results of our CFS assay showed that secreted proteins from both *L. salivarius* and *L. fermentum* were not heat stable indicating another antimicrobial peptide/protein.

Using MALDI TOF MS/MS, we detected a range of secreted proteins that have similarity to other studies investigating the secretome of different *Lactobacillus* strains (Turner *et al.* 2004; van Pijkeren *et al.* 2006). Amongst these, several candidate proteins with potential antimicrobial activity could be identified: an LysM domain protein in both a protein peptidase M23B in *L. salivarius* and an APF-like surface protein in *L. fermentum*.

Proteins can be anchored to the cell envelope by LysM domains, which bind to the peptidoglycan in the bacterial cell wall. van Pijkeren *et al.* (2006) identified nine proteins with such a domain in *L. salivarius* strain UCC118. Most LysM-containing proteins known to date are peptidoglycan hydrolases that are involved in bacterial cell degradation (Buist *et al.* 2008). The best-characterized LysM-containing protein is the N-acetylgulcosaminidase AcmA of *L. lactis* (Buist *et al.* 1995), which binds to the cell wall and initiated lysis. Our identified LysM domain protein is also a peptidoglycan binding protein, which may cause lysis of *S. aureus* after binding to its cell wall, but does not affect lactobacilli.

The protein peptidase M23B (Stohl *et al.* 2012) is a metalloproteinase, which is known to cleave bacterial cell wall peptidoglycans in *Neisseria gonorrhoeae* (Stohl *et al.* 2012). In general, peptidases of family M23 are used by certain bacteria to lyse cell walls of other bacteria, either as a defensive or feeding mechanism. In lactobacilli, there are no reports so far.

There is also no report in *L. salivarius* or *L. fermentum* of an aggregation-promoting factor protein APF which are proteins associated with a diverse number of functional roles in lactobacilli, including self-aggregation, the bridging of conjugal pairs, co-aggregation with other commensal or pathogenic bacteria and maintenance of cell shape (Boris, Suárez and Barbés 1997). Recently, an aggregation-promoting factor in *L. plantarum* has been described with a potential role in interaction with other pathogens (Hevia *et al.* 2013).

Although these proteins have the potential to provide anti-staphylococcal activity, further studies including knockout and complementation analyses in *L. salivarius* isolates and recombinant protein production followed by cell-free anti-staphylococcal testing of candidate proteins need to be performed. However, being that *L. salivarius* showed a remarkable anti-staphylococcal effect compared to the well-studied *L. fermentum*, the decision of past and present clinical studies (Glück and Gebbers 2003; Eggers *et al.* 2016) to focus on the utilization of non-*L. salivarius* lactobacilli for the reduction in *S. aureus* carriage may need to be revisited.

In summary, we were able to demonstrate that *L. salivarius*—and with weaker effect *L. fermentum*—had a strong killing effect on planktonic *S. aureus*. *Lactobacillus salivarius* was furthermore effective against biofilm *S. aureus,* hence making it a promising candidate for the treatment of chronic infections. Although further studies are needed to evaluate the potential of the identified proteins, the data contained herein may aid developing new anti-staphylococcal strategies through the use of *L. salivarius* or its secreted proteins.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSPD* online.

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