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Characterization of biosurfactants produced by *Lactobacillus* spp. and their activity against oral streptococci biofilm

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Abstract Lactic acid bacteria (LAB) can interfere with pathogens through different mechanisms; one is the production of biosurfactants, a group of surface-active molecules, which inhibit the growth of potential pathogens. In the present study, biosurfactants produced by *Lactobacillus reuteri* DSM 17938, *Lactobacillus acidophilus* DDS-1, *Lactobacillus rhamnosus* ATCC 53103, and *Lactobacillus paracasei* B21060 were dialyzed (1 and 6 kDa) and characterized in term of reduction of surface tension and emulsifying activity. Then, aliquots of the different dialyzed biosurfactants were added to *Streptococcus mutans* ATCC 25175 and *Streptococcus oralis* ATCC 9811 in the culture medium during the formation of biofilm on titanium surface and the efficacy was determined by agar plate count, biomass analyses, and flow cytometry. Dialyzed biosurfactants showed abilities to reduce surface tension and to emulsifying paraffin oil. Moreover, they significantly inhibited the adhesion and biofilm formation on titanium surface of *S. mutans* and *S. oralis* in a dose-dependent way, as demonstrated by the remarkable decrease of cfu/ml values and biomass production. The antimicrobial properties observed for dialyzed biosurfactants produced by the tested lactobacilli opens future prospects for their use against microorganisms responsible of oral diseases.

Eleonora Ciandrini and Raffaella Campana contributed equally to the research.

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Introduction

Biosurfactants (BSFs) are surface-active compounds synthesized by diverse groups of microorganisms, which exist in a wide variety of chemical compounds, such as fatty acids, neutral lipids, phospholipids, glycolipids, and lipopeptides. Biosurfactants have attracted much interest as hydrocarbon dissolution agents in late 1960s (Francy et al. 1991), but over the past five decades, they have greatly extended their applications in food, pharmaceutical, and oil industries, thanks to their peculiar properties. For instance, they have been proposed as an improved alternative to synthetic surfactants, such as carboxylates, sulphonates, and sulfate acid esters.

Some advantages of biosurfactants are represented by their specific action, lower toxicity, higher biodegradability, and effectiveness at a wide range of temperatures, pH, and salinity (Rodrigues et al. 2006a; Saharan et al. 2012).

The most commonly isolated biosurfactants are represented by glycolipids and lipopeptides, but rhamnolipids produced by *Pseudomonas aeruginosa* (Nitschke et al. 2005), sophorolipids released from *Candida* species (Daverey and Pakshirajan 2009), and surfactin and iturin from *Bacillus subtilis* (Ahimou et al. 2000) are also included. The biosurfactants production yields by these microorganisms are relatively high (2–10 g/l), and their ability to reduce the water surface tension to values below 30 mN/m was reported (Ahimou et al. 2000; Daverey and Pakshirajan 2009; Nitschke et al. 2005).

On the other hand, biosurfactants were found to be also released by different species of lactobacilli, also called lactic

acid bacteria (LAB). They are classified as "Generally Recognized As Safe" (GRAS) bacteria and are commensal microorganism involved in several homeostatic processes in different body districts. Lactobacilli produced biosurfactants in lower amounts (20–100 mg/l) (Rodrigues et al. 2006b) and are generally less effective to lower the air-water surface tension (around 36–40 mN/m). Such biosurfactants have been characterized as complex mixtures, whose composition has been only partially identified. However, several authors reported the interesting properties of biosurfactants produced by lactobacilli to inhibit the adhesion of pathogens to biomaterial and/or cell surfaces, highlighting their key role in the biofilm formation process (Saravanakumari and Mani 2010; Gudiña et al. 2010; Sharma et al. 2014; Sharma et al. 2015).

A biofilm is a thin layer of microorganisms adhering to the surface of an organic or inorganic structure, together with their secreted "extracellular polymeric substances" (EPS). Biofilms represent the predominant phenotype of nearly all bacteria in their natural habitat, whether pathogenic or environmental. For instance, biofilm formation by oral pathogens, such as *Streptococci* spp., on the solid surface of the enamel or the root of the teeth is considered to play a major role in the pathogenesis of dental caries (Socransky and AD 2002). Moreover, the presence of bacterial biofilm on the dental implants can cause peri-implantitis inflammatory disease and/or implant loss (Fürst et al. 2007; Elter et al. 2008). The application of antimicrobial agents seems to be a useful tool for controlling the formation and growth of oral biofilms (Ntrouka et al. 2011). However, bacteria self-organized in a cooperating biofilm can be up to 1000 times more resistant to the common antibiotics and antimicrobials than the same bacteria circulating in a planktonic state (Reffuveille et al. 2013). For this reason, in the last decade, the attention was paid to new class of antimicrobial agents, which have demonstrated antimicrobial activity against cariogenic bacteria (Jeon et al. 2011), or new strategies to control biofilm formation. Among these, the study of the effects of probiotics in the pathogenesis of oral diseases is a relatively new research area in oral health (Haukioja et al. 2008; Söderling et al. 2011). Particularly, in the recent years, the attention of researchers has turned up to the ability of lactobacilli to interfere with the adhesion of oral pathogens to abiotic surfaces (Teanpaisan et al. 2011; Söderling et al. 2011; Marttinen et al. 2013). The inhibition of pathogen adhesion and, consequently, of biofilm formation is generally attributed to the activity of biosurfactants produced by different species of bacteria such as lactobacilli (Gudiña et al. 2010; Tahmourespour et al. 2011).

Data in literature are mostly referred to the so called "cell-bound biosurfactants," which are commonly extracted at the end of the fermentation by washing the centrifuged bacteria cells with phosphate buffer under gentle stirring at room temperature (Rodrigues et al. 2006b; Gudiña et al. 2010; Tahmourespour et al. 2011). However, few data are available for the so called "excreted biosurfactants," which are released in the culture media during the fermentation process (Sharma and Saharan 2014). For this reason, the aim of the present study was to characterize in terms of surface tension reduction and emulsifying activity, the "excreted biosurfactants," produced by selected *Lactobacillus* spp., purified through dialysis using membranes at two different molecular weight cut-off (1 and 6 kDa). Moreover, the anti-biofilm effect of each dialyzed biosurfactant against *Streptococcus mutans* ATCC 25175 and *Streptococcus oralis* ATCC 9811 was investigated by plate count agar, biomass analyses, and flow cytometry (FCM).

Materials and methods

Bacteria and culture conditions

Seven *Lactobacillus* spp. were used in this study: *Lactobacillus reuteri* DSM 17938 (Reuflor, Italchimici, Italy), *Lactobacillus acidophilus* DDS-1 (Nutratec, Urbino, Italy), *Lactobacillus paracasei* B21060 (Floratec, Bracco, Italy), *Lactobacillus rhamnosus* ATCC 53103, *Lactobacillus rhamnosus* ATCC 7469, *Lactobacillus casei* ATCC 15008, and *Lactobacillus salivarius* ATCC 11741. All these strains were grown in Man Rogosa and Shape agar (MRS) (Oxoid, UK) at 37 °C for 24–48 h under microaerophilic conditions (5 % O_2; 10 % CO_2; 85 % N_2).

In addition, *S. mutans* ATCC 25175 and *S. oralis* ATCC 9811, two reference human oral pathogens, were used as pathogen bacteria model. These strains were routinely grown on Sheep blood agar base (Oxoid, UK) with 5 % of Sheep blood (Oxoid, UK) at 37 °C for 24 h.

Stock cultures of each strain were keep at −80 °C in Nutrient broth (Oxoid, UK) with 15 % of glycerol.

Biosurfactants preparation and assessment of the antimicrobial activity by time-killing studies

LAB strains overnight cultures (15 ml) were inoculated to 600 ml of MRS broth (Oxoid, UK) and incubated at 37 °C for 48 h under microaerophilic conditions. For the recovery of the crude excreted biosurfactants (BSF), bacterial cultures were centrifuged at 17,000 rpm for 15 min at 4 °C, and the supernatants were filtered through a 0.22-μm pore size filter (Millipore).

To test the antimicrobial effect of the crude excreted biosurfactants, *S. mutans* ATCC 25175 and *S. oralis* ATCC 9811 were grown in brain-heart infusion broth (BHI) (Oxoid, UK) supplemented with 1 % yeast extract at 37 °C for 18 h. Then, 500 μl of each bacterial culture (about 10^8 cfu/ml) and 500 μl of each biosurfactant were combined in 24-well-polystyrene plates and incubated at 37 °C. At the baseline time (0 h) and after 3, 6, and 24 h, different aliquots were

aseptically removed, serially diluted in physiological saline solution, and plated on Sheep agar base with 5 % sheep blood at 37 °C for 24–48 h. At the end of incubation, plates were observed to calculate the colony-forming unit (cfu/ml). Each experiment was performed in duplicate.

Dialysis of biosurfactants

Four *Lactobacillus* spp. (*L. reuteri* DSM 17938, *L. acidophilus* DDS-1, *L. rhamnosus* ATCC 53103, and *L. paracasei* B21060) were selected on the basis of the antimicrobial activity determined by time-killing studies, and their excreted biosurfactants were purified through dialysis. The dialyses were performed against demineralized water at room temperature using Spectra/Por® membranes at two different molecular weight cut-offs, 1 and 6 kDa (Spectrum® Laboratories, Inc.). The solutions containing the biosurfactants were finally freeze-dried.

Characterization of dialyzed biosurfactant surface properties

The following experiments were conducted to determine biosurfactant activities of dialyzed/freeze-dried biosurfactants in terms of reduction in air-water surface tension and oil-water emulsion ability.

The reduction in air-water surface tension (ST) of biosurfactants was determined by the Ring method (Kim et al. 2000) using a tensiometer (DCA-100 Contact Angle Tensiometer—First Ten Angstroms, Inc., USA) equipped with a 1.9-cm De Noüy platinum ring at room temperature. About 15 ml of each sample was withdrawn, and surface tension was measured at 0.5, 1, 2.5, 5, and 10 mg/ml. MRS broth was also analyzed at the same concentrations for comparison.

The emulsification activity of biosurfactants is expressed as emulsification index (E24) (Kuiper et al. 2004). Equal volumes of the aqueous phase containing the dialyzed biosurfactant (1 and 10 mg/ml) and paraffin oil were vigorously mixed with a vortex for 2 min, and allowed 24 h to settle. The emulsification index was calculated by the following equation:

$$E24 = \frac{\text{height of an emulsion layer}}{\text{total height}} \times 100$$

Each test was performed in duplicate.

Biofilm formation

Biofilm formation on titanium surface of *S. mutans* ATCC 25175 and *S. oralis* ATCC 9811 was obtained as described by Ciandrini et al. (Ciandrini et al. 2014) with some modification. First, sterilized titanium disks were placed on 24-well-polystyrene cell culture plates (Cellstar®, Greiner Bio-One, Germany) and incubated for 4 h by gentle shaking at room temperature with human saliva, collected from four healthy volunteers (with their informed consent). Saliva was previously clarified by centrifugation (15,000×*g* for 15 min at 4 °C) and sterilized by 0.22-μm filtration. The disks were finally washed with 10 mM PBS at pH 7. At this point, titanium disks were covered with 1.6 ml of BHI and infected with 200 μl (10^6 cell/ml) of each bacterial suspension after incubation in BHI broth for *S. mutans* ATCC 25175 and *S. oralis* ATCC 9811. Plates were, then, incubated at 37 °C for 24 h. The bacteria adherent to titanium disks were harvested by vigorous vortexing for 2 min in physiological saline solution, then serially diluted and plated on Sheep agar base with 5 % sheep blood (Oxoid, UK). The plates were incubated at 37 °C under the adequate conditions for 24–48 h, and the colony-forming units per milliliter (cfu/ml) were counted.

To assess biofilm formation, biomass analysis was carried out using Crystal Violet (CV) staining. Briefly, at each time point, titanium disks were removed, washed with PBS, and immersed in a new well containing 0.1 % (*v*/*v*) of CV (Sigma, Italy) for 15 min. Then, disks were washed again with PBS, air-dried, and the remaining CV was dissolved in 85 % ethanol for 15 min at room temperature. Finally, 200 μl from each well was transferred into a 96-well plate to be spectrophotometrically analyzed at 570 nm, using a Multiscan Ex Microplate Reader (Thermo Scientific). Data point was averaged from at least eight replicate per well, and each experiment was performed three times using independent cultures.

Anti-biofilm effect of dialyzed biosurfactants

To evaluate the anti-biofilm activity of excreted biosurfactants of *L. reuteri* DSM 17938, *L. acidophilus* DDS-1, *L. rhamnosus* ATCC 53103, and *L. paracasei* B21060 strains, each dialyzed biosurfactant (1 and 6 kDa) was tested at two concentrations (1 and 10 mg/ml) against *S. mutans* ATCC 25175 and *S. oralis* ATCC 9811 during biofilm formation on titanium surface.

For this purpose, saliva-conditioned titanium disks, once positioned into a 24-wells polystyrene cell culture plate, were covered with 200 μl (10^6 cell/ml) of *S. mutans* ATCC 25175 and *S. oralis* ATCC 9811 cultures and 1 ml of each dialyzed biosurfactant, at the mentioned concentrations. For each microorganism, wells, inoculated with 200 μl (10^6 cell/ml) of *S. mutans* ATCC 25175 and *S. oralis* ATCC 9811 in BHI broth, were also included as controls. Plates were incubated at 37 °C for 24 h to allow biofilm formation and, at the end of the incubation, adherent bacteria were harvested as described above for plate count enumerations.

Two additional series of 24-wells polystyrene cell culture plates were prepared using the same described procedure, to

perform biomass analysis and flow cytometry (FCM), respectively. For biomass analysis, after *S. mutans* ATCC 25175 and *S. oralis* ATCC 9811 biofilm formation, wells were washed twice in PBS and stained by CV 0.1 % (*v*/*v*) (Sigma, Milan, Italy) for 15 min. Disks were PBS washed and air-dried; at this point, the remaining CV was dissolved in 85 % ethanol for 15 min at room temperature and 200 μl from each well was transferred into a 96-well plate and analyzed by a Multiscan Ex Microplate Reader spectrophotometrically at 570 nm.

For FCM analysis, the titanium adherent bacteria were harvested by vigorous vortexing for 2 min in physiological saline solution. Each sample, diluted in the same buffer, was labeled with SYBR Green I (1/10,000 *v*/*v*) and Propidium Iodide (PI) (10 μg/ml) (Barbesti et al. 2000), incubated in the dark for 15 min at room temperature and immediately processed by FACSCalibur flow cytometer (BD Biosciences, USA) equipped with a 488 nm laser. All experiments were performed in triplicates. Multi-parametric analyses were performed on both scattering signals as forward-scattered light (FSC) and side-scattered light (SSC) and fluorescence emission in FL1/FL3 channels. In particular, the green fluorescence of SYBR Green I was detected in the FL1 (530/30) channel while PI red fluorescence in the FL3 (>670) channel. Threshold levels were set on FSC in order to eliminate noise, due to the presence of cellular debris which contribute much smaller than intact cells to the overall signal. Bacterial cells were gated according to FSC/SSC parameters. The data were analyzed using CellQuest™ Pro software (BD Biosciences, USA).

Statistical analysis

Statistical analysis was performed using Prism version 5.0 (GraphPad Inc., USA). All experiments were performed in duplicate, and the standard error of the mean was calculated from the combined measurements. Data points were analyzed through one-way analysis of variance (ANOVA) with Bonferroni post hoc test unless the assumptions for the parametric test were not respected. In this case, Kruskal-Wallis non-parametric test with Dunnett's multiple comparison test was applied. P values <0.05 were considered to be statistically significant.

Results

Antimicrobial activity of crude excreted biosurfactants in killing studies

The antimicrobial activities of all *Lactobacillus* spp. crude excreted biosurfactants against oral streptococci were determined by time-kill assays (Fig. 1). In general, the results show that BSFs were able to reduce the growth of *S. mutans* ATCC 25175 and *S. oralis* ATCC 9811 up to 24 h with variable ability depending on the producing LAB strain. In detail, the biosurfactants from *L. acidophilus* DDS-1, *L. paracasei* B21060, and *L. rhamnosus* ATCC 53103 induced 1-log reduction in cfu/ml values after 3 h of incubation for *S. mutans* ATCC 25175, and these values remained essentially unchanged after 6 h reaching 2-log reduction after 24 h of incubation. Biosurfactant from *L. reuteri* DSM 17938 showed the greatest antimicrobial activity, with a cfu/ml reduction of 2-log after 3 h, 3-log after 6 h, and even 8-log after 24 h (Fig. 1a).

Reduction of cfu/ml values also occurred toward *S. oralis* ATCC 9811, but, in this case, biosurfactants of *L. acidophilus* DDS-1, *L. paracasei* B21060, and *L. rhamnosus* ATCC 53103 induced a reduction of 2-log after 3 and 6 h of incubation, reaching 5.4-log after 24 h. The biosurfactant of *L. reuteri* DSM 17938 showed the greatest antimicrobial activity, with a decrease of 3-, 4-, and 8-log after 3, 6, and 24 h, respectively (Fig. 1b). Biosurfactants excreted by *L. rhamnosus* ATCC 7469, *L. casei* ATCC 15008, and *L. salivarius* ATCC 11741 showed to be less active in reducing the growth of *S. mutans* ATCC 25175 and *S. oralis* ATCC 9811.

For this reason, only the BSFs of *L. reuteri* DSM 17938, *L. acidophilus* DDS-1, *L. paracasei* B21060, and *L. rhamnosus* ATCC 53103 were chosen for subsequent dialysis procedure.

Screening assays for dialyzed biosurfactants production

Dialyzed (1a and 6 kDa) biosurfactants excreted by *L. reuteri* DSM 17938, *L. acidophilus* DDS-1, *L. rhamnosus* ATCC 53103, and *L. paracasei* B21060 strains were tested using qualitative and quantitative methods (Table 1).

All dialyzed biosurfactants were able to decrease air-water surface tension (Fig. 2). The reduction in surface tension was quite linear in the range of the tested concentrations (1–10 mg/ml) and clearly more pronounced for the biosurfactants with respect to the growth medium (MRS broth), analyzed for comparison. In fact, the measured surface tension for MRS broth was 48 mN/m at 10 mg/ml, different from the biosurfactants, which showed lower values (35–40 mN/m) (Table 1). No marked differences among biosurfactants can be observed despite, in general, a slightly higher surface activity has been shown by the 1 kDa dialyzed compounds.

The estimation of emulsification activity (E24) against paraffin oil was used, together with the reduction of air-water surface tension to assess the biosurfactant activity. Data obtained from the differently dialyzed biosurfactants (Table 1) were compared with those of distilled water, as negative control, and 1 % SDS, a common chemical surfactant, as positive control. All the dialyzed biosurfactants showed dose-dependent emulsifying activities, with the highest E24 index of 50 and 61.11 % for the 1 and 6 kDa dialyzed biosurfactants from *L. paracasei* B21060, respectively.

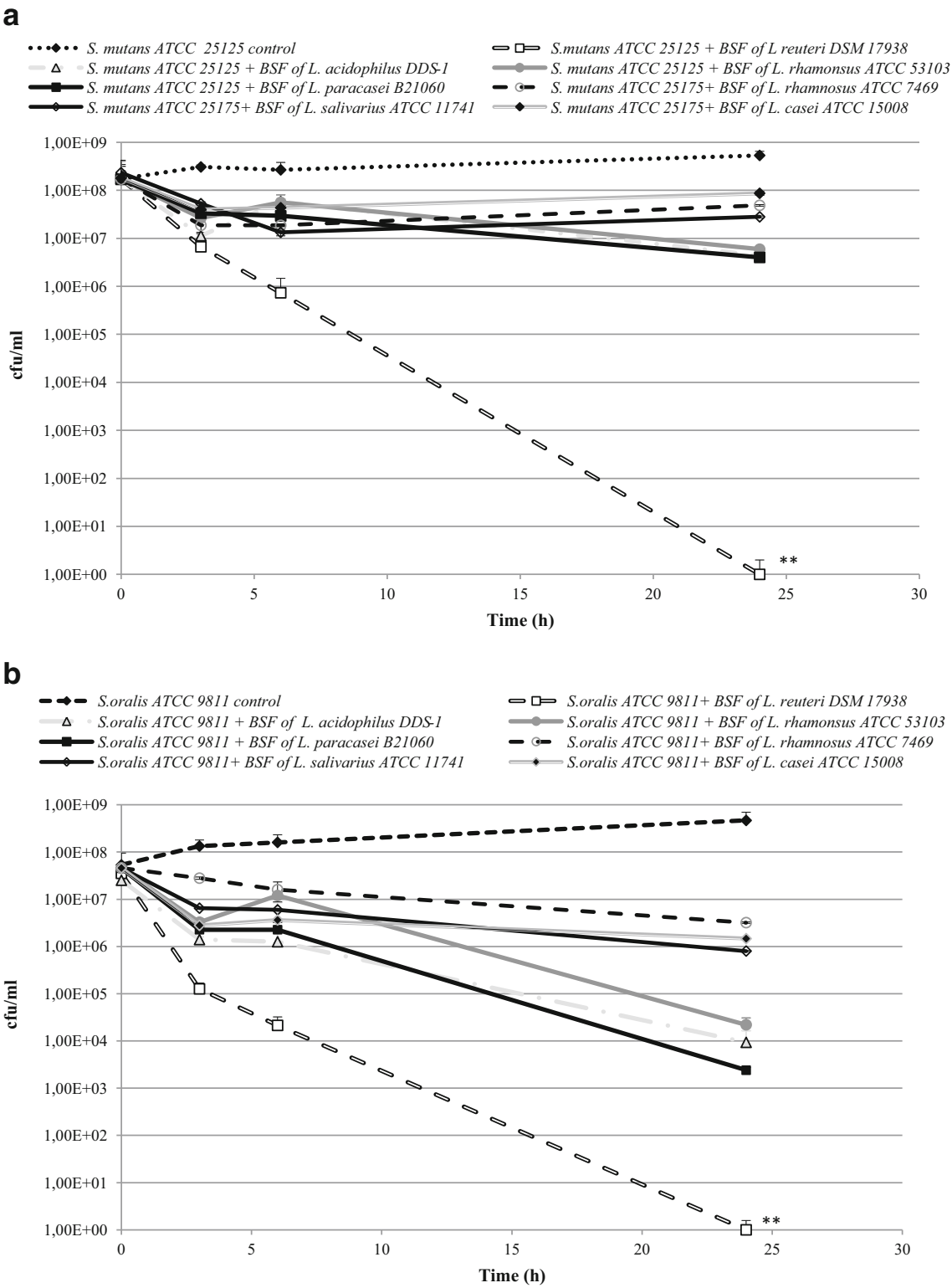

Fig. 1 Antimicrobial activity of biosurfactants produced by *L. reuteri* DSM 17938, *L. acidophilus* DDS-1, *L. rhamnosus* ATCC 53103, and *L. paracasei* B21060 against **a** *S. mutans* ATCC 25175 and **b** *S. oralis* ATCC 9811 assessed by time-kill studies

However, all the 6 kDa dialyzed biosurfactants, with the exception of that from *L. paracasei* B21060, showed E24 values higher than those of the correspondent 1 kDa dialyzed biosurfactants.

Table 1 Surface tension and emulsification activity against paraffin oil of dialyzed biosurfactants (1 and 6 kDa) produced by selected *Lactobacillus* spp.

	Surface tension[a]				Percent of emulsification (E24 index)[b]			
	Dialyzed 1 kDa		Dialyzed 6 kDa		Dialyzed 1 kDa		Dialyzed 6 kDa	
Strains	10 mg/ml	1 mg/ml	10 mg/ml	1 mg/ml	10 mg/ml	1 mg/ml	10 mg/ml	1 mg/ml
L. reuteri DSM 17938	37.67 ± 0.33	42.98 ± 0.40	39.65 ± 0.75	45.38 ± 0.80	42.86	46.67	58.82	35.29
L. acidophilus DDS-1	37.71 ± 0.44	42.61 ± 0.41	39.04 ± 1.08	44.59 ± 0.13	46.67	37.50	53.33	47.06
L. rhamnosus ATCC 15023	34.81 ± 0.31	38.53 ± 0.48	39.27 ± 2.36	45.75 ± 0.42	43.75	37.50	58.82	52.94
L. paracasei B21060	38.90 ± 0.34	41.24 ± 0.45	37.62 ± 0.82	47.92 ± 0.58	50.00	41.18	61.11	41.17

[a] Surface tension of MRS broth was 53.0 mN/m; surface tension of water was 72.2 mN/m

[b] Emulsification index of 1 % SDS was 65.52 % and that of distilled water was 1.72 %

Effect of dialyzed biosurfactants on streptococci biofilm formation

The anti-biofilm activity of dialyzed biosurfactants (1 and 6 kDa) against streptococci was assessed by the plate counts agar and biomass analysis. Data are presented in Fig. 3. As shown, all 1 and 6 kDa dialyzed BSFs were effective to inhibit the biofilm growth of *S. mutants* ATCC 25175 and *S. oralis* ATCC 9811. In particular, BSFs from *L. acidophilus* DDS-1, *L. paracasei* B21060, and *L. rhamnosus* ATCC 53103, tested at 10 mg/ml, induced a 3-log reduction in *S. mutans* ATCC 25175 cfu/ml values, while that from *L. reuteri* DSM 17938 determined a reduction less than 2-log. Moreover, the lower logarithmic decrease of the cfu/ml values of *S. mutans* ATCC 25175 treated with 1 kDa dialyzed BSFs at the concentration 1 mg/ml highlighted their dose-dependent effect (Fig. 3a). In the case of *S. oralis* ATCC 9811 biofilm, the 1 kDa dialyzed BSF from *L. acidophilus* DDS-1 (10 mg /ml) was the most effective, showing a cfu/ml reduction of 3-log, while those of *L. reuteri* DSM 17938, *L. rhamnosus* ATCC 53103, and *L. paracasei* B21060 caused a modest decrease of cfu/ml values in *S. oralis* ATCC 9811 (Fig. 3a). Regarding anti-biofilm activity of 6 kDa dialyzed biosurfactants, BSFs from *L. reuteri* DSM 17938, *L. acidophilus* DDS-1, and *L. paracasei* B21060, tested at 10 mg/ml, induced 1-log reduction in cfu/ml values of *S. mutans* ATCC 25175, while that of *L. rhamnosus* ATCC 53103 determined 3-log reduction (Fig. 3b). Against *S. oralis* ATCC 9811, the BSFs 6 kDa of *L. acidophilus* DDS-1 and *L. rhamnosus* ATCC 53103 (10 mg/ml) exhibited good antimicrobial activity with a 3-log reduction, while those of *L. reuteri* DSM 17938 and *L. paracasei* B21060, at the same

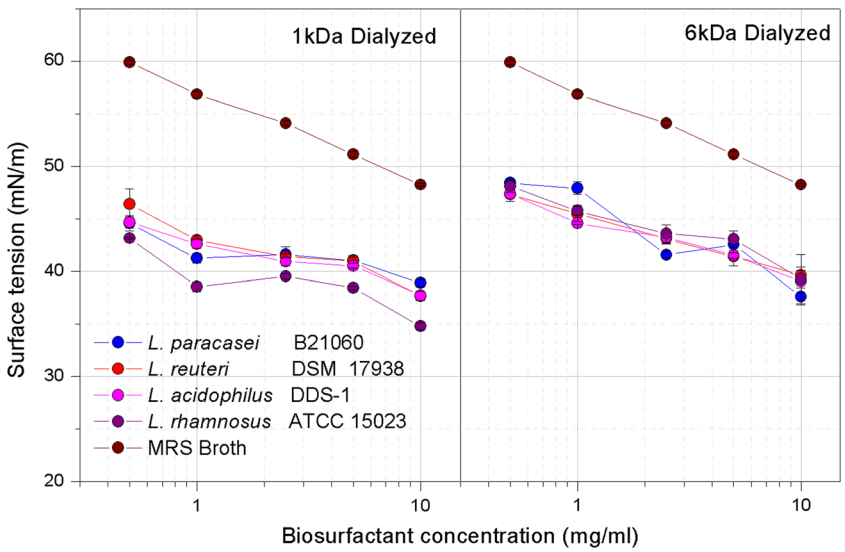

Fig. 2 Effect of dialyzed (1 and 6 kDa) biosurfactants on the air/water surface tension (mN/m) at different concentrations, obtained from *L. reuteri* DSM 17938, *L. acidophilus* DDS-1, *L. rhamnosus* ATCC 53103, *L. paracasei* B21060, and the starting MRS broth used. The reference surface tension value was 72.2 mN/m. Results represent the average of three independent measurements, and *error bars* represent standard deviations of the mean values

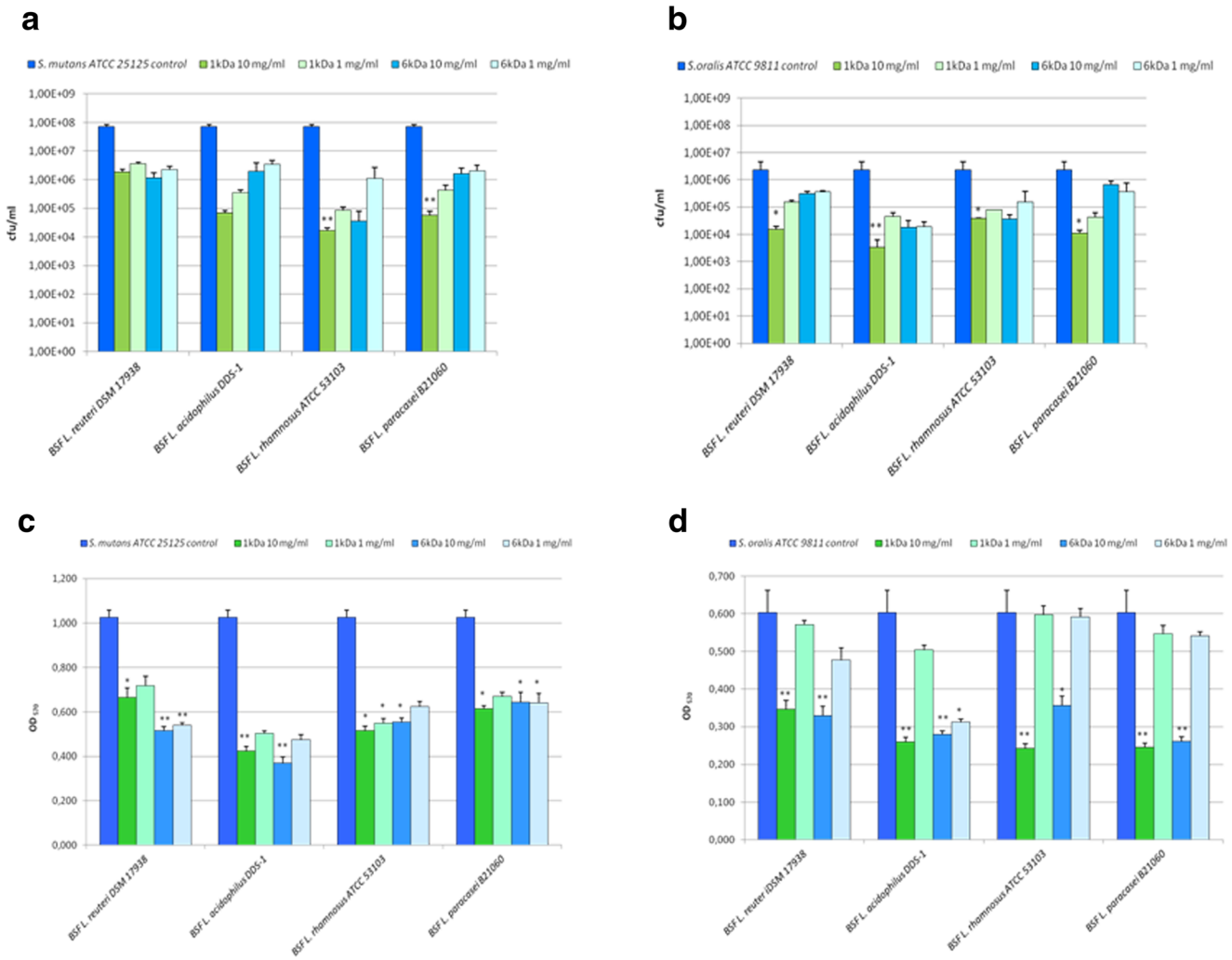

Fig. 3 Activity of dialyzed biosurfactants (1 and 6 kDa) produced by *L. reuteri* DSM 17938, *L. acidophilus* DDS-1, *L. rhamnosus* ATCC 53103, and *L. paracasei* B21060 at two concentrations (10 and 1 mg/ml) against *S. mutans* ATCC 25175 and *S. oralis* ATCC 9811 biofilm formation assessed by plate counts agar (**a**, **b**) and CV staining at 570 nm (**c**, **d**)

concentration, induced only 1-log reduction in cfu/ml values (Fig. 3b). A dose-dependent effect was also confirmed for the 6 kDa BSFs against *S. mutans* ATCC 25175 and *S. oralis* ATCC 9811.

In general, biomass analysis on biofilms treated with 1 and 6 kDa dialyzed BSFs confirmed the data previously obtained by plate count agar. In particular, the most effective dialyzed 1 kDa BSFs against *S. mutans* ATCC 25175 were those from *L. acidophilus* DDS-1 and *L. rhamnosus* ATCC 53103 (10 mg/ml) with optical density of 0.423 (±0.022) and 0.514 (±0.022), respectively, in comparison to 1.024 (±0.034) of the control (Fig. 3c). Dialyzed 1 kDa biosurfactants (10 mg/ml) were also active toward *S. oralis* ATCC 9811 biofilm, with optical density of 0.259 (±0.012) for BSF of *L. acidophilus* DDS-1, 0.243 (±0.011) for *L. rhamnosus* ATCC 53103, and 0.254 (±0.011) for *L. paracasei* B21060; lower activity was observed for *L. reuteri* DSM 17938 BSF (0.347 ± 0.023) (Fig. 3d). The 6 kDa dialyzed BSFs also possess anti-biofilm activity against *S. mutans* ATCC 25175 and *S. oralis* ATCC 9811, although more noticeable in the case of *S. oralis* ATCC 9811 with optical density of 0.329 (±0.026) for BSF of *L. reuteri* DSM 17938, 0.280 (±0.009) for *L. acidophilus* DDS-1, 0.356 (±0.025) for *L. rhamnosus* ATCC 53103, and 0.261 (±0.012) for *L. paracasei* B21060. The biosurfactant showing the most relevant decrease in optical density (0.312 ± 0.008) against *S. oralis* ATCC 9811 at low concentration (1 mg/ml), in comparison to the control (0.603 ± 0.060), was the BSF from *L. acidophilus* DDS-1 (Fig. 3d). Biomass analysis highlighted the dose-dependent effect of all the dialyzed biosurfactants (1 and 6 kDa), particularly remarkable in the case of biofilm formation inhibition of *S. oralis* ATCC 9811.

FCM analysis, assessed by double staining with SYBR Green I and PI, showed a remarkable biofilm inhibition percentages in term of total viable cells in each treated sample (Table 2). In particular, all the 1 kDa dialyzed biosurfactants

Table 2 Percentages of *S. mutans* ATCC 25175 and *S. oralis* ATCC 9811 biofilm formation inhibition induced by *L. reuteri* DSM 17938, *L. acidophilus* DDS-1, *L. rhamnosus* ATCC 53103, and *L. paracasei* B21060 dialyzed biosurfactants (1 and 6 kDa) at two concentrations (10 and 1 mg/ml) as determined in FCM after double staining with SYBR Green I and PI

		Biofilm formation inhibition (%)	
Dialyzed biosurfactants of		*S. mutans* ATCC 25175	*S. oralis* ATCC 9811
L. reuteri DSM 17938:			
1 kDa	10 mg/ml	99.18	98.19
	1 mg/ml	95.13	65.59
6 kDa	10 mg/ml	94.24	94.79
	1 mg/ml	92.82	84.60
L. acidophilus DDS-1:			
1 kDa	10 mg/ml	98.25	95.74
	1 mg/ml	94.15	77.35
6 kDa	10 mg/ml	96.43	95.52
	1 mg/ml	90.14	75.45
L. rhamnosus ATCC 53103:			
1 kDa	10 mg/ml	98.36	96.48
	1 mg/ml	93.82	90.81
6 kDa	10 mg/ml	92.52	95.13
	1 mg/ml	76.64	74.17
L. paracasei B21060:			
1 kDa	10 mg/ml	98.95	92.32
	1 mg/ml	96.15	80.32
6 kDa	10 mg/ml	92.86	95.19
	1 mg/ml	92.19	85.28

10 mg/ml showed the higher inhibition percentages of biofilm formation against *S. mutans* ATCC 25175, with values ranging from 98.25 % with BSF from *L. acidophilus* DDS-1 to 99.18 % with that from *L. reuteri* DSM 17938. In the case of *S. oralis* ATCC 9811, the percentages of biofilm formation inhibition ranged from 92.32 % with BSF from *L. paracasei* B21060 to 98.19 % with BSF from *L. reuteri* DSM 17938. As regards 6 kDa biosurfactants, biofilm inhibition percentages against *S. mutans* ATCC 25175 ranged from 92.52 % with BSF from *L. rhamnosus* ATCC 53103 to 96.43 % with BSF from *L. acidophilus* DDS-1. Similarly, biofilm inhibition percentages toward *S. oralis* ATCC 9811 varied from 94.79 % with BSF from *L. reuteri* DSM 17938 to 95.52 % with that from *L. acidophilus* DDS-1. FCM analysis also registered the dose-dependent effect of 1 and 6 kDa dialyzed biosurfactants with less activity at 1 mg/ml concentration against *S. mutans* ATCC 25175 and *S. oralis* ATCC 9811.

Discussion

Several studies have shown the beneficial properties of lactobacilli for humans in various anatomic districts, including the gastrointestinal and the genito-urinary tracts (Gomaa 2013). In addition, these bacteria have been recognized as fundamental in the maintenance of homeostasis in the oral cavity (Meurman and Stamatova 2011). Oral diseases, in fact, represent important human infections and are widespread in all age groups with the least success to control the actual infection. The ability, showed by streptococci, to steadily adhere to the surfaces of salivary protein-coated teeth and to dental implants, and to create a biofilm is fundamental for the promotion of the oral lesion development leading, at least, to dental or implant loss.

An important protective role was demonstrated for lactobacilli in the biofilm process (Meurman and Stamatova 2011; Teanpaisan et al. 2011). Lactobacilli were demonstrated to reduce the growth in the oral cavity of pathogens, such as streptococci (Çaglar et al. 2006; Çaglar et al. 2008), and to inhibit the biofilm formation (Jalasvuori et al. 2012). Lactobacilli are also involved in the process of biofilm formation on oral surfaces, by exerting an antimicrobial and anti-adhesive effect against pathogen bacteria through the production of specific substances, such as bacteriocins and biosurfactants and, at the same time, by competing with them for the colonization (Söderling et al. 2011).

In this work, the antimicrobial activity of the crude excreted biosurfactants from different *Lactobacillus* spp. toward *S.*

mutans ATCC 25175 and *S. oralis* ATCC 9811 was initially investigated in time-killing studies. The obtained data showed a time-dependent decrease of *S. mutans* ATCC 25175 and *S. oralis* ATCC 9811 growth, particularly remarkable in the presence of the supernatant from *L. reuteri* DSM 17938 that is able to completely inhibit the growth of both streptococci up to 24 h. Conversely, Söderling et al. (2011) found that the antimicrobial effect of two *L. reuteri* strains was weaker and less efficient in reducing the viability (6.25 log cfu/ml) of tested *S. mutans* strains after 60 min of exposure. Our results suggest that *L. reuteri*, reuterin-producer in optimal conditions, showed antimicrobial activity also against oral streptococci other than against gut microorganisms (Spinler et al. 2008).

Since biosurfactants produced by different lactobacilli are complex biological mixtures, in this study, crude excreted biosurfactants, selected on the basis of their antimicrobial activity in killing studies, were dialyzed. The two obtained fractions (1 and 6 kDa cut-off), after freeze-drying, were characterized in terms of surface tension reduction and emulsifying ability and demonstrated to possess surface-active characteristics, such as the ability to bring down the air-water surface tension or to emulsify a water/oil mixture. Data published in the literature reported that bacteria with high surface activity and emulsifying properties represent promising microbial candidates for biosurfactant production (Banat et al. 2000).

Regarding the surface tension observed for all 1 and 6 kDa dialyzed, obtained after 48 h of incubation, a reduction of the interfacial tension from 47.92 to 34.81 mN/m was observed when compared to the surface tension of MRS broth (53.0 mN/m), with a slight higher surface activity in the 1 kDa fractions. This decrease of surface tension confirmed the production of biosurfactants by the isolates and accumulation within the media. These data are in agreement with those reported by Sharma and Saharan (Sharma and Saharan 2014), showing similar values of ST for excreted biosurfactants produced by a different *Lactobacillus* strains, with a surface tension reduction of the production media to 40.8 mN/m from the initial value of 53 mN/m. Moreover, in this study, another approach for screening potential biosurfactant-producing lactobacilli was the determination of the emulsification activity (% E24). The highest emulsifying activity was obtained from the dialyzed 6 kDa biosurfactant of *L. paracasei* B21060 (61.11 %), comparable with 58 and 57 % referred by Sharma and Saharan.

Another characteristic of a biosurfactant is the ability to reduce pathogen adhesion and subsequent biofilm formation on different surfaces, such as plastic materials or glass (Rodrigues et al. 2006a; Gudiña et al. 2010; Tahmourespour et al. 2011; Gomaa 2013). For this, in the present study, the antimicrobial activity of dialyzed biosurfactants produced by selected *Lactobacillus* spp. was evaluated during biofilm formation of *S. mutans* ATCC 25175 and *S. oralis* ATCC 9811 on titanium surface through three different techniques as agar plate count, biomass analysis, and flow cytometry. Our results indicate an inhibition of the biofilm produced by both the examined oral pathogens when the dialyzed BSFs were added in the culture medium, as demonstrated by decrease of cfu/ml, biomass production, as well as FCM values. The inhibitory effect showed a dose-dependence for both the 1 and 6 kDa BSFs against *S. mutans* ATCC 25175 and *S. oralis* ATCC 9811. These findings are in agreement with data reported by Tahmourespour et al. (Tahmourespour et al. 2011), which referred that biosurfactant of *L. acidophilus* interfered in the adhesion and biofilm formation of *S. mutans* to glass slide. Moreover, anti-adhesive effect depending on *L. paracasei* biosurfactant concentration toward several bacteria, including *S. mutans* and *S. oralis*, on plastic tissue culture plate was reported by Gudiña et al. (Gudiña et al. 2010).

The precise mechanisms of biosurfactant antimicrobial effect have not yet been explained, even if, as observed in our study, it seems to be highly dependent on biosurfactant type and the target bacteria. In fact, the antimicrobial activity of biosurfactants has not been observed in all cases (Rodrigues et al. 2006b; Walencka et al. 2008). Some authors have observed anti-adhesive activity with biosurfactants against several pathogenic microorganisms such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Streptococcus agalactiae*, but not against *Escherichia coli*, *Candida albicans*, and *P. aeruginosa* (Gudiña et al. 2010).

In conclusion, surface interactions are mediated by the amphiphilic nature of molecules with hydrophilic (acid, peptide cations, anions, or polysaccharides) and hydrophobic regions (unsaturated or saturated hydrocarbon chains or fatty acids), allowing them to act as surfactants at the interfaces (Myers 2005; Banat et al. 2010). Biosurfactants can be excreted in the culture broth or remain attached to the cell wall of bacteria, but most data in literature are referred to cell-bound biosurfactants (Sharma et al. 2014; Sharma et al. 2015), and few reports are available on biosurfactants contained in culture media (Saravanakumari and Mani 2010; Sharma and Saharan 2014). This research represents the first work in which the excreted biosurfactants of different LAB strains were dialyzed and characterized for their surface ability prior to be tested for their antimicrobial activity. In addition, we have demonstrated that active biosurfactant molecules are released by LAB in the culture media, from which can be separated by a simple dialysis method, and the obtained dialyzed fractions, in particular those with 6 kDa molecular weight, possess antimicrobial and anti-biofilm activities against oral streptococci.

Our results confirm that LAB strains are biosurfactant producers and, since these microorganisms are considered as GRAS, their biosurfactants are safe for human consumption and biomedical applications. In particular, biosurfactants of LAB origin, reducing the ability of streptococci to adhere and develop biofilm on oral surfaces, may contribute to

prevent oral diseases. These findings are encouraging and could suggest the application of lactobacilli excreted biosurfactants as surface active agents in oral hygiene formulations, or as suitable alternative to conventional antimicrobials. Further studies are undergoing to better understand the chemical structural characterization of these excreted biosurfactants.

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Compliance with ethical standards

Ethical statement

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

Conflict of interest The authors declare that they have no conflict of interest.

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