RESEARCH ARTICLE

Biologically-relevant *Staphylococcus aureus* biofilm phenotype characterisation and liability to novel antibiofilm drugs

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Abstract

Objectives: To characterise the biofilm matrix composition of a newly described *Staphylococcus aureus* biofilm phenotype .

Method: This experimental study was conducted at the Faculty of Pharmacy, Helwan University, Cairo, Egypt, from January 2021 to March 2022, and comprised methicillin-resistant *Staphylococcus aureus* and methicillin-susceptible *Staphylococcus aureus* biofilm-forming clinical isolates which were allowed to construct biofilms under two distinct culture conditions; one a commonly used condition, and the other one a novel, more biologically-relevant condition. The formed biofilms were analysed for matrix composition through treatment with proteinase, sodium meta-periodate, and streptokinase. The efficacy of Cis-2-Decenoic acid and hamamelitannin on the biologically-relevant biofilms was evaluated using biofilm viability assay based on a colorimetric assay for measuring cell metabolic activity and scanning electron microscope imaging. Data was analysed using GraphPad Prism 5.01.

Results: Of the 58 isolates, 45(77.6%) were methicillin-resistant *Staphylococcus aureus* and 13(22.4%) were methicillin-susceptible *Staphylococcus aureus*. There was significant difference in responses to streptokinase, proteinase and sodium meta-periodate ($p<0.05$) among the differentially-developed biofilms in methicillin-resistant *Staphylococcus aureus* isolates. Regarding the methicillin-susceptible *Staphylococcus aureus* isolates, the differentially-developed biofilms showed significantly different liabilities to streptokinase only ($p<0.05$). Mean biofilm inhibition for Cis-2-Decenoic acid was 54.27±27.93% and mean biofilm dispersion was 71.92±11.59% while the corresponding values for hamamelitannin were 83.03±13.95% and 70.48±7.116% against the newly described methicillin-resistant *Staphylococcus aureus* biofilm phenotype.

Conclusion: Applying biologically-relevant culture conditions on staphylococci biofilms and antibiofilm drugs is recommended.

Keywords: *Staphylococcus aureus*, Fibrinolytic, Hamamelitannin, Polysaccharide, Polymeric, Fibrin.

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Introduction

Biofilm formation in staphylococci is a crucial virulence determinant that rigorously affects treatment of related infections.[1-3] Several biofilm formation mechanisms have been reported in *staphylococci*, involving ica-dependent and ica-independent mechanisms.[4] The ica-dependent biofilm is the first and well-characterised biofilm phenotype in *Staphylococcus*[5]. spp. The production of biofilm relies on and regulated by the Intercellular adhesion ADBC operon (icaADBC operon) which consists of the biosynthetic genes icaA, icaB, icaC, icaD and the repressor icaR. It encodes the polysaccharide intercellular adhesin (PIA) that is also known as polymeric N-acetyl-glucosamine (PNAG). PIA is composed of β-1, 6-linked N-acetyl glucosamine polymer. Ica-dependent biofilm was initially outlined in *S. epidermidis* and consequently it was revealed that methicillin-susceptible *Staphylococcus aureus* (MSSA) isolates also frequently depend on icaADBC-encoded PIA in biofilm formation.[5, 6] Another biofilm phenotype was described in methicillin-resistant *Staphylococcus aureus* (MRSA) isolates, where the biofilm matrix tends to be more proteinaceous. In MRSA isolates, the major constituents of the biofilm matrix are surface adhesin proteins and extracellular deoxyribonucleic acid (eDNA). The surface adhesin proteins involve fibronectin binding proteins (FnBPs), the cell wall anchored clumping factors A (ClfA) and ClfB, and *S. aureus* surface protein C (SasC) and SasG as well as other proteins. The major autolysin (Atl) and autolysin-released eDNA play roles in the early stages of PIA-independent biofilm development by MRSA isolates. Cytoplasmic proteins have also been implicated in this biofilm phenotype[4, 6]. Another biofilm phenotype was described in *S. aureus*[6] when a physiologically relevant conditions were applied during the biofilm development. In these circumstances, fibrin biofilms that rely on the coagulase-mediated conversion of fibrinogen into fibrin were developed. Fibrin biofilms were regulated by the two-component system *S. aureus* exoprotein expression (SaeRS), that regulates Coagulase (Coa) production as well.[4, 6]

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The use of antibiofilm drugs is a novel promising approach to combat biofilm-related infections caused by multidrug resistant (MDR) strains. Different natural and synthetic antibiofilm drugs have been identified.[7,8] One of the proposed antibiofilm drugs is Cis-2-Decenoic acid (CDA), a biofilm dispersion autoinducer identified in *Pseudomonas (P.) aeruginosa*. CDA signalling system was found to regulate multiple genes involved in different virulence and metabolic pathways, including chemotaxis, cell attachment, motility, iron uptake, tricarboxylic acid (TCA) cycle, and respiration.[9] Three different mechanisms have been reported regarding the ability of CDA to control biofilms; impairing biofilm formation, promoting biofilm dispersion and altering the persister cell sub-population.[10,] Moreover, CDA shows interspecies and interkingdom activity, and is able to induce biofilm dispersion in other pseudomonads, *Escherichia coli or E. coli, Klebsiella pneumoniae or K. pneumoniae, S.pyogenes, S. aureus* and *Candida albicans or C. albicans*.[11,12]

Another promising antibiofilm drug is hamamelitannin (HAM), which is a natural product present in the leaves and the bark of *Hamamelis virginiana* (witch hazel). HAM is considered as a natural inhibitor of the RAP/TRAP quorum sensing system that regulates biofilm formation and virulence in *S. aureus*. .[13, 14]

The current study was planned to characterise the biofilm matrix composition of a newly described *S. aureus* fibrin biofilm phenotype constructed under biologically-relevant conditions, and to evaluate its liability to CDA and HAM as promising antibiofilm drugs.

Materials and Methods

This experimental study was conducted at the Faculty of Pharmacy, Helwan University, Cairo, Egypt, from January 2021 to March 2022, and comprised fourty five MRSA and thirteen MSSA isolates that had previously formed detectable biofilms under different laboratory conditions and were preserved by the Department of Microbiology and Immunology. The study was approved by the Scientific Research Ethical Committee, Faculty of Pharmacy, Helwan University, and was conducted in accordance with its rules.

Proteinase from *Aspergillus melleus*, streptokinase C from *streptococcus spp.*, sodium meta-periodate, HAM, CDA and 3 - (4,5-dimethylthiazol-2-yl) - 2,5-diphenyl tetrazolium bromide (MTT) (Sigma Aldrich, UK) and other chemicals of analytical grade from local resources were subsequently used.

Microbiological media included brain heart infusion (BHI) (Biolife, Italy), and Roswell Park Memorial Institute (RPMI) 1640 (Sigma Aldrich, UK). Media were prepared using distilled water and sterilisation by autoclaving at 121°C for 15min.

The test isolates were allowed to construct biofilms either in the conditions commonly applied in microbiological laboratories using 96-well microtiter plates in BHI supplemented with 2.5% glucose, or in more biologically-relevant conditions that were achieved by conditioning the biofilm supporting surfaces with human plasma prior to establishing biofilms in RPMI-1640, which is an iron-limiting medium. Biofilms were developed as previously described.[6] For BHI-supported biofilms, the test organisms were grown overnight in the same medium. The overnight cultures were diluted 200 folds and 200µl of the diluted suspension were added to each well. Then, the plates were incubated at 37°C for 48h.

For RPMI-1640-supported biofilms, the test organisms were grown in RPMI-1640 and adjusted to optical density of 0.1-0.2. Wells were conditioned with 20% human plasma, collected and supported by Kasr Al Ainy Hospital, Cairo, in 50 mM carbonate buffer pH 9.6. The added plasma was discarded after incubating the plates for 1h at 37°C and then 100µl of the overnight culture and 100µl of the plain medium were added to each well. The plates were incubated for 48h at 37°C to allow the construction of biofilms. Afterwards, the planktonic bacteria were thrown out, and the plates were washed twice with distilled water and were allowed to air-dry.

To determine the relative composition of the biofilms formed by the isolates under different developmental conditions, symbolised by BHI and RPMI, the formed biofilms were treated with either proteinase 200µg/ml in 20mM Tris hydrochloride pH 7.5, or sodium meta-periodate 1mg/ml.[15] Furthermore, the liability of the formed biofilms to fibrinolytic drugs was also estimated by treating the biofilms with streptokinase 1,000IU/ml. The plates were incubated with the specific treatment at 37°C for 1h. Then, the wells were washed twice with distilled water and the remaining biofilms were stained for 15min with 0.5% crystal violet and quantified as previously described[6] using a microtiter plate reader (Biotech®, Elx 808). Dispersion of the biofilms was expressed as a percent of control wells incubated with phosphate buffered saline (PBS) pH 7.4 instead of the dispersing agent. Mean values of triplicate readings were calculated in all cases.

For biofilm dispersion assays, biofilms were formed on the surface of 24-well plates in RPMI-1640 under the biologically-relevant conditions. The formed biofilms were treated with either HAM 250µM in distilled water or CDA 0.62, 1.47, 14.7, or 147µM in 10% ethyl alcohol, and incubated for 3h at 37°C. Afterwards, the plates were

washed twice with PBS. The remaining biofilm viability in the test wells was estimated using the MTT assay and was expressed as a percent of the biofilm viability of control wells treated with the corresponding solvent for each drug. Each condition was represented by triplicate wells.

To measure the viability of biofilms, 0.5ml of MTT (0.5mg/ml) in RPMI-1640 was added to each well, and the plates were incubated for additional 2h. Then, MTT was discarded, and the plates were washed once with PBS. Afterwards, Dimethyl-sulphoxide was added to dissolve the formed formazan crystals and incubated for 15min at 37°C prior to the measurement of absorbance at 570nm using the spectrophotometer (GeneQuant, Biochrom Ltd, UK).

For biofilm formation assays, the biofilms were developed in the presence of CDA 0.62µM or HAM 250µM, and treated in the same manner.

For scanning electron microscope (SEM) assays, biofilms were formed under biologically-relevant conditions on six-well microtiter plates that contained glass cover slips. Then, the growth medium was discarded, and the formed biofilms were washed twice with distilled water. Subsequently, the biofilms formed on the coverslips were treated with either HAM 250, 500µM, CDA 0.62, 1.47, 14.7, 147 and 1470µM, or the corresponding solvent as a control. The treated biofilms were then incubated with the drugs for 3h at 37°C. Afterwards, the drugs were discarded and the biofilms were prepared for SEM examination as described elsewhere.[20] Briefly, the treated biofilms were carefully washed twice with distilled water and were fixed through incubation 2h at 4°C with glutaraldehyde 2.5% v/v in 0.1M phosphate buffer, pH 7.4. Then, glutaraldehyde was discarded, and the biofilms were washed with distilled water and dehydrated using a graded series of ethanol 25, 50, 75 and 100%. After air-drying, the remaining biofilms were sputter coated using a gold sputter coating apparatus (S150A, Edwards, England) and examined by SEM (Quanta GEF, Netherlands).

Inhibition *S. aureus* biofilm formation and dispersion of the biofilms that were established in the newly-described biologically-relevant conditions using the previously reported antibiofilm concentrations of CDA and HAM[21] was evaluated.

CDA at nanomolar concentration 0.62µM has previously been reported to be effective in the dispersion of tryptic soy broth (TSB)-supported S. aureus biofilms.[22] The same concentration was tested for its ability to disperse biofilms that were formed in the newly-described biologically-relevant conditions. Viability of the treated biofilms were measured by the MTT assay and compared to that of untreated control wells.

Data was analysed using GraphPad Prism 5.01. Data distribution was tested using the Kolmogorov-Smirnov test. Unpaired t test and Mann-Whitney test were used to compare two independent groups with parametric and non-parametric data, respectively. Wilcoxon matched pairs test was used for paired data. Variances were compared using F test. For more than two groups, Kruskal-Wallis test was used. $P \leq 0.05$ was considered statistically significant.

Results

Of the 58 isolates, 45(77.6%) were MRSA and 13(22.4%) were MSSA. Biofilms formed by MRSA isolates under the tested conditions were significantly different in composition and sensitivity to streptokinase (Figure 1). Regarding MSSA isolates, the difference was only significant with streptokinase-treated biofilms. The means of biofilm dispersion were 78.58±17.91% and 55.59±17.54% for RPMI-1640 and BHI biofilms, respectively (Figure 2).

Both CDA and HAM were effective in inhibiting biofilm formation, but HAM showed significantly higher inhibitory effect (p=0.04) with a mean biofilm formation inhibition of 83.03±13.95% compared to 54.27±27.93% for CDA. HAM at the same concentration was tested for biofilm dispersion activity against MRSA biofilms and showed mean biofilm dispersion of 70.48±7.11%.

In the biofilm dispersion assay, CDA at the previously reported antibiofilm concentration (0.62µM) showed no significant difference compared to untreated control wells (p=0.06).

To optimise the least satisfactory biofilm dispersing CDA concentrations, 1.47, 14.7 and 147µM were tested for their ability to induce biofilm dispersion (Figure 3). There was mean reduction in the treated MRSA biofilm viability of 50.78±12.02 and 71.92±11.59%, for 14.7 and 147µM, respectively. The efficacy of CDA 147µM to induce MRSA biofilm dispersion was further compared to that of HAM 250µM, and the difference was not statistically significant (p=1.0), indicating comparable efficacy.

The biofilm dispersion activities of both CDA and HAM were further investigated by SEM. CDA at concentrations of 0.62µM and 1.47µM showed undetectable reduction in cell density compared to untreated control, but its effect was obvious at 14.7 and 147µM, while, in contrast, a huge reduction in cell density and deformity of the remaining cells were observed at 1470µM (Figure 4). Regarding the SEM micrographs of HAM-treated MRSA-0194 biofilms, reduction in the cell density without morphological changes was observable for both the tested concentrations.

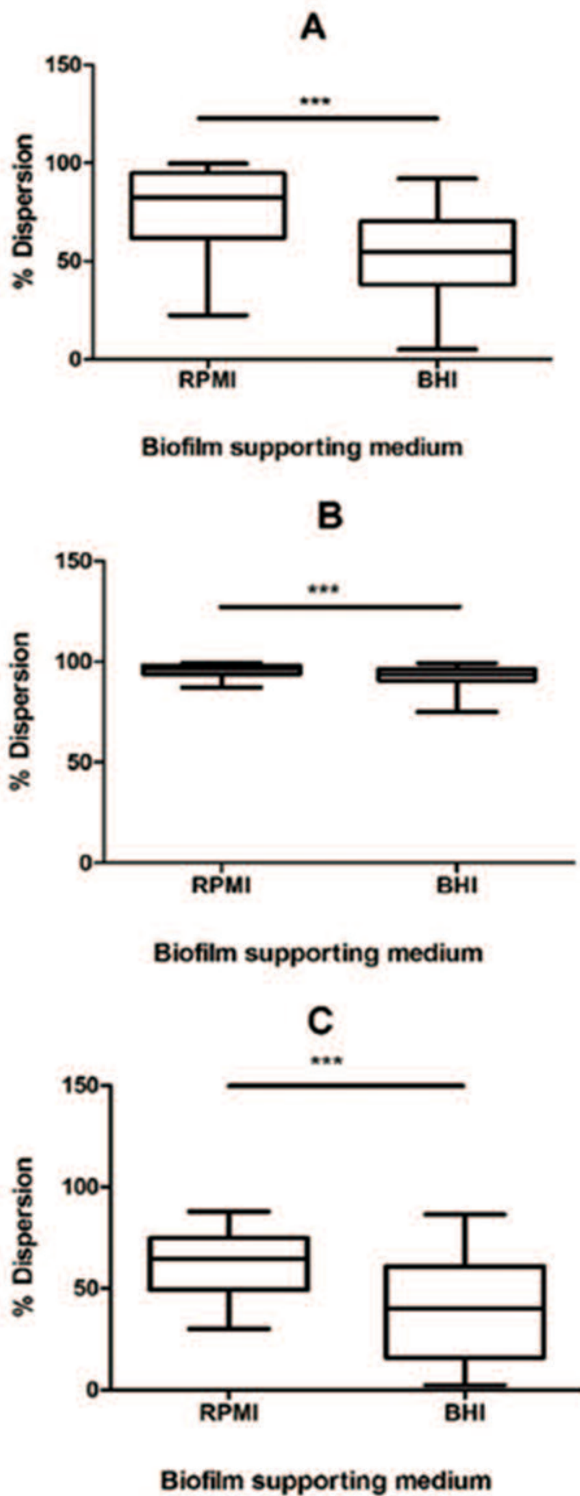

Figure 1: Dispersion of biofilm formed by MRSA clinical isolates grown in RPMI-1640 vs BHI under specified conditions (n=45). A) Streptokinase, Mann Whitney test, p<0.0001. B) Proteinase, Mann Whitney test, p=0.0008. C) Periodate, unpaired t test, p<0.0001. Biofilm dispersion was calculated as a percent of the control wells that were treated with PBS. MRSA: Methicillin-resistant *Staphylococcus aureus*, RPMI: Roswell Park Memorial Institute, BHI: Brain Heart Infusion, PBS: Phosphate buffered saline

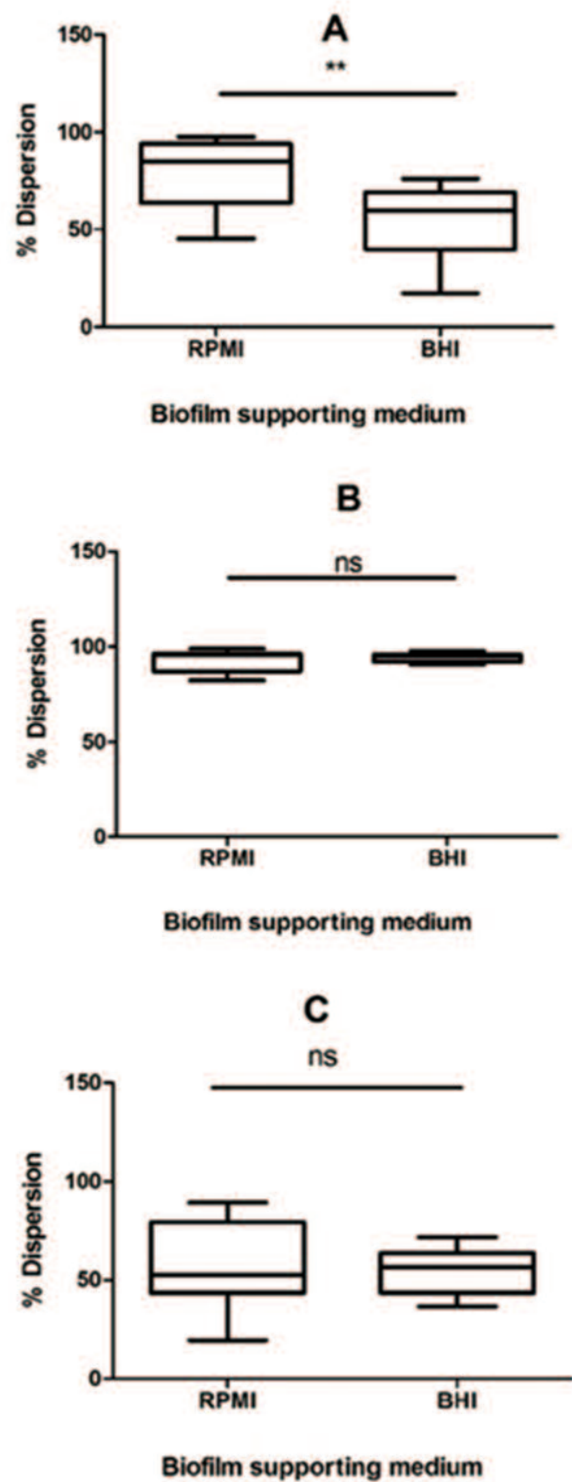

Figure 2: Dispersion of biofilm formed by MSSA clinical isolates grown in RPMI-1640 vs BHI under specified conditions (n=13). A) Streptokinase, unpaired t test, p=0.003. B) Proteinase, Mann Whitney test, p=0.8777. C) Periodate, unpaired t test, p=0.5587. Biofilm dispersion was calculated as a percent of the control wells that were treated with PBS. MSSA: Methicillin-susceptible *Staphylococcus aureus*, RPMI: Roswell Park Memorial Institute. BHI: Brain Heart Infusion, PBS: Phosphate buffered saline.

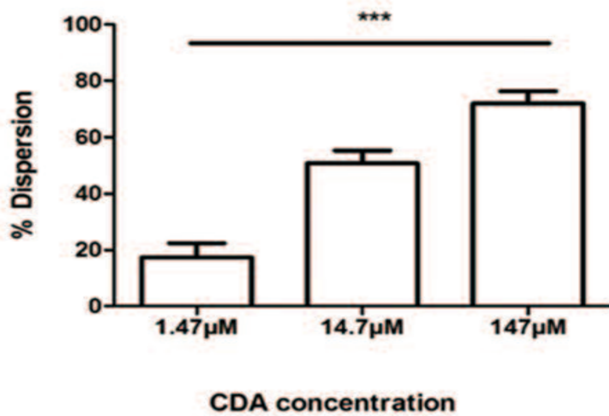

Figure 3: Effect of different concentrations of CDA on dispersion of biofilms formed by MRSA clinical isolates grown in RPMI-1640 under specified conditions and calculated as percent dispersed compared to control wells of the same isolates that were treated with plain solvent (n=7). Kruskal Wallis test, p=0.0003. Error bars represent the standard error of the mean.
CDA: Cis-2-Decenoic acid, MRSA: Methicillin-resistant *Staphylococcus aureus*, RPMI: Roswell Park Memorial Institute.

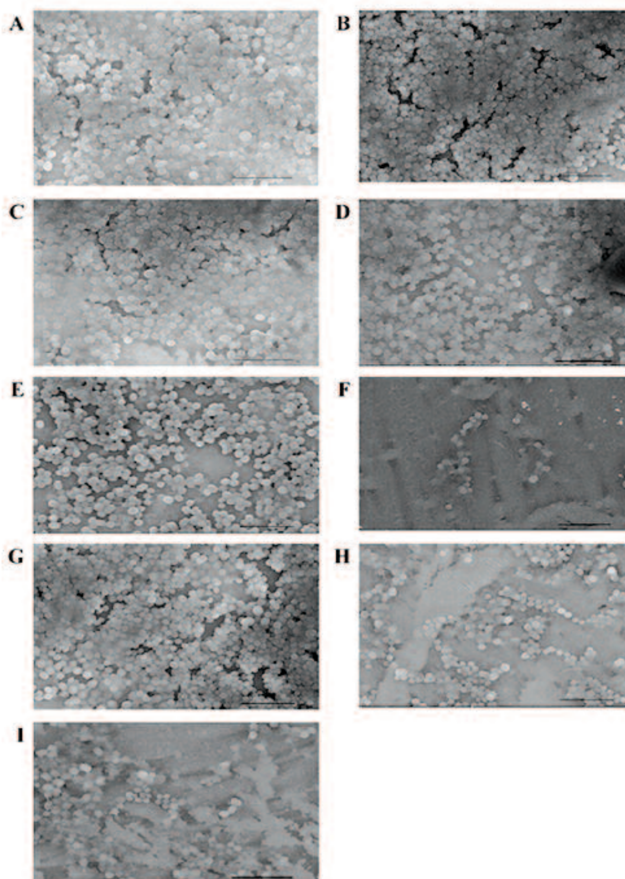

Figure 3: Figure 4: Scanning electron microscope (SEM) micrographs for biofilms formed by isolate MRSA 0194. A) 10% ethyl alcohol treated control, B-F) Treated with CDA 0.62, 1.47, 14.7, 147 or 1470µM, respectively. G) Distilled water-treated control. H-I) Treated with HAM 250 and 500µM, respectively. Scale bars 5µm; magnification power 15000X.
MRSA: Methicillin-resistant *Staphylococcus aureus*, CDA: Cis-2-Decenoic acid, HAM: Hamamelitannin

Discussion

Different mechanisms of biofilm formation have been identified in *S. aureus*. These biofilms vary greatly phenotypically and are differentially regulated.[4] The biofilm matrix is the main component of the biofilm mass that determines the biofilm properties and sensitivity to various treatments.[19,20] Variability in biofilm matrix composition may reflect different response to antibiofilm drugs.

It was proved that when *S. aureus* was allowed to construct biofilms under specific laboratory conditions that mimic the physiological environments, the formed biofilms were quantitatively comparable to that formed in the ordinarily used microbiological media, but were differentially regulated.[6] The objective of the current study was to assess the structure of the biofilm matrix produced by MRSA and MSSA clinical isolates under distinct culture environments, and to appraise the effectiveness of novel antibiofilm drugs in MRSA biofilms under biologically-relevant circumstances.

Under both the tested conditions, MRSA biofilms were highly proteinaceous as indicated by their liability to treatment with proteinase with mean biofilm dispersion of 95.78±2.95% and 92.54±5.53% for RPMI-1640 and BHI biofilms, respectively. However, analysis revealed significantly different medians (Figure 1). Biofilms formed in biologically-relevant conditions were more liable to dispersion by streptokinase compared to that developed in BHI, strongly confirming the previously reported shift of *S. aureus* biofilm from FnBPs-dependent biofilm phenotype to the Coa-dependent fibrin biofilm phenotype when physiological culture conditions were applied.[6] Their responses to sodium meta-periodate also had significantly different means (Figure 1). Biofilms formed in BHI had a wider range of response to sodium meta-periodate. The diverse responses of biofilms formed by different MRSA isolates to sodium meta-periodate were previously reported by Glatthardt T et al.[15] who used TSB supplemented with glucose as a biofilm-supporting medium, and the tested isolates showed dissimilar behaviour and, finally, their biofilms were classified as either proteinaceous, mixed or even polysaccharide.

The responses of MSSA biofilms formed in RPMI-1640 to sodium meta-periodate were more variable compared to that formed in BHI biofilms (p=0.039). Percentages of biofilm dispersion upon treatment with sodium meta-periodate ranged from 19.49% to 89.35% with a median of 52.52% for RPMI-1640-based biofilms compared to a range of 36.51%-71.84% and a median of 56.41% for BHI-based biofilms. This may indicate more homogenous biofilms formed by different MSSA isolates in BHI compared to that

formed in RPMI-1640. The current findings regarding the MSSA biofilms that were formed in BHI support the results reported by Glatthardt T et al. and Chaignon P et al.[15, 21] Moreover, Chaignon P et al.[21] justified this lack of response to be a result of the multi-step mechanism of sodium meta-periodate de-polymerisation of the PNAG macromolecules which involves several modifications followed by mild acidic hydrolysis. This may not be achieved by simple treatment with sodium meta-periodate and subsequent washing.

The current results have proved the existence of proteins and polysaccharides in both MSSA and MRSA biofilm matrix in different ratios, depending on the strain and the culture condition. The different behaviour of MRSA biofilms formed in RPMI-1640 and BHI to streptokinase, proteinase and sodium meta-periodate may indicate different sensitivity to antibiofilm drugs. The use of RPMI-1640 under the specified conditions to support biofilm formation reflects more biologically-relevant situation and, hence, the antibiofilm activity of CDA and HAM on the newly described MRSA biofilm phenotype was evaluated. CDA at 0.62µM has previously been reported to be effective in the dispersion of TSB-supported *S. aureus* biofilms.[18] However, according to our results, at this concentration CDA was sufficient to inhibit *S. aureus* fibrin biofilm formation, but not to induce its dispersion. Furthermore, SEM micrographs showed detectable biofilm dispersion activity without morphological changes on the remaining cells at 14.7 and 147µM. Higher concentration 1470µM caused deformity of the remaining cells that may indicate detergent effect rather than direct biofilm dispersion activity.

Conclusion

Staphylococci biofilm matrix composition and response to antibiofilm drugs varied greatly with the surrounding environment. Therefore, to thoroughly resemble the actual biofilm composition and responsiveness to antibiofilm drugs in the in-vivo infection environment, applying the newly-described biologically-relevant biofilm-supporting conditions during the laboratory studies on *S. aureus* biofilms is strongly recommended.

Limitation: The current study has a limitation as the sample size was not calculated and that can have an effect on the power of the study.

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Disclaimer: The study is part of a PhD thesis.

Conflict of Interest: None.

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