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CLINICAL MICROBIOLOGY - RESEARCH PAPER

Biofilm formation and antibiotic susceptibility of *Staphylococcus* and *Bacillus* species isolated from human allogeneic skin

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Abstract
Human skin banks around the world face a serious problem with the high number of allogeneic skins that are discarded and cannot be used for grafting due to persistent bacterial contamination even after antibiotic treatment. The biofilm formation capacity of these microorganisms may contribute to the antibiotic tolerance; however, this is not yet widely discussed in the literature. Thisstudy analyzed bacterial strains isolated from allogeneic human skin samples,which were obtained from a hospital skin bank that had already been discardeddue to microbial contamination. Biofilm formation and susceptibility topenicillin, tetracycline, and gentamicin were evaluated by crystal violetbiomass quantification and determination of the minimum inhibitoryconcentration (MIC), minimum biofilm inhibitory concentration (MBIC), andminimum biofilm eradication concentration (MBEC) by the broth microdilutionmethod with resazurin dye. A total of 216 bacterial strains were evaluated, and204 (94.45%) of them were classified as biofilm formers with varying degrees ofadhesion. MBICs were at least 512 times higher than MICs, and MBECs were atleast 512 times higher than MBICs. Thus, the presence of biofilm in allogeneicskin likely contributes to the inefficiency of the applied treatments as antibiotictolerance is known to be much higher when bacteria are in the biofilmconformation. Thus, antibiotic treatment protocols in skin banks shouldconsider biofilm formation and should include compounds with antibiofilmaction.

Keywords Allograft contamination · Biofilms · Skin banks · Antibiotic tolerance · Resazurin · Minimum inhibitory concentration

Introduction

Human allogeneic skin grafts are mainly used on patients with severe burns [1], and they promote the temporary closure of chronic wounds and second- and third-degree burns when it is not possible to obtain the patient's skin for autografting or prepare the compromised site to receive the autografting [2–4]. The skin allograft forms a mechanical and biological barrier during the early cicatrization of lesions and reduces the loss of water, electrolytes, proteins, and heat, as well as reducing the rate of infection and exchange of dressings [3, 5, 6]. However, the application of allogeneic skin grafts is limited due to the lack of cadaveric donors and the presence of potential pathogens, such as bacteria of endogenous (donor's own microbiota) or exogenous origin (mortuary environment, tissue recovery team [7], or surgical environment [8]), in the tissue [5].

To avoid transmission of pathogens through skin grafting [5,6], skins are stored in skin banks where a series of microbiological tests are performed to maintain quality and safety control [2, 3, 9]. To date, there is no standardization among skin banks worldwide regarding the methodology, including the antibiotics used and their concentrations [1, 10], for decontamination of human allogeneic skin. Many skin banks worldwide face the problem of having to discard skins due to persistent bacterial contamination [3, 4, 11]. In our previous study using in vitro disk diffusion and minimum inhibitory concentration (MIC) gradient strip methods, we observed that most of the bacteria isolated from allogeneic skins were susceptible to the antibiotics used by the skin banks where they were stored [12]. Therefore, factors other

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than resistance to antibiotics may influence the persistence of these microorganisms after antibiotic treatment.

Bacteria in biofilms exhibit 10–100 times higher antibiotic tolerance [13] than bacteria in the planktonic state [14–19]. The mechanisms of biofilm tolerance involve its development and structure, including a negatively charged extracellular matrix, nutrient and oxygen concentration gradients, easy transfer of resistance genes, expression of efflux pumps, and the presence of persister cells [15, 16, 20, 21]. In addition, current antibiotics were developed to act on planktonic cells; thus, they are ineffective for cells within a biofilm architecture [13, 22, 23].

The presence of bacterial biofilms in other types of grafts, such as prosthetic vascular [24] and bone [25, 26], has been described in previous studies. *Staphylococcus* and *Bacillus*, the most prevalent genera in allogeneic skin [3, 6, 27], are biofilm-forming species [28–31]; however, no studies have evaluated biofilm formation by bacteria isolated from human allogeneic skin.

The objective of this research was to detect and quantify the biofilm formation capacity of *Staphylococcus* and *Bacillus* isolated from allogeneic skin samples that were discarded due to bacterial contamination at the Dr. Roberto Corrêa Chem Skin Bank of the Hospital Santa Casa de Misericórdia, Porto Alegre-RS, and to evaluate the minimum biofilm inhibitory concentration (MBIC) and the minimum biofilm eradication concentration (MBEC) of the antibiotics gentamicin, penicillin, and tetracycline on these biofilms. We aimed to better understand the contribution of biofilm formation on persistent bacterial contamination of allogeneic skin.

Material and methods

Bacterial strains

The 216 strains, including 103 *Staphylococcus* sp. and 113 *Bacillus* sp., used in this study were isolated and identified in a previous study of human skin samples from cadaveric donors provided by the Dr. Roberto Corrêa Chem Skin Bank of the Santa Casa Hospital in Porto Alegre, RS [12]. All the samples had been discarded due to microbial contamination with non-acceptable microorganisms or due to persistence of bacterial contamination after a first cycle of treatment with penicillin (1000 U/mL) and streptomycin (200 mg/mL) or after a second cycle of treatment with vancomycin (50 mg/mL).

Detection and quantification of biofilm biomass

Quantification of biofilm formation by the 216 bacterial strains was performed by the method of Stepanovic et al. [32] with modifications. The strains were recovered on tryptone soya agar (TSA) at 37 °C for 24 h and then adjusted with 0.9% sterile saline to a turbidity of 0.5 according to the McFarland standard. These suspensions were mixed with tryptone soya broth (TSB) to a final concentration of 1.5×10^7 CFU/mL in a total volume of 200 µL and then dispensed in octuplicate into 96-well flat bottom polystyrene tissue culture plates. For the positive control, a suspension of *Staphylococcus epidermidis* (ATCC 35,984) in sterile saline was added to TSB, and 200 µL of TSB broth was used for the negative control. The plates were incubated at 37 °C for 24 h. Afterwards, the wells were aspirated, washed three times with sterile saline, and the plates were shaken lightly to remove all non-adherent bacteria. The remaining adhered cells were fixed with 99% methanol. After 15 min, the methanol was removed and the plates were dried before staining with 2% crystal violet dye. Excess dye was removed by washing with tap water, the plates were dried, and then the adhered, stained cells were solubilized with 160 µL of 100% ethanol. The optical density (OD) of each well was measured in a spectrophotometer at 570 nm.

The strains were classified into four adhesion levels according to the OD values obtained. The OD cutoff point (ODc) was defined as three standard deviations above the average OD of the negative control. The classification was as follows: $OD \leq ODc$, non-adherent; $ODc < OD \leq 2 \times ODc$, weakly adherent; $2 \times ODc < OD < 4 \times ODc$, moderately adherent; $4 \times ODc < OD$, strongly adherent [32]. This classification was used to screen strains for planktonic and biofilm cell viability.

Determination of planktonic bacteria cell viability in the presence of antibiotic

Twelve strains were evaluated for their viability in the planktonic state by determining the MICs of gentamicin, tetracycline, and penicillin by broth microdilution according to the Clinical and Laboratory Standards Institute (CLSI) [33] and CLSI-M4530[34] for *Bacillus* sp. and by the modified resazurin dye method according to Pettit et al. [35] MIC assays were performed in triplicate. The strains *Staphylococcus aureus* ATCC® 29,213 and *Enterococcus faecalis* ATCC® 29,212 were used for quality control ranges.

The antibiotics tetracycline and gentamicin were effective against all the allogeneic skin bacterial strains tested [12]; thus, they were used to compare antibiotic tolerance between cells in the planktonic and biofilm form. Penicillin, one of the most widely used antibiotics among skin banks [10], was also used to evaluate treatments already performed in skin banks.

The antibiotics gentamicin, tetracycline, and penicillin were serially diluted in Mueller Hinton cation-adjusted broth (CMHII), and final concentrations ranged from 512 to 1 µg/mL. The strains were recovered on TSA at 37 °C for 24 h and then adjusted with 0.9% sterile saline to a turbidity of

0.5 according to the McFarland standard. In 96-well flat-bottom polystyrene tissue culture plates, bacterial suspensions were added to 100 µL of diluted antibiotic to a final concentration of 5×10^5 CFU/mL. For the untreated positive controls, the strains were added to 100 µL of antibiotic-free CMHII. For the negative control, 105 µL of CMHII with antibiotic was added to each dilution to detect a possible interaction between the antibiotic and the dye, which would produce a false positive. After incubation for 24 h at 35 °C, 10.5 µL of 20 µM resazurin dye was added to each well and the plates were incubated at 37 °C for various, specific times determined by standardization (data not shown). Then, the plates were assayed visually and by measuring absorbances at 570 nm and 600 nm.

The percentage difference in dye reduction between the treated well and the untreated positive control was used to determine the MIC that is defined as the lowest antibiotic concentration that inhibits ≥ 80% of the metabolic activity in the test well relative to the untreated positive control and is determined by the following formula:

$$\frac{(\varepsilon_{OX})\lambda_2 A\lambda_1 - (\varepsilon_{OX})\lambda_1 A\lambda_2 \text{ of the treated test well}}{(\varepsilon_{OX})\lambda_2 A^\circ\lambda_1 - (\varepsilon_{OX})\lambda_1 A^\circ\lambda_2 \text{of the untreated positivecontrol}} \times 100$$

in which ε_{ox} = the molar extinction coefficient of the oxidized form of the resazurin dye, A = absorbance of the test well, A° = absorbance of the untreated positive control, λ_1 = 570 nm, and λ_2 = 600 nm.

Determination of biofilm bacteria cell viability in the presence of antibiotic

Determination of the viability of strains in the biofilm form was performed according to the methods of Pettit et al. [35] and Flemming et al. [36] with modifications. The MBIC was determined by the broth microdilution method with resazurin dye, and the MBEC was determined by the plating method. The same bacterial strains that were evaluated for their planktonic viability were evaluated for their viability in the biofilm form in the presence of the same antibiotics (penicillin, tetracycline, and gentamicin).

Bacterial strains were cultured on TSA and incubated at 37 °C for 24 h, adjusted to 1.8×10^9 CFU/mL with TSB, and dispensed into the wells of 96-well flat bottom polystyrene tissue culture plates. Then, 200 µL of TSB was added to each well and plates were incubated for 24 h at 37 °C to allow for biofilm formation. For the positive control, 200 µL of TSB was added to the strain without treatment, and 200 µL of TSB was used for the negative control. After incubation, plates were washed with 200 µL of 0.9% sterile saline three times to remove planktonic cells and then dried. Then, 200 µL of CMHII containing gentamicin, tetracycline, or penicillin at concentrations ranging from 512 to 1 µg/mL was added to each well. To the positive control wells, 200 µL of CMHII without antibiotic was added. To the negative control wells, 200 µL CMHII with antibiotic at each dilution was added to detect possible interaction between the antibiotic and the dye.

After incubation for 24 h at 35 °C, 20 µM resazurin dye was added to the wells at 10% of the total volume and incubated for 1 h at 37 °C. The plates were assayed visually and by measuring absorbances at wavelengths of 570 nm and 600 nm. The percentage difference in dye reduction between the treated well and the untreated control was used to determine the MBIC; the same formula described above was used. MBIC was defined as the lowest antibiotic concentration that inhibits ≥ 80% of the metabolic activity in the test well relative to the positive control.

The wells with blue staining (indicating absence of cell viability) were scraped and seeded onto standard plate count agar (PCA). Because resazurin dye is non-toxic, cells can be plated directly from wells after absorbances were measured [36]. After incubation for 24 h at 37 °C, the MBEC was determined. The minimum antibiotic concentration in which there was no growth (0 CFU/mL) on PCA represents the absence of new biofilm growth, which may be extrapolated to the absence of biofilm recolonization capacity in the host [17]. The MBIC and MBEC assays were performed in triplicate.

Statistical analysis

To evaluate associations among the variables analyzed, Pearson's correlation coefficient in Sigma Plot software (13.0) was used. Differences were considered statistically significant when $P < 0.05$.

Results

Detection and quantification of biofilm biomass

Of the 216 bacterial strains evaluated, 204 (94.5%) formed biofilms with some degree of adhesion, and only 12 (5.6%) did not produce biofilms. It was observed that 117 (54.2%) of the strains were classified as strongly adherent, 13 (6.0%) as moderately adherent, and 74 (34.3%) as weakly adherent (Table 1). Among the strongly adherent bacteria, the *Bacillus* sp. strains stood out followed by *Staphylococcus epidermidis*. Most of the *B. cereus* and *S. lugdunensis* strains formed weakly adherent biofilms. Only *B. cereus*, *S. aureus*, and *S. haemolyticus* strains did not form biofilms.

Table 1 Biofilm classification of the analyzed strains

Strain (*n*)	Adhesion level			
	Strong *N* (%)	Moderate *N* (%)	Weak *N* (%)	Non-adherent *N* (%)
S. epidermidis (*n*: 42)	24 (20.5%)	5 (38.5%)	13 (17.6%)	0
S. aureus (*n*: 19)	16 (13.7%)	1 (7.7%)	0	2 (16.7%)
S. haemolyticus (*n*: 13)	9 (7.7%)	0	3 (4.1%)	1 (8.3%)
S. lugdunensis (*n*: 13)	1 (0.9%)	1 (7.7%)	11 (14.9%)	0
S. capitis (*n*: 12)	11 (9.4%)	1 (7.7%)	0	0
S. saprophyticus (*n*: 2)	2 (1.7%)	0	0	0
Staphylococcus sp. (*n*: 2)	2 (1.7%)	0	0	0
B. cereus (*n*: 54)	0	1 (7.7%)	44 (59.5%)	9 (75.0%)
B. subtilis (*n*: 17)	15 (12.8%)	1 (7.7%)	1 (1.3%)	0
B. vallismortis (*n*: 4)	2 (1.7%)	1 (7.7%)	1 (1.3%)	0
B. licheniformis (*n*: 4)	4 (3.4%)	0	0	0
B. pumilus (*n*:1)	0	0	1 (1.3%)	0
Bacillus sp.* (*n*:33)	31 (26.5%)	2 (15.4%)	0	0
Total (*n*: 216)	117 (54.2%)	13 (6.0%)	74 (34.3%)	12 (55.5%)

*Strain could not be identified by MALDI-TOF.

Bacteria cell viability determined by MIC, MBIC, and MBEC

Twelve strains of varying biofilm formation capacity (four of each classification, two species of each genus) were analyzed for cell viability in the presence of antibiotic. The MIC, MBIC, and MBEC data are shown in Table 2. MIC values ranged from < 1 to 128 µg/mL for penicillin, < 1 to 4 µg/mL for tetracycline, and < 1 to 16 µg/mL for gentamicin. Penicillin had the highest MIC values at 128 µg/mL for two *B. cereus* strains (7.9.4 and 8.4.2), 64 µg/mL for *S. aureus* (25.4.2), and 32 µg/mL for *S. lugdunensis* (32.4.2) and *S. capitis* (14.8.2). The highest tetracycline MIC was 4 µg/mL for two *B. subtilis* strains (3.12.2 and 36.10.2). The highest gentamicin MICs were 16 µg/mL for *S. lugdunensis* and 8 µg/mL for *S. capitis*.

The MBICs were 512-fold higher than the MICs for all the antibiotics tested. MBIC values for penicillin ranged from 64 to > 512 µg/mL, which were 4–512 times higher than the MICs for penicillin. The MBIC values for tetracycline ranged from 1 to > 512 µg/mL, which were 1–512 times higher the MIC values. MBIC values for gentamicin

Table 2 MIC, MBIC, and MBEC values for penicillin, tetracycline, and gentamicin against the twelve strains tested

Biofilm	Strain	Species	Penicillin			Tetracycline			Gentamicin		
			MIC	MBIC	MBEC	MIC	MBIC	MBEC	MIC	MBIC	MBEC
				µg/mL			µg/mL			µg/mL	
WEAK	15.6.2	*S. haemolyticus*	< 1	> 512	> 512	< 1	> 512	> 512	< 1	> 512	> 512
	25.4.2	*S. aureus*	64	> 512	> 512	< 1	32	256	< 1	> 512	> 512
	7.9.4	*B. cereus*	128	> 512	> 512	< 1	4	256	< 1	16	> 512
	23.1.2	*B. pumilus*	< 1	64	64	< 1	1	512	< 1	1	256
MODERATE	27.13.4	*S. epidermidis*	16	> 512	> 512	< 1	2	256	< 1	1	> 512
	32.4.2	*S. lugdunensis*	32	> 512	> 512	1	2	64	16	1	> 512
	8.4.2	*B. cereus*	128	> 512	> 512	< 1	1	128	< 1	2	> 512
	16.7.2	*B. subtilis*	4	> 512	> 512	1	> 512	512	1	> 512	128
STRONG	14.8.2	*S. capitis*	32	> 512	> 512	< 1	> 512	256	8	> 512	> 512
	18.7.2	*S. saprophyticus*	< 1	> 512	> 512	< 1	> 512	256	< 1	> 512	> 512
	3.12.2	*B. subtilis*	< 1	256	> 512	4	32	> 512	< 1	< 1	128
	36.10.2	*B. subtilis*	< 1	64	64	4	8	32	< 1	< 1	64

MIC minimum inhibitory concentration, *MBIC* minimum biofilm inhibitory concentration, *MBEC* minimum biofilm eradication concentration.

also ranged from 1 to > 512 µg/mL; however, there were no differences between the MIC and MBIC values for the moderately adherent *S. lugdunensis* strain (32.4.2) and two strongly adherent *B. subtilis* strains (3.12.2 and 36.10.2), whereas the gentamicin MBIC values for weakly adherent *S. haemolyticus* (15.6.2) and *S. aureus*, moderately adherent *B. subtilis*, and strongly adherent *S. saprophyticus* were at least 512 times higher than the gentamicin MICs. The lowest MBIC value was 64 µg/mL for penicillin against weakly adherent *B. pumilus* and strongly adherent *B. subtilis* strain.

MBEC values were also at least 512 times higher than MBIC values. For penicillin, both MBEC and MBIC values ranged from 64 to > 512 µg/mL. The lowest MBEC value was 64 µg/mL for penicillin against weakly adherent *B. pumilus* (23.1.2) and moderately adherent *B. subtilis* (36.10.2). For tetracycline, the MBEC values ranged from 32 to > 512 µg/mL. The MBIC and MBEC values were the same for weakly adherent *S. haemolyticus* (15.6.2), whereas the MBECs were 4–512-fold higher than the MBICs for the other strains. For tetracycline, the lowest MBEC values were 64 µg/mL for moderately adherent *S. lugdunensis* and 32 µg/mL for strongly adherent *B. subtilis*.

Regarding gentamicin, MBEC values ranged from 64 to > 512 µg/mL, which was 32–512 times higher than the MBIC values for all the strains except weakly (15.6.2 and 25.4.2) and strongly adherent (14.8.2 and 18.7.2) *Staphylococcus*. The lowest MBEC value was 64 µg/mL for strongly adherent *B. subtilis* (36.10.2). The MBEC values were lower than the MBIC values for tetracycline and/or gentamicin with strongly adherent *Staphylococcus* strains (14.8.2 and 18.7.2) and moderately adherent *B. subtilis* (16.7.2). The MBIC value was > 512 µg/mL and the MBEC value was 256 µg/mL for tetracycline against strongly adherent *Staphylococcus* strains (14.8.2 and 18.7.2). For moderately adherent *B. subtilis* (16.7.2), the tetracycline MBIC and MBEC were > 512 µg/mL and 512 µg/mL, respectively, and the gentamicin MBIC and MBEC were > 512 µg/mL and 128 µg/mL, respectively.

No correlation was observed between the crystal violet quantified biomass and metabolic activity evaluated by resazurin dye for penicillin ($P = 0.668$), tetracycline ($P = 0.102$), or gentamicin ($P = 0.275$).

Discussion

The high number of human allogeneic skins, which are used for grafting, that are discarded due to bacterial contamination is a serious worldwide problem among skin banks [37, 38]. Even after antibiotic treatment, bacterial contamination of skins persists [3, 4, 11]. Bacteria in the biofilm form are known to be more antibiotic tolerant than bacteria in the planktonic form [13–19]. Studies have identified some of the bacterial species present in human allogeneic skin [3, 6, 27], and other studies [28–31] have shown that these species form biofilms. However, no studies have determined whether biofilm formation by bacterial strains isolated from human allogeneic skin affect susceptibility to the antibiotic treatments currently used in skin banks, thereby contributing to the unsolved bacterial contamination problems.

This study quantified biofilm formation by bacterial strains isolated from human allogeneic skin using the crystal violet staining method. Most of the strains (94.44%) formed biofilms with varying degrees of adherence. Moreover, the majority (54.17%) of the biofilm formers were strongly adherent. These data support the hypothesis that the biofilm form is one of the factors responsible for persistent bacterial contamination in skin banks.

To evaluate the action of the antibiotics gentamicin, penicillin, and tetracycline on the viability of bacterial strains isolated from contaminated human allogeneic skins, MIC and MBIC assays were performed on planktonic and biofilm forms, respectively, using the broth microdilution method with resazurin dye. Antibiotic MICs were least 512 times higher for biofilm forms than planktonic forms of most of the strains, regardless of their weak or strong adherence. The MIC-MBIC ratios could have been found to be greater than 512 times if concentrations below 1 µg/mL and above 512 µg/mL had been tested; for example, some species of *Staphylococcus* (*S. haemolyticus* 15.6.2, weakly adherent *S. aureus* 25.4.2, *S. capitis* 14.8.2, and strongly adherent *S. saprophyticus* 18.7.2) exhibited MICs < 1 µg/mL and MBICs > 512 µg/mL for the antibiotics tested. The MIC and MBIC values did not differ and were < 1 µg/mL for moderately adherent *S. lugdunensis* 32.4.2 and strongly adherent *Bacillus* species 3.12.2 and 36.10.2; however, if concentrations below 1 µg/mL were tested, there may have been a fold difference in these values. Our results generally agree with studies on *Staphylococcus* sp. that show an increase in antibiotic tolerance when cells are in the biofilm form, thus requiring higher antibiotic concentrations for elimination. Ciofu et al. [39]observed a twofold increase in the MBIC compared to the MIC. Pettit et al. [35] reported MBICs at least seven times higher than MICs. Pettit et al. [40] observed MBIC/MIC ratios of at least fivefold. Mottola et al.[41] found the MBICs to be 2–5000 times higher than the MICs and the MBECs to be twofold higher than the MBICs for gentamicin.

We are aware of the limitation of the study concerning the combined used of penicillin and streptomycin in the routine of the skin bank and our "in vitro" testing with a single antimicrobial. In a previous study, we had observed that although streptomycin did not have reference values for the bacterial isolates analyzed, among the antimicrobials used in the skin bank, it presented the second highest value of MIC_{90} (16 µg/mL). On the other hand, amikacin,

gentamicin, and tetracycline presented MIC_{90} values less than the susceptibility breakpoints established for the isolates, being much more effective at lower concentrations than the antimicrobial concentrations already used in the skin bank [12]. Therefore, we had decided to test the antimicrobials penicillin, gentamicin, and tetracycline separately, since this study was all based on the minimum inhibitory concentration of the tested antibiotics and the interpretation of the results, on the CLSI documents. In another study from our group, the antimicrobials penicillin (widely used in skin banks), ceftazidime (described in other studies), gentamicin, and tetracycline (which showed high inhibitory potential against the isolates) were selected to be tested in six different combinations on bacterial isolates in planktonic state (manuscript in preparation).

For grafting, skin allografts should be sterile because recipient patients are often immunosuppressed [6]. Therefore, it is extremely important to determine the MBEC, which represents the absence of the colonization capacity of the biofilm [41]. For the same bacterial strain analyzed, the MBEC was generally much higher than the MBIC for the respective antibiotic. In the case of *Bacillus* strains, which form spores that are not eliminated by antibiotic therapies, upon plating for the MBEC analysis, the spores may germinate leading to the formation of new colonies [42]. Another contributing factor may be the presence of persister cells with slow or absent growth that are also not eliminated by antibiotic treatment [14, 21, 43].

This study showed that higher concentrations of penicillin (higher MBECs) are necessary to eradicate bacterial cells in the biofilm state compared to the other two antibiotics analyzed. This may be due to the higher penicillin resistance of the planktonic cells observed by the high MIC values or due to the presence of β-lactamases in the biofilm matrix that increase biofilm tolerance to β-lactam antibiotics by inactivating them [18, 21]. This finding is extremely relevant because penicillin is one of the antibiotics most used in skin banks at a concentration of 625 μg/mL, and the MBECs for all the strains analyzed were > 512 μg/mL for penicillin except for two strains (*B. pumilus* 23.1.2 and *B subtilis* 36.10.2).

Considering both MBEC assays for biofilm eradication (no growth) and MBIC assays for ≥ 80% inhibition of cellular metabolic activity, MBEC values were expected to be higher than MBIC values [17]. However, strongly adherent *Staphylococcus* species (14.8.2 and 18.7.2) had higher MBIC than MBEC values for tetracycline, and moderately adherent *B. subtilis* (16.7.2) had higher MBIC than MBEC values for tetracycline and gentamicin. Positively charged antibiotics, such as aminoglycosides and tetracycline, may bind to the negatively charged biofilm matrix and be carried along with biofilm bacterial cells during plating for the MBEC analysis, thereby inhibiting new growth. To avoid this problem, cells must be washed prior to plating [14].

The lack of correlation between the crystal violet quantified biofilm adherence level and cell viability after antibiotic therapy has been observed previously [44, 45]. However, another study did find a correlation between these variables and described a difference in the adherence classification as a possible cause of the discrepancy [36].

In this study, most of the isolates were classified as biofilm formers with different degrees of adhesion, MBIC values were at least 512 times higher than MIC values, and MBEC values were at least 512 times higher than MBIC values. Thus, the presence of biofilms in allogeneic skins may be a contributing factor to the inefficiency of the applied treatments as antimicrobial tolerance is known to be much higher when bacteria are in the biofilm conformation. The treatments currently performed in skin banks are based on the existence of cells in the planktonic form only [13, 22, 23]. However, MIC determination is not an adequate method to determine appropriate antibiotic concentrations for skin allograft treatment protocols. Thus, treatment protocols used in skin banks should be designed considering the presence of biofilm-forming bacteria and should include compounds with antibiofilm action [46–48]. In addition, the implementation of sonication techniques and agents, such as peracetic acid [1], that are already known to be more effective against bacteria in the biofilm form would certainly improve treatment.

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Declarations

Ethics approval and consent to participate This study was approved by the Research Ethics Committees of Universidade Federal do Rio Grande do Sul (protocol CAAE 36949514.8.0000.5347) and of Irmandade da Santa Casa de Misericórdia de Porto Alegre (protocol CAAE 45100215.1.0000.5335).

Consent for publication All authors agree to publish this study and to publish its results.

Competing interests The authors declare no competing interests.

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