Influence of Probiotic Culture Supernatants on In Vitro Biofilm Formation of Staphylococci

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Background: The effects of cell-free culture supernatants of probiotic *Lactobacillus rhamnosus* GG and *Streptococcus salivarius* K12 on replication and biofilm forming *of Staphylococcus aureus* and *S. epidermidis* were assessed in vitro.

Methods: S. aureus and *S. epidermidis* strains were exposed to cell-free culture supernatants of *L. rhamnosus* GG and *S. salivarius* K12 at different concentrations starting at 0, 4, and 24 h after the onset of incubation. Bacterial amplification was measured on microplate readers, as well as biofilm growth after safranine staining. Scanning electron microscopy was performed for visualization of biofilm status.

Results: The *S. salivarius* K12 culture supernatant not only reduced or prevented the formation and maturation of fresh biofilms but even caused a reduction of preformed *S. epidermidis* biofilms. The *L. rhamnosus* GG culture supernatant did not show clear inhibitory effects regardless of concentration or time of addition of supernatant, and even concentration-depending promotional effects on the planktonic and biofilm growth of *S. aureus* and *S. epidermidis* were observed.

Conclusion: In particular, the inhibitory effects of the *S. salivarius* K12 culture supernatant on the formation of staphylococcal biofilms are of potential relevance for biofilm-associated diseases and should be further assessed by in vivo infection models.

Keywords: probiotics, biofilm, *Lactobacillus rhamnosus*, *Streptococcus salivarius*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, cell-free supernatant, prevention

Introduction

Cell-free supernatants from microbial cultures were known for decades to show inhibitory effects on the growth of bacteria [1–7]. Depending on the bacterial species and clone, as well as on the incubation conditions, highly diverse biologically active substances are secreted and can therefore be detected in the culture supernatant. These include antimicrobial agents, such as bacteriocins [8], and metabolic products, such as organic acids [9], hydrogen peroxide [10], or biosurfactants [11], all with activity against planktonic and biofilm-bound bacteria, as well as immunomodulatory substances [12–14]. From a teleological standpoint, it is debatable whether at least some of the abovementioned compounds could be regarded as metabolic waste products with a random activity against other bacterial species or as purposely produced substances to shape the environment of a given bacterium.

For the probiotic bacterium *Lactobacillus rhamnosus*, culture supernatants exhibit in vitro activity against a number of Gram-positive and Gram-negative bacteria such as *Clostridium* spp., *Enterobacter* spp., and staphylococci [15]. In vivo, *Lactobacillus rhamnosus* GG was shown to temporarily colonize the human gastrointestinal tract [16] with supportive effects in the case of diarrhea in children [17] and curative effects against antibiotic-induced disorders in the gastrointestinal tract [18]. Also, the preventive addition of *L. rhamnosus* to milk can reduce the risk of developing dental caries [19]. The use of *L. rhamnosus* GG as a probiotic substance has been extensively assessed regarding its safety profile and is therefore considered as relatively harmless for patients [20]. In vitro studies also provided evidence for *L. rhamnosus* GG induced effects on biofilm formation in various oral microorganisms [21].

Streptococcus salivarius K12 produces bacteriocin-like inhibitory substances (BLIS). Among these antimicrobial peptides, the lantibiotics, salivaricin A2 and salivaricin B, have been investigated for their biochemical characteristics and functions at a cellular level [22–24]. They inflict growth inhibition on bacteria like *Streptococcus pyogenes* and *Haemophilus influenzae*, as well as *Candida albicans* [25–26]. The preventive use of *S. salivarius* K12 leads to a significantly reduced recurrence of both bacterial and viral pharyngotonsillitis and otitis media [27]. Its use as a probiotic in immunocompetent people is characterized by a good safety profile with reliable antibiotic activity and rare side effects [28].

Sensitivity of staphylococcal biofilms towards antimicrobial peptides has been described [29], although the majority of studies were focused on growth inhibition rather than reduction of formed and differentiated biofilms. In the presented study here, the effects of cell-free culture supernatants of *L. rhamnosus* GG and *S. salivarius* K12 on both planktonic and biofilm growth of *S. aureus* and *S. epidermidis* strains, as well as their reductive capacity for existing biofilms, were

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assessed. Compared to former publications, the novel aspects were *S. salivarius*-mediated effects on staphylococci in general and *L. rhamnosus*-mediated effects on preformed staphylococcal biofilms.

Methods

Bacterial Strains. Experiments were performed using the strains *S. aureus* (ATCC 25923), *S. epidermidis* (ATCC 35984), *L. rhamnosus* GG (ATCC 53103), and *S. salivarius* K12 (kindly provided by Dr. J. Tagg, Otago, NZ; see also ref. 26).

Production of Cell-Free Supernatants of Probiotic Bacteria. Overnight cultures of *L. rhamnosus* GG and *S. salivarius* K12 were incubated in 18-mL brain heart infusion (BHI) broth (BectonDickinson, Heidelberg, Germany) at 37 °C and 5% CO_2. Afterwards, centrifugation was performed for 15 min at 4000 rpm (rotations per minute) at room temperature with subsequent filter sterilization of the recovered culture supernatants using a 0.22-μm filter (B. Braun, Melsungen, Germany).

Then, 15 mL aliquots out of the initial 18-mL volumes were subjected to twice-repeated freeze-drying using a Christ Alpha 1–4 system (Martin Christ Gefriertrocknungsanlagen, Osterode/Harz, Germany). The obtained powder was later dissolved in bi-distilled and autoclaved water to desired concentrations. For comparison, the sterile BHI broth was subjected to these procedures. The pH values of 10 independent preparations of sterile and spent media were measured with a pH 720 inoLab instrument and a SenTix Microelectrode (WTW, Weilheim, Germany) before and after freeze-drying and reconstitution. In addition, the masses of the freeze-dried substances from 10 independent preparations of sterile and spent media were measured.

General Procedures for Assessing Staphylococcal Growth and Biofilm Formation in the Presence of Cell Free Supernatants. For a gross assessment of potential effects of cell free supernatants on staphylococcal growth, the *S. epidermidis* and *S. aureus* strains were incubated overnight in BHI broth at 37 °C and under ambient atmosphere. The liquid cultures were adjusted to MacFarland standard of 0.5 by the addition of sterile 0.9% NaCl solution, and 100 μL aliquots of the bacterial suspensions were spread onto Columbia base agar plates supplemented with 5% sheep blood (BectonDickinson). Then sterile filter discs with 5-mm diameter were placed onto the inoculated agar surface. On each filter disc, 10 μL aliquots of the cell free culture supernatants at 1-fold to 15-fold concentrations were pipetted. The plates were incubated overnight at 37 °C and ambient atmosphere. The following day, potential growth inhibition zones around the discs were scrutinized by macroscopic inspection.

For assessment of biofilm formation, experiments were performed in 96-well microtiter plates coated with human fibronectin [30] because of an inherent binding affinity of staphylococci to fibronectin [31]. Generally, endpoint measurements of optical density were carried out on a microtiter plate reader.

In the endpoint measurements of bacterial amplification, the transmission of light with a wavelength of 600 nm (optical density at 600 nm = OD 600 nm) was measured for the bacterial suspensions or media. Based on the absorbance, which correlates with the degree of turbidity of the bacterial culture, the growth of the bacteria in the respective test cavities could be determined.

The readout of the results for biofilm formation by means of safranine staining was carried out analogously. In detail, safranine O was used for the staining. All wells were rinsed twice with 1× PBS buffer (Becton Dickinson) by gently pipetting up and down. After a short drying period of 10 min, 200 μL of a 0.1% safranine solution was applied to each test cavity and remained there for 20 min at room temperature. Subsequently, the described washing process with PBS buffer was repeated. After the cavities had been dried, intensity measurement of the colored cavity bottoms were carried out using the microtiter plate reader at OD 492 nm. The detection limit for biofilm structures was set >0.05 for an OD of 492 nm [32–34].

Quantitative Procedures for Assessing Staphylococcal Growth and Biofilm Formation in the Presence of Spent Media. Planktonic growth and the biofilm formation and masses of *S. aureus* and *S. epidermidis* were investigated by the addition of different concentrations of culture supernatants on the microtiter plate.

On day 1, overnight cultures of *S. aureus* were grown in CASO broth (CB) medium (Carl Roth GmbH, Karlsruhe, Germany) and of *S. epidermidis* in tryptic soy broth (TSB) medium (Becton Dickinson). In parallel, coating of the microtiter plates with human fibronectin was performed.

On day 2, dilution series of 15-fold, 7.5-fold, 3.75-fold, 1.8-fold, 0.9-fold, and 0.46-fold concentrated probiotic cell-free culture supernatants were incubated with 10^5 colony forming units (cfu) per well of the assessed staphylococcal strains.

On day 3, the evaluation was performed in the microtiter plate reader as end point and absorption measurement.

Each series was duplicated as technical replica with 4 biological replicates each. Positive controls (inoculated growth media with supplements of freeze-dried sterile BHI medium) and negative controls (sterile growth media with supplements of freeze-dried sterile spent media) were included.

Experiments on Delayed Application of Culture Supernatants to Biofilm-Associated Staphylococci. To investigate whether the timing of addition of the culture supernatants had an influence on staphylococcal growth, biofilm formation, and masses, the probiotic culture supernatants were added at 0, 4, and 24 h after the incubation of the bacteria in the test wells had started. The total incubation time for these experiments was 48 h.

On day 1, overnight cultures of staphylococci were grown, and fibronectin coating of the microtiter plates was performed as described above.

On day 2, the bacterial suspensions were prepared using the respective nutrient medium (CB for *S. aureus* and TSB for *S. epidermidis*) and aliquoted at 100-μL volumes (corresponding to 10^5 colony forming units [cfu] per well) in the microtiter plates, except in the case of the negative control.

At the indicated time points, 100 μL of a 30× concentrated probiotic cell-free culture supernatant were added directly to the cavities and cautiously mixed by repeated pipetting. Thus, each well then contained a 1:1 mixture of bacterial culture and culture supernatants in a ratio of 1:1 and probiotic culture supernatants in 15-fold concentration.

The incubation at 37 °C and 5% CO_2 was performed for a total of 48 h with short breaks at the times of addition of the probiotic cell-free culture supernatant at 4 or 24 h to the respective wells after the onset of the incubation.

Two specific positive controls were prepared by adding 100 μL of the respective sterile freeze-dried growth medium per cavity instead of the probiotic cell-free culture supernatant at time points 0 h and 24 h of each experimental series and thus incubated for another 48 h or 24 h, respectively.

The negative controls, each containing 100 μL of sterile medium and 100 μL of sterile culture supernatant, were incubated for 48 h.

Statistics. Statistical assessments of the data were performed using the software Microsoft Excel (Microsoft,

Redmond, WA, USA) and IBM SPSS Statistics (IBM Corporation, New York, USA) applying the two-sided Mann–Whitney U test. Significance was accepted in the case of $P < 0.05$.

Scanning Electron Microscopy (SEM). Scanning electron microscopy (SEM) was performed using a Merlin VP compact system (Carl Zeiss Microscopy GmbH, Jena, Germany) as described by the manufacturer with the following modifications. In detail, cover slips served as sample carriers for the assessment of biofilm growth. The staphylococci were inoculated in uncoated 24-well microtiter plates, each containing a round, sterile, plastic cover slip coated with human fibronectin and were incubated in the absence or presence of cell-free culture supernatants for 24 or 48 h under aerobic conditions.

After completion of the desired incubation period, the cover slips were carefully removed from the cavities using tweezers, were rinsed once with 1× PBS, and were afterwards subjected to further sample preparation for scanning electron microscopy as described by Patenge et al. [30].

Ethics. Ethical clearance was not necessary for this study, because neither patient data nor assessments of sample materials from patients were presented.

Results

Basic Data of the Spent Media Before and After Freeze-Drying. As a prerequisite for interpretation of the results, pH values of the culture supernatants before and after freeze drying as well as the masses of the freeze-dried powder were assessed.

The pH values from the *L. rhamnosus* sterile spent media were 5.65 ± 0.02 and 5.99 ± 0.06, respectively, after filtering but before freeze-drying and after reconstitution of the freeze-dried substances by addition of sterile water. After freeze-drying, a culture volume of 18 mL resulted in dried powder masses of 561 ± 30 mg. In order to obtain a neutral pH, 234 ± 16 μL 1 N NaOH had to be added to the reconstituted spent media.

The corresponding pH values for the filtered *S. salivarius* spent media were 5.10 ± 0.00 and 5.66 ± 0.06, respectively, before freeze-drying and after reconstitution. Freeze-drying resulted in powder masses of 530 ± 20 mg. 285 ± 24 μL 1 N NaOH had to be added for neutralization of the reconstituted culture supernatant. Thus, in terms of the dissolved powder masses, the concentrations of the reconstituted cell free culture supernatants used in the experiments for both bacterial species varied approximately between 17 (0.45-fold concentration) and 560 (15-fold concentration) g/L.

When subjecting the sterile culture media to the same procedures as the spent media, before sterile filtering the pH values were 7.54 ± 0.03 and 7.30 ± 0.02 before freeze-drying and after reconstitution, respectively, with a mass of 740 ± 12 mg of the dried powder. After sterile filtering the corresponding pH values were 7.49 ± 0.02 and 7.15 ± 0.01, respectively, and the dried powder mass was 530 ± 30 mg. For neutralization of the reconstituted media, 15 or 50 μL 1 N NaOH, respectively, had to be added when prepared before or after sterile filtering.

The different amounts of basic fluid needed for neutralization of media before and after filtering and before and after freeze-drying indicated that the filters absorbed some basic molecules while freeze-drying evaporated some acidic molecules.

Quantitative Growth Inhibition Measurements. When using the reconstituted culture supernatants for classical agar diffusion assay techniques, no inhibition zones could be recorded around the impregnated filter discs irrespective of the concentrations of the cell free culture supernatants used in this experiment (data not shown). Therefore, the more refined technique described below was used for further analysis.

At all but the highest concentration of *L. rhamnosus* GG culture supernatant, the ODs of *S. aureus* cultures rose with increasing concentration. Using 15-fold concentrated supernatant, the OD of *S. aureus* culture was lower than that in the associated positive control. By measuring the absorbance of the safranine dye, an OD 492 nm > 0.05 suggesting biofilm growth could be detected for each amount of culture supernatants added to the assay. Only when using culture supernatants at the maximum concentration, the biofilm mass was lower than that in the positive control (Table 1; Figure 1).

In turn, when *S. aureus* cultures were incubated with *S. salivarius* K12 supernatant, an inhibitory effect as demonstrated by low optical density (OD 600 nm) could be observed already for the use of 1.8-fold concentrated culture supernatant. The effect correlated to the amount of supernatants except for the 15-fold concentrate. The relationship between increasing concentration of the culture supernatant and decreasing biofilm mass was already visible for the lowest concentration of the culture supernatant and stayed consistent across all concentration levels (Table 1; Figure 2).

Table 1. Measured optical densities in cased of different concentrations of culture supernatant (n.s. = not significant)

Concentration of cell-free supernatant	15-fold	7.5-fold	3.75-fold	1.8-fold	0.9-fold	0.46-fold	Positive control	Negative control
Measurement of optical density (OD) at 600 nm to assess planktonic growth (OD values ± standard deviation) (P = significance level compared with positive control; n.s. = not significant)								
S. aureus culture with	0.43 ± 0.10;	0.90 ± 0.12;	0.99 ± 0.26;	0.97 ± 0.21;	0.71 ± 0.15;	0.72 ± 0.12;	0.66 ± 0.06;	0.38 ± 0.07;
L. rhamnosus GG supernatant	$P < 0.001$	$P < 0.05$	$P < 0.01$	$P < 0.01$	n.s.	n.s.	reference	$P < 0.001$
S. aureus culture with	0.62 ± 0.32;	0.33 ± 0.06;	0.28 ± 0.03;	0.53 ± 0.06;	0.64 ± 0.15;	0.61 ± 0.05;	0.62 ± 0.07;	0.46 ± 0.07;
S. salivarius K12 supernatant	n.s.	$P < 0.01$	$P < 0.01$	$P < 0.05$	n.s.	n.s.	reference	$P < 0.05$
S. epidermidis culture with	0.77 ± 0.22;	1.08 ± 0.05;	0.86 ± 0.05;	0.75 ± 0.05;	0.67 ± 0.07;	0.61 ± 0.06;	0.61 ± 0.04;	0.40 ± 0,04;
L. rhamnosus GG supernatant	n.s.	$P < 0.001$	$P < 0.001$	$P < 0.001$	n.s.	n.s.	reference	$P < 0.001$
S. epidermidis culture with	0.40 ± 0.06;	0.30 ± 0.05;	0.53 ± 0.08;	0.69 ± 0.06;	0.62 ± 0.07;	0.57 ± 0.18;	0.62 ± 0.03;	0.45 ± 0.06;
S. salivarius K12 supernatant	$P < 0.001$	$P < 0.001$	$P < 0.01$	$P < 0.05$	n.s.	n.s.	reference	$P < 0.001$
Measurement of optical density (OD) at 492 nm to assess biofilm growth (OD values ± standard deviation) (P = significance level compared with positive control; n.s. = not significant)								
S. aureus culture with	0.11 ± 0.13;	0.73 ± 0.32;	0.36 ± 0.05;	0.71 ± 0.67;	0.52 ± 0.31;	0.57 ± 0.52;	0.58 ± 0.68;	0.05 ± 0.03;
L. rhamnosus GG supernatant	$P < 0.05$	n.s.	n.s.	n.s.	n.s.	n.s.	reference	$P < 0.001$
S. aureus culture with	0.04 ± 0.02;	0.02 ± 0.01;	0.03 ± 0.03;	0.13 ± 0.07;	0.19 ± 0.04;	0.24 ± 0.19;	0.48 ± 0.57;	0.04 ± 0.01;
S. salivarius K12 supernatant	$P < 0.001$	$P < 0.001$	$P < 0.001$	n.s.	n.s.	n.s.	reference	$P < 0.001$
S. epidermidis culture with	0.07 ± 0.04;	0.14 ± 0.06;	0.14 ± 0.11;	0.05 ± 0.03;	0.08 ± 0.04;	0.20 ± 0.09;	0.30 ± 0.08;	0.05 ± 0.02;
L. rhamnosus GG supernatant	$P < 0.001$	$P < 0.01$	$P < 0.01$	$P < 0.001$	$P < 0.001$	$P < 0.05$	reference	$P < 0.001$
S. epidermidis culture with	0.03 ± 0.02;	0.03 ± 0.02;	0.28 ± 0.19;	0.11 ± 0.04;	0.06 ± 0.02;	0.15 ± 0.07;	0.33 ± 0.09;	0.06 ± 0.03;
S. salivarius K12 supernatant	$P < 0.001$	$P < 0.001$	n.s.	$P < 0.001$	$P < 0.001$	$P < 0.01$	reference	$P < 0.001$

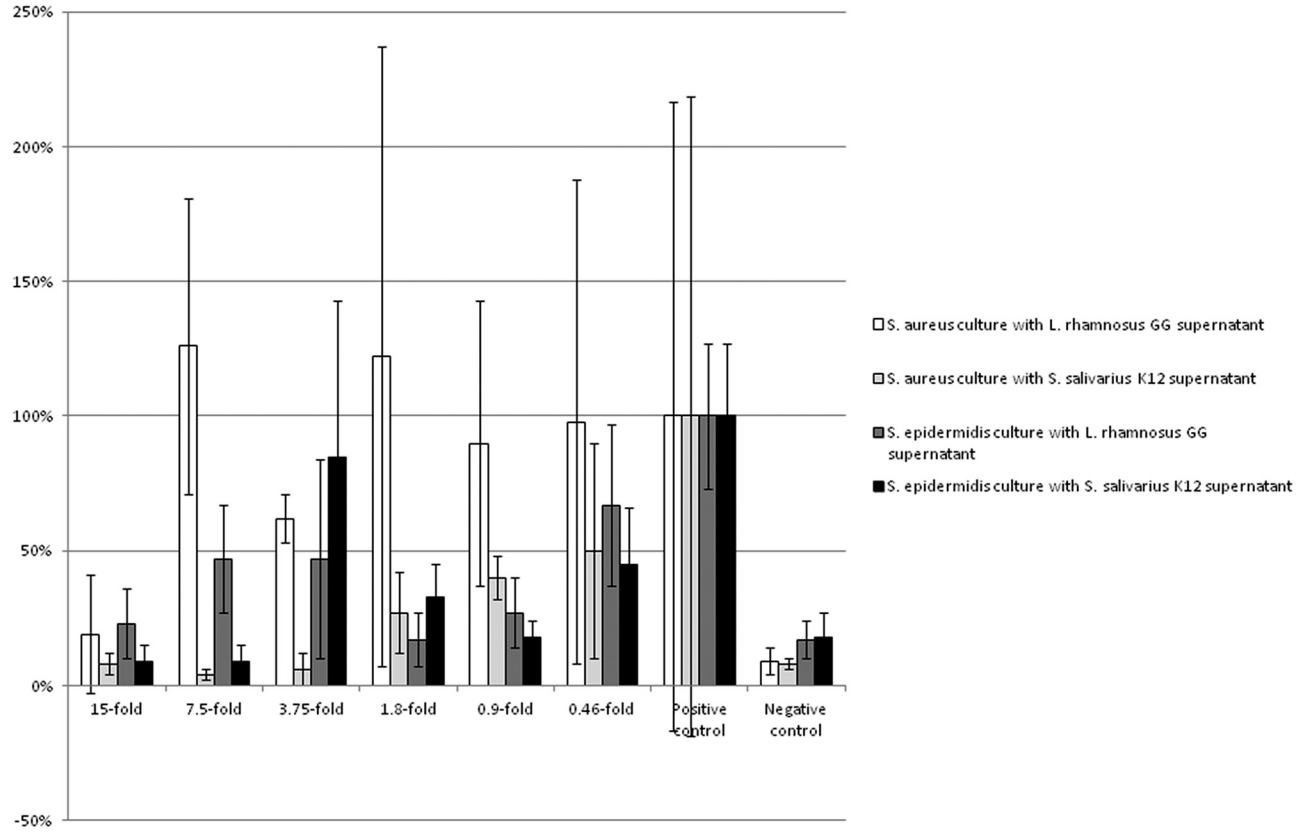

Figure 1. Measured optical densities in the case of different concentrations of culture supernatants. Measurement of optical density (OD) at 492 nm to assess biofilm growth (OD values ± standard deviation)

When the *L. rhamnosus* GG culture supernatant was used on *S. epidermidis* cultures, they grew to a higher optical density than that in the positive control wells. Up to a 7.5-fold concentration, this effect increased steadily. Then, only at 15-fold concentration, it dropped again. The use of the *L. rhamnosus* GG culture supernatant resulted in reduced

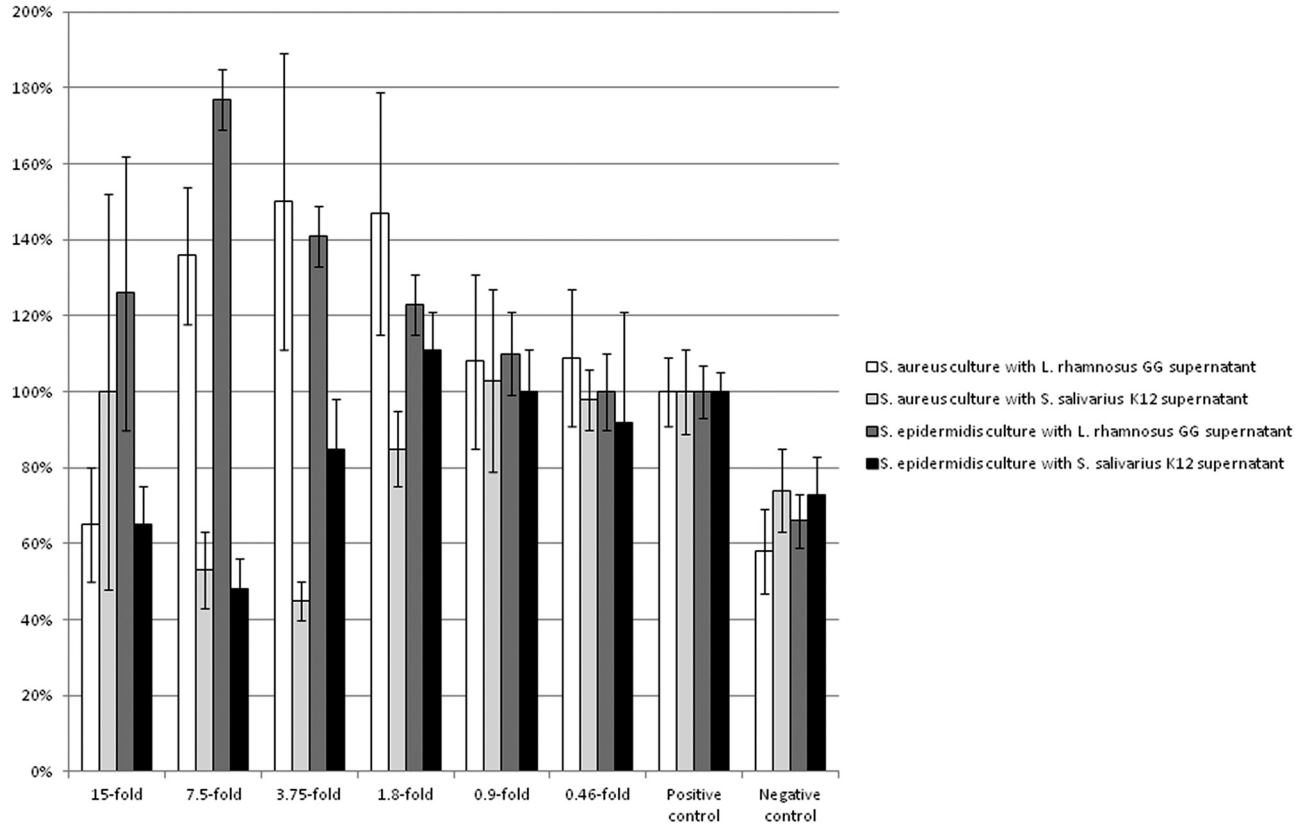

Figure 2. Measured optical densities in the case of different concentrations of culture supernatants. Measurement of optical density (OD) at 600 nm to assess planktonic growth (OD values ± standard deviation)

biofilm growth compared to the positive control. However, varying and even contradictory effects were observed for different concentrations of supernatants (Table 1; Figure 1).

According to measurement results of the OD 600 nm, *S. epidermidis* growth with the *S. salivarius* K12 culture supernatant suggested a tendency for a concentration-dependent effect. Small amounts of culture supernatants had no or even a slight growth-promoting effect. But from 3.75-fold concentration on, an increasingly growth-inhibiting influence was observed. Biofilm formation was obviously reduced due to culture supernatant, and this effect increased with increasing concentrations of the *S. salivarius* K12 culture supernatant in spite of the outlier at 3.75-fold concentration (Table 1; Figure 2).

Experiments on Delayed Application of Culture Supernatants. Overall, co-incubation with the *L. rhamnosus* GG culture supernatant resulted in a significantly higher optical density of the *S. aureus* culture than that in the positive controls. This effect was consistent for all three addition times. This observation for bacterial amplification rates correlated with the results for biofilm formation. Increased planktonic and biofilm growth of *S. aureus* after addition of the *L. rhamnosus* GG supernatant was observed independently of the time of addition to the medium (Table 2; Figures 3 and 4).

Upon early addition of the *S. salivarius* K12 culture supernatant to the *S. aureus* culture at 0 and 4 h after the start of culture, respectively, there was a considerably lower optical density compared to both positive controls. If the addition occurred 24 h after the onset of culture, then the optical density of the *S. aureus* culture was higher compared to that for earlier addition time-points, but still lower than that of the positive control incubated for 48 h.

In the case of immediate addition of the *S. salivarius* K12 culture supernatant and in the case of 4-h latency of addition to the *S. aureus* culture, no biofilm formed by *S. aureus* could be detected. Associated absorption measurements at OD 462 nm were lower than those of the positive controls after 24 or 48 h of incubation. Only the addition of culture supernatants after previous incubation of the *S. aureus* culture for 24 h resulted in increased biofilm mass in comparison to the positive control that was incubated for 48 h (Table 2, Figure 3).

Irrespective of the time point, a higher optical density compared to the positive controls resulted in the addition of the *L. rhamnosus* GG culture supernatant to *S. epidermidis* cultures. *S. epidermidis* grew independently of the time of addition of supernatants in the form of a biofilm. The biofilm mass was reduced compared to the positive controls when supernatants were added immediately or 4 h after the start of incubation but it was increased if a supernatant was added with 24-h latency (Table 2, Figure 3).

The optical density of *S. epidermidis* cultures varied with the time of *S. salivarius* K12 addition. The lowest OD values were measured in the case of immediate and 4-h-delayed addition of the culture supernatant to the *S. epidermidis* culture. These values were lower compared to those of the two positive controls. If the addition of the supernatant occurred with 24-h latency, the optical density of the culture at the end of the study was higher than that in case of earlier addition, but still lower than that of the 24-h positive control. At all times of addition of supernatant, reduced absorption values were measured at OD 462 nm. If the addition was made immediately or after 4 h, no biofilm growth could be detected. Biofilms could be detected in the case of an addition of the culture supernatant that was delayed by 24 h, but this biofilm still had less mass than the biofilms in the control samples. In comparison with the 24-h positive control, a reduction of an already preformed biofilm was demonstrated (Table 2, Figure 3).

Scanning Electron Microscopy (SEM). Biofilms could be demonstrated for all experimental settings with the exception of co-incubation of staphylococci with the *S. salivarius* K12 supernatant at 15-fold concentration that was added immediately or 4 h after the onset of incubation (Figure 5**A**). Examples of weak (Figure 5B) and strong (Figure 5C, D) biofilm growth are presented to visualize the above described quantitative effects of the cell-free culture supernatants.

Table 2. Measured optical densities after delayed application of culture supernatant (n.s. = not significant)

	0-h delay of application (measurement after 48 h)	4-h delay of application (measurement after 48 h)	24-h delay of application (measurement after 48 h)	24-h positive control	48-h positive control	48-h negative control
Measurement of optical density (OD) at 600 nm to assess planktonic growth (OD values ± standard deviation) (P_{24} = significance level compared with 24-h positive control; P_{48} = significance level compared with 48-h positive control; n.s. = not significant)						
S. aureus culture with *L. rhamnosus* GG supernatant	1.03 ± 0.23; $P_{24} < 0.001$; P_{48} = n.s.	1.07 ± 0.27; $P_{24} < 0.001$; P_{48} = n.s.	1.15 ± 0.10; $P_{24} < 0.001$; $P_{48} < 0.05$	0.55 ± 0.12; reference 24 h	0.97 ± 0.19; reference 48 h	0.37 ± 0.08; significance not assessed
S. aureus culture with *S. salivarius* K12 supernatant	0.32 ± 0.03; $P_{24} < 0.001$; $P_{48} < 0.001$	0.34 ± 0.03; $P_{24} < 0.001$; $P_{48} < 0.001$	0.77 ± 0.15; P < 0.01; $P_{24} < 0.01$; $P_{48} < 0.05$	0.55 ± 0.12; reference 24 h	0.97 ± 0.19; reference 48 h	0.37 ± 0.08; significance not assessed
S. epidermidis culture with *L. rhamnosus* GG supernatant	1.13 ± 0.14; $P_{24} < 0.001$; $P_{48} < 0.001$	1.17 ± 0.05; $P_{24} < 0.001$; $P_{48} < 0.001$	1.18 ± 0.09; $P_{24} < 0.001$; $P_{48} < 0.001$	0.74 ± 0.12; reference 24 h	0.64 ± 0.11; reference 48 h	0.39 ± 0.07; significance not assessed
S. epidermidis culture with *S. salivarius* K12 supernatant	0.33 ± 0.06; $P_{24} < 0.001$; $P_{48} < 0.001$	0.36 ± 0.03; $P_{24} < 0.001$; $P_{48} < 0.001$	0.56 ± 0.10; $P_{24} < 0.01$; P_{48} = n.s.	0.74 ± 0.12; reference 24 h	0.64 ± 0.11; reference 48 h	0.39 ± 0.07; significance not assessed
Measurement of optical density (OD) at 492 nm to assess biofilm growth (OD values ± standard deviation) (P_{24} = significance level compared with 24-h positive control; P_{48} = significance level compared with 48-h positive control; n.s. = not significant)						
S. aureus culture with *L. rhamnosus* GG supernatant	0.32 ± 0.20; $P_{24} < 0.001$; $P_{48} < 0.001$	0.40 ± 0.26; $P_{24} < 0.001$; $P_{48} < 0.001$	0.47 ± 0.34; $P_{24} < 0.01$; $P_{48} < 0.01$	0.10 ± 0.01; reference 24 h	0.09 ± 0.03; reference 48 h	0.06 ± 0.03; significance not assessed
S. aureus culture with *S. salivarius* K12 supernatant	0.02 ± 0.01; $P_{24} < 0.001$; $P_{48} < 0.001$	0.03 ± 0.01; $P_{24} < 0.001$; $P_{48} < 0.001$	0.17 ± 0.09; $P_{24} < 0.05$; $P_{48} < 0.05$	0.10 ± 0.01; reference 24 h	0.09 ± 0.03; reference 48 h	0.06 ± 0.03; significance not assessed
S. epidermidis culture with *L. rhamnosus* GG supernatant	0.14 ± 0.01; $P_{24} < 0.05$; $P_{48} < 0.05$	0.18 ± 0.18; P_{24} = n.s.; P_{48} = n.s.	0.28 ± 0.19; P_{24} = n.s.; P_{48} = n.s.	0.24 ± 0.07; reference 24 h	0.24 ± 0.08; reference 48 h	0.07 ± 0.04; significance not assessed
S. epidermidis culture with *S. salivarius* K12 supernatant	0.03 ± 0.01; $P_{24} < 0.001$; $P_{48} < 0.001$	0.03 ± 0.01; $P_{24} < 0.001$; $P_{48} < 0.001$	0.12 ± 0.06; $P_{24} < 0.01$; $P_{48} < 0.01$	0.24 ± 0.07; reference 24 h	0.24 ± 0.08; reference 48 h	0.07 ± 0.04; significance not assessed

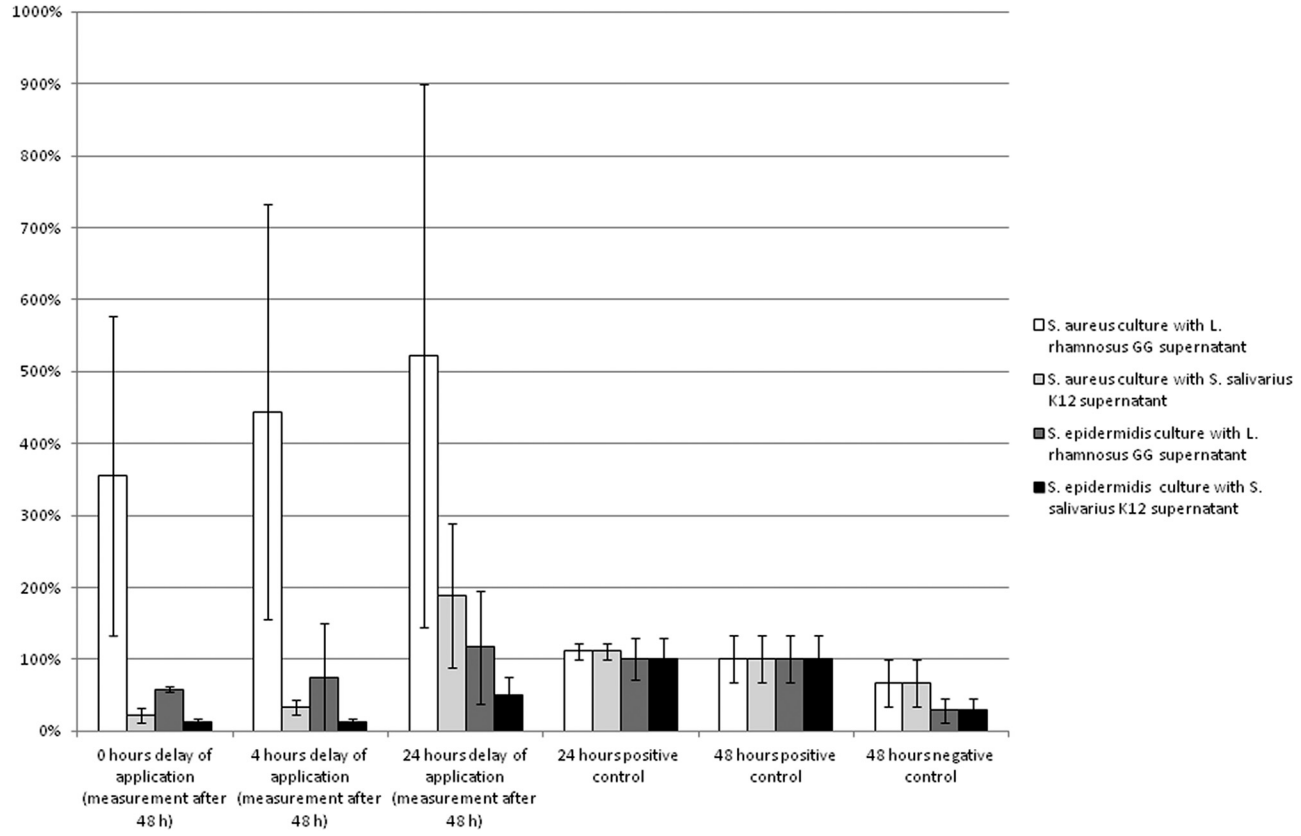

Figure 3. Measured optical densities after delayed application of culture supernatants. Measurement of optical density (OD) at 492 nm to assess biofilm growth (OD values ± standard deviation)

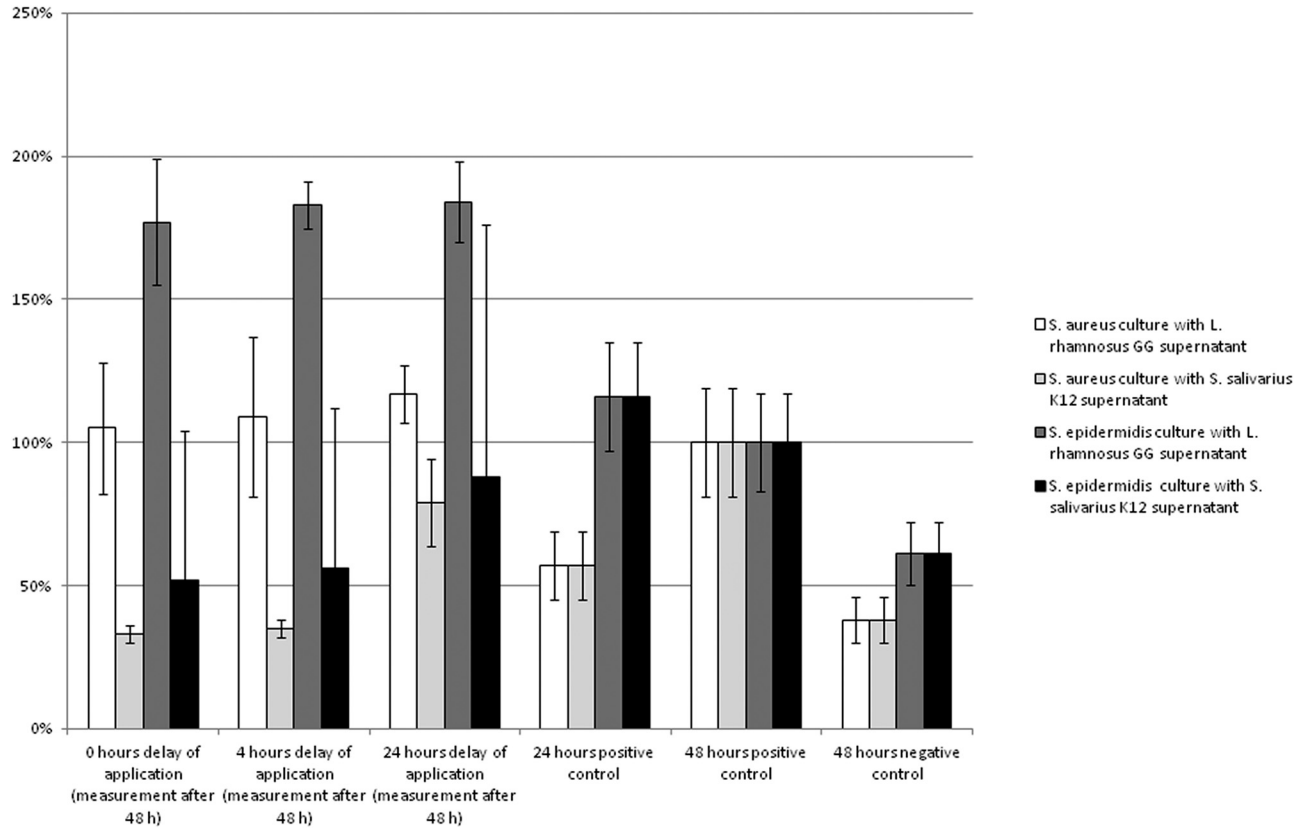

Figure 4. Measured optical densities after delayed application of culture supernatant. Measurement of optical density (OD) at 600 nm to assess planktonic growth (OD values ± standard deviation)

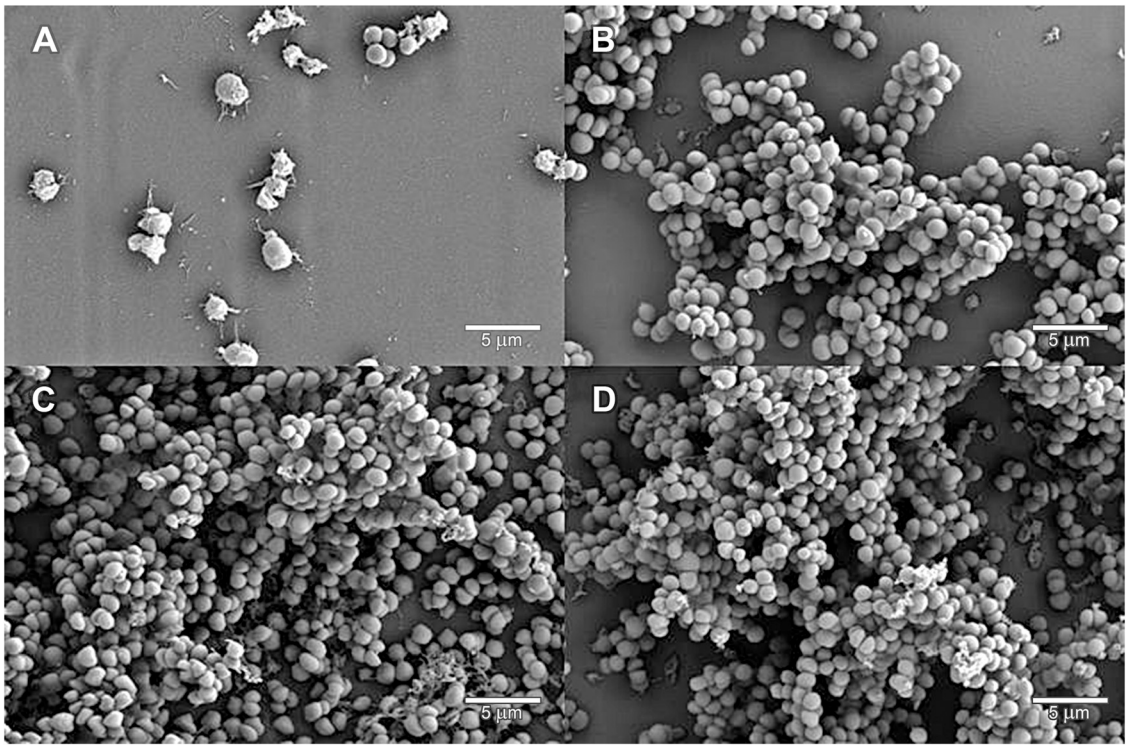

Figure 5. Exemplary demonstration of biofilm growth using SEM. (A) Absence of biofilm in the case of co-incubation of *S. epidermidis* with the *S. salivarius* K12 supernatant at 15-fold concentration that was added immediately after the onset of incubation. (B) Weak biofilm formation in the case of co-incubation of *S. epidermidis* with the *S. salivarius* K12 supernatant at 15-fold concentration that was added 24 h after the onset of incubation. (C and D) Intense biofilm growth of *S. epidermidis* in the positive control samples, which were incubated without the *S. salivarius* K12 supernatant, 24 h and 48 h after the onset of incubation, respectively. Magnification: 5000×

Discussion

This study focused on the identification and quantification of inhibitory effects (minimum inhibitory concentration [MIC]) of culture supernatants from established probiotic bacteria on planktonic and biofilm growth of staphylococci. Similar to susceptibility testing of bacteria to antibiotics, experiments were carried out to identify gross inhibitory effects and, thereafter, the minimum inhibitory concentration of probiotic culture supernatants. Not only the detection of an inhibitory effect of the culture supernatants per se but also the necessary concentration and the optimal time of addition were of interest in these experiments. Furthermore, in contrast to conventional antibiotic susceptibility testing, not only the effect on planktonic bacterial growth was investigated, but special attention was also given to biofilm growth.

The results of the influence of culture supernatants on the planktonic forms of both assessed *Staphylococcus* species, i.e., *S. aureus* and *S. epidermidis*, could represent genus-wide effects due to the similarity of results for strains of both species. The *L. rhamnosus* GG culture supernatant increased the optical density of staphylococcal cultures, while the *S. salivarius* K12 culture supernatant reduced their optical density.

Regardless of the time of delayed addition of *L. rhamnosus* GG culture supernatant, generally there was a mild growth-promoting effect for *S. aureus* and a pronounced one for *S. epidermidis*. On the other hand, the *S. salivarius* K12 culture supernatant was able to inhibit both staphylococcal cultures in the case of early addition. After 24-h latency of the addition, a slight inhibitory effect could still be noted.

In addition to the observations for the planktonic form of *S. aureus* and *S. epidermidis*, a species-specific but also concentration-dependent effect was observed regarding the influence on biofilm formation in the MIC experiments. Overall, the addition of *L. rhamnosus* GG and *S. salivarius* K12 culture supernatants resulted in markedly reduced *S. epidermidis* biofilm growth. The two highest concentrations of the *S. salivarius* K12 culture supernatant thoroughly suppressed biofilm formation.

In contrast, this reducing or suppressing effect on *S. aureus* biofilm formation was observed only when using the *S. salivarius* K12 culture supernatant. The *L. rhamnosus* GG culture supernatant did show ambiguous effects on biofilms formed by *S. aureus*.

Focusing on time-dependent influences on the formation of biofilms in the presence of the *L. rhamnosus* GG culture supernatant, an increasingly growth-promoting effect on *S. aureus* biofilms was seen in the case of increasingly delayed addition in comparison to low biofilm masses in the positive controls. One potential explanation for the low biofilm masses in the positive controls may be the detachment of the biofilm as part of the washing steps prior to staining with safranine [35]. Although there is no general recommendation on the number of washing steps to be performed, two steps are usually considered to be the absolute minimum and have therefore been used in this study [31–33, 36–38]. Also, some inconsistencies between the concentrations of the applied spent media and their activities on biofilm masses may result from the safranine staining technique. As recently shown [39–40], extraction of stains from air-dried biofilms and subsequent measurements of the supernatants as opposed to direct measurements of the in-situ stained biofilms could be the superior technique.

The *L. rhamnosus* GG culture supernatant promoted the biofilm formation of *S. epidermidis* after a 24-h lag compared to the onset of cultural growth of the staphylococci in the experiment. In contrast, also at other time points, the *S. salivarius* K12 culture supernatant demonstrated marked inhibitory effects. In the case of early addition, biofilm growth was suppressed for both *S. aureus* and *S. epidermidis* by *S. salivarius* K12 culture supernatant. For *S. aureus*, this reduction effect

was no longer detectable when added with 24-h latency, whereas it could still be confirmed for *S. epidermidis*. This apparently subsequent reduction of the biofilm mass due to culture supernatants could be caused by destabilization and subsequent release of the loosened biofilm, which is promoted by the washing steps [41].

The scanning electron microscopic evaluation of the biofilm formation allowed the direct visualization of the grown biofilm and thus supported the determined quantitative results. Only the two highest concentrations of the co-administered *S. salivarius* K12 culture supernatant were associated with undetectable biofilms, while biofilms were formed by S. *aureus* and *S. epidermidis* at all time points of investigation upon addition of the *L. rhamnosus* GG culture supernatant.

The main findings, namely, the proof of a concentration-dependent and time-dependent effect of probiotic culture supernatants on planktonic and biofilm growth of staphylococci, correspond to the results in the literature in particular for *S. salivarius* K12. The observed increased effect by higher concentrations of the culture supernatants was also described by others [2, 14, 42]. Further, the inhibitory effect largely depends on the status of the biofilm. Generally, it seems to be much easier to achieve effects on fresh biofilms than on already grown and matured ones as previously suggested elsewhere [43].

This study did not analyze the molecular background of the observed growth inhibiting and biofilm-dissolving effects of the probiotic culture supernatants. The acidic pH values of the spent media could be an explanation, since acidification has a long standing tradition, e.g., for prevention of staphylococcal food poisoning [44]. Systematic neutralization, e.g., by adding sodium hydroxide to all supernatants, was not performed, an admitted limitation of the study. However, both probiotic bacterial strains acidify their culture supernatants, but the application of their spent media displayed marked differences in terms of the exposed bacterial species and their status as being planktonic or biofilm-associated. As mentioned above, probiotic antibacterial activity relies on several modes of action, and the present results point in that direction as well.

Probiotic bacteria have successfully been used on a routine basis to fight the growth of staphylococci in the human environment or on human mucosal surfaces [44]. In contrast, counteracting established staphylococcal biofilms still remains at an experimental level [45]. Since therapy in a real clinical scenario usually has to deal with already existing biofilms, the effect of a substance or a mix of substances which reduce an existing biofilm mass would be extremely valuable. However, this goal has not been satisfyingly reached irrespective of the application methods or compounds utilized so far [46–50]. Alternatively, the inhibitory effect on the growth of fresh biofilms could be used and even plays a role in implant materials that have been covered by biofilm-reducing substances [51, 52]. For this reason, probiotic bacteria or antimicrobials secreted by them into the culture supernatants could be used both for preventive and therapeutic purposes. Probiotic culture supernatants as described in this study, mainly the supernatants of *S. salivarius* K12, should be prospectively tested for their antimicrobial properties in in vivo models of implant infections.

Conclusions

Time and concentration-depending effects of cell-free supernatants of the probiotic bacteria *L. rhamnsosus* GG and *S. salivarius* K12 on amplification and biofilm formation of staphylococci could be confirmed. The growth-inhibiting effects of the *S. salivarius* K12 supernatants were more pronounced. The mode of action appears to rely on more than just acidification of the growth media.

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Authors' Contribution

HF and AP wrote the article. CK performed the experiments. PW, SR, and AP planned the study and supervised the experiments. All authors have read and corrected the article prior to publication.

Conflict of Interest

There is no conflict of interest to report.

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