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Original Article

High reduction of staphylococcal biofilm by aqueous extract from marine sponge-isolated *Enterobacter* sp.

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ABSTRACT

Staphylococcus aureus and *Staphylococcus epidermidis* are among the most important bacterial species responsible for biofilm formation on indwelling medical devices, including orthopaedic implants. The increasing resistance to antimicrobials, partly attributed to the ability to form biofilms, is a challenge for the development of new antimicrobial agents. In this study, the cell-free supernatant obtained from sponge-associated *Enterobacter* strain 84.3 culture inhibited biofilm formation (>65%) and dissociated mature biofilm (>85%) formed by *S. aureus* and *S. epidermidis* strains. The culture supernatant was subjected to solvent partitioning and the aqueous extract presented a concentration-dependent antibiofilm activity for each strain with a minimum biofilm eradication concentration (MBEC) ranging from 16 to 256 μg/mL. The effect of the aqueous extract on mature *S. aureus* biofilm was analyzed by confocal scanning laser microscopy, showing a significant reduction of the biofilm layer as well as diminished interactions among the cells. This extract is not toxic for mammalian cells (L929 cell line). Studies targeting substances with antibiofilm activity gained significant attention in recent years due to difficult-to-treat biofilm infections. Here, sponge-associated *Enterobacter* 84.3 proved to be a source of substances capable of eradicating staphylococcal biofilm, with potential medical use in the future.
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1. Introduction

Staphylococci are currently the major agents of medical device-related infections [1,2]. In orthopaedic prosthetic infections, the most commonly Gram-positive cocci isolated are *Staphylococcus aureus* and coagulase-negative staphylococci (CNS) [3]. *S. aureus* strains causing implant infections show high rates of antimicrobial resistance, and there is an alarming increase of antimicrobial resistance observed in other species, such as *Staphylococcus epidermidis* [1,3,4]. Implant infection causing bacteria generally form biofilms, in which bacterial aggregates tightly adhere to the biomaterial surface and are responsible for the persistence of implant infections. Biofilm serves as a bacterial strategy to evade the host's immune system and is often a source of bacterial spread to other body sites. Biofilms show an inherent resistance to antimicrobials [5,6] and an increased rate of horizontal genetic transfer leading to the acquisition and spread of multidrug-resistance [1–3].

Today there are no therapies that effectively target staphylococcal biofilms, and none of the known modulators of virulence has been approved yet for clinical use [6]. This indicates the need for new antibiofilm molecules. Marine sponge-associated bacteria represent a still underexploited source of biodiversity able to synthesize a broad range of bioactive substances, including antibiofilm agents with good performance against clinical isolates [7–10].

In a previous study, we built a collection of culturable marine sponge-associated bacteria isolated from samples of the marine sponge *Oscarella* spp. from Cabo Frio, SE Brazil [11]. In search of potential antimicrobial substances, 85 bacterial isolates were tested against indicator bacteria of medical importance in agar

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diffusion assays, where cells experience *quorum sensing*. Twenty-seven (31.7%) of them showed inhibitory activity against at least one clinical indicator strain assayed, including some biofilm producers [11]. Aiming to find substances that eradicate biofilm rather than bacterial growth, supernatants of the remaining 58 strains grown under planktonic conditions were evaluated for antimicrobial activity against the reference strains *S. aureus* ATCC 29213, *S. aureus* ATCC 25923 and *S. epidermidis* ATCC 35984. The cell-free supernatant from *Enterobacter* strain 84.3 showed no inhibitory activity against the staphylococcal strains (data not shown). Thus, this strain was selected as potential producer of antibiofilm agents, because these agents may impose a weaker selective pressure for the development of drug resistance relative to current antibiotics. Besides, antibiofilm substances may be co-administered with antibiotics and some promising results *in vitro* have been reported [12]. Therefore, in this study *Enterobacter* strain 84.3 was screened for the capacity to inhibit biofilm formation and to dissociate mature biofilm of *Staphylococcus* spp. reference strains as well as clinical strains.

2. Materials and methods

2.1. Bacterial strain and culture condition

Sponge-associated *Enterobacter* strain 84.3 (MK780772.1) was isolated from the Brazilian sponge *Oscarella* spp. as previously described [11]. Cell-free culture supernatants were prepared by inoculating 10^7 cells of *Enterobacter* 84.3 in 3 mL of Brain Heart Infusion (BHI) medium (Difco, MI, USA). After incubation at 25 °C for 24 h, 22 mL of BHI containing 1% glucose were added and the culture further incubated at 25 °C for 48 h. After centrifugation at 4000×*g* for 20 min (Eppendorf centrifuge 5804 R, Hamburg, Germany) the supernatant was sterilized by filtration (Millipore 0.22 μm) and kept at 4–7 °C until use.

Six strains classified as strong biofilm producers were included in the study as indicator strains and positive control for the antibiofilm assays: three reference strains of the American Type Culture Collection (ATCC), *S. aureus* ATCC 29213, *S. aureus* ATCC 25923 and *S. epidermidis* ATCC 35984; and three clinical strains isolated from orthopaedic prosthetic infections, *S. aureus*1117a and *S. aureus*11123a, and from surgical site infection, *S. epidermidis* 24 [13,14]. All *Staphylococcus* strains were grown on BHI agar for 18 h at 37 °C or in BHI broth supplemented with 1% glucose (BHIg).

2.2. Metabolite extraction

Metabolite extraction was performed as previously described [15] with minor modifications. Briefly, total biomass obtained from a 500 mL culture in BHI medium was centrifuged (11,000 × g for 15 min). The supernatant was collected and thoroughly mixed with 250 mL of ethyl acetate (Tedia, OH, USA) in a separation funnel and partitioned overnight into ethyl acetate and aqueous phases. After removal of the upper organic phase, partition of the aqueous phase was repeated twice with 250 mL each of ethyl acetate. The pooled organic phases were evaporated to dryness in a rotary vacuum evaporator (HeiVap, Heidolph, Schwabach, Germany) and the weight was determined. The delipidated aqueous phase was filtered (Millipore 0.22 μm), lyophilized and weighed. For the assays, the lipid extract was dissolved in BHIg supplemented with 10% dimethyl sulfoxide (DMSO) at a concentration of 5 mg/mL and the lyophilized aqueous residue at the same concentration in BHIg medium.

2.3. Antibiofilm activity

2.3.1. Cell-free supernatant

Marine *Enterobacter* culture supernatant was evaluated for its capacity to either inhibit biofilm formation or to disrupt biofilm previously formed by the reference and clinical strains. Antibiofilm activity was performed as previously described [16] with modifications. Briefly, in the wells of a 96-well microtiter plate (TPP, Trasadingen, Switzerland) 20 μL of the indicator strain culture (~10^8 CFU/mL) were mixed with either 180 μL of the cell-free supernatant or 180 μL of BHIg medium (negative control). All tests were carried out in triplicate and the plate was incubated aerobically for 24 h at 37 °C. After incubation, the wells were washed three times with phosphate-buffered saline (PBS; pH 7.4). The remaining attached bacteria were fixed by exposure to hot air at 60 °C for 60 min, and the wells were stained with 0.2% crystal violet for 15 min at room temperature. The plate was air-dried, and the dye retained by adherent cells was dissolved in 95% ethanol. The optical density of each well was measured at 570 nm using a microtiter plate reader (model 680, Bio-Rad Laboratories, Hertfordshire, UK). The inhibition of biofilm formation of each strain was expressed as percentage of biofilm reduction, by applying the following formula: $(OD_{control} - OD_{sample}/OD_{control}) \times 100$. Three independent experiments were performed in triplicate and therefore each data point was averaged from a total of nine values obtained and the standard deviation (SD) was calculated.

Enterobacter 84.3 supernatant was also evaluated for its capacity to dissociate mature biofilm produced by the reference strains. As mentioned above, three independent experiments were carried out in triplicate. Twenty microliters of indicator bacteria (~10^8 CFU/mL) in 180 μL of BHIg were loaded into wells of a microtiter plate and incubated at 37 °C for 24 h. Planktonic cells were removed by washing the plate three times with PBS. To the remaining preformed staphylococcal biofilm, 200 μL of the cell-free supernatant were added per well. Control samples of each staphylococcal biofilm were kept without the addition of cell-free supernatant. After another 24 h at 37 °C the plates were again washed in PBS, heat-fixed and stained as described above. The ability to dissociate biofilm was also expressed as antibiofilm activity (%), by applying the above-mentioned formula.

2.3.2. Extracts

To determine the antibiofilm activity, the extracts were tested in serial dilutions ranging from 1024 to 1 μg/mL in BHIg medium and used for antibiofilm activity assays as described above with few modifications. In a 96-well microtiter plate containing 20 μL (~10^8 CFU/mL) of each reference strain culture was inoculated with the BHIg containing the diluted extracts or in BHIg without addition of the extracts (negative control wells). All tests were carried out in triplicate and the plate was incubated aerobically for 24 h at 37 °C. After incubation plates were treated as described in section *2.3.1*.

The extracts diluted in BHIg were also evaluated for their capacity to dissociate mature biofilm of the reference strains, as described for the cell-free supernatant in the previous section.

The minimum biofilm eradication concentration (MBEC) was determined considering the lowest concentration of each extract capable of inhibiting biofilm formation or dissociating mature biofilm calculated by applying the formula shown in section *2.3.1*.

2.3.3. Statistical analysis

For biofilm production, statistical analyses were performed on raw optical density data of three biological replicates. An unpaired Student's t test was performed to determine the *p*-values between control and treatment by GraphPad Prism 8 (GraphPad Software,

Inc., CA, USA). Results with a *p*-value $\leq$ 0.05 were considered statistically significant.

2.4. Confocal microscopic observation of antibiofilm activity

In order to directly observe the multicellular structures in the biofilm in the presence or absence of bioactive extract, Confocal Laser Scanning Microscopy (CLSM), with specific fluorescent markers using the Filmtracer™ LIVE/DEAD™ Biofilm Viability Kit (Thermo Fisher Scientific, MA, USA) was performed according to the instructions of the manufacturer. As previously described, aliquots of overnight culture (~10^8 CFU/mL) of *S. aureus* ATCC 25923 strain were distributed in chamber slides (Nunc Inc., IL, USA) for CLSM observations. After incubation at 37 °C for 24 h, bacteria that remained in suspension were removed by aspiration and the remaining adherent cells on the slide were washed with sterile distilled water and treated with the bioactive extract at the previously established MBEC. Untreated biofilm control was included on each slide. After heat fixation, cells were incubated with SYTO 9 and propidium iodide nucleic acid stains provided in the Filmtracer™ LIVE/DEAD™ Biofilm Viability Kit. Samples were observed on a Zeiss Axioplan microscope (Carl Zeiss, Jena, Germany) equipped for fluorescence microscopy. Live bacteria with intact cell membranes show green fluorescence and dead bacteria with compromised membranes show red fluorescence.

2.5. Cytotoxicity bioassays

A microassay for cytotoxicity in the L929 cell line (mouse fibroblast) was performed with the method known as “dye-uptake”, using neutral red dye [17], with minor modifications. One-hundred microliters of L929 cell line suspension at a concentration of 5×10^5 cells/mL were seeded in 96-well microplates. Cells were incubated for 24 h at 37 °C in a 5% CO2 atmosphere to allow attachment. The active extract was added to the cell culture at concentrations ranging from 500 μg/mL to 7.8 μg/mL, and the cells were incubated for 24 h at 37 °C in a 5% CO_2 atmosphere. Then, 100 μL of 0.01% neutral red solution were added for 2 h at 37 °C in a 5% CO_2 atmosphere. After incubation, the medium was removed and the cell monolayers washed with PBS and the dye incorporated by the viable cells was eluted using a mixture of methanol/acetic acid/water (50:1:49). The dye uptake was determined by measuring the optical density of the eluate at 490 nm in an automatic spectrophotometer (ELx800TM, Bio-TeK Instruments, Inc., VT, USA). The 50% cytotoxic concentration (CC_{50}) was defined as the concentration of the extract which caused 50% reduction in the number of viable cells.

3. Results

3.1. Effects of enterobacter 84.3 supernatants on biofilms

Antibiofilm activity was evaluated by microtiter plate assay using the cell-free supernatants obtained from *Enterobacter* strain 84.3 cultures. The results indicated that the marine strain produces substances able to reduce biofilm formation and to dissociate mature biofilm formed by the tested indicator strains. For all reference strains, the cell-free supernatant inhibited biofilm formation at a high percentage, with a maximum value of 93.4% for *S. aureus* ATCC 25923, followed by 93.0% for *S. aureus* ATCC 29213 and of 89.0% for *S. epidermidis* ATCC 35984 (Fig. 1A–C). Biofilm formation by the three clinical strains isolated from orthopaedic prosthetic or surgical site infections was also inhibited and ranged from 80.1% for *S. aureus* 11123a over 82.9% for *S. aureus* 1117a to 66.2% for *S. epidermidis* 24 (Fig. 1D–F).

In addition to inhibiting biofilm formation, the *Enterobacter* 84.3 culture supernatant showed a high capacity to dissociate mature biofilm formed by *Staphylococcus* spp. strains. The biofilm eradication levels were similar to those observed for inhibitory activity of biofilm formation. Eradication activity was 86.1% for *S. aureus* ATCC 25923, 85.3% for *S. aureus* ATCC 29213 and 76.1% for *S. epidermidis* ATCC 35984 (Fig. 2).

3.2. Antibiofilm activity of aqueous extract

To investigate whether the antibiofilm activity of cell-free supernatant from *Enterobacter* 84.3 culture might be due to the lipidic or aqueous fraction, the respective fractions were evaluated at different concentrations. No inhibition or dissociation effect on the biofilms was observed for the lipid extract. However, the aqueous extract acted by inhibiting and dissociating biofilms of the reference strains at all concentrations tested (from 1 to 1024 μg/mL) and antibiofilm activity rates and the MBEC are listed in Table 1.

For all reference strains, the aqueous extract inhibited biofilm formation at a high percentage (Fig. 3A–C). *S. aureus* ATCC 25923 biofilm formation was inhibited by 94.0% in the presence of the 16 μg/mL of the aqueous extract and it was dissociated by 85.3% when treated with 32 μg/mL of the same bioactive extract. Furthermore, a strong inhibitory effect ($\geq$77%) on biofilms has also been observed against *S. aureus* ATCC 29213 and *S. epidermidis* ATCC 35984, as well as promising disruption rates ($\geq$68%) of the mature biofilms.

The aqueous extract showed a concentration-dependent antibiofilm activity both for the inhibitory effect on biofilm formation and for the dissociation effect of mature biofilm from *S. aureus* and *S. epidermidis*. Fig. 3D represents this observation on the mature biofilm of *S. aureus* ATCC 25923. The effect of aqueous extract at 32 μg/mL on pre-formed *S. aureus* ATCC 25923 biofilm was also visualized on glass surfaces by confocal laser scanning microscopic imaging. This approach confirmed that the aqueous extract significantly reduced the biofilm biomass, average thickness, and substrate coverage as compared to the untreated control (Fig. 4), thus having a major effect on mature biofilm.

3.3. Cytotoxicity of the active extract

The aqueous extract was subjected to cytotoxicity assay in order against the mammalian L929 cell line. When the cells were treated with concentration from 7.8 to 500 μg/mL, the observed CC_{50} was higher than the maximum concentration tested (where CC_{50} stands for 50% cytotoxic concentration, defined as the concentration required to reduce the cell number by 50% compared with that for the untreated controls). These results suggest that the antibiofilm substance present in the aqueous extract from *Enterobacter* 84.3 is not toxic for L929 cells. Thus, as the CC_{50} for mammalian cells was higher than the observed MBEC (16–128 μg/mL) for *S. aureus* strains, the use of this substance for inhibiting or dissociate biofilms could be recommended in the future.

4. Discussion

Staphylococci are currently the most common cause of nosocomial infections, mainly related to orthopaedic devices on which the bacteria form biofilm [3–5]. Staphylococcal biofilms are preserved from host defenses and often display dramatic decrease in antimicrobial susceptibility, resulting frequently in the development of persistent and chronic infections [3,5].

In the past years many antibiofilm agents have been identified from various sources, including from sponge-associated bacteria, which represent promising producers of substances for

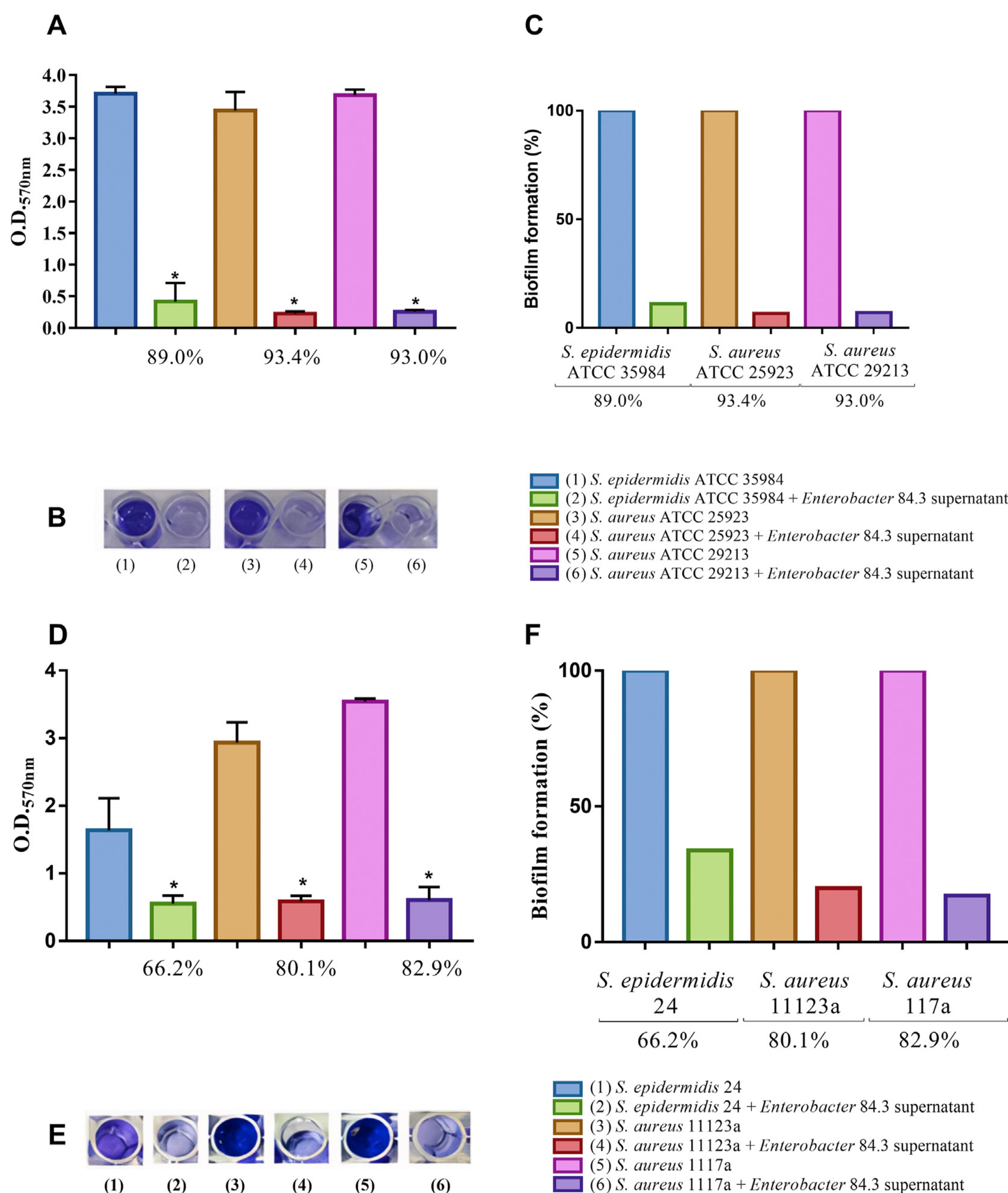

Fig. 1. Antibiofilm activity of the cell-free supernatant from *Enterobacter* sp. 84.3 culture on *Staphylococcus* spp. biofilm formation. Biofilm produced in absence of supernatant was used as control. The inhibition of biofilm formation is expressed as optical density of each well measured at 570 nm (A and D), $*p < 0.0001$. The ratio of biofilm absorbance/planktonic absorbance was calculated and this value used to calculate the "biofilm formation" in percentage on the y axis (C and F). Wells of 96-well plate showing biofilms in the absence and presence of supernatant after crystal violet staining (B and E). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

biotechnological and pharmaceutical applications [7–10,18–21]. However, none of them has entered the market. Hence, there is a large unmet need for the development of antibiofilm formulations to tackle this problem [7].

In a previous study, antibacterial tests were performed with bacteria isolated from *Oscarella* sponges and their cell-free culture supernatants [11], and the marine strain *Enterobacter* 84.3 was selected because no bacteriostatic or bactericidal activity against the target strains were observed, in spite of strong antibiofilm activity. The current study aimed to analyze for the first time the antibiofilm activities of sponge-associated *Enterobacter* 84.3 on staphylococcal biofilms. Our findings showed that *Enterobacter* 84.3 substances were particularly effective at both inhibiting biofilm development and disaggregating the mature biofilm, but without killing the *Staphylococcus* strains or inhibiting their growth. *S. aureus and S. epidermidis* infections associated with biofilm development are estimated to reach 250,000 cases per year in the USA with a mortality rate of up to 25%. These infections represent an important social and economic burden worldwide [2]. The interest in the development of innovative approaches for prevention and treatment of staphylococcal adhesion and biofilm formation capabilities has therefore increased. A viable approach should target the staphylococcal adhesive properties without affecting bacterial viability in order to avoid the rapid appearance of escape mutants [21,22].

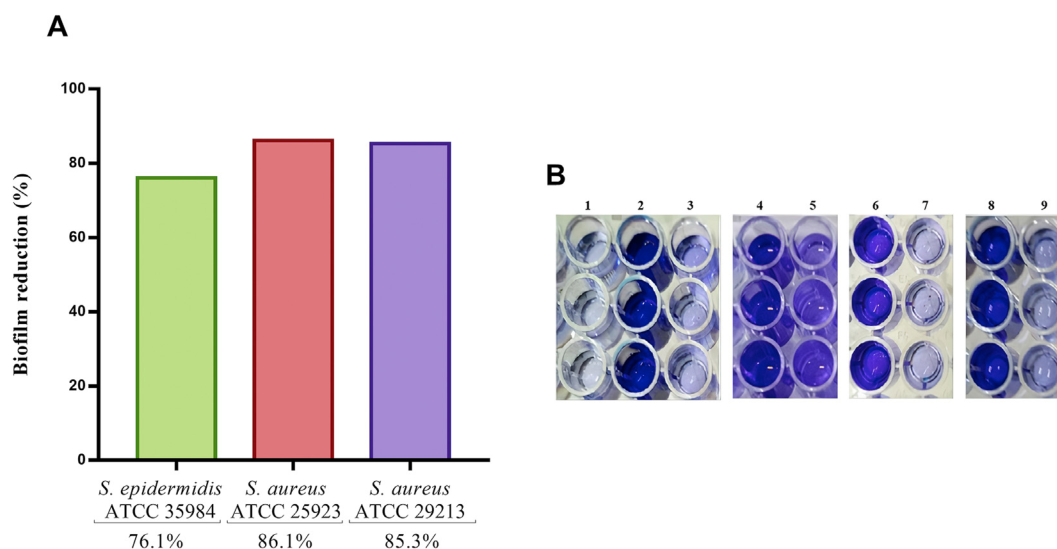

Fig. 2. Antibiofilm activity of the cell-free supernatant from *Enterobacter* sp. 84.3 culture on mature biofilms. Percentages of biofilm biomass reduction of reference staphylococcal strains (A). Wells of 96-well plate showing mature biofilms untreated and treated after crystal violet staining (B). Control wells: 1. sterile growth medium (BHIg); 2. *S. aureus* ATCC 25923, indicator strain producing strong biofilm; 3. only cell-free supernatant. Test wells: 4. *S. aureus* ATCC 25923; 5. *S. aureus* ATCC 25923 treated with cell-free supernatant; 6. *S. aureus* ATCC 29213; 7. *S. aureus* ATCC 29213 treated with cell-free supernatant; 8. *S. epidermidis* ATCC 35984; 9. *S. epidermidis* ATCC 35984 treated with cell-free supernatant. Values below the wells 5, 7 and 9 show the percentages of mature biofilm biomass reduction. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Antibiofilm activity of the aqueous extract from *Enterobacter* sp. 84.3 culture on staphylococcal biofilm.

Strains	Antibiofilm activity	
	Inhibitory rate[a] (MBEC[b] - μg/mL)	
	Inhibition	Dissociation
S. aureus ATCC 25923	94.0% (16)	85.3% (32)
S. aureus ATCC 29213	87.3% (64)	79.6% (128)
S. epidermidis ATCC 35984	77.0% (128)	68.2% (256)

[a] Biofilm reduction rate (%).
[b] Minimum Biofilm Eradication Concentration (MBEC).

Our results showed high rates of antibiofilm activity (from 66% to 94%), including clinical strains isolated from orthopaedic prosthetic and surgical site infections [13,14]. Sayem and colleagues [10] reported that cell-free supernatants obtained from sponge associated *Bacillus licheniformis* SP1 showed inhibition effects of almost 90% on biofilm formation by *S. aureus.* Interestingly, *S. aureus* biofilms were more affected than *S. epidermidis* biofilms, as also observed in our study. In general, the cell-free supernatant from *Enterobacter* 84.3 culture inhibited *S. aureus* biofilm formation by more than 75%, while acting on *S. epidermidis* biofilm at a 10% lower rate. This result could be explained by differences in the extracellular matrix components of each strain, which comprise a complex mixture of polysaccharides, lipids, extracellular DNA (eDNA) and proteins [1,20]. Studies have revealed how distinct constituents of the biofilm matrix contribute to its architectural stability and functionality. These findings will provide the basis for developing novel therapeutics that can effectively target components of the biofilm matrix and modulate biofilm stability [1,5,23].

In this study, we observed that the biofilm degradation activity from *Enterobacter* 84.3 was caused by water-soluble substances. The aqueous extract presented minimum biofilm eradication concentration values smaller than the CC_{50} observed for L929 cells (higher than 500 μg/mL). These are some of the features that make it of great biotechnological interest.

Confocal laser scanning microscopy images also confirmed that the bioactive extract was able to dissociate the mature biofilm. This characteristic shows that the antibiofilm agent could be applied both for prevention of formation and removal of mature biofilm on indwelling medical devices. In addition, its use could also be employed in a combination therapy with antibiotics of interest, since the bacterial cells will end up unprotected from the extracellular matrix (biofilm) [21,22]. This model of action was also proposed for pentadecanoic acid, a molecule derived from pentadecanal produced by the Antarctic bacterium *Pseudoalteromonas haloplanktis* TAC125 [21,24]. Pentadecanoic acid is a good candidate for combined therapies with conventional antibiotics, because it prevented staphylococcal biofilm formation and positively modulated the antimicrobial activity of vancomycin [21].

In the case of infections related to orthopaedic prosthetic biofilm, the cost is very high since it involves long hospitalization time, surgery to change the prosthesis and antimicrobial administration as prophylaxis agents of nosocomial infections [3]. Biofilm dispersal agents generally interfere with chemical pathways or processes, such as *quorum sensing*, which are required for bacteria to maintain the mode of existence as biofilm [5,25]. As dispersed cells are generally more susceptible to antimicrobial treatment than biofilm-residing cells, this strategy has recently become an intense area of study [1,12,21].

Based on the findings, we hypothesize that antibiofilm substances present in aqueous extract might interfere with the cell surface, thus influencing cell—cell interactions as pre-requisite for biofilm formation [23], or with other steps of biofilm assembly. Since the antibiofilm substances are found in the aqueous phase, they might be polysaccharides. Polysaccharides have been previously reported to produce anti-adherence effects between microorganisms and surfaces [10,23].

In conclusion, the cell-free culture supernatant from sponge-associated *Enterobacter* 84.3 contains water-soluble molecules that can provide a tool for better exploration of novel antibiofilm substances. Inhibiting biofilm formation and dissociating mature biofilm of strains of *S. aureus* and *S. epidermidis* without affecting their growth represent a special feature of the antibiofilm activity

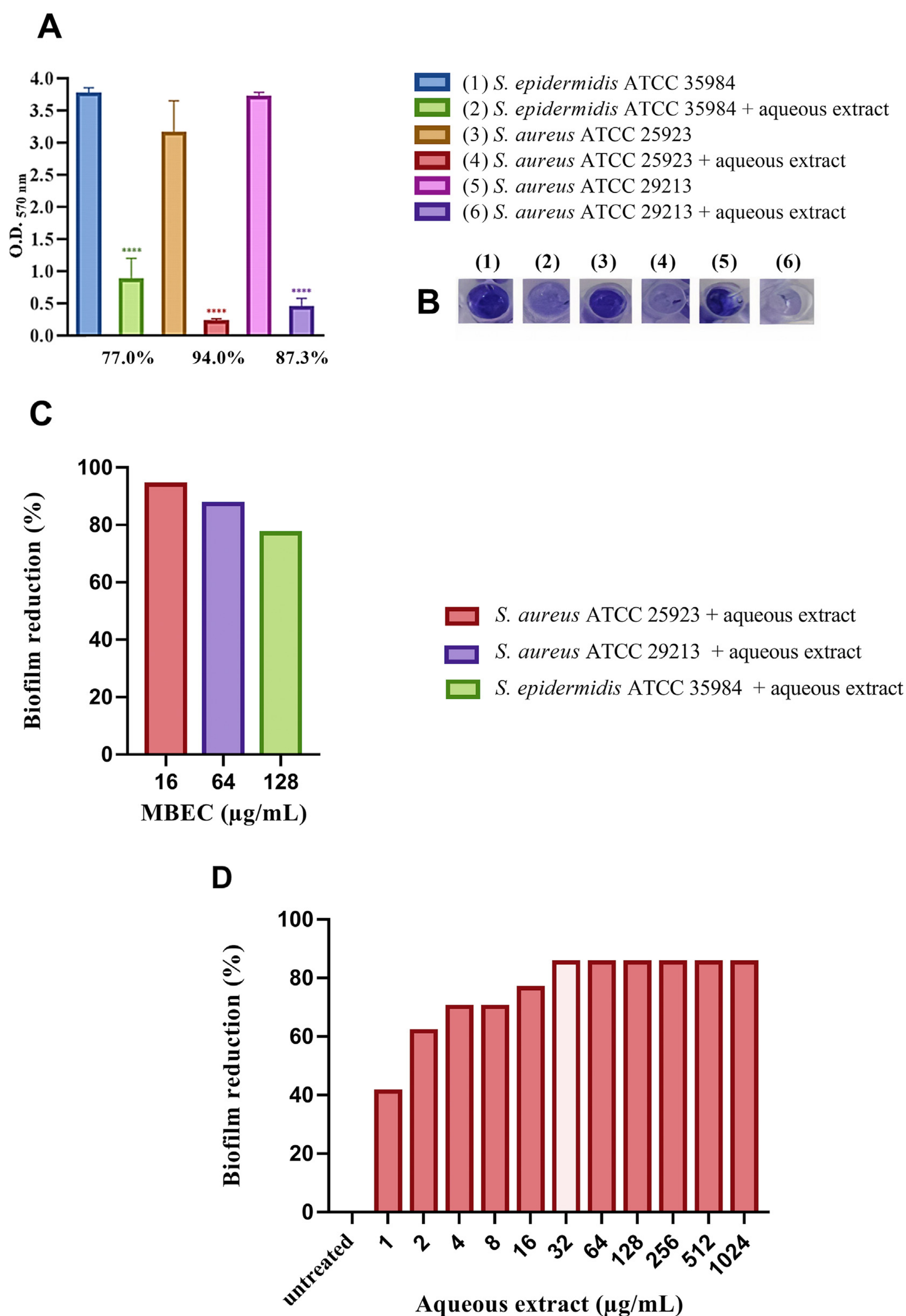

Fig. 3. Antibiofilm activity of the aqueous extract from *Enterobacter* sp. 84.3 culture on *Staphylococcus* spp. biofilm formation. The assays were performed at minimum biofilm eradication concentration (MBEC) for each reference strain and the activity is concentration-dependent. The inhibition of biofilm formation is expressed as optical density of each well measured at 570 nm (A), $*p < 0.0001$. Wells of 96-well plate showing biofilms in the absence and presence of aqueous extract after crystal violet staining (B). The absorbance values were used to calculate the antibiofilm activity in percentage applying the formula shown in section 2.3.1(C). MBEC was defined at 32 µg/mL on pre-formed *S. aureus* ATCC 25923 biofilm (D). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

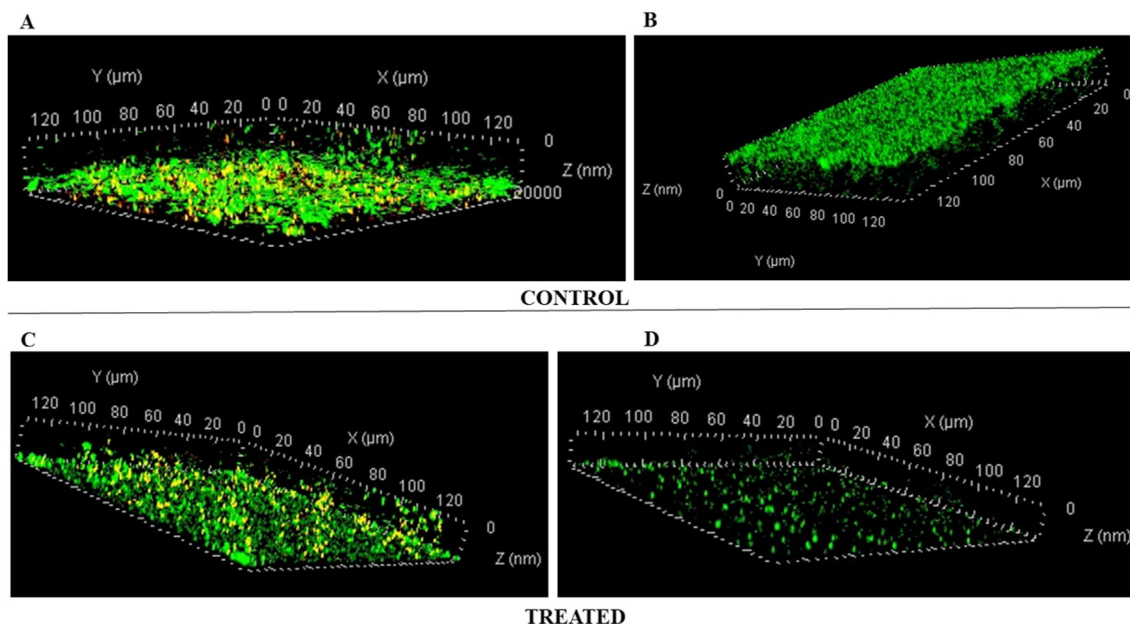

Fig. 4. Representative confocal laser scanning microscopy images of *S. aureus* ATCC 25923 biofilm in the presence of the bioactive aqueous extract (32 µg/mL), visualized by fluorescence vital dye. 3D read out of the stained biofilm: (top) untreated biofilm control, and (bottom) treated biofilm. Biofilm incubated with SYTO 9 and propidium iodide nucleic acid stains (A and C) and with FilmTracer stain (B and D). Green cells and clusters indicate bacterial cells with intact membranes (live). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

described in this report. Further research on such bioactive aqueous extract might help developing new antibiofilm agents active against staphylococcal biofilms on indwelling medical devices.

Declaration of competing interest

No conflict of interest is declared.

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References

[1] Høiby N, Bjarnsholt T, Givskov M, Molin S, Ciofu O. 2010. Antibiotic resistance of bacterial biofilms. Int J Antimicrob Agents 2010;35:322–32.
[2] Figueiredo AMS, Ferreira FA, Beltrame CO, Côrtes MF. The role of biofilms in persistent infections and factors involved in *ica*-independent biofilm development and gene regulation in *Staphylococcus aureus*. Crit Rev Microbiol 2017;43:602–20.
[3] Arciola CR, Campoccia D, Montanaro L. Implant infections: adhesion, biofilm formation and immune evasion. Nat Rev Microbiol 2018;16:397–409.
[4] Li B. Webster TJ Bacteria antibiotic resistance: new challenges and opportunities for implant associated orthopedic infections. J Orthop Res 2018;36:22–32.
[5] Otto M. Staphylococcal infections: mechanisms of biofilm maturation and detachment as critical determinants of pathogenicity. Annu Rev Med 2013;64:175–88.
[6] Gajdács M. The concept of an ideal antibiotic: implications for drug design. 2019. Molecules 2019;24:892.
[7] Balasubramanian S, Othman EM, Kampik D, Stopper H, Hentschel U, Ziebuhr W, et al. Marine Sponge-derived *Streptomyces* sp. SBT343 extract inhibits staphylococcal biofilm formation. Front Microbiol 2017;8:236.
[8] Indraningrat A, Smidt H, Sipkema D. Bioprospecting sponge-associated microbes for antimicrobial compounds. Mar Drugs 2016;4:E87.
[9] Kiran GS, Sabarathnam B, Selvin J. 2010. Biofilm disruption potential of a glycolipid biosurfactant from marine *Brevibacteriumcasei*. FEMS Immunol Med Microbiol 2010;59:432–8.
[10] Sayem S, Manzo E, Ciavatta L, Tramice A, Cordone A, Zanfardino A, et al. Anti-biofilm activity of an exopolysaccharide from a sponge-associated strain of *Bacillus licheniformis*. Microb Cell Factories 2011;10:74.
[11] Laport MS, Bauwens M, de Oliveira Nunes S, Willenz P, George I, Muricy G. Culturable bacterial communities associated to Brazilian *Oscarella* species (Porifera: Homoscleromorpha) and their antagonistic interactions. Antonie Leeuwenhoek 2017;110:489–99.
[12] Verderosa AD, Totsika M, Fairfull-Smith KE. Bacterial biofilm eradication agents: a current review. Front Chem 2019;7:824.
[13] Schuenck RP, Cavalcante FS, Emery E, Giambiagi-de Marval M, dos Santos KR. *Staphylococcus aureus* isolates belonging to different multilocus sequence types present specific virulence gene profiles. FEMS Immunol Med Microbiol 2012;65:501–4.
[14] Iorio NL, Caboclo RF, Azevedo MB, Barcellos AG, Neves FP, Domingues RM, et al. Characteristics related to antimicrobial resistance and biofilm formation of widespread methicillin-resistant *Staphylococcus epidermidis* ST2 and ST23 lineages in Rio de Janeiro hospitals, Brazil. Diagn Microbiol Infect Dis 2012;72:32–40.
[15] Santos OCS, Soares AR, Machado FL, Romanos MT, Muricy G, Giambiagi-deMarval M, et al. Investigation of biotechnological potential of sponge-associated bacteria collected in Brazillian coast. Lett Appl Microbiol 2015;60:140–7.
[16] Stepanovic S, Vukovic D, Hola V, Di Bonaventura G, Djukic S, Cirkovic I, et al. Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. APMIS 2007;115:891–9.
[17] Borenfreund E, Puerner J. Toxicity determined in vitro by morphological alterations and neutral red absorption. Toxicol Lett 1985;24:119–24.
[18] Rajput A, Thakur A, Sharma S, Kumar M. aBiofilm: a resource of anti-biofilm agents and their potential implications in targeting antibiotic drug resistance. Nucleic Acids Res 2018;46:D894–900.
[19] Santos-Gandelman JF, Giambiagi-deMarval M, Oelemann WMR, Laport MS. Biotechnological potential of sponge-associated bacteria. Curr Pharmaceut Biotechnol 2014;15:143–55.
[20] Artini M, Papa R, Vrenna G, Lauro C, Ricciardelli A, Casillo A, et al. Cold-adapted bacterial extracts as source of anti-infective and antimicrobial compounds against *Staphylococcus aureus*. Future Microbiol 2019;14:1369–82.
[21] Ricciardelli A, Casillo A, Papa R, Monti DM, Imbimbo P, Vrenna G, et al. Pentadecanal inspired molecules as new anti-biofilm agents against *Staphylococcus epidermidis*. Biofouling 2018;34:1110–20.
[22] Papa R, Selan L, Parrilli E, Tilotta M, Sannino F, Feller G, et al. Anti-biofilm activities from marine cold adapted bacteria against Staphylococci and *Pseudomonas aeruginosa*. Front Microbiol 2015;6:1333.
[23] Casillo A, Papa R, Ricciardelli A, Sannino F, Ziaco M, Tilotta M, et al. Anti-biofilm activity of a long-chain fatty aldehyde from Antarctic

Pseudoalteromonas haloplanktis TAC125 against *Staphylococcus epidermidis* biofilm. Front Cell Infect Microbiol 2017;7:46.

[24] Vacca I. Biofilms: building up the matrix. Nat Rev Microbiol 2017. https://doi.org/10.1038/nrmicro.2017.9.

[25] McDougald D, Rice SA, Barraud N, Steinberg PD, Kjelleberg S. Should we stay or should we go: mechanisms and ecological consequences for biofilm dispersal. Nat Rev Microbiol 2011;10:39–50.