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Research Letter

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Antibacterial properties of biosurfactants against selected Gram-positive and -negative bacteria

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One sentence summary: Biosurfactants are promising compounds for inhibition and/or disruption of biofilms formed by many bacterial cultures an ability that may be enhanced by the presence of short-chain organic acids.

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ABSTRACT

The antibacterial properties and ability to disrupt biofilms of biosurfactants (rhamnolipids, sophorolipids) and sodium dodecyl sulphate (SDS) in the presence and absence of selected organic acids were investigated. *Pseudomonas aeruginosa* PAO1 was inhibited by sophorolipids and SDS at concentrations >5% v/v, and the growth of *Escherichia coli* NCTC 10418 was also inhibited by sophorolipids and SDS at concentrations >5% and 0.1% v/v, respectively. *Bacillus subtilis* NCTC 10400 was inhibited by rhamnolipids, sophorolipids and SDS at concentrations >0.5% v/v of all three; the same effect was observed with *Staphylococcus aureus* ATCC 9144. The ability to attach to surfaces and biofilm formation of *P. aeruginosa* PAO1, *E. coli* NCTC 10418 and *B. subtilis* NCTC 10400 was inhibited by sophorolipids (1% v/v) in the presence of caprylic acid (0.8% v/v). In the case of *S. aureus* ATCC 9144, the best results were obtained using caprylic acid on its own. It was concluded that sophorolipids are promising compounds for the inhibition/disruption of biofilms formed by Gram-positive and Gram-negative microorganisms and this activity can be enhanced by the presence of booster compounds such as caprylic acid.

Keywords: biosurfactants; biofilms; rhamnolipids; sophorolipids; antimicrobial

INTRODUCTION

Biosurfactants are surface-active biomolecules with emulsifying activities. These molecules tend to accumulate at the interface between phases (liquid-liquid/air-liquid) that show different degrees of polarity and hydrogen bonding. These compounds have been used in a wide range of applications, including use in agriculture, the food industry and in industrial processes involving the flotation and leaching of minerals (Banat *et al.* 2010). The surface and interfacial tension reducing properties of surfactants produce excellent detergency and emulsifying, foaming and dispersing traits, making them some of the most versatile products for use in chemical processes (Greek 1991). The advantages of biosurfactants over their chemical counterparts are their specific action, low toxicity, high biodegradability, widespread applicability, their good performance at extreme temperatures, pH and salinity and additionally their unique structures that could show new properties and future applications.

Different kinds of biosurfactants have different properties and show a wide range of physiological functions depending

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on the microorganisms producing them. Significant among all these attributes is the solubilization of hydrophobic compounds, heavy metal binding, virulence factors, cell signalling (quorum sensing) and biofilm formation (Franzetti *et al.* 2011; Díaz De Rienzo *et al.* 2015a). Biosurfactants with antimicrobial activity against Gram-positive microorganisms include sophorolipids, produced by *Starmerella bombicola* (Banat, Díaz De Rienzo and Quinn 2014; Díaz De Rienzo *et al.* 2015a), against Gram-negative bacteria surfactin produced by *Bacillus subtilis* strains (Ahimou, Jacques and Deleu 2000) and rhamnolipids from *Pseudomonas aeruginosa* have been reported to have biofilm disruption abilities against *Bordetella bronchiseptica* (Irie, O'Toole and Yuk 2005). In recent years, the interest in the properties of rhamnolipids produced by *P. aeruginosa* has led to them becoming a target for production at commercial scale. The use of rhamnolipids is considered safe in a range of different industrial applications. The applications of rhamnolipids have been studied mainly in the fields of bioremediation (Banat *et al.* 2010) and metal removal (Díaz De Rienzo *et al.* 2015b), but information on their use as antimicrobial agents, and as disruptors of biofilm is still quite sketchy. Recently, organic acids like acetic acid, citric acid and lactic acid have been used as basic materials for controlling *Shigella* species, with a potential for use in the development of 'green' technologies for microbial load reduction (In *et al.* 2013). Caprylic acid has also been reported to have effective antimicrobial properties against microorganisms like *Staphylococcus aureus* and various species of *Streptococcus* (Nair *et al.* 2005).

The aim of this study was to determine the antibacterial properties of different kinds of biosurfactants in combination with selected organic acids against a group of pathogenic microorganisms and evaluate the effect of the surfactants on biofilm disruption of selected Gram-positive and -negative microorganisms.

MATERIALS AND METHODS

Microorganisms and culture conditions

Pseudomonas aeruginosa PAO1, *Escherichia coli* NCTC 10418, *B. subtilis* NCTC 10400 and *S. aureus* ATCC 9144 were maintained in Luria- Bertani (LB) broth plus 20% glycerol at −20°C. Bacterial growth from a nutrient agar slant incubated for 24 h at 37°C was used to obtain a bacterial suspension with an optical density (570 nm) adjusted to give 10^8 cfu mL^{-1} for each of the strains used. One milliliter of this suspension was inoculated into a 250 mL Erlenmeyer flask containing 50 mL of LB broth for antimicrobial assays. The inoculum was incubated for 24 h at 37°C on a rotary shaker at 180 rpm.

Rhamnolipid characteristics

A rhamnolipid containing solution of 10% (w/v) mixture of monorhamnolipids and dirhamnolipids (surface tension 28 mN m^{-1}, CMC: 20 mg L^{-1}) was obtained from Jeneil Biosurfactant Inc. (Saukville, Wisconsin).

Sophorolipid characteristics

The sophorolipid containing 50% hydrophile (Sophorose) plus 50% lipophile (fatty acid), surface tension: 38 mN m^{-1}, CMC: 40 mg L^{-1}, was obtained from MG Intobio Co Ltd (Incheon, South Korea).

Table 1. Concentrations and combinations of biosurfactants and adjuvants for determination of MCD.

Treatment	Concentration (v/v)
Rhamnolipids	1%
Sophorolipids	1%
Citric acid	0.8%
Caprylic acid	0.8%
Lactic acid	0.8%
Citric acid/rhamnolipids	0.8%/1%
Citric acid/sophorolipids	0.8%/1%
Caprylic acid/rhamnolipids	0.8%/1%
Caprylic acid/sophorolipids	0.8%/1%
Lactic acid/rhamnolipids	0.8%/1%
Lactic acid/sophorolipids	0.8%/1%

Determination of minimum inhibitory concentration of sophorolipids, rhamnolipids and sodium dodecyl sulfate

The antibacterial activity of sophorolipids, rhamnolipids and sodium dodecyl sulfate (SDS) against *P. aeruginosa* PAO1, *E. coli* NCTC 10418, *B. subtilis* NCTC 10400 and *S. aureus* ATCC 9144 was determined using the standard clear zone inhibition halo method. An overnight culture of each strain was diluted to 10^8 cfu mL^{-1} and LB plates were inoculated. Equidistant holes were punched in the solidified inoculated agar using a standard cork borer to cut wells. Sixty (60) microliters of each surfactant at different concentrations were added to each agar well. The treated agar plates were left at room temperature for 4 h and thereafter they were incubated at 37°C for 24 h. The antimicrobial activity of the biosurfactants was determined by measuring the inhibition zone diameter against each microorganism. Minimum inhibitory concentration (MIC) values were determined by measuring OD at 570 nm.

Determination of MIC of surfactants with added caprylic acid

The same methodology described above was followed; however, this time each individual surfactant system, at different concentrations, was used in the presence of an adjuvant compound (caprylic acid 0.8% v/v) and was compared to the caprylic acid with SDS (pH 3.5). MIC values were determined by measuring OD at 570 nm.

Determination of minimum concentration for disinfection of sophorolipids, rhamnolipids and SDS

The bacterial suspension was adjusted to 10^8 cfu mL^{-1} for each strain and 1 mL of this suspension was inoculated into a 250 mL Erlenmeyer flask containing 50 mL of LB broth and growth was measured at different time intervals (0, 60, 120 min) under different culture conditions (Table 1). Minimum concentration for disinfection (MCD) values were determined by measuring the OD at 570 nm.

To evaluate the effect of the biosurfactants/adjuvants during the exponential phase of growth, measurements were taken at 0, 60 and 120 min without treatment and then the cultures were treated with the biosurfactants/boosters for three more hours to evaluate disinfection *in vivo*.

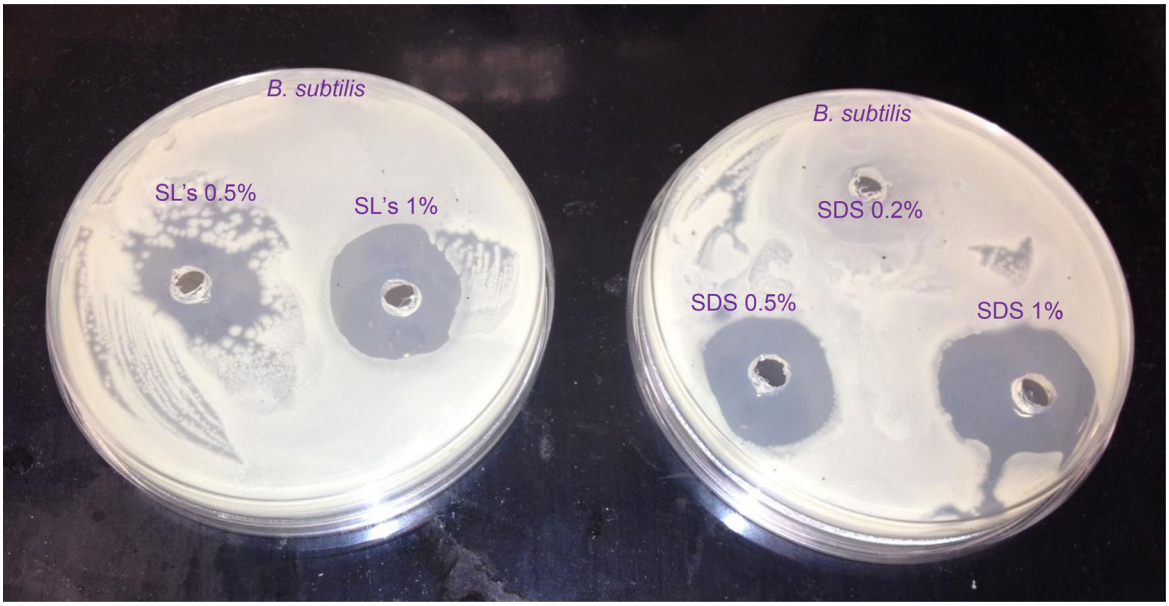

Figure 1. Antimicrobial activity of sophorolipids (0.5 and 1% v/v) and SDS (0.2, 0.5 and 1% v/v) against *B. subtilis* NCTC 10400.

Preparation of biofilms

Pseudomonas aeruginosa PAO1, *E. coli* NCTC 10418, *B. subtilis* NCTC 10400 and *S. aureus* ATCC 9144 were grown overnight and diluted 100-fold with LB broth following which 2 mL samples were dispensed in triplicate to fill the 12-well plates, biofilms were formed on sterile, glass coverslips (18 mm × 18 mm) which were put into the 12 well plates (vertically) and were incubated at 37°C for 24 h. Biofilms were stained with 1% w/v crystal violet for 10 min, washed with deionized water and the structure was observed using a phase contrast microscope with a 40× objective lens.

Anti-adhesive effect of biosurfactants against different microorganisms on glass surfaces

The anti-adhesive activity of biosurfactants against *P. aeruginosa* PAO1, *E. coli* NCTC 10418, *B. subtilis* NCTC 10400 and *S. aureus* ATCC 9144 was estimated in 12-well plates. The coverslip glasses were incubated in different concentrations of each (bio)/surfactant (0.2–5% v/v) overnight. The cells were allowed to attach to the glass coverslips pretreated for 24 h at 37°C as described above. Cells incubated in medium without surfactants were used as controls. After the incubation time, the medium was decanted and the coverslips were gently rinsed with phosphate-buffered saline pH 7 and stained with 1% w/v crystal violet for 10 min, and observed using a phase contrast microscope with a 40× objective.

Disruption of pre-formed biofilms by biosurfactants

Biofilms of *P. aeruginosa* PAO1, *E. coli* NCTC 10418, *B. subtilis* NCTC 10400 and *S. aureus* ATCC 9144 were allowed to form in 12-well plates as described earlier. After a period of 24 h, planktonic cells were removed and fresh medium (LB) was added containing different concentrations of biosurfactants (0.5–5%). The plates were incubated at 37°C for 24 h. The planktonic cells were discarded after the incubation time and the biofilms were stained using the crystal violet assay described earlier. Cells untreated with surfactants were controls for these experiments. The values were expressed in terms of percent biofilm formed in comparison with those untreated.

RESULTS AND DISCUSSION

Determination of MIC of sophorolipids, rhamnolipids and SDS

Surfactants both of biological and chemical origin are characterized by the formation of aggregate structures termed micelles, at their critical micelle concentration (CMC), and their foaming and detergent abilities (Chen *et al.* 2010a,b; 2011; Penfold *et al.* 2011). Biosurfactants have been reported as antimicrobial agents against *B. subtilis*, *S. epidermidis* and *Propionibacterium acnes* at low MIC levels (<1.6 mM) (Lang, Katsiwela and Wagner 1989). In this study, the growth of *P. aeruginosa* PAO1 on agar plates was inhibited by sophorolipids and SDS at concentrations >5% v/v, while the growth of *E. coli* NCTC 10418 was inhibited by sophorolipids and SDS at concentrations >5% v/v and 0.1%, respectively.

The effect on Gram-positive cells like *B. subtilis* NCTC 10400 and *S. aureus* ATCC 9144 was different. The growth of *B. subtilis* NCTC 10400 was inhibited by rhamnolipids, sophorolipids and SDS at lower concentrations >0.5% v/v for all three surfactants (Fig. 1); the same effect was observed for *S. aureus* ATCC 9144 (Fig. 2); all the MIC results are summarized in Table 2. These results showed resistance of *P. aeruginosa* PAO1 and *E. coli* NCTC 10418, cells towards rhamnolipids at concentrations higher than those reported earlier (Lang, Katsiwela and Wagner 1989; Dusane *et al.* 2010) and an inhibitory effect on Gram-positive microorganisms such as *B. subtilis* NCTC 10400 and *S. aureus* ATCC 9144. While, sophorolipids had an inhibitory effect on all tested bacterial strains (both Gram positive and negative).

Combinations of commercial/biosurfactants/adjuvants for microbial inhibition

Some organic acids exhibit antimicrobial properties against foodborne pathogens (Beauchat and Colden 1989). Citric acid,

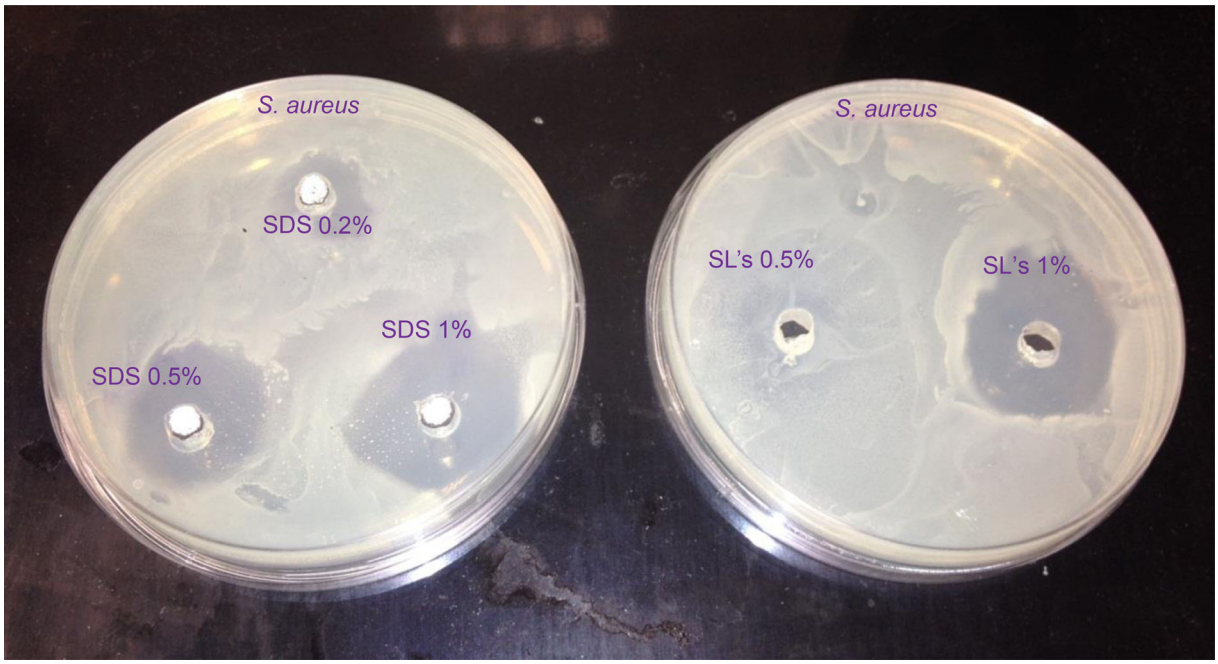

Figure 2. Antimicrobial activity of sophorolipids (0.5 and 1% v/v) and SDS (0.2, 0.5 and 1% v/v) against *S. aureus* ATCC 9144.

Table 2. MIC of surfactants towards cells of different microorganisms.

Microorganisms	MIC (% v/v) Rhamnolipds	Sophorolipids	SDS
Pseudomonas aeruginosa PAO1	–	1	1
Escherichia coli NCTC 10418	–	1	0.2
Bacillus subtilis NCTC 10400	0.5	0.5	0.2
Staphylococcus aureus ATCC 9144	0.5	1	0.2

(–) No inhibition.

acetic acid and caprylic acid are among the most organic acids categorized as 'safe for human use' (Nair *et al.* 2005; In *et al.* 2013). Caprylic acid is the common name for the eight-carbon saturated fatty acid also known as octanoic acid. Caprylic acid is used commercially in the production of esters used in perfumery and in the manufacture of dyes (Beare-Rogers, Dieffenbacher and Holm 2001). Because of its short chain length, it has no difficulty in penetrating the cell wall membranes, making it more effective against certain lipid-coated bacteria, such as *S. aureus* and various species of *Streptococcus* (Nair *et al.* 2005). In this study, citric acid, lactic acid and caprylic acid did not shown any effect on the grow of the selected microorganisms (data not shown). However, when caprylic acid (0.8% v/v) was combined with rhamnolipids (0.2% v/v) or SDS (0.2% v/v), the growth of *B. subtilis* NCTC 10400 and *S. aureus* ATCC 9144 were inhibited. In addition, caprylic acid (0.8% v/v) in the presence of sophorolipids at 1% v/v also inhibited the growth of *P. aeruginosa* PAO1, *E. coli* NCTC 10418, *B. subtilis* NCTC 10400 and *S. aureus* ATCC 9144 (Table 3). These results demonstrate that rhamnolipids are unable to inhibit growth of Gram-negative *P. aeruginosa* PAO1 and *E. coli* NCTC 10418 either on its own or in presence of any organic acids tested in this work. Rhamnolipids however (in presence or absence of caprylic acid) showed an inhibitory effect towards the Gram-positive bacteria tested in this work. The best treatment for inhibiting the growth of all the selected bacterial strains (as evaluated on agar plates) was to use sophorolipids in the presence of caprylic acid. Using these results as a starting point, the most effective treatments were tested in liquid medium culture and inhibition of growth evaluated at different times. The effect of adding the antibacterial agent to the culture medium at time zero as well as during exponential phase of growth was evaluated.

In this study, the results suggest that rhamnolipids and sophorolipids may have different mechanisms of action against microorganisms; rhamnolipids inhibit the growth in the exponential phase, which suggests that they may have an influence on cell division, while the antimicrobial effects of sophorolipids

Table 3. Effect of Caprylic acid (CA) on the growth of different microorganisms in the presence of surface active compounds.

Microorganisms	Rhamnolipids (0.2% v/v) + CA (0.8% v/v)	Sophorolipids (0.2% v/v) + CA (0.8% v/v)	SDS (0.2% v/v) + CA (0.8% v/v)
Pseudomonas aeruginosa PAO1	–	+[a]	–
Escherichia coli NCTC 10418	–	+[a]	–
Bacillus subtilis NCTC 10400	+	+	+
Staphylococcus aureus	+	+	+

[a]SL 1% v/v.
(+)Inhibition/(–) no inhibition.

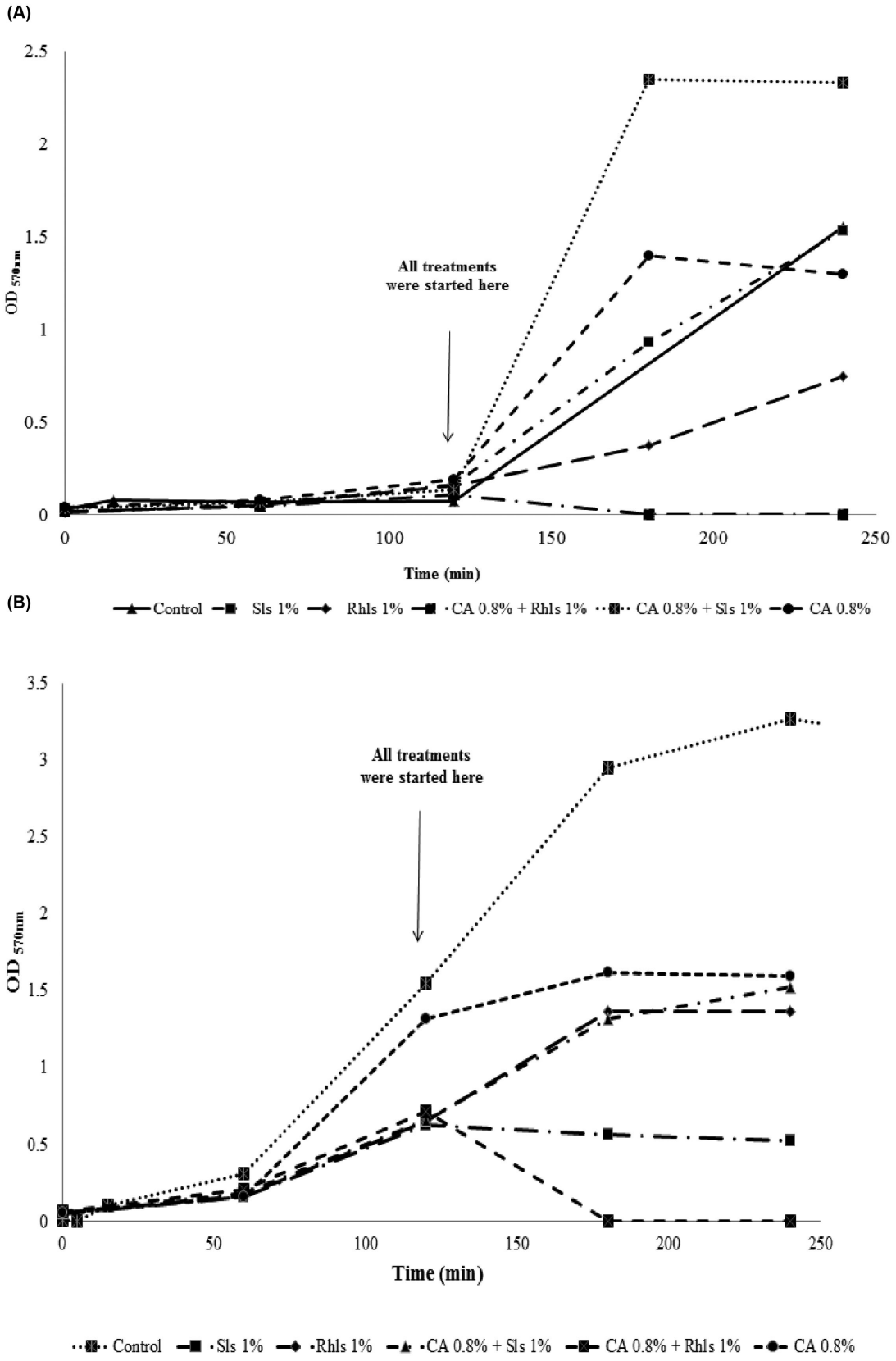

Figure 3. Growth of *S. aureus* ATCC 9144 (A) and *P. aeruginosa* PAO1 (B) in LB medium in presence of different surfactant and/or adjuvant.

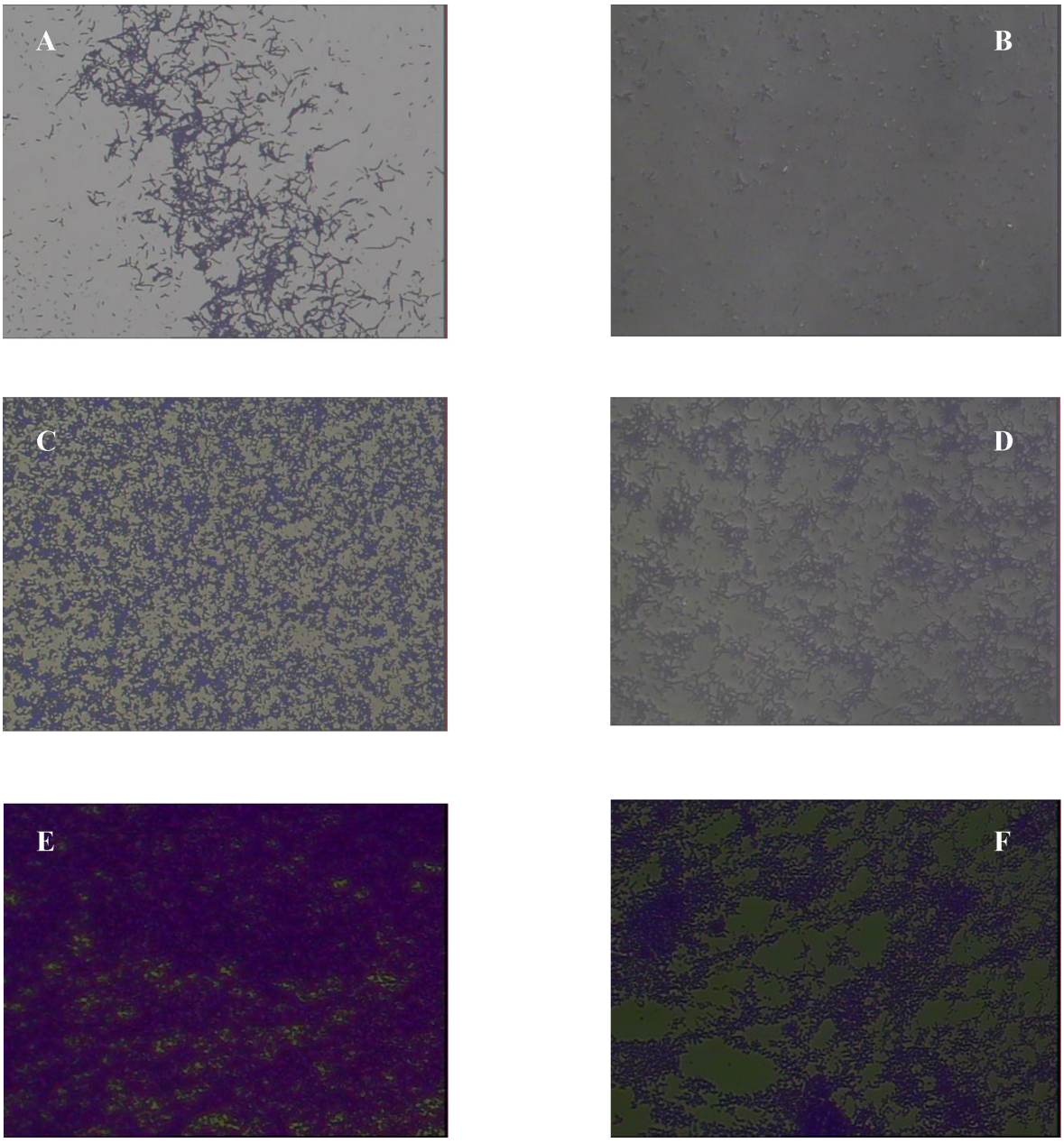

Figure 4. Representative images depicting the effect of biosurfactants on biofilms of Gram-positive and Gram-negative microorganisms. **(A, C, E)** Controls: *B. subtilis* NCTC 10400, *E. coli* NCTC 10418 and *P. aeruginosa* PAO1, respectively; **(B, D, F)** after treatment with sophorolipids (1% v/v) in presence of the caprylic acid (0.8% v/v) for the same organism respectively.

occur between the exponential and stationary phases (Fig. 3). These results predicate a possible use of sophorolipids in the presence of caprylic acid at pH5 as an antimicrobial agent, because they are comparable to conventional antimicrobials used in the agriculture and healthcare industries (Kim *et al.* 2002).

Anti-adhesive properties of biosurfactants/surfactants

Biosurfactants such as sophorolipids are also reported to have biofilm disruption abilities (Díaz De Rienzo *et al.* 2015a). Regardless of their potential, there are few studies on biosurfactants and their interaction with bacterial cells (Rodrigues *et al.* 2006a,b; Rivardo *et al.* 2009). In this study, we tested the anti-adhesion property of sophorolipids (1% and 5% v/v), rhamnolipids (1% and 5% v/v) and SDS (1% and 5% v/v) in the presence and absence of caprylic acid (0.8% v/v) against *P. aeruginosa* PAO1, *E. coli* NCTC 10418, *B. subtilis* NCTC 10400 and *S. aureus* ATCC 9144. The attachment of *P. aeruginosa* PAO1, *E. coli* NCTC 10418 and *B. subtilis* NCTC 10400 was inhibited by sophorolipids (1% v/v) in the presence of caprylic acid (0.8% v/v) (Fig. 4). In the case of *S. aureus* ATCC 9144, the best results were obtained using caprylic acid (0.8% v/v) on its own (data not shown).

Previous studies have shown an effect of a co-incubation of *P. aeruginosa* with 0.25 mM rhamnolipids in the prevention of adhesion and the following biofilm formation (Davey, Caiazza and O'Toole 2003). Dusane *et al.* (2010) have demonstrated about 80% inhibition of *B. pumilus* cell attachment to polystyrene surfaces observed after 1 h of treatment with low

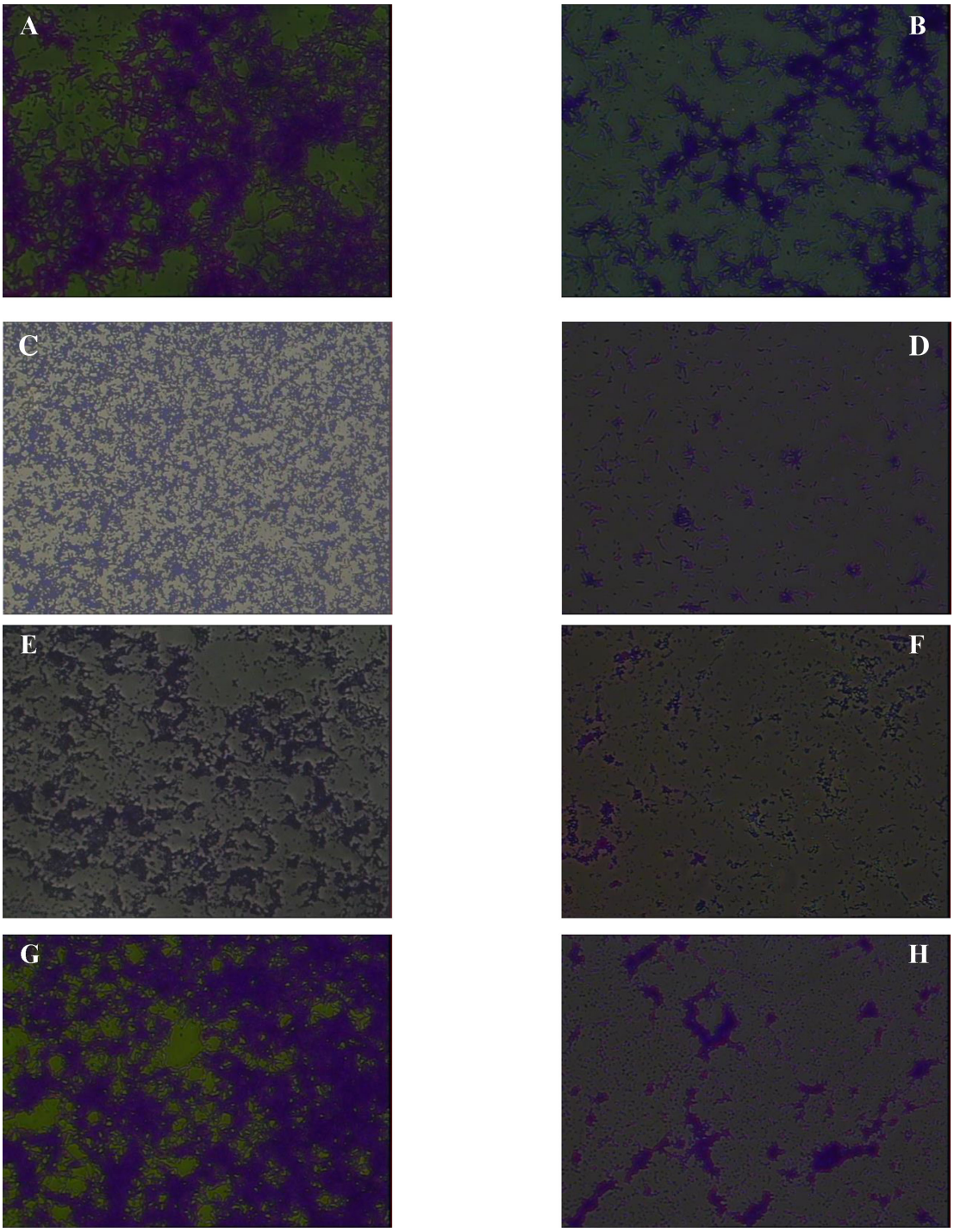

Figure 5. Representative images depicting the effect of biosurfactants on pre-formed biofilms of Gram-positive and Gram-negative microorganisms. (**A, C,E,G**) Controls: *B. subtilis* NCTC 10400, *E. coli* NCTC 10418, *P. aeruginosa* PAO1 and *S. aureus* ATCC 9144 (**B,D,F,H**) after treatment with sophorolipids (5% v/v).

concentrations of rhamnolipids. However, in this study the surface treatment with rhamnolipids did not stop the cells from growing on it. This may be due to rhamnolipids properties, which make them easily rinsed during the washing step. In comparison, the anti-adhesive effect of sophorolipids towards Gram-positive and Gram-negative microorganisms has not been previously reported, and is being highlighted in this work as a good antimicrobial for selected bacterial strains both on its own and in the presence of caprylic acid.

Disruption of biofilm formed by selected microorganisms

The deposition of microorganisms on solid surfaces and subsequent biofilm formation is a phenomenon that occurs naturally but is also part of the microorganism's strategy to protect itself from external toxic factors (Pereira *et al.* 2007). These biofilms are serious health hazards due to their resistance to antibiotics (Dusane *et al.* 2010). In this study, preformed biofilms of

P. aeruginosa PAO1, *E. coli* NCTC 10418, *B. subtilis* NCTC 10400 and *S. aureus* ATCC 9144 on glass coverslips were disrupted with sophorolipids (5%) in the absence of adjuvants (Fig. 5).

Earlier studies have also demonstrated that rhamnolipids (100 mM) can disrupt biofilms formed by *B. pumilus* (Dusane *et al.* 2010), surfactin produced by *B. subtilis* has also been shown to inhibit biofilm formation by *Salmonella enterica*, *E. coli* and *Proteus mirabilis* (Mireles, Toguchi and Harshey 2001). The results of this study indicate that sophorolipids have a great potential to be used individually or in combination with caprylic acid for the disruption of biofilms.

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Conflict of interest. None declared.

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