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ORIGINAL ARTICLE

Rhamnolipids and lactonic sophorolipids: natural antimicrobial surfactants for oral hygiene

M. Elshikh[1], I. Moya-Ramírez[2], H. Moens[3], S. Roelants[3,4], W. Soetaert[3,4], R. Marchant[1] and I.M. Banat[1]

1 School of Biomedical Sciences, Ulster University, Coleraine, UK
2 Chemical Engineering Department, Faculty of Sciences, University of Granada, Granada, Spain
3 Bio Base Europe Pilot Plant, Gent, Belgium
4 Laboratory for Industrial Biotechnology and Biocatalysis (InBio.be), Faculty of Bioscience Engineering, University of Ghent, Ghent, Belgium

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Correspondence
Ibrahim M. Banat, Ulster University, Cromore Rd, Coleraine, Londonderry BT52 1SA, UK.
E-mail: im.banat@ulster.ac.uk

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Abstract

Aims: To assess the efficacy of rhamnolipid (mixture of monorhamnolipid and dirhamnolipid congeners), purified monorhamnolipid, dirhamnolipid and lactonic sophorolipid biosurfactants against pathogens important for oral hygiene.
Methods and Results: Acquired and produced biosurfactants were fully characterized to allow the antimicrobial activity to be assigned to the biosurfactant congeners. Antimicrobial activity was assessed using the resazurin-aided microdilution method. Mixed rhamnolipid JBR425 (MR) and lactonic sophorolipids (LSLs) demonstrated the lowest minimum inhibitory concentration (MIC) which ranged between 100 and 400 μg ml^{-1} against *Streptococcus mutans*, *Streptococcus oralis*, *Actinomyces naeslundii*, *Neisseria mucosa* and *Streptococcus sanguinis*. Combining these biosurfactants with standard antimicrobial agents namely chlorhexidine, sodium lauryl sulphate, tetracycline HCl and ciprofloxacin showed a dramatic drop in the MIC values. In addition, *in vitro* studies demonstrated the biosurfactants' ability to prevent and disrupt oral pathogens biofilms. The increased permeability of microorganisms treated with biosurfactant, as shown using bisbenzimide dye, in part explains the inhibition effect.
Conclusion: The results demonstrate that rhamnolipids and LSLs have the ability to inhibit oral pathogens both in planktonic and oral biofilm states.
Significance and Impact of the Study: The findings indicate the potential value of biosurfactants for both oral hygiene and the pharmaceutical industries since there is a serious need to reduce the reliance on synthetic antimicrobials and antibiotics.

Introduction

Rhamnolipids and sophorolipids are amphipathic natural surfactants produced by some microorganisms. These glycolipids comprise hydrophilic and hydrophobic segments attached via covalent bonds (Banat *et al.* 2010). This unique structural arrangement allows these biomolecules to demonstrate a wide spectrum of desirable properties, useful in pharmaceutical and cosmetic product development such as emulsification, detergency, solubilization and low toxicity (Fracchia *et al.* 2015). Recently, there has been more attention paid to the biomedical-related functionalities of biosurfactants, such as antimicrobial (Bharali *et al.* 2013), antiviral (Borsanyiova *et al.* 2016) and anticancer properties (Shao *et al.* 2012). Rhamnolipids are mainly produced by *Pseudomonas aeruginosa* or other species in the genus *Burkholderia*. Structurally, rhamnolipids are classified according to the number of rhamnose sugar moieties into mono- or di- (linked together through α-1,2-glycosidic linkage), in all cases attached via O-glycosidic linkage to one or two fatty acid chains which vary in length typically from C_8 to C_{14}

carbons and most commonly C_{10}, to produce monorhamnolipids and dirhamnolipids (Fig. 1), respectively (Abdel-Mawgoud *et al.* 2010). Sophorolipids, on the other hand, are mainly produced by yeast species such as *Starmerella bombicola* and *Candida bastistae* (Chen *et al.* 2006). Like rhamnolipids, sophorolipids are composed of a disaccharide sophorose, which can be acetylated on the 6′ and/or 6″ positions, which is β-glycosidically attached to a fatty acid chain typically C_{16}–C_{18} carbon length. Configuration of the fatty acid chain determines the subtype of sophorolipid; if it remains free, acid sophorolipids are formed, whereas when esterified at the 4″ position, lactonic sophorolipids (LSLs) are produced (Fig. 1) (Callaghan *et al.* 2016).

In a recent review discussing applications of biosurfactants in oral-related health (Elshikh *et al.* 2016a), it was concluded that using biosurfactants in oral-related applications represents a promising area of development. It was also concluded that to enhance research in this field qualitatively and quantitatively, there is a requirement to assess the antimicrobial effect of a wide range of well-characterized biosurfactant types. Previous research has focussed on lipopeptide/lipoprotein biosurfactants and often the products were neither well purified nor characterized.

To add rigorous information to this field, this research has assessed the antimicrobial effect of mixed congener rhamnolipids (MR), purified monorhamnolipid (Mono-RL) and dirhamnolipid (Di-RL) and purified LSLs, against microorganism's representative of the oral microflora namely *Streptococcus mutans*, *Streptococcus oralis*, *Streptococcus sanguinis*, *Neisseria mucosa* and *Actinomyces naeslundii*. These selected microbial strains together with some others contribute to the main architecture of oral biofilms, which may arise from poor diet and oral hygiene habits, eventually leading to tooth decay (Kolenbrander *et al.* 2010).

Materials and methods

Microorganisms and culture conditions

Microorganisms used are as follows: *S. mutans* (DSM-20523), *S. oralis* (DSM-20627); *A. naeslundii* (DSM-43013); *N. mucosa* (DSM-4631) and *S. sanguinis* (NCTC 7863). Microorganisms were stored in a mixture of glycerol and brain heart infusion (BHI) medium (1 : 1) at −80°C. Subcultures were prepared on nutrient agar plates and stored at 4°C. For LSL production, *S. bombicola* lactone esterase overexpression strain (oe *sble*) (Roelants *et al.* 2016) was maintained as described in the production section.

Production, extraction and purification of lactonic sophorolipids

The production process to obtain diacetylated LSLs was performed at the 150 l scale as described by Delbeke *et al.* (2016) using the *S. bombicola* lactone esterase overexpression strain (oe *sble*) (Delbeke *et al.* 2016). The medium

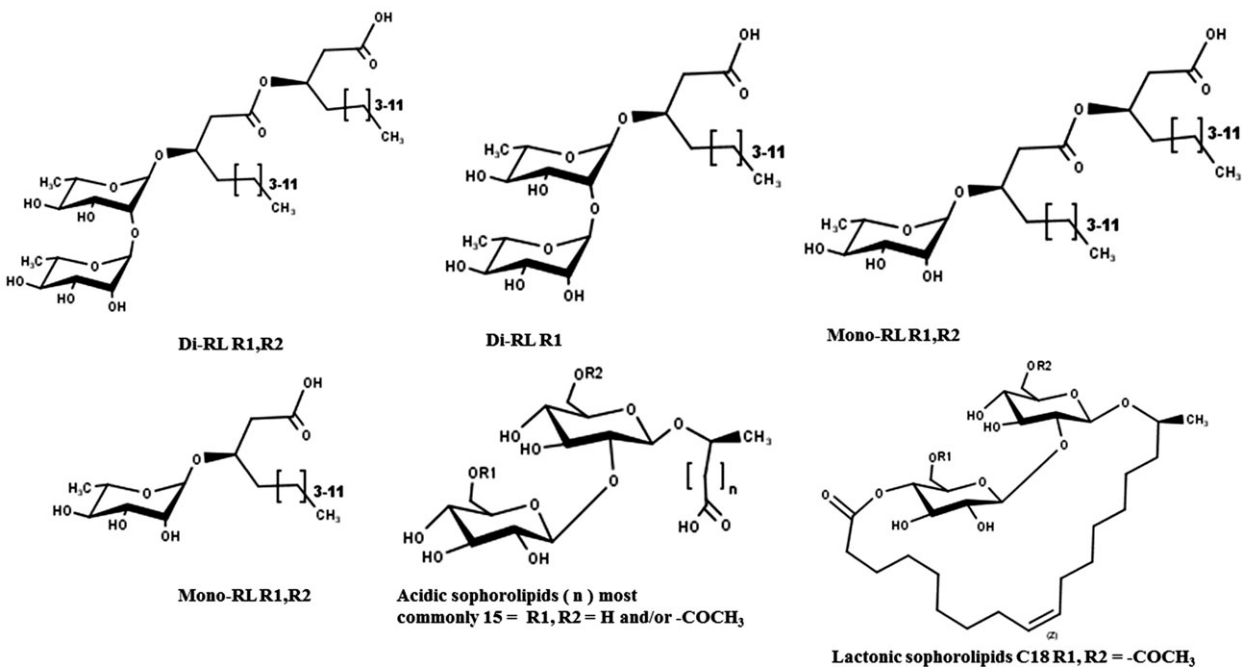

Figure 1 Representative chemical structures of monorhamnolipids (Mono-RL) dirhamnolipids (Di-RL) lactonic sophorolipids and acidic sophorolipids.

described by Lang *et al.* (2000) was used for the seed strains containing in g l^{-1}: 132 glucose, 4 yeast extract (YE), 5 Na-citrate.$2H_2O$, 1·5 NH_4Cl, 1·0 KH_2PO_4, 0·16 K_2HPO_4, 0·7 $MgSO_4$, 0·5 NaCl, 0·27 $CaCl_2.2H_2O$. The first seed cultures (50 ml, 30 h, 30°C, 200 rev min^{-1}) were inoculated from cryovials and transferred to 500 ml of the same medium (48 h, 30°C, 200 rev min^{-1}). These were inoculated (3%) in the main reactor (150 l, Sartorius DCU-3, Goettingen, Germany) containing 60 l of the production medium, based on corn steep liquor (CSL) instead of YE (100 g l^{-1} glucose.H_2O, 5 g l^{-1} dried CSL, 1 g l^{-1} K_2HPO_4, 4 g l^{-1} $(NH_4)_2SO_4$, 0·5 g l^{-1}, $MgSO_4.7H_2O$). Twelve g l^{-1} of rapeseed oil was added at the start. Aeration and stirring were set to 20 l min^{-1} and 500 rev min^{-1} respectively. After 20 h of batch fermentation, the continuous addition of rapeseed oil was started (0·8 g l^{-1} h^{-1}). The continuous glucose feed was started when its concentration dropped below 30 g l^{-1} and adjusted to keep the concentration in the reactor between 30 and 50 g l^{-1}. Temperature was maintained constant at 25°C, pH at 3·5 by automatic NaOH addition after a spontaneous drop during growth. The dissolved oxygen concentration was kept above 30%. Fermentation was stopped after 260 h.

The purification of diacetylated LSLs was based on melting the SLs and crystallization in (cold) water, which has been described in literature for the wild type for an SL product enriched in lactonic SLs (Hu and Ju 2001; Roelants *et al.* 2016). Sedimentation inside the reactor was achieved by heating to 70°C with sedimentation overnight (12 h). The bottom 'oily' phase consisting of the melted lactonic SLs was filtered at 70°C and filter aid (dicalite speedplus) was applied to facilitate filtration. The SL 'oil' was subsequently washed with reverse osmosis water (3×) and sedimented again. Finally, a sub-batch of 2 l of this product was crystallized (4°C, 200 rev min^{-1}) and the resulting 'slurry' (65% DM) was freeze-dried after determining some conditions important for freeze-drying, that is, the eutectic point (−30°C), the max drying point (25°C) and the minimum pressure (0·07 mbar).

Rhamnolipids investigated in this study

Mixed congener JBR425 Rhamnolipid (MR) (provided by Jeneil Biotech Inc., Saukville, WI), was prepared by incubating the readily solubilized rhamnolipids for 24 h at 100°C to ensure the evaporation of any residual solvents and to allow preparation of stock solutions with greater accuracy.

Separation of rhamnolipid congeners

The separation of monorhamnolipid and dirhamnolipid congeners of the mixed rhamnolipids was carried out through phase flash chromatography, as detailed by Rudden *et al.* (2015), using a Varian IntelliFlash 310 Flash Chromatography (Analogix Semiconductor Inc., Santa Clara, CA) system equipped with an Agilent SuperFlash™ SF40–240 g normal phase silica column (40·6 mm × 37·9 cm, 50 μm). A flow rate of 60 ml min^{-1} was set constant throughout the procedure. The mixed rhamnolipids, dissolved in chloroform, were injected into a hexane conditioned column, and followed by a washing step with hexane and chloroform to remove lipid material until no colour was detected in the washing solvents. A solvent mixture of chloroform and methanol of (97 : 3) ratio was used to elute monorhamnolipids followed by (50 : 50) solvent ratio for dirhamnolipid elution. Efficiency of the separation was confirmed by UPLC-MS.

Biosurfactant chemical analysis

Rhamnolipids under study were quantified by UPLC-MS. Analytical detection was carried out using a UPLC Waters Acquity H-Class chromatograph (Waters Corporation, Milford, MA), coupled to a mass spectrometer (Waters Xevo-TG-S, Oxford, UK). A Waters UPLC BEH C-18 column was used where the mobile phase consisted of 70% (v/v) water and 30% (v/v) acetonitrile. The flow rate of the mobile phase was adjusted to 0·5 ml min^{-1} and the sample injection volume was 3·0 μl (Moya-Ramírez *et al.* 2015). LSLs were analysed as previously described by Smyth *et al.* (2010) using HPLC connected to an LCQ quadrupole ion-trap mass spectrometry and a Gemini C18 column (250 mm × 4·6 mm × 5 μm) (Phenomenex, Cheshire, UK) for compound elution. The mobile phase consisted of 30% acetonitrile and 70% water. The injection volume was set at 20 μl with a flow rate of 0·5 ml min^{-1}. All mass spectrometry analyses were carried out using negative mode.

Determination of critical micelle concentration

Surface tension (ST) and critical micelle concentration (CMC) of the biosurfactants were determined using a KRUSS KI0T Tensiometer (Hamburg, Germany) with a platinum ring. To determine the CMC, a series of concentrations of rhamnolipids or sophorolipids diluted in distilled water, were prepared and their ST measured. ST was then plotted against concentration, where the CMC was determined from the extrapolated intercept of the two sections of the graph as described by Fracchia *et al.* (2010).

Antimicrobial activity of biosurfactants

Antimicrobial activities of the biosurfactants used were quantified by determining minimum inhibitory

concentration (MIC) using a method based on Clinical and Laboratory Standards Institute (CLSI) guidelines and described by Elshikh *et al.* (2016b). Biosurfactant MIC was assessed using Muller Hinton medium. LSLs and monorhamnolipids, were dissolved in <0·1% DMSO. Where DMSO was used, an untreated control was incubated with a similar concentration. Experimentally, two-fold serial dilutions were carried out by transferring 50 μl across 96-well plate from vertical rows 1–10 (3·12–0·006 mg ml^{-1}) for dirhamnolipids and MR and (1·56–0·003 mg ml^{-1}) for the monorhamnolipid fraction and LSL. A 50 μl suspension of the selected bacterial strain with an adjusted optical density (OD) was added to columns 1–10 to give a final concentration of *c.* 5×10^5 CFU per ml. Column 11 contained broth only and column 12 contained untreated bacteria. Resazurin (0·015%) was prepared and added to wells as described in the method and MIC was determined according to the colour status of the dye. To elucidate the mode action of biosurfactants (bacteriostatic or biocidal), 100 μl from wells showing no bacterial growth (MIC) was serially diluted and plated alongside the untreated bacteria to calculate the log reduction and percentage of inhibition.

Combination effect of biosurfactant-antibiotic/antimimicrobial on MIC

The use of LSLs and MR individually, in combination with antimicrobials (chlorhexidine and sodium lauryl sulphate), and in combination with antibiotics (tetracycline HCl and ciprofloxacin) has been investigated as described earlier (Elshikh *et al.* 2016b). In all cases, plates were prepared to contain final concentration of 125–0·03 μg ml^{-1} of the standard compound +50 μg ml^{-1} per well of biosurfactant in columns 1–10. In addition, OD adjusted bacteria were prepared to a final density of *c.* 5×10^5 CFU per ml (final well content was 100 μl).

Biofilm studies

Microorganisms under investigation were first assessed for their ability to form biofilm, using the O'Toole protocol (O'Toole 2011) (data not shown). Assessment included growing bacteria in BHI with and without supplementation of 1% sucrose for 24 and 48 h, incubated at 37°C in a 5% CO_2 atmosphere. Biofilm growth conditions that led to the most robust biofilm were utilized to test the efficacy of MR and LSLs, against microorganisms associated with oral biofilm formation. Efficacy of biosurfactants to interfere with oral biofilms was investigated from different perspectives: (i) Co-incubation experiment to assess prevention of biofilm formation. (ii) Disruption of existing biofilm. (iii) Pre-coating experiment to assess prevention of biofilm formation on a coated surface.

Preparation of overnight culture

Freshly streaked bacteria on BHI agar plates were incubated overnight at 37°C in 5% CO_2 atmosphere. A loopful of colonies was used to inoculate 10 ml of fresh BHI medium, which was incubated overnight at 37°C in 5% CO_2 atmosphere. On the day of experiment, the latter was diluted with fresh BHI culture to an OD at 600 nm equivalent to 10^8 CFU per ml. In all biofilm experiments 1 : 100 dilution of the fresh bacterial culture was prepared in BHI + 1% sucrose (optimal medium condition for biofilm growth).

Co-incubation assay

The assay was performed as described by Fracchia *et al.* (2010). Bacterial density was adjusted to 10^6 CFU per ml (as described earlier). In this experiment, a range of MR concentrations (0·1–0·8 mg ml^{-1}) and LSLs (0·05–0·6 mg ml^{-1}), were added to an adjusted OD culture, then 200 μl per well of this mixture was used. Plates were incubated for 48 h at 37°C in 5% CO_2 atmosphere. Plates were then processed and biofilms were analysed using crystal violet as detailed by O'Toole (2011).

Anti-adhesion assay

Assay performed according to Elshikh *et al.* (2017), with different concentrations (200 μl) of MR (0·8–12·5 mg ml^{-1}) and LSLs (0·025–0·4 mg ml^{-1}) added to six wells per concentration per replicate (except columns 1 and 2). Plates containing MR were incubated at 40°C overnight. Plates containing LSLs were also incubated overnight, but at room temperature, to avoid the irreversible bonding of LSLs with the plate surface. Upon completion of the incubation period, the plates were washed with PBS and sterilized under UV light. In all, 200 μl of bacterial suspension adjusted to 10^6 CFU per ml was added to coated wells and further incubated for 48 h and subsequently processed in accordance with the protocol of O'Toole (2011).

Biofilm disruption assay

This assay was carried out based on the method of Janek *et al.* (2012) with some modification. Biofilm was grown in 96-well plates, six wells per replicate, for 12 h followed by careful removal of medium (BHI + 1% sucrose), which was replaced with MR concentration range (0·1–1 mg ml^{-1})

and LSLs concentration range (0·1–1·0 mg ml^{-1}), both prepared in the same medium. The immature treated biofilm was further incubated for 36 h at 37°C in 5% CO_2 atmosphere. Plates were processed and analysed using the crystal violet method as described above.

The following equation was used to calculate the percentage of biofilm formation for all anti-biofilm studies:

$$\%\,\text{Biofilm formation} = [(OD_T/OD_C)] \times 100$$

where OD_T is the optical density of the rhamnolipid-treated biofilm and the OD_C the optical density of the untreated biofilm.

Bisbenzimide accumulation assay

The permeabilization effect of treating bacteria with biosurfactants was measured according to the method of Coldham *et al.* (2010) using a bacterial culture adjusted to an OD equivalent to 10^6 CFU per ml. Cells were co-inoculated with different concentrations of MR (0·5–5–50 mg ml^{-1}) and LSLs (0·25–25–25 mg ml^{-1}). A volume of 180 μl per biosurfactant-treated bacterium was placed in 96-well plates and was further incubated for 4 h then examined by adding 20 μl of H33342 bisbenzimide to a final concentration of 2·5 μmol l^{-1}. Fluorescence was read on a FLUOstar plate reader from the top of the wells using excitation and emission filters of 355 and 460 nm.

Imaging

The effect of biosurfactant treatment on oral pathogens was visualized using the Live/Dead™ BacLight staining kit. Biofilms were grown on sterile glass coverslips using BHI+1% sucrose as a supplement. Once the incubation period (48 h at 37°C and 5% CO_2) was complete, biofilms were washed with PBS buffer, incubated for a further 30 min with 12·5 and 25 mg ml^{-1} of LSLs and MR respectively. The biofilms were viewed using a fluorescence microscope at 40× magnification to assess the extent of the disruption of the biofilm and the status of the bacteria.

Statistical analysis

All data in this study were analysed using Graphpad Prism ver. 5.0 (GraphPad Software, La Jolla, CA). Experiments were performed at least in duplicate. Data are presented as mean ± SEM. One-way ANOVA analysis of variance with Bonferroni post-test. Significance of variances is indicated as follows: ns (non-significant), $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$.

Results

Biosurfactant production, acquisition and characterization

Analysis of the mixed rhamnolipids and LSLs are presented in Tables 1 and 2 respectively. Relative abundance analysis of MR showed a 1 : 1 ratio of monorhamnolipid to dirhamnolipid congeners, where **Rh-C_{10}-C_{10}** represents the highest percentage (38·74%, MW 504·3 g mol^{-1}) of monorhamnolipid and **Rh-Rh-C_{10}-C_{10}** is the most abundant (31·52%, MW 650·3 g mol^{-1}) of the dirhamnolipid congeners. Analysis of the purified monorhamnolipid fraction showed that it comprised over 99% of monorhamnolipid congeners, with the highest relative abundance of 68·87% for **Rh-C_{10}-C_{10}** (MW 504·3 g mol^{-1}). The dirhamnolipid purified fraction had an overall dirhamnolipid congener content of >98% where **Rh-Rh-C_{10}-C_{10}** (MW 650·3 g mol^{-1}) was the most abundant dirhamnolipid congener; 65·22% of the total sample. LSL analysis showed an overall content of lactonic sophorolipid >99%, mainly consisting of 91·73% diacetyl **C18:1** (MW 688·4 g mol^{-1}), 5·75% diacetyl **C18:2** (MW 686·3 g mol^{-1}) and a smaller percentage (2·5%) of monodiacetyl **C18:1** (MW 646·4 g mol^{-1}).

CMCs values for Mono-RL, Di-RL, MR, and LSLs were 32, 31, 48 and 22 μg ml^{-1}, respectively.

Biosurfactant efficacy, alone and in combination with antimicrobials

Biosurfactants were tested for antimicrobial efficacy against some oral pathogens as described earlier (findings

Table 1 HPLC chemical analysis of rhamnolipids investigated in terms of relative abundance of congeners based on peak intensities. Showing highest concentration in bold

Congeners	*m/z*	Mono-RL%	Di-RL%	MR%
Rh-C_8-C_{10}	476·3	10·550	0·050	5·105
Rh-C_{10}-C_{10}	504·3	**68·870**	1·230	**38·749**
Rh-C_{10}-$C_{12:1}$	530·3	**19·960**	0·078	6·047
Rh-Rha-C_8-C_{10}	622·3	0·033	9·520	4·542
Rh-Rh-C_{10}-C_{10}	650·3	0·475	**65·220**	**31·522**
Rh-Rh-C_{10}-$C_{12:1}$	676·3	0·034	6·930	4·064
Rh-Rh-C_{10}-C_{12}	678·3	0·069	**16·950**	9·971

Table 2 HPLC chemical analysis of Lactonic Sophorolipids investigated in terms of relative abundance of congeners based on peak intensities. Showing highest concentration in bold

LSLs Congeners	*m/z*	LSLs %
C18:1, 2Ac	688·4	**91·734**
C18:2, 2Ac	686·3	5·755
C18:1, 1Ac	646·3	2·510

summarized in Table 3). It is noticeable that the rhamnolipid mixture (MR) is more effective than the purified monorhamnolipid and dirhamnolipid fractions against all tested microorganisms. It is also interesting to note that dirhamnolipids demonstrated a greater inhibitory effect compared with the monorhamnolipid fraction. Based on the MIC results; MR was used for further investigations, as the separate monorhamnolipid and dirhamnolipid did not show a lower MIC compared to the mixture. Lactonic SLs, on the other hand, demonstrated a similar or better effect than that shown by MR.

To quantify the inhibitory action of biosurfactants (MR and LSLs), wells demonstrating no growth were serially diluted and plated alongside untreated control, allowing measurement of log reduction and % inhibition (Table 4). The results show LSLs have a better killing effect as most log reductions ranged between 4 and 5 as compared with 2–4 log reductions, resulting from treating bacteria with MR. We further investigated the combination effect of biosurfactants (MR–LSLs) with standard antimicrobials and antibiotics agents, namely chlorhexidine, sodium lauryl sulphate, tetracycline HCl and ciprofloxacin, on their inhibitory ability (Table 5). The results clearly demonstrated that the presence of a small concentration of biosurfactant decreased the MIC for antibiotics and standard antimicrobials significantly compared to using standard antimicrobial agents alone.

Biofilm prevention assessment

The ability of biosurfactants to prevent biofilm formation by microorganisms was assessed in two ways: (i) simultaneous incubation of biosurfactants and oral pathogens. (ii) assessing the ability of surface coating (antiadhesion in 96-well plates) with biosurfactants to prevent formation of biofilms. In the case of co-incubation assessment (Fig. 2a, b), using as little as 0·2 mg ml^{-1} MR resulted in more than 80–90% growth inhibition for all microorganism tested except for around 60% of *S. mutans*. Using 0·2 mg ml^{-1} of LSL caused 90% biofilm inhibition against all the organisms used. The results of the second assessment methods (Fig. 3a,b) show that using 3·12 mg ml^{-1} MR resulted in 80–90% of biofilm growth prevention for *S. oralis, S. sanguinis, N. mucosa* and *A. naeslundii*, and to a lesser extent (70%) growth inhibition in the case of *S. mutans*. However, in the pre-coating experiment using 0·2 mg ml^{-1} Lactonic SL prevented more than 80% biofilm formation of all microorganisms investigated, except *S. mutans* which required a higher concentration of 0·4 mg ml^{-1} to achieve 40% growth inhibition.

Table 4 Log reduction and inhibition percentage of oral pathogens after treatment with biosurfactants (compared to untreated controls)

Strains	Log reduction of MR (CFU per ml) (% Inhibition)	Log reduction LSLs (CFU per ml) (% Inhibition)
Streptococcus mutans	2·66 ± 0·001 (99·89)	5·53 ± 0·012 (99·99)
Streptococcus oralis	4·17 ± 0·055 (99·99)	4·34 ± 0·007 (99·99)
Streptococcus sanguinis	3·99 ± 0·041 (99·99)	4·07 ± 0·198 (99·99)
Actinomyces naeslundii	3·20 ± 0·102 (99·99)	5·06 ± 0·318 (99·99)
Neisseria mucosa	3·69 ± 0·137 (99·99)	4·80 ± 0·183 (99·99)

Biofilm disruption assessment

Further to the two aspects mentioned earlier, the ability of biosurfactants to disrupt an immature biofilm (12 h) was investigated. Results (Fig. 4a,b) demonstrated excellent potency of rhamnolipids and LSLs to restrict developing biosurfactant. Using MR, 0·2 mg ml^{-1} eliminated preformed biofilms of *S. oralis, S. sanguinis* and *A. naeslundii,* while *N. mucosa* and *S.mutans* biofilms were inhibited by 75 and 25%, respectively, when 0·4 and 1·0 mg ml^{-1} were used, compared to the untreated control. When developing biofilms of *S. oralis, S. sanguinis, N. mucosa* and *A. naeslundii* were treated with 0·2 mg ml^{-1} LSLs, 90% inhibition was achieved while it required 1·0 mg ml^{-1} to reduce the biofilm of *S. mutans* by 60%.

Effect of biosurfactants on permeability

The bisbenzimide fluorescent probe was used to measure cellular accumulation in treated bacteria. With increasing

Table 3 Minimum inhibitory concentration (MIC) and minimum biocidal concentration (MBC) (mg ml^{-1}) of oral pathogens treated with biosurfactants

Strains	MR		Mono-RL		Di-RL		LSLs	
Concentration (mg ml^{-1})	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Streptococcus mutans	390	780	780	1560	390	780	195	390
Streptococcus oralis	97·5	195	780	1560	780	1560	97·5	195
Streptococcus *sanguinis*	97·5	195	780	1560	390	780	195	390
Actinomyces naeslundii	195	390	1560	1560	390	780	97·5	390
Neisseria mucosa	390	390	780	1560	390	780	195	780

Table 5 Minimum inhibitory concentration (MIC) of standard antimicrobial compounds alone and in combination with biosurfactants

Antimicrobial/antibiotic (μg ml^{-1})	*Streptococcus mutans*	*Streptococcus oralis*	*Streptococcus sanguinis*	*Neisseria mucosa*	*Actinomyces naeslundii*
Tetracycline HCl	0·24	0·24	0·24	0·24	0·24
MR + Tetracycline HCl	0·12	0·03	0·03	0·03	0·03
LSLs + Tetracycline HCl	0·03	0·03	0·03	0·03	0·03
Ciprofloxacin	3·9	3·9	3·9	3·9	3·9
MR + Ciprofloxacin	0·97	0·03	0·03	0·03	0·03
LSLs + Ciprofloxacin	0·03	0·03	0·03	0·03	0·03
Chlorhexidine	15·6	15·6	31·2	15·6	15·6
MR + Chlorhexidine	1·9	0·03	0·03	0·03	0·03
LSLs + Chlorhexidine	0·03	0·03	0·03	0·03	0·03
Sodium lauryl sulphate (SLS)	62·5	62·5	62·5	62·5	62·5
MR + SLS	15·6	0·03	0·03	0·03	0·03
LSLs + SLS	0·03	0·03	0·03	1·9	0·03

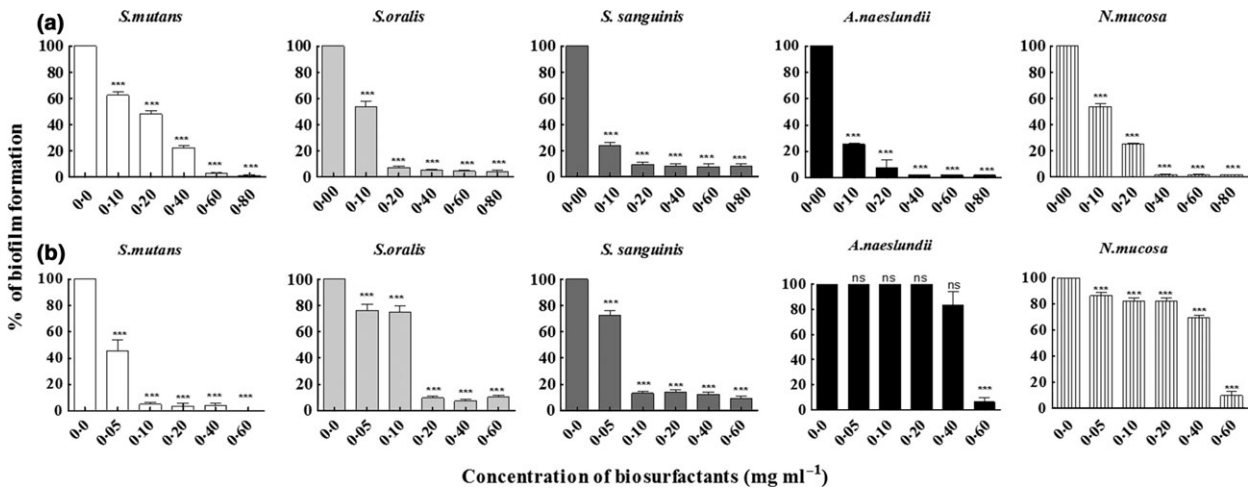

Figure 2 Biofilm formation percentage of different bacteria (namely *Streptococcus mutans, Streptococcus oralis, Streptococcus sanguinis, Neisseria mucosa* and *Actinomyces naeslundii*) treated in co-incubation experiment with different biosurfactant concentrations (a: MR; b: LSLs). Results are of duplicate experiments presented as mean values with error bars ± SD (n = 12). Significance of variances is indicated as follows: ns (Non-significant), *P<0·05, **P<0·01, ***P<0·001).

concentrations of rhamnolipids mixture (0·5–5–50) mg ml^{-1} and Lactonic SL (0·25–2·5–25) mg ml^{-1}, a direct relationship was seen with increasing concentrations of dye in the bacterial cells (Fig. 5).

Discussion

Chemical analysis of MR (Table 1) agrees with analysis by Wang *et al.* (2007), a good indicator of the consistency of rhamnolipid product composition. Chemical analysis of purified rhamnolipid fractions (mono- and di-) confirms the efficiency of the method to produce products with >98% purity. The CMC for MR (48 μg ml^{-1}) shows close agreement with that reported previously of 30 μg ml^{-1} (Roshtkhari and Mulligan 2016). In the case of LSLs (L-C18:1 diacetylated), a value of 31 μg ml^{-1} (Ribeiro *et al.* 2015) can be compared to our reported value of 22 μg ml^{-1}. Variation in CMC and also methodology values can be attributed to sample impurities as well as sample composition (Kłosowska-Chomiczewska *et al.* 2017).

Antimicrobial capacity of biosurfactants was assessed by a microdilution method enhanced with resazurin. This proved especially advantageous in comparison with the standard microdilution method, as LSLs and monorhamnolipids have a tendency to precipitate after a period of incubation. Applying resazurin dye in this assay is equally beneficial in the case of a mixture of rhamnolipids, due to the dark colour of the solution. Antimicrobial activity results (Table 3) suggest that MR works with increased efficacy against the microorganisms studied, when compared with the purified congeners. Discussing antimicrobial activity findings can be challenging for several

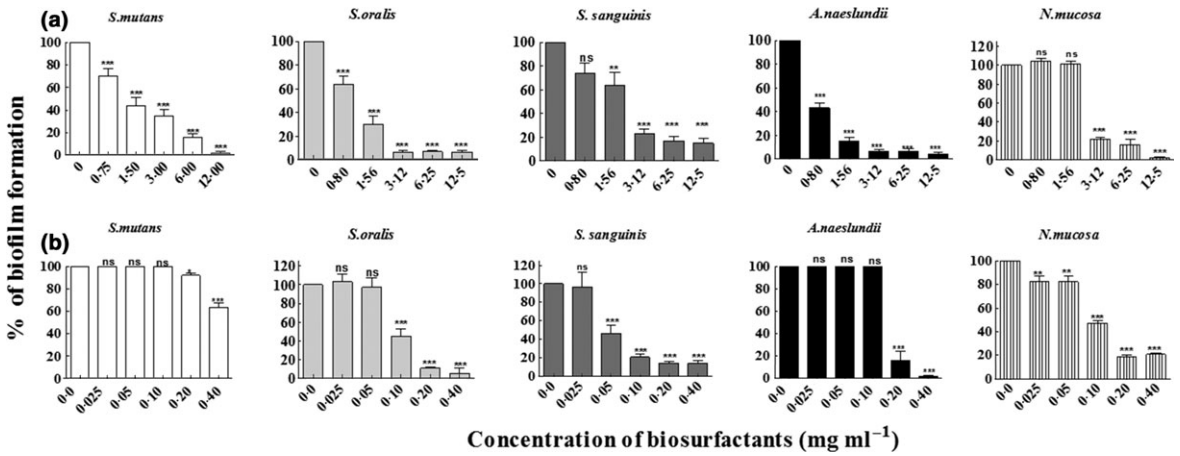

Figure 3 Biofilm formation percentage of different bacteria (namely *Streptococcus mutans, Streptococcus oralis, Streptococcus sanguinis, Neisseria mucosa* and *Actinomyces naeslundii*) grown on treated surface (96-well plate) with different biosurfactant concentrations (a: MR; b: LSLs). Results are of duplicate experiments presented as mean values with error bars ± SD (n = 12). Significance of variances is indicated as follows: ns (Non-significant), *P<0·05, **P<0·01, ***P<0·001).

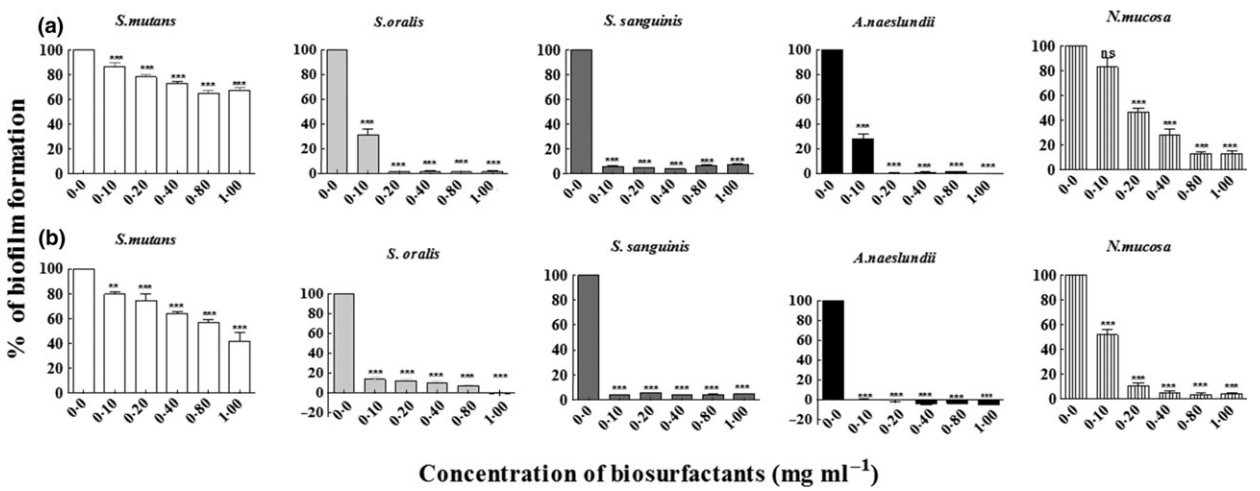

Figure 4 Immature biofilm (12 h) formation percentage of different bacteria (namely *Streptococcus mutans, Streptococcus oralis, Streptococcus sanguinis, Neisseria mucosa* and *Actinomyces naeslundii*) treated with different biosurfactants concentrations (a: MR; b: LSLs). Results are of duplicate experiments presented as mean values with error bars ±SD (n = 12). Significance of variances is indicated as follows: ns (Non-significant), *P<0·05, **P<0·01, ***P<0·001).

reasons such as the variation amongst researchers in assessment method (i.e. microdilution, disc diffusion, etc.) (Elshikh *et al.* 2016b) and variation in rhamnolipid or sophorolipid composition (directly related to their structural activity). It has recently been reported that the majority of biosurfactants investigated in relation to antimicrobial activities are lipopeptides and lipoproteins (Elshikh *et al.* 2016a). An example of this are lipoprotein from *Lactobacillus paracasei* A20, which had shown good antimicrobial and antiadhesive effect against some oral-related opportunistic microorganisms at concentrations around ≥25 mg ml^{-1}, a far higher concentration than was demonstrated in this study, (Gudiña *et al.* 2010). In an effort to make relevant comparisons, if we compare reported MIC values of Caprylic acid (C8:0) and Capric acid (C10:0) (most relevant fatty acid chain lengths to the rhamnolipids being studied), against *Staphylococcus aureus*, *Staphylococcus epidermidis* and other oral pathogens (Batovska *et al.* 2009), these were mostly 500 μg ml^{-1} or higher, indicating the role the sugar moiety of biosurfactants may play in the penetration of the bacterial cell membrane.

Mixed rhamnolipids JBR425 have occasionally been studied for their antibacterial properties. Díaz De Rienzo

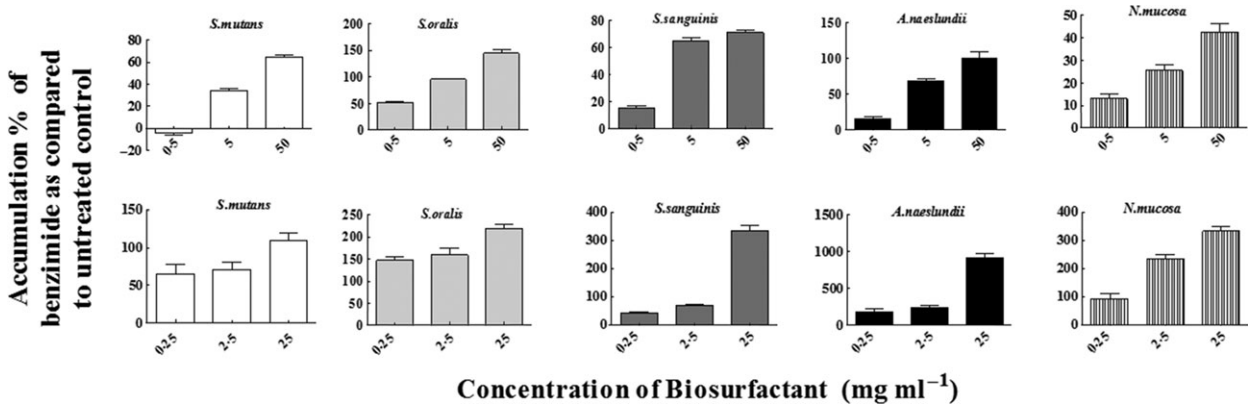

Figure 5 Effect of different MR: (a) concentrations (0·5–5–50 mg ml^{-1}) and different LSLs (b) concentrations (0·25–2·5–25 mg ml^{-1}) on the permeabilization of investigated oral pathogens (namely *Streptococcus mutans*, *Streptococcus oralis, Streptococcus sanguinis, Neisseria mucosa* and *Actinomyces naeslundii*), presented as a percentage of accumulation of fluorescence benzimide dye as compared to untreated cells. Mean values with error bars ±SD (n = 12).

et al. (2016a) reported potent activity for the combination of 0·4 g l^{-1} and caprylic acid at 0·1 g $l^{-1,}$ against *P. aeruginosa* ATCC 15442 biofilms where inhibition was attributed to disruption of bacterial cell–cell interactions and bacterial cell–substratum interactions. These results are also in agreement with previous findings (Davey *et al.* 2003). Other studies have also implicated rhamnolipids with antimicrobial and antibiofilm inhibitions (Das *et al.* 2014; Díaz De Rienzo and Martin 2016b). Regarding LSLs, a recent review on the application of biosurfactants for oral health applications (Elshikh *et al.* 2016a), concluded that these biomolecules had not been investigated thoroughly. Based on a robust method to determine MIC and a well-characterized LSL product, our MIC values were much lower than those reported before for antimicrobial activity. It remains difficult to make accurate comparison as the types of studied bacteria and the sophorolipids reported previously are different to those presented in this study.

Ashby *et al.* (2011) assessed antimicrobial activity of sophorolipids (98% latonic SL mixture composed of C18:1 and C18:0) against *Acne vulgaris*, to demonstrate inhibitory action at 2·4 mg ml^{-1}. Kapjung *et al.* (2002) assessed mixed sophorolipid samples (mixture of acidic and lactonic) obtained using different sugars as carbon source, reported to have an antibacterial effect (expressed as MIC value) on *Bacillus subtilis* KCTC1028, *Propionibacterium acne* ATCC 6919 and *S. mutans* ATTC 35668 of 4, 1 and 0·5 μg ml^{-1} respectively. Recent research (Díaz De Rienzo *et al.* 2015) has shown the effect of a mixed acidic-lactonic sample on Gram-negative *Cupriavidus necator* ATCC 17699 and Gram-positive *Bacillus subtilis* BBK006, both inhibited at concentrations of 50 mg ml^{-1}.

The bactericidal or bacteriostatic nature of an antimicrobial agent (in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines) defines a bactericidal substance in terms of its ability to cause 3 log reductions as compared with untreated bacteria (after 24 h of contact) (Barry *et al.* 1999). The log reduction data (Table 4) clearly demonstrate the biocidal nature of LSLs in achieving log reductions in the range of 4–5, while the rhamnolipid mode of inhibition depended on the type of microorganism and produced a log reduction of 2–4.

The combination of biosurfactant at sub-inhibitory concentrations of 50 μg ml^{-1} (above CMC values of both surfactants) with different concentrations of the standard antimicrobial agents, noticeably contributed to achieving very low MIC values, compared with use of antimicrobial agents alone (Table 5). This observation can be attributed (in addition to the antimicrobial efficacy of the biosurfactants), to the stabilization of the standard antibacterial agent (Gaysinsky *et al.* 2005) and increasing bioavailability to the microorganism (Kasturi and Prabhune 2013).

Biofilm formation conditions were optimal with BHI + 1% sucrose and 48 h incubation in 5% CO_2 atmosphere (data not shown). The sucrose presence compared with other sugars is thought to aid development of an extracellular matrix and lectin binding (Yang *et al.* 2006). In the co-incubation experiment, a noticeable inhibition effect occurred at even sub-MIC levels (Fig. 2a, b). The need for a slightly higher concentration of LSLs to achieve inhibition against *N. mucosa* and *A. naeslundii* biofilms is also clear, which can be understood in the context of the reduced susceptibility of some bacterial cells to antimicrobial agents when they are present in a biofilm state due to the shielding effect offered by the biofilm matrix (Frank *et al.* 2007), another factor which

can account for the increase in the effective dosage of LSLs against certain types of biofilms is their reduced solubility in aqueous media which can make them less available.

The antiadhesion assay was conducted as prescribed by previous authors, however, based on some preliminary experiments, it was noticed that temperature and contact time required for coating are important to enhance the antiadhesion properties of the biosurfactant; therefore, the method was modified as detailed earlier. Researchers have related the antiadhesive property of biosurfactants to surface energy alteration, as demonstrated when a surface coated with rhamnolipid-like biosurfactants discouraged adhesion of *S. mutans* due to repulsive forces induced by the coating substance (van Hoogmoed *et al.* 2006). In support of this finding, Fracchia *et al.* (2010) noticed that lipoprotein-like biosurfactant (derived from *Lactobacillus* sp. CV8LAC) has effective antiadhesion properties at 2500 μg ml^{-1} against different *Candida albicans* strains. A similar observation was made when a biosurfactant crude extract produced by *Lactobacillus lactis 53* was tested against oral health-related pathogens such as *S. epidermidis*, *Streptococcus salivarius*, *S. aureus and Rothia dentocariosa.* In addition, however, our results suggest that the antimicrobial effect of biosurfactants may also play a role in antiadhesion, given their antimicrobial efficacy (Rodrigues *et al.* 2006).

Treatment of immature biofilm with biosurfactants has shown promising results, with most bacterial biofilms disrupted at concentrations as low as 200 μg ml^{-1}. This efficacy, beside the direct killing effect of biosurfactants on the bacteria, can be explained in terms of the ability of the biosurfactant to disrupt the biofilm matrix, as has been demonstrated (Díaz De Rienzo and Martin 2016b). This opens a new avenue for using biosurfactant in adjuvant therapy, to reduce dosage of antibiotics and to enhance efficacy (Kasturi and Prabhune 2013). Visualization of biosurfactant-treated biofilm in this study (Fig. 6) supports this suggestion as it can be noticed that short-term treatment has caused a colour shift to red/orange, an indication of increasing dysfunction or dead cells plus a change in the uniformity of biofilms, compared to those untreated. In support of the Live/Dead stain information, other data have been presented in Figs S1–S3, where Fig. S1 demonstrates quantifiable effect of investigated biosurfactants against biofilms grown on plastic

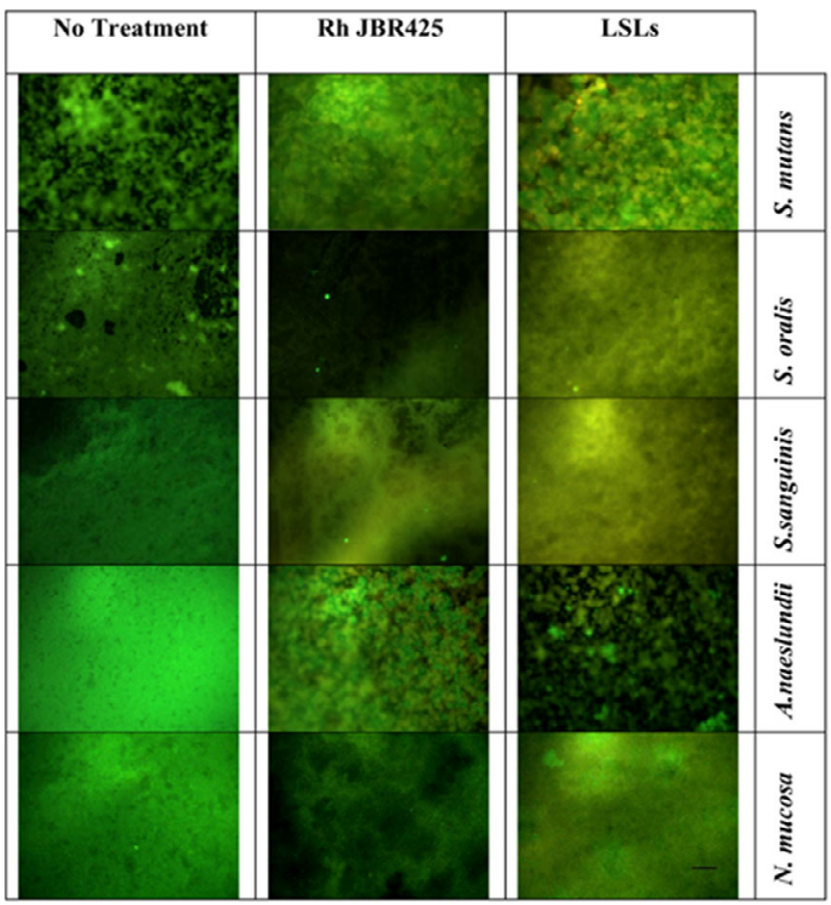

Figure 6 Biofilm of bacteria (namely, *Streptococcus mutans, Streptococcus oralis, Streptococcus sanguinis, Neisseria mucosa* and *Actinomyces naeslundii*) grown in BHI medium supplemented with 1% sucrose and formed on coverslips. First column—untreated control of microorganism. Second column—bacteria treated with 25 mg ml^{-1} MR and third column—bacteria treated with 12·5 mg ml^{-1} LSLs. Exposure time to treatment—30 min. Bacteria stained with Live/Dead™ to visualize bacterial viability using fluorescence microscope at 409 nm. Scalebar represents 20 μm for all images. [Colour figure can be viewed at wileyonlinelibrary.com]

discs where log reduction was observed at 30 min. The effects of this exposure on all our strains were also captured using scanning electron microscopy (SEM). To illustrate these effects, only two selected representative treatment one for the Gram-positive *S. oralis* and one for the Gram-negative *N. mucosa,* relative to untreated control were included (Figs S2 and S3, respectively). It is noticeable that morphological changes occurred due to biosurfactants treatment including cell membrane damages which allows the release of cellular material (as in the case of *S. oralis*) or reduction/removal of extra cellular polymeric substance density (as in the case of *N. mucosa*), which render cells more susceptible to antimicrobial agents. The latter effect can be also associated with signs of cell stress such as cells swelling.

Measuring the percentage accumulation of bisbenzimide dye in biosurfactant-treated bacteria supports the view that biosurfactants exert their antimicrobial effect, in part, due to the permeabilization effect on the bacterial membrane (Magalhães and Nitschke 2013).

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Conflict of Interest

None.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Log reduction quantification of biosurfactants-treated biofilm.

Figure S2. Scanning electron microscopy of biosurfactants-treated biofilms.

Figure S3. Scanning electron microscopy of biosurfactants-treated *Neisseria mucosa* biofilms.