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Antimicrobial and antibiofilm activities of marine sponge-associated bacteria against multidrug-resistant *Staphylococcus* spp. isolated from canine skin

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A B S T R A C T

Dogs play important roles in our society, thus the concern for their health becomes imperative. *Staphylococcus* spp. are commensal bacterium frequently isolated from canine skin and recognized as zoonotic agents. These bacteria have been becoming increasingly resistant to antimicrobials used to treat infections and to produce biofilm, which further increases their virulence capability and resistance. In this context, sponges-associated bacteria are known as prolific sources of substances with antimicrobial activities, representing a potential to integrate the arsenal of drugs for clinical use. In this study, 121 strains of *Staphylococcus* isolated from healthy or infected dogs were characterized according to their resistance to antimicrobials, as well as to their biofilm production ability. From the total of strains, 82 were resistant to at least one antimicrobial and 40 were multidrug-resistant (MDR). Furthermore, 117 out of 121 were capable to produce biofilm, and within those 36 were classified as strong biofilm producers. A set of fifteen bacterial strains previously isolated from marine sponges were also evaluated for antimicrobial and antibiofilm activities. Among the marine bacteria with antimicrobial activity, eight inhibited the growth of more than 50% of the MDR *Staphylococcus*. In addition, the cell-free supernatant obtained from five sponge-associated bacteria cultures was able to disaggregate more than 50% of the mature biofilm staphylococcal cells. The organic extracts (256 μg/mL) from two potential strains, *Pseudomonas fluorescens* H40 and H41, dissociated the biofilm of a strain classified as MDR and strong biofilm producer in 88.5% and 91.3%, respectively. These marine *Pseudomonas* strains also exhibited a strong activity of antimicrobial and antibiofilm substances. The results suggest that the sponge-associated bacteria analyzed could be potential sources of antimicrobial and antibiofilm substances against MDR and biofilm producers *Staphylococcus* isolated from canine skin.

1. Introduction

Staphylococci is part of canine microbiota, but some species can cause opportunistic infections. *Staphylococcus pseudintermedius* is the predominant species, although the *Staphylococcus schleiferi* and coagulase-negative species are present as well, especially on the skin, upper respiratory tract, ear and mucosal tissues [1,2]. This bacterial genus is considered the most common opportunistic pathogen in dogs, frequently causing dermatitis, otitis externa and pyoderma [3,4].

A major concern is methicillin-resistant staphylococci since these strains usually acquire resistance to other antimicrobial classes [5,6]. Resistant and multidrug-resistant *Staphylococcus* presented in canine microbiota and in canine infections have increased over the years and represent big challenges to empiric treatment, to limitation of antimicrobial options and to the potential for zoonotic transmission [1,5,7,8].

The ability to form biofilm is one of the main virulence determinants studied nowadays in bacteria because it facilitates the adherence to biotic and/or to abiotic surfaces [9,10]. In veterinary medicine, biofilm production of staphylococcal isolates has been registered by several studies. Most of them reported a high rate of biofilm formation by *Staphylococcus* isolated from dogs, with no significant difference related to the origin (body site, healthy or infected dogs) as well as the level of

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antimicrobial susceptibility of the strains [11–15].

The scarce antimicrobial options for the treatment of veterinary medicine infections caused by multidrug-resistant and biofilm-producing *Staphylococcus* spp. leads to the search of novel effective drugs. Marine sponge-associated bacteria has emerged in the last years as a rich source of natural substances with diverse bioactivities, such as antibacterial and antibiofilm with good results against clinical isolates [16–20]. Thus, this study was designed to evaluate the antimicrobial and antibiofilm activities of marine sponge-associated bacteria against multidrug-resistant and biofilm producers *Staphylococcus* isolated from canine skin.

2. Materials and methods

2.1. Bacterial strains

Staphylococcus spp. samples analyzed in this study compose the culture collection of the Molecular Microbiology Laboratory of the Institute of Microbiology at UFRJ, Brazil. One-hundred and twenty-one isolates were selected from the following characteristics: collected from unmedicated adult dogs (1–8 years of age) of both sexes that were either healthy (n = 22), or diagnosed with pyoderma (n = 47) or otitis externa (n = 52). The pure cultures from the laboratory culture collection were reactivated onto BHI (Brain Heart Infusion; Difco, MI, USA) and all isolates were previously identified as: *S. pseudintermedius* (63), *S. schleiferi* (38), *Staphylococcus aureus* (5), *Staphylococcus sciuri* (5), *Staphylococcus cohnii* (4), *Staphylococcus simulans* (2), *Staphylococcus auricularis* (1), *Staphylococcus capitis* (1), *Staphylococcus epidermidis* (1) and *Staphylococcus* sp. (1) [8].

In addition, the reference strains of the American Type Culture Collection (ATCC) *S. aureus* ATCC 29213 and *S. epidermidis* ATCC 35984 were included in the study as indicator and positive control of the antimicrobial and antibiofilm tests, respectively. Besides these, 15 bacterial strains isolated from marine sponges and characterized as antimicrobial producers in previous studies were used: *Bacillus algicola* Mm95 M, *Bacillus circulans* Mm98 M, *Bacillus pumillus* Cc94 M, *B. pumillus* Pc31, *B. pumilus* Pc32, *Bacillus* sp. Ca31 M, *Kocuria* sp. Mm37 M, *Pseudomonas denitrifcans* Mm84 M, *Pseudomonas fluorescens* H40, *P. fluorescens* H41, *Pseudomonas putida* H51, *Pseudovibrio ascidiaceicola* Pm31 M, *Pseudovibrio denitrificans* Cc98 M, *P. denitrificans* Hh95 M, *Pseudovibrio* sp. Cc93 M [19,21,22].

2.2. Susceptibility antimicrobial test

Staphylococcal strains isolated from canine skin were tested for susceptibility to antimicrobial agents by the agar disc diffusion method on Mueller-Hinton Agar (Difco). Twelve commercial antimicrobials (Sensifar, São Paulo, Brazil) commonly used in our local geographic area were included in this study: cefoxitin (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), clindamycin (2 μg), erythromycin (15 μg), gentamicin (10 μg), mupirocin (200 μg), oxacillin (1 μg) penicillin (10 μg), rifampin (5 μg), tetracycline (30 μg), trimethoprim-sulfamethoxazole (25 μg). After following recommendations from the CLSI-VET [23] and measuring the antimicrobial zone diameters, the strains were categorized as either susceptible or resistant. Given the lack of breakpoints for mupirocin in CLSI-VET, susceptibility and differentiation of levels of resistance to this antibiotic were interpreted following the criteria established by CLSI, with no zone of inhibition around 200 μg mupirocin discs defining mupirocin-resistant strains [24]. Multidrug-resistant (MDR) strains were defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories [25].

2.3. Assay for antimicrobial activity

The assay for antimicrobial substance production was performed as described previously by Marinho et al. [26]. Briefly, 20 μL of the bacterial suspension (~10^7 cells/mL) from the marine strain cultures at stationary phase of growth were spotted onto BHI or alternatively Marine (2216; Difco) agar, according to the inability of the strain to grow in BHI. After the growth of each strain at 25 °C, they were exposed to chloroform vapors for 30 min. Right after, 10^5 cells/mL of the indicator strains (*S. aureus* ATCC 29213 and *Staphylococcus* spp. categorized as MDR in susceptibility test) at exponential phase of growth in 3 mL of BHI soft agar were poured over the plates. The plates were incubated at 37 °C for 18 h and the inhibition zones around the spotted strain were measured. An indicator strain was considered susceptible to the activity of a marine strain when it exhibited a clear inhibition zone (≥8 mm). To screen marine bacteria with antimicrobial activity, *S. aureus* ATCC 29213 was used as an indicator strain inhibited by marine bacteria, positive control. All tests were performed in duplicate or triplicate when necessary.

2.4. Evaluation of biofilm production

Biofilm production of all *Staphylococcus* strains was assessed by the quantification method according to Stepanovic et al. [27]. The bacterial suspension (~10^8 CFU/mL) was added to the growth medium (BHI) and loaded into wells of a polystyrene microtiter plate (TPP, Switzerland). The negative control wells contained broth only. The plate was incubated aerobically for 24 h at 37 °C. After incubation, the wells were washed three times with phosphate-buffered saline (PBS; pH7.4). The remaining attached bacteria were heat-fixed by exposure to hot air at 60 °C for 60 min. The plate was stained with 0.2% crystal violet for 15 min at room temperature. After the plate was air-dried, the dye bound to the 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).

All tests were carried out in triplicate, and the strains were divided into the following categories: no biofilm, weak, moderate, or strong biofilm producers [27]. For this, it was necessary to establish the cutoff value (ODc). The ODc was defined as three standard deviations (SD) above the mean OD of the negative control (uninoculated medium): ODc = average OD of negative control + (3 × SD of negative control). Based upon the OD values, the classification was determined as follows: OD ≤ ODc = no biofilm producer, ODc < OD ≤ 2 × ODc = weak biofilm producer, 2 × ODc < OD ≤ 4 × ODc = moderate biofilm producer, and 4 × ODc < OD = strong biofilm producer.

2.5. Antibiofilm effects of marine strain cell-free supernatants

2.5.1. Pre-screening: cell-free supernatants

In a preliminary step, cell-free supernatants of 15 bioactive marine strains were evaluated for their capacity to dissociate mature biofilm of the *S. epidermidis* ATCC 35984 (positive control for biofilm production) and one clinical strain classified as a strong biofilm producer, as aforementioned described. The following procedure was performed: 10^7 cells/mL of the marine strains were inoculated in 3 mL of their respective culture media (BHI or Marine). After 24 h, the grown ones were transferred to 22 mL of the same broth media and incubated at 25 °C for 48 h. The cultures were centrifuged at 4000×*g* for 20 min (Eppendorf, Hamburg, Germany) to separate the cell pellets from the media. To remove all bacterial cells, supernatants were filtered through a 0.22 μm filter.

The detection of antibiofilm activity was performed by the same method according to Stepanovic et al. [27] with modifications. Following an incubation at 37 °C for 24 h, 96-well plates containing pre-formed staphylococcal biofilms were washed three times with PBS. Two-hundred microliters of the cell-free supernatants from the bioactive bacteria cultures were added to the wells and control samples of each staphylococcal biofilm were kept without the addition of bioactive supernatants. Subsequently to another incubation round (24 h, 37 °C), the plates were again washed in PBS, heat-fixed and stained as described

above. The ability to dissociate biofilm was expressed as antibiofilm activity (%), by applying the following formula: ($OD_{control}$ - OD_{sample}/$OD_{control}$) × 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.

In order to perform the same tests on all strains of *Staphylococcus* spp. classified as strong biofilm producers, the marine bacterial strains with the greatest potential for antibiofilm activity were selected according to the following criteria: those cell-free supernatants that were able to dissociate at least 25% of the biofilm compared to the control of the same untreated strain.

2.5.2. Screening: biofilm dissociation of strong biofilm-producing *Staphylococcus spp.*

The ability to dissociate the mature biofilm of the strong biofilm-producing *Staphylococcus* spp. was analyzed considering the marine strains selected as explained above. The biofilm dissociation was assessed spectrophotometrically in the presence of or without the cell-free supernatants from bioactive marine bacteria. The reduction of biofilm formation of each clinical strain was also expressed as antibiofilm activity (%). 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.

2.6. Metabolite extraction of the cell-free supernatants

The cell-free supernatant from each marine strain that strongly dissociated the mature biofilm of the strong biofilm-producing *Staphylococcus* spp. was selected for initial chemical characterization by extraction of bioactive compounds. The metabolite extraction was performed as previously described [19]. Briefly, total biomass obtained from a 500 mL culture grown in BHI medium for 24 h at 25 °C was centrifuged (11000 × *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 aqueous phase was filtered (Millipore 0.22 μm), lyophilized and weighed. For the assays, the organic extract was dissolved in BHI supplemented with 10% dimethyl sulfoxide (DMSO) at a concentration of 5 mg/mL and the lyophilized aqueous residue at the same concentration in BHI medium.

2.7. Determination of the minimum biofilm eradication concentration

Each extract was tested against a clinical MDR and strong biofilm-producing *Staphylococcus* spp. in serial dilutions ranging from 1024 to 1 μg/mL in BHI medium as described for the cell-free supernatants in the previous section. All tests were carried out in triplicate and the plate was incubated aerobically for 24 h at 37 °C. The minimum biofilm eradication concentration (MBEC) was determined considering the lowest concentration of each extract capable of dissociating mature biofilm calculated by applying the formula shown in section 2.5.1.

3. Results

3.1. Susceptibility test of Staphylococcus isolated from canine skin

From 121 staphylococcal strains, 82 (67.8%) were resistant to at least one tested antimicrobial. None of the antimicrobials used showed any activity against all *Staphylococcus*, each of them found at least five resistant strains. Penicillin was the less active antimicrobial (56 resistant strains) and mupirocin, the most active (five resistant strains). In addition, 40 (33.0%) *Staphylococcus* spp. isolates were characterized as MDR bacteria, among these 31 (77.5%) were *S. pseudintermedius*. The highest number of MDR strains came from canine pyoderma cases, 22 isolates; whereas 12 bacteria were isolated from otitis cases and 6 isolated from healthy dogs (Table 1).

In general, the isolates were resistant mainly to penicillin and the second largest resistance rate was detected to trimethoprim-sulfonamide and macrolide, followed by the classes tetracycline, lincosamide and aminoglycoside. Resistance rates of less than 20% were observed for the classes quinolone, rifamycin and amphenicol.

3.2. Antibacterial activity of sponge-associated bacteria

To evaluate the inhibitory activity of the 15 sponge-associated bacteria, 40 *Staphylococcus* strains characterized as MDR were used as indicators strains in this assay. Thus, nine marine bacteria presented antibacterial activity (Suppl. Fig. 1). However, the strains *B. pumillus* Pc31 and Pc32, *P. denitrifcans* Mm84 M, *P. fluorescens* H40 and H41, *P. putida* H51, *P. denitrificans* Cc98 M and Hh95 M inhibited more than 50% of the MDR strains from canine skin. Among them, *P. fluorescens* H40 and H41 were the most effective bacteria: they inhibited the growth of 37 (92.5%) MDR *Staphylococcus* spp. Only two *Staphylococcus* strains isolated from pyoderma cases, *S. schleiferi* 43d and *S. pseudintermedius* 57d, were resistant to the activity of all the marine bacteria (Table 2).

3.3. Evaluation of biofilm production

All staphylococcal strains were evaluated for quantitative biofilm production (Suppl. Table 1). Among 121 staphylococcal strains, 117 (97.0%) were able to produce biofilm *in vitro* (Fig. 1A). Among the biofilm producers, 39 (33.3%) strains were also classified as MDR. Considering the 36 strains as strong biofilm producers, half of them were also MDR. None of the 47 staphylococcal strains from pyoderma were characterized as non-biofilm producers, most of them were strong biofilm producers (Fig. 1B).

3.4. Effects of marine strains supernatants on biofilms

Only sponge-associated bacteria able to grow in BHI were selected for antibiofilm activity tests against *Staphylococcus* spp., because Marine media is not suitable for staphylococcal biofilm formation tests and showed changes in pre-formed biofilms when it was added after 24 h of growth (data not shown). Therefore, each one of the bioactive cell-free supernatants from marine strains *P. fluorescens* H40, *P. fluorescens* H41, *P. putida* H51, *B. pumillus* Pc31 and *B. pumillus* Pc32 were used for antibiofilm activity pre-screening against *S. epidermidis* ATCC 35984 and *S. pseudintermedius* I2 (isolated from canine otitis externa), both strong biofilm producers. It was considered a positive antibiofilm activity dissociation ≥25% compared to control (sterile BHI media added to staphylococcal mature biofilm). The results indicated that the cell-free supernatants from the marine strains had bioactive substances able to dissociate mature biofilm formed by the tested *Staphylococcus* spp. strains. In particular, *P. fluorescens* H40 and *P. fluorescens* H41 showed a strong antibiofilm activity (≥60% of biofilm dissociation) against *S. epidermidis* ATCC 35984 and of *S. pseudintermedius* I2 (Fig. 2). The bioactive supernatants from the marine strains H51, Pc31 and Pc32 did not reduce the optical density of mature biofilm of the *S. epidermidis* ATCC 35984 or *S. pseudintermedius* I2 strains by at least 25%.

Then, the bioactive cell-free supernatants from marine strains *P. fluorescens* H40 and H41 were selected to be tested against all *Staphylococcus* spp. classified as strong biofilm producers (Suppl. Table 1). In general, the cell-free supernatants from these marine strains showed some biofilm dissociation level against 35 of the 36 isolates tested (Fig. 3). Only one clinical strain, *S. schleiferi* subsp. *coagulans* isolated from pyoderma case, was resistant to antibiofilm activity of the *P. fluorescens* H40 and H41. Even the H41 bioactive cell-free supernatant strongly dissociated (>75%) the mature biofilm of three strains of

Table 1
Resistance profile to antimicrobial of *Staphylococcus* isolated from canine skin.

canine skin	isolates (n = 121)	antimicrobial											
		cfo	cip	cli	clo	eri	gen	mup	oxa	pen	rif	sut	tet
Healthy (n = 22)	*S. aureus* (1)	-	-	-	-	-	-	-	-	1	-	-	-
	S. capitis (1)	-	-	-	-	-	-	-	-	-	-	-	-
	S. cohnii (2)	1	-	-	-	-	-	-	1	1	-	-	-
	S. epidermidis (1)	-	-	-	-	-	-	1	-	-	-	-	1
	S. pseudintermedius (9)	-	2	2	1	5	1	-	-	6	1	5	3
	S. schleiferi (5)	-	-	-	-	-	-	-	-	1	-	-	-
	S. sciuri (2)	-	-	-	-	1	-	1	-	2	-	-	-
	Staphylococcus sp. (1)	-	-	-	-	-	-	1	-	1	-	-	-
Subtotal		**1**	**2**	**2**	**1**	**6**	**1**	**3**	**1**	**12**	**1**	**5**	**4**
Otitis (n = 52)	*S. aureus* (3)	1	-	-	-	-	-	-	1	3	1	2	-
	S. auricularis (1)	-	1	-	1	1	1	-	-	1	-	1	1
	S. cohnii (1)	-	-	-	-	1	-	-	-	-	-	-	-
	S. pseudintermedius (21)	1	5	5	1	8	6	1	1	11	2	7	7
	S. schleiferi (22)	-	1	1	-	3	8	-	-	1	-	-	-
	S. sciuri (2)	1	-	2	-	1	-	-	1	1	-	-	-
	S. simulans (2)	-	-	-	-	-	1	-	-	-	-	-	-
Subtotal		**3**	**7**	**8**	**2**	**14**	**16**	**1**	**3**	**17**	**3**	**10**	**8**
S aureus (1)	-	-	-	-	1	-	-	-	1	-	-	-	
Pyoderma (n = 47)	*S. cohnii* (1)	-	-	1	-	1	-	-	-	1	-	-	-
	S. pseudintermedius (33)	-	7	12	4	13	6	-	-	20	3	19	14
	S. schleiferi (11)	2	1	3	1	4	2	1	2	4	2	5	3
	S. sciuri (1)	-	-	-	-	-	-	-	-	1	-	-	-
	2	**8**	**16**	**5**	**19**	**8**	**1**	**2**	**27**	**5**	**24**	**17**	
Total		**6**	**17**	**26**	**8**	**39**	**25**	**5**	**6**	**56**	**9**	**39**	**29**
Resistance rate (%)		**5.0**	**14.0**	**21.5**	**6.6**	**32.2**	**20.7**	**4.1**	**5.0**	**46.3**	**7.4**	**32.2**	**24.0**

cfo: cefoxitin, cip: ciprofloxacin, cli: clindamycin, clo: cloramphenicol, eri: erythromycin, gen: gentamicin, mup: mupirocin, oxa: oxacillin, pen: penicillin, rif: rifampin, sut: trimethoprim-sulfamethoxazole, tet: tetracycline.
cfo: cefoxitin, cip: ciprofloxacin, cli: clindamycin, clo: chloramphenicol, eri: erythromycin, gen: gentamicin, mup: mupirocin, oxa: oxacillin, pen: penicillin, rif: rifampin, sut: trimethoprim-sulfamethoxazole, tet: tetracycline; -: strains resistant to activity of the all marine bacteria.

S. pseudintermedius isolated from pyoderma cases and two of which were also classified as MDR.

3.5. Antibiofilm activity of extracts

To investigate whether the antibiofilm activity from *P. fluorescens* H40 and *P. fluorescens* H41 cultures on mature biofilm might be due to the organic or aqueous fraction, the respective fractions were evaluated at different concentrations against *S. pseudintermedius* I2, a strain classified as strong biofilm producer and MDR. No or weak (≤25% compared to control) dissociation effect on the biofilm were observed for the aqueous fraction from *P. fluorescens* H40 and H1. However, both organic extracts acted by dissociating biofilm of the *S. pseudintermedius* I2 at all concentrations tested (from 1 to 1024 μg/mL). The antibiofilm activity rates and the MBEC are listed in Suppl. Table 2. The mature biofilm of the *S. pseudintermedius* I2 was dissociated by 88.5% and 91.3% in the presence of the 256 μg/mL of the bioactive extracts from the *P. fluorescens* H40 and H41 cultures, respectively. In this context, ethyl acetate extracts of these marine strains showed antibiofilm activity against clinical strains biofilm producers and MDR.

4. Discussion

Marine sponge-associated bacteria are rich sources of bioactive compounds and are well known for their antimicrobial activity. However, reports are scanty for their antibiofilm activity [16,17,20,28]. Furthermore, marine bacterial extracts are yet to be tapped for their antimicrobial and antibiofilm activities against veterinary clinical strains, including *Staphylococcus* spp. from canine skin.

The isolates analyzed in this study are known as the major staphylococcal species in canine infections and the antimicrobial resistance was common. All species isolated, mainly *S. pseudintermedius* followed by *S. schleiferi,* were resistant to at least one antimicrobial drug, except one strain of *S. capitis* from a healthy dog. The degree of multidrug-resistance was similar to that of the previous studies, as well as the highest number of MDR *S. pseudintermedius* strains that came from canine pyoderma and otitis cases [1,5,7,13,29]. The high rates of MDR isolates could be explained by the fact that the antimicrobials tested in this work are commonly encountered in the principal formulations available for skin infections treatment. Then, the increased or even indiscriminate use of those drugs may be leading to the selection of resistant strains of staphylococci from dogs [29].

The production of the enzyme beta-lactamase is the major mechanism by which staphylococci acquire resistance [5,7]. Therefore, the incidence of resistance to penicillin observed in the present study was not surprising, since it has reported data of the *S. pseudintermedius* strains of canine origin producing beta-lactamases [4,29]. In this study, an important portion of isolates showed also a pattern of resistance to drugs belonging to classes trimethoprim-sulfonamide, macrolide, tetracycline, lincosamide and aminoglycoside. Antimicrobial resistance to fluoroquinolone, rifamycin and amphenicol were also observed among the strains analyzed. Our results are in accordance with other reports about the antimicrobial resistance in *Staphylococcus* spp. isolated from dogs [7,13]. The increase in resistance to antimicrobials in these isolates, especially in cases of canine dermatitis, presents a great challenge in controlling infections by limiting therapeutic options. Therefore, the search for new substances that will strengthen the arsenal to fight these infections is necessary. In this context, marine sponges and their associated microorganisms are gaining prominence, with several substances with antimicrobial action already described [18,19,30,31].

The marine strains *B. pumillus* Pc31 and Pc32, *P. denitrifcans* Mm84 M, *P. fluorescens* H40 and H41, *P. putida* H51, *P. denitrificans* Cc98 M and Hh95 M inhibited more than 50% of the MDR strains from canine skin. Among them, *P. fluorescens* H40 and H41 were the most effective bacteria. They inhibited the growth of 92.5% of the *Staphylococcus* isolates. This high frequency of activity against *Staphylococcus* strains was also observed in a previous study [21]. The bioactive fractions of *P. fluorescens* H40 and H41 cultures were obtained and one of

Table 2
Staphylococcus spp. multidrug-resistant isolated from canine skin and the susceptibility profile to sponge-associated bacteria.

Dog	Isolates	MDR profile	susceptibility profile to sponge-associated bacteria
Healthy (n = 6)	*S. pseudintermedius* P1	pen, rif, tet	Cc98 M, H40, H41, H40, H41, H51, Mm84 M, Pc31, Pc32
	S. pseudintermedius P4	eri, pen, sut	Cc98 M, H40, H41, H40, H41, H51, Hh95 M, Mm84 M, Pc32
	S. pseudintermedius P7	eri, pen, sut, tet	Cc98 M, H40, H41, H40, H41, H51, Hh95 M, Mm84 M, Pc32
	S. pseudintermedius P8	cip, eri, gen, pen, sut	Cc98 M, H40, H41, H40, H41, H51, Mm84 M, Pc31, Pc32
	S. pseudintermedius P33	cip, eri, pen, sut	Cc98 M, H40, H41, H40, H41, H51, Mm84 M, Pc31, Pc32
	S. pseudintermedius P35	eri, pen, sut	Cc98 M, H40, H41, H40, H41, H51, Hh95 M, Mm84 M, Pc32
Otitis externa (n = 12)	*S. aureus* 116	cfo, pen, oxa, sut	Cc98 M, H40, H41, H51, Hh95 M, Mm84 M, Pc31, Pc32
	S. aureus 117	pen, rif, sut	Cc98 M, H40, H41, H51, Hh95 M, Mm84 M, Pc31, Pc32
	S. auricularis 107	cip, clo, eri, gen, pen, sut, tet	H40, H41, H51, Mm84 M
	S. pseudintermedius 101	cip, cli, eri, gen, pen, rif, sut, tet	H40, H41, H51, Mm84 M, Pc31, Pc32
	S. pseudintermedius 106	cip, cli, clo, eri, gen, pen, sut, tet	Cc98 M, H40, H41, H51, Hh95 M, Mm84 M, Pc31, Pc32
	S. pseudintermedius 83	eri, pen, sut, tet	Cc98 M, H40, H41, H51, Mm84 M, Pc31, Pc32
	S. pseudintermedius 93	eri, cip, cli, pen, sut, tet	Cc98 M, H40, H41, H51, Mm84 M, Pc31, Pc32
	S. pseudintermedius 95	cip, cli, eri, gen, pen, rif, sut, tet	Cc98 M, H40, H41, H51, Hh95 M, Mm84 M, Pc31, Pc32
	S. pseudintermedius I2	cip, eri, pen, sut	Cc98 M, H40, H41, H51, Mm84 M, Pc31, Pc32
	S. pseudintermedius I9	cfo, eri, mup, pen, oxa, tet	Cc98 M, H40, H41, H51, Hh95 M, Mm84 M, Pc31, Pc32
	S. schleiferi I12	cip, eri, gen,	Cc98 M, H40, H41, H51, Hh95 M, Mm84 M, Pc31, Pc32
	S. sciuri 85	cfo, cli, eri, oxa, pen	Cc98 M, H40, H41, H51, Hh95 M, Mm84 M
Pyoderma (n = 22)	*S. cohnii* 67d	cli, eri, pen	H40, H41, H51, Mm84 M, Pc31, Pc32
	S. pseudintermediu 81d	cip, clo, eri, gen, pen, sut, tet	Cc93 M, H40, H41, H51, Hh95 M, Mm84 M, Pc31, Pc32
	S. pseudintermedius 24d	cli, clo, eri, gen, pen, tet	Cc93 M, H40, H41, Hh95 M, Mm84 M, Pc31, Pc32
	S. pseudintermedius 25d	cli, eri, gen, pen, rif, sut	H40, H41, Hh95 M, Mm84 M, Pc31, Pc32
	S. pseudintermedius 26ad	cip, cli, eri, gen, pen, sut	Cc98 M, H40, H41, Hh95 M, Mm84 M, Pc31, Pc32
	S. pseudintermedius 26d	cip, cli, clo, eri, pen, rif, sut, tet	Cc98 M, H40, H41, Hh95 M, Pc31
	S. pseudintermedius 27d	cip, pen, sut	Cc98 M, H40, H41, H51, Pc31, Pc32
	S. pseudintermedius 29ad	cip, eri, pen, sut	Cc98 M, H40, H41, Pc31, Pc32
	S. pseudintermedius 44d	cip, cli, eri, pen, rif, sut, tet	H40, H41, Pc31, Pc32
	S. pseudintermedius 54d	pen, sut, tet	Cc98 M, H40, H41, H51, Hh95 M, Mm84 M, Pc31, Pc32
	S. pseudintermedius 55d	cip, eri, pen, sut, tet	Cc98 M, H40, H41, Hh95 M, Pc31, Pc32
	S. pseudintermedius 57d	eri, pen, sut	–
	S. pseudintermedius 62d	eri, pen, sut, tet	Cc98 M, H40, H41, H51, Hh95 M, Mm84 M, Pc31
	S. pseudintermedius 64d	gen, pen, sut, tet	H40, H41, H51, Mm84 M, Pc31, Pc32
	S. pseudintermedius 65d	eri, pen, tet	Cc98 M, H40, H41, H51, Hh95 M, Mm84 M, Pc31, Pc32
	S. pseudintermedius 68d	pen, sut, tet	Cc93 M, Cc98 M, H40, H41, Hh95 M, Mm84 M, Pc31, Pc32
	S. pseudintermedius 71d	cli, eri, sut	Cc93 M, H40, H41, H51, Hh95 M, Mm84 M, Pc31, Pc32
	S. pseudintermedius 76d	clo, eri, gen, pen, sut, tet	Cc93 M, H40, H41, H51, Mm84 M, Pc31, Pc32
	S. schleiferi 22d	cip, clo, eri, pen, rif, sut	Cc93 M, Cc98 M, H40, H41, H51, Hh95 M, Mm84 M, Pc31, Pc32
	S. schleiferi 43d	cfo, cli, eri, gen, oxa, pen, rif, sut, tet	–
	S. schleiferi 50d	cfo, eri, oxa, pen, sut	H40, H41, H51
	S. schleiferi 58d	cli, mup, sut	H40, H41, H51

cfo: cefoxitin, cip: ciprofloxacin, cli: clindamycin, clo: chloramphenicol, eri: erythromycin, gen: gentamicin, mup: mupirocin, oxa: oxacillin, pen: penicillin, rif: rifampin, sut: trimethoprim-sulfamethoxazole, tet: tetracycline; -: strains resistant to activity of the all marine bacteria.

antimicrobial substances was identified as diketopiperazine cyclo-(-L-Leu-L-Pro). This substance was bactericidal for strains of *S. aureus* and *Pseudomonas aeruginosa* [19]. Other analysis related to *P. fluorescens* H41 demonstrated that genes involved in the biosynthesis of the pyoverdine siderophore are related to the antimicrobial activity [32]. Our results confirm that sponge-associated bacteria, mainly the previously characterized *P. fluorescens* H40 and H41 strains, have a high potential for producing a wide array of antimicrobial substances active against multidrug-resistant bacteria. Additionally, our work also comes to complement the fewer reports on the antibiofilm potential of marine *Pseudomonas* strains [33,34].

In addition to the problem of the difficulty in treating animals with staphylococcal infections resistant to conventional antimicrobials, biofilm-forming strains also contribute to therapeutic failure. Biofilm formation is currently recognized as an important virulence factor in *Staphylococcus* [9]. Through biofilm the bacteria are able to evade the host's immune system. It also is characterized by an inherent resistance to antimicrobials and by an increased rate of horizontal genetic transfer leading to the acquisition and spread of antimicrobial resistance and multidrug-resistance [35]. In this study, an elevated frequency (97%) of *Staphylococcus* spp. biofilm producers was observed whether the dog was healthy or sick (pyoderma or otitis externa). Furthermore, a correlation of 33% was found between multidrug-resistance and the ability to produce biofilm, suggesting that MDR staphylococci are more prone to produce large quantities of the extracellular matrix [12,13].

Considering the problem of therapeutic failure in the treatment of infections caused by biofilm-forming bacteria, we also evaluated the potential of substances produced by marine bacteria as an alternative to

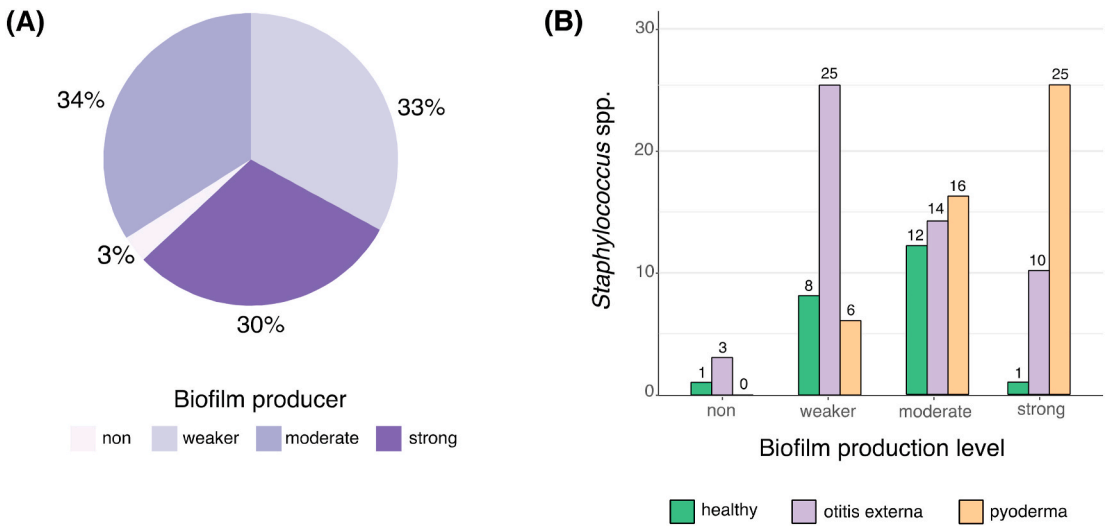

Fig. 1. Biofilm production by *Staphylococcus* spp. isolated from canine skin. (A) Distribution of the isolates according to biofilm production. (B) Distribution of staphylococcal strains isolated from different origins according to biofilm production.

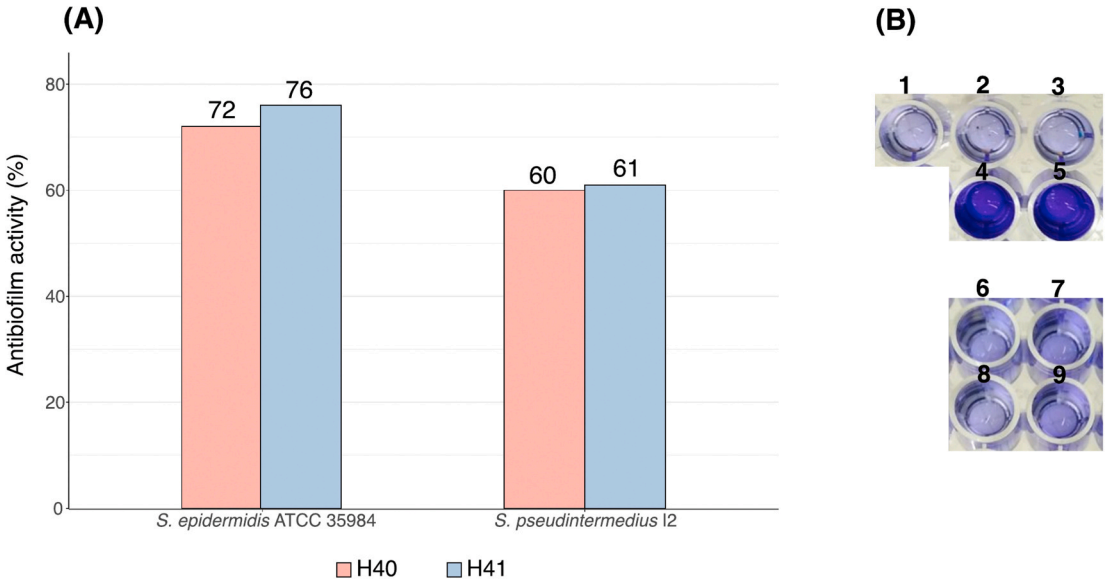

Fig. 2. Antibiofilm activity of the cell-free supernatants from *P. fluorescens* H40 and H41 cultures on mature biofilms. (A) Percentages of the antibiofilm activities against biofilms of *S. epidermidis* ATCC 35984 and *S. pseudintermedius* I2 strains. (B) 96-well plate showing mature biofilms untreated and treated after crystal violet staining. Control wells: (1) sterile growth medium (BHI); (2 and 3) *P. fluorescens* H40 and H41 cell-free supernatants, respectively; (4) *S. epidermidis* ATCC 35984; (5) *S. pseudintermedius* I2. Test wells: (6 and 8) *S. epidermidis* ATCC 35984 treated with H40 and H41 cell-free supernatants, respectively; (7 and 9) *S. pseudintermedius* I2 treated with H40 and H41 cell-free supernatants, respectively.

dissociate these bacterial communities present in a mature biofilm. The potential of the marine strains *P. fluorescens* H40 and H41 was demonstrated one more time when the cell-free supernatants and organic extracts from their cultures showed a good antibiofilm activity against *Staphylococcus* spp. strong biofilm producers including MDR strains isolated from canine skin. The active organic extracts were obtained from a medium polarity solvent considered environmentally friendly. Ethyl acetate is commonly employed in the extraction of microbial natural products yielding a broad range of bioactive substances from different classes of secondary metabolites. As described in previous study of our research group, these organic extracts presented minimum biofilm eradication concentration value similar to the CC_{50} observed for Vero cell and Hep-2 cancer cell lines [19]. Despite the isolation and identification of the antibiofilm substances have not been pursued in this study, previous data suggest that the extracts of *P. fluorescens* H40 and H41 are complex mixtures of compounds of different polarities. Moreover, the antibiofilm activity observed were not due to a direct inhibition of the growth rate of the target *Staphylococcus* strains, but to a dissociation of their extracellular matrix (mature biofilm). Based on these results, the development of new antiseptic or antibiotic topical preparations from these substances is an important and worthwhile task for the future.

Other few sponge-associated bacteria have been found to possess antibiofilm activities against pathogenic bacteria [17,20,28,36]. However, this is the first study that demonstrated an antibiofilm activity due to the breakdown of mature biofilm. This is a differential among other studies since the animal will start the antimicrobial therapy when the symptomatic infection is already installed. The destruction of a pre-formed biofilm will hopefully guarantee an increased efficacy of the antimicrobial treatment, especially if the own antibiofilm agent also

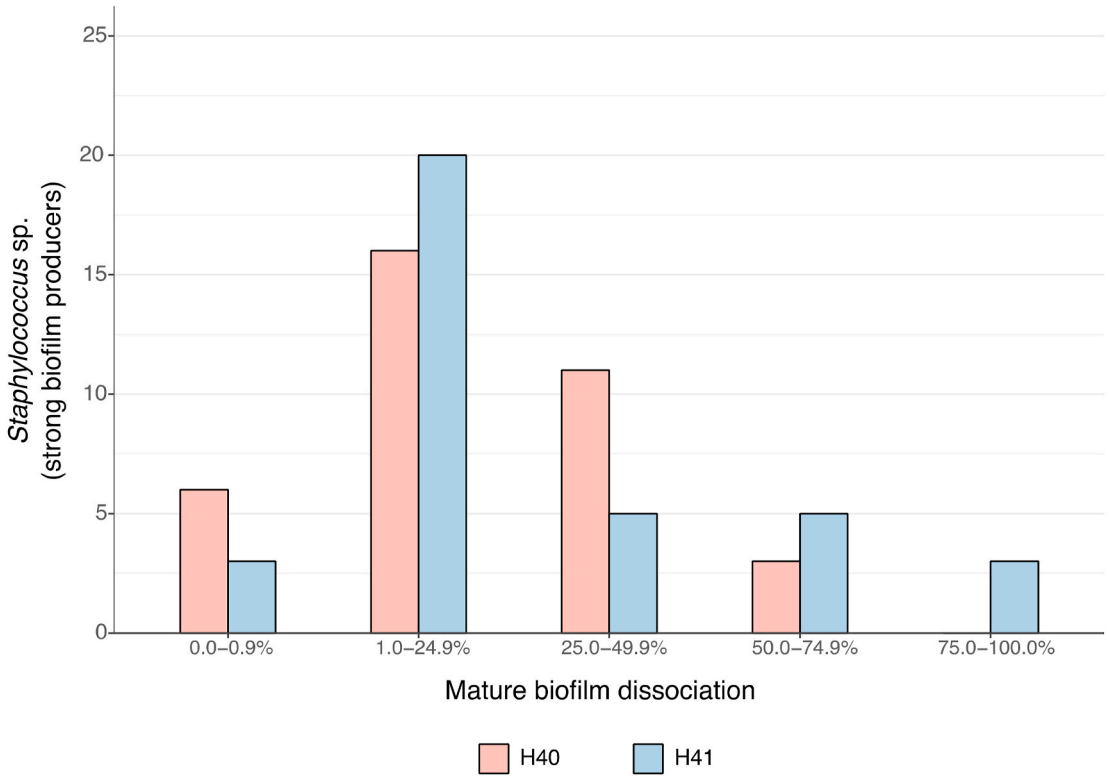

Fig. 3. Biofilm dissociation level caused by cell-free supernatants from marine strains *P. fluorescens* H40 and *P. fluorescens* H41 against strong biofilm-producing *Staphylococcus* spp.

possess an antimicrobial activity. It is known that the occurrence of veterinary biofilm-mediated infections as well as the antimicrobial resistance of staphylococcal isolates from cases of canine skin infections appear to be increasing worldwide [11–13,29]. To the best of our knowledge, this is the first report on marine sponge-associated bacteria that produce antimicrobial and antibiofilm substances with potential for the control and treatment of the skin staphylococcal infections in dogs, opening up new possibilities for the applications of bioactive metabolites from sponge-associated microorganisms in veterinary medicine.

Credit author statement

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Declaration of competing interest

No conflict of interest is declared.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micpath.2020.104612.

References

[1] C.L. Cain, Antimicrobial resistance in staphylococci in small animals, Vet. Clin. North Am. - Small Anim. Pract. 43 (2013) 19–40.
[2] A. Moodley, N. Chandra, S.C. Ba, S. Saxmose, L. Guardabassi, *Staphylococcus pseudintermedius* colonization patterns and strain diversity in healthy dogs: a cross-sectional and longitudinal study Vet, Microbiol. 160 (2012) 420–427.
[3] B. Penna, W. Mendes, R.F. Rabello, W. Lilenbaum, Isolation of meticillin-resistant staphylococci in canine skin infections in Rio de Janeiro, Brazil, Vet. Dermatol. 24 (2013) 373–375.
[4] M. Ruzauskas, N. Couto, A. Pavilonis, I. Klimiene, R. Siugzdiniene, M. Virgailis, L. Vaskeviciute, L. Anskiene, C. Pomba, Characterization of *Staphylococcus pseudintermedius* isolated from diseased dogs in Lithuania, Pol. J. Vet. Sci. 19 (2016) 7–14.
[5] N. Beça, L.J. Bessa, A. Mendes, J. Santos, L. Leite-Martins, A.J. Matos, P.M. da Costa, Coagulase-positive *Staphylococcus*: prevalence and antimicrobial resistance, J. Am. Anim. Hosp. Assoc. 51 (2015) 365–371.
[6] J.S. Weese, E. van Duijkeren, Methicillin-resistant *Staphylococcus aureus* and *Staphylococcus pseudintermedius* in veterinary medicine, Vet. Microbiol. 140 (2010) 418–429.
[7] B. Duim, K.M. Verstappen, E.M. Broens, L.M. Laarhoven, E. Van Duijkeren, J. Hordijk, Changes in the population of methicillin-resistant *Staphylococcus pseudintermedius* and dissemination of antimicrobial-resistant phenotypes in The Netherlands, J. Clin. Microbiol. 54 (2016) 283–288.
[8] C.C. Rossi, B.A.B. Salgado, E.M. Barros, P.A. de Campos-Braga, M.N. Eberlin, W. Lilenbaum, M. Giambiagi-deMarval, Identification of *Staphylococcus epidermidis* with transferrable mupirocin resistance from canine skin, Vet. J. 235 (2018) 70–72.
[9] L. de Vor, S.H.M. Rooijakkers, J.A.G. van Strijp, Staphylococci evade the innate immune response by disarming neutrophils and forming biofilms, FEBS Lett. 594 (2020) 2556–2569, https://doi.org/10.1002/1873-3468.13767.

[10] A.D. Verderosa, M. Totsika, K.E. Fairfull-Smith, Bacterial biofilm eradication agents: a current review, Front. Chem. 7 (2019) 824. https://doi: 10.3389/fchem.2019.00824.
[11] P. Casagrande-Proietti, V. Stefanetti, D.R. Hyatt, M.L. Marenzoni, S. Capomaccio, M. Coletti, A. Bietta, M.P. Franciosini, F. Passamonti, Phenotypic and genotypic characterization of canine pyoderma isolates of *Staphylococcus pseudintermedius* for biofilm formation, J. Vet. Med. Sci. 77 (2015) 945–951.
[12] J.-I. Han, C.-H. Yang, H.-M. Park, Emergence of biofilm-producing *Staphylococcus pseudintermedius* isolated from healthy dogs in South Korea, Vet. Q. 35 (2015) 207–210.
[13] G. Meroni, J.F. Soares-Filipe, L. Drago, P.A. Martino, Investigation on antibiotic-resistance, biofilm formation and virulence factors in multi drug resistant and non multi drug resistant *Staphylococcus pseudintermedius*, Microorganisms 16 (7) (2019) E702.
[14] A. Singh, M. Walker, J. Rousseau, J.S. Weese, Characterization of the biofilm forming ability of *Staphylococcus pseudintermedius* from dogs, BMC Vet. Res. 9 (2013) 93.
[15] N. Velázquez-Guadarrama, A.L. Olivares-Cervantes, E. Salinas, L. Martínez, M. Escorcia, R. Oropeza, I. Rosas, Presence of environmental coagulase-positive staphylococci, their clonal relationship, resistance factors and ability to form biofilm, Rev. Argent. Microbiol. 49 (2017) 15–23.
[16] A. Indraningrat, H. Smidt, D. Sipkema, Bioprospecting sponge-associated microbes for antimicrobial compounds, Mar. Drugs 4 (2016) E87.
[17] G.S. Kiran, B. Sabarathnam, J. Selvin, Biofilm disruption potential of a glycolipid biosurfactant from marine *Brevibacterium casei*, FEMS Immunol. Med. Microbiol. 59 (2010) 432–438.
[18] J.F. Santos-Gandelman, M. Giambiagi-deMarval, W.M.R. Oelemann, M.S. Laport, Biotechnological potential of sponge-associated bacteria, Curr. Pharmaceut. Biotechnol. 15 (2014) 143–155.
[19] O.C.S. Santos, A.R. Soares, F.L. Machado, M.T. Romanos, G. Muricy, M. Giambiagi-deMarval, M.S. Laport, Investigation of biotechnological potential of sponge-associated bacteria collected in Brazillian coast, Lett. Appl. Microbiol. 60 (2015) 140–147.
[20] S. Sayem, E. Manzo, L. Ciavatta, A. Tramice, A. Cordone, A. Zanfardino, M. de Felice, M. Varcamonti, Anti-biofilm activity of an exopolysaccharide from a sponge-associated strain of *Bacillus licheniformis*, Microb. Cell Factories 10 (2011) 74.
[21] O.C.S. Santos, P.V.M.L. Pontes, J.F.M. Santos, G. Muricy, M. Giambiagi-deMarval, M.S. Laport, Isolation, characterization and phylogeny of sponge-associated bacteria with antimicrobial activities from Brazil, Res. Microbiol. 161 (2010) 604–612.
[22] J.F. Santos-Gandelman, O.C.S. Santos, P.V.M. Pontes, C.L. Andrade, E. Korenblum, G. Muricy, M. Giambiagi-deMarval, M.S. Laport, Characterization of cultivable bacteria from Brazilian sponges, Mar. Biotechnol. 15 (2013) 668–676.
[23] CLSI, Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals Document VET08 Ed4, Clinical and Laboratory Standards Institute, Wayne, PA, 2018.
[24] CLSI, Performance Standards for Antimicrobial Susceptibility Tests. Ed30. CLSI Supplement M100, Clinical and Laboratory Standards Institute, Wayne, PA, 2020.
[25] A.P. Magiorakos, A. Srinivasan, R.B. Carey, Y. Carmeli, M.E. Falagas, C.G. Giske, S. Harbarth, J.F. Hindler, G. Kahlmeter, B. Olsson-Liljequist, D.L. Paterson, L. B. Rice, J. Stelling, M.J. Struelens, A. Vatopoulos, J.T. Weber, D.L. Monnet, Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance, Clin. Microbiol. Infect. 8 (2012) 268–281.
[26] P.R. Marinho, A.P. Moreira, F.L. Pellegrino, G. Muricy, M.C. Bastos, K.R. Santos, M. Giambiagi-deMarval, M.S. Laport, Marine *Pseudomonas putida*: a potential source of antimicrobial substances against antibiotic-resistant bacteria, MI 104 (2009) 678–682.
[27] S. Stepanovic, D. Vukovic, V. Hola, G. Di Bonaventura, S. Djukic, I. Cirkovic, F. Ruzicka, Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci, APMIS 115 (2007) 891–899.
[28] S. Balasubramanian, J. Skaf, U. Holzgrabe, R. Bharti, K.U. Förstner, W. Ziebuhr, U. H. Humeida, U.R. Abdelmohsen, T.A. Oelschlaeger, A new bioactive compound from the marine sponge-derived *streptomyces* sp. SBT348 inhibits staphylococcal growth and biofilm formation, Front. Microbiol. 9 (2018) 1473.
[29] B. Penna, R. Varges, L. Medeiros, G.M. Martins, R.R. Martins, W. Lilenbaum, Species distribution and antimicrobial susceptibility of staphylococci isolated from canine otitis externa, Vet. Dermatol. 21 (2010) 292–296.
[30] M.S. Laport, O.C.S. Santos, G. Muricy, Marine sponges: potential sources of new antimicrobial drugs, Curr. Pharmaceut. Biotechnol. 10 (2009) 86–105.
[31] M.S. Laport, P.R. Marinho, O.C.S. Santos, P. Almeida, M.T.V. Romanos, G. Muricy, M.A.V.P. Brito, M. Giambiagi-deMarval, Antimicrobial activity of marine sponges against coagulase-negative staphylococci isolated from bovime mastitis, Vet. Microbiol. 155 (2012) 362–368.
[32] L. Ye, J.F. Santos-Gandelman, C.C. Hardoim, I. George, P. Cornelis, M.S. Laport, Antibacterial activity and mutagenesis of sponge-associated *Pseudomonas fluorescens* H41, Antonie Leeuwenhoek 108 (2015) 117–126.
[33] V. Ramalingam, R. Rajaram, C. Premkumar, C. Santhanam, P. Dhinesh, S. Vinothkumar, K. Kaleshkumar, Biosynthesis of silver nanoparticles from deep sea bacterium *Pseudomonas aeruginosa* JQ989348 for antimicrobial, antibiofilm and cytotoxic activity, J. Basic Microbiol. 54 (2014) 928–936.
[34] I. Doghri, F. Brian-Jaisson, M. Graber, A. Bazire, A. Dufor, M.N. Bellon-Fontaine, J. M. Herry, A.C. Ferro, V. Sopena, I. Lanneluc, S. Sablé, Antibiofilm activity in the culture supernatant of a marine *Pseudomonas* sp. bacterium, Microbiology 166 (2018) 239–252.
[35] T. Bjarnsholt, The role of bacterial biofilms in chronic infections, APMIS Suppl. 136 (2013) 1–51.
[36] N. Kamarudheen, K.V.B. Rao, Fatty acyl compounds from marine *Streptomyces griseoincarnatus* strain HK12 against two major bio-film forming nosocomial pathogens; an *in vitro* and *in silico* approach, Microb. Pathog. 127 (2019) 121–130.