Antimicrobial activity of ear cleanser products against biofilm and planktonic phases of Staphylococcus spp. and Pseudomonas spp. isolated from canine skin and ear infections.

Abish Samuel Stephen (a.s.stephen@qmul.ac.uk)
Queen Mary University of London
Vanessa Chala
Virbac (France)
Céline S Nicolas
Virbac (France)
Pierre Jasmin
Virbac (France)
Robert Patrick Allaker
Queen Mary University of London

Research Article

Keywords: canine, otitis, biofilm, antimicrobial, cleanser, MRSA, staphylococcus, pseudomonas

Posted Date: August 10th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2159480/v1

Abstract

Background: *Staphylococcus* spp., and *Pseudomonas* spp., including multidrug resistant staphylococci are frequent isolates from canine otitis externa and atopic dermatitis. Biofilm formation by these organisms may be important in otitis chronicity. With ear cleanser products commonly used to control microbial overgrowth, it is important to measure their antibiofilm effects. Six ear cleansers (Epiotic SIS, Epiotic Advanced, Cleanaural , Otifree, Peptivet and Sonotix) were evaluated against infection isolates of *Pseudomonas aeruginosa*, methicillin resistant and sensitive *Staphylococcus aureus* and *Staphylococcus pseudintermedius*. Antibiofilm activity was measured colorimetrically via the presence of viable cells as detected by the reduction of a thiazolyl blue tetrazolium bromide compound (MTT). Minimum inhibitory concentration (MIC) of Epiotic SIS and Epiotic Advanced were measured using a broth micro-dilution assay to ascertain inhibition in planktonic phase.

Results: Epiotic (SIS and Advanced), Cleanaural and Peptivet showed strong antibiofilm activity, with Otifree and Sonotix showing moderate to low antibiofilm activity. Differences in inhibition between the methicillin resistant and sensitive staphylococcal isolates were also observed between the products with Otifree showing significantly less inhibition of the resistant isolate of *S. aureus* compared to the sensitive isolate. *P. aeruginosa* biofilms were less effectively disrupted by some ear cleansers compared to Epiotic , and the MIC results indicated that less diluted solutions were required to inhibit this isolate compared to the staphylococci. Differences in the antibacterial effects between Epiotic SIS and Epiotic Advanced solutions could also be detected from the MIC assays suggesting differences in formulations can affect antimicrobial efficacy.

Conclusions: Commonly used canine ear cleanser products showed activity against multidrug resistant and sensitive *Staphylococcus*spp. and *P. aeruginosa* isolates in both biofilm and planktonic phases. Differences between strains and cleansers were observed that should enable better targeted use of these products.

Background

Atopic dermatitis and the frequently associated otitis externa are some of the most common cutaneous primary inflammatory conditions in dogs, often associated with secondary bacterial infections (1–5). Besides hypersensitivity and the resulting inflammation, additional host factors such as keratinisation and abnormal cerumen production are also thought to contribute to the pathogenesis of these conditions. Yeasts, staphylococci and *Pseudomonas* spp, are among the most frequent isolates found from infected canine skin and ears (6–9). These infections are typically polymicrobial in nature, with a proportion involving potentially multidrug resistant staphylococci and/or biofilm forming *Pseudomonas* spp (10, 11). These can result in chronic or recurrent infections leading to severe outcomes with treatment requiring multiple courses of antimicrobials and ear cleaning. Topical ear cleanser products are sold commercially in a variety of formulations containing different components and technologies including antimicrobial, anti-inflammatory, surfactant and cerumenolytic compounds (12–14). Some provide good

cleansing properties and help control bacterial proliferation, as demonstrated *in vitro* and *in vivo* (15). However, their possible role in controlling biofilms that are clinically relevant has not been tested yet. Indeed, the ability of the bacterial strains to form biofilm can alter their sensitivity to antimicrobials and antibiotics, a common cause of treatment failure (16–19). Cleaning ears with a product able to disrupt and maintain bactericidal effects on biofilms could be an important step to prevent more serious diseases. This study therefore aimed to compare the antibiofilm activity of a selection of commercial ear cleanser products. Used *in vivo*, especially in clinically affected dogs, cleansers can be diluted by various wax and secretions, the minimum inhibitory concentration (MIC) for the two Virbac products (Epiotic SIS and Epiotic Advanced) were determined to ascertain dilutions of the different products that are inhibitory.

Results

Antibiofilm Effects

Epiotic SIS, Epiotic advanced, Peptivet and Cleanaural solutions showed significant antibiofilm activities against all isolates tested ($p < 0.001$ when compared to control, Fig. 1–3). Survival of strains in biofilm was indeed decreased by at least 79% with both Epiotic formulations and by at least 66% and 73% with Cleanaural and Peptivet respectively (Fig. 1–4). No significant difference was observed between these four products for any strain tested. The antibiofilm effect of the two other products (Otifree and Sonotix) was not as strong and varied between strains. Otifree was ineffective against *P. aeruginosa* (Fig. 1) and methicillin resistant *Staphylococcus aureus* (MRSA, Fig. 2) but could significantly reduce survival of the other strains in biofilm (% of decrease versus control varying between 41% and 58%, $p < 0.001$, Fig. 2–4). Sonotix was also ineffective against *P. aeruginosa* (Fig. 1) but could reduce the survival of the other strains in biofilm (% of decrease varying between 57% and 76%, $p < 0.001$, Fig. 2–4). When comparing products between them, a significantly lower efficacy of Otifree ($p < 0.05$) was observed compared to Epiotic SIS, Epiotic advanced, Peptivet or Cleanaural for all strains tested, and a significant lower efficacy of Sonotix ($p < 0.05$) compared to the same four ear cleansers for *P. aeruginosa*, MRSA and MRSP.

Minimum Inhibitory Concentration of Epiotic ear cleansers

Significant growth inhibition was observed on the staphylococci strains in up to 24-fold diluted Epiotic SIS and 12-fold diluted Epiotic Advanced solutions, whereas *P. aeruginosa* was susceptible in up to 6-fold diluted ear cleanser (Fig. 5). No significant differences in MIC values could be observed for both ear cleanser solutions between methicillin sensitive and resistant strains of the staphylococci. However, there were some differences in the MIC for Epiotic SIS compared to Epiotic Advanced, with the former showing efficacy at higher dilutions than the latter (Fig. 5).

Discussion

Staphylococci are some of the most abundant organisms present in the canine skin microbiota, and strains belonging to the genera *Staphylococcus* spp., and *Pseudomonas* spp. are frequently isolated from

canine atopic dermatitis and otitis externa (20–23). A high proportion of *Pseudomonas* spp, isolates from these infections can form biofilms and thus contribute to its virulence and persistence (10, 11, 16–19). It is therefore important to evaluate the antibiofilm activities of available canine ear cleanser products to help inform use. In this study, a differential antibiofilm effect for each ear cleanser was observed on the bacterial strains (Fig. 1–4). Epiotic SIS, Epiotic Advanced, Cleanaural and Peptivet showed high efficacy against both methicillin resistant and sensitive staphylococci strains, with Otifree and Sonotix showing moderate to low efficacy. All products tended to show a slightly reduced efficacy against the resistant staphylococcal strains compared to the sensitive strains. It has been shown that there is an association between multi-drug resistance and biofilm forming ability in *S. aureus* (18, 19). It is possible that MRSA and MRSP strains form higher biomass biofilms that are not easily disrupted.

Small differences in the MIC curves between sensitive and resistant strains could also be detected in the MIC assays, with the methicillin resistant strains reaching higher OD in the dilutions under the MIC compared to the sensitive strains (Fig. 5). The antibiofilm assay in this study would detect biofilm disruption and bactericidal activity as a cumulative effect as, any cells not adhering to the wells will be removed when the ear cleanser is aspirated from the wells after treatment. So, comparison with MIC curves can suggest differences in biofilm disruption and bactericidal activity as the MIC would predominantly measure bactericidal activity of the cells in planktonic phase. Results for the staphylococcal strains in this study showed that methicillin sensitive strains were more susceptible in the antibiofilm and MIC assays compared to the resistant strains, indicating that there are no major differences in the activity of Epiotic SIS and Epiotic Advanced ear cleansers against biofilm and planktonic phase. However, across all *Staphylococcus* spp., strains studied in the MIC assay, higher dilutions of Epiotic SIS showed activity compared to Epiotic Advanced (Fig. 5), suggesting formulation differences can have differential effects on antibiofilm and bactericidal efficacies.

The Epiotic SIS and Epiotic Advanced ear cleansers showed high antibiofilm efficacy against the gram-negative *P. aeruginosa* strain comparable to the *Staphylococcus* spp. strains, whereas the other ear cleansers showed reduced efficacy relative to the staphylococci. Comparing these data with MIC curves suggested that the Epiotic products showed similar MIC values, although requiring higher concentrations of the solution than the staphylococci to show effective inhibition. Whereas the *P. aeruginosa* strains were inhibited up to 6-fold dilutions, the staphylococci were still inhibited up to 24-fold dilutions of Epiotic SIS & Epiotic Advanced (Fig. 5). A high antibiofilm activity, even with high dilutions (6-to-24-fold dilutions), suggests that these ear cleansers may disrupt staphylococcal and *P. aeruginosa* biofilms effectively in clinical situations.

Conclusion

Several commercially available products may have good antimicrobial and/or wax elimination properties, however, this study has highlighted important differences in antibiofilm activities between products. Further, differences observed between antibiotic resistant and sensitive isolates indicates that the degree of antibiofilm activity of a product in addition to identification of the isolates responsible for the infection

should also be criteria in treatment considerations. Recurrent and/or chronic infections may require an adapted approach in products selection by considering the presence of biofilms on canine skin and ears.

Methods

The following ear cleansers were evaluated in this study: Epiotic SIS (Virbac), Epiotic Advanced (Virbac), Cleanaural (Dechra), Otifree (Vetoquinol), Peptivet (Vetruus), Sonotix (Vetoquinol).

Organisms & culture conditions

Bacterial strains used in this study are listed in Table 1. All strains are infection isolates kindly supplied by the Royal Veterinary College, UK. The strains were maintained on Blood Agar (with 5% v/v defibrinated horse blood) at 37●C aerobically (5% CO_2). Liquid cultures used to make bacterial suspensions for antibiofilm or Minimum Inhibitory Concentration (MIC) assays were grown overnight on Tryptone Soya Broth.

Biofilm formation & treatment

Bacterial cultures incubated aerobically at 37°C for 24 hours were pelleted by centrifugation (3500 rpm for 10 min at 20°C). The pellets were resuspended in broth and standardized at a concentration of 10^7 colony-forming units per ml at an optical density of 600nm (OD_{600}). Biofilms were then formed in wells of micro titre plates by adding 100µL of standardized inoculum for a period of 90 minutes at 37°C under shaking (75 rpm) to obtain pre-adhesion. Following this period, the supernatant was discarded, and 200µL broth for biofilm growth added and incubated aerobically for 24 hours. The biofilms were then separately exposed to the 100µL of test substances, chlorhexidine (0.2% w/v), and negative control (deionised water) for 15 minutes at 37°C. Experiments were repeated twice with sextuplicate biofilms per experiment.

Quantification of cell viability of microbial biofilms

The percentage of surviving cells after exposure to the antimicrobial agents was verified by the analysis of bacterial metabolism of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Abcam) according to the manufacturer's instructions. Briefly, test solutions were removed by pipetting from the wells, then 50µL of MTT solution and 50µL of medium was added and the plate incubated (at 37°C for 1 hour) under light protection aerobically. After incubation, 150µL of MTT solvent solution was added for the solubilisation of products derived from biochemical activity promoted by the biofilm viable cells. After 15 minutes of incubation at 37°C with shaking in an orbital shaker at approx. 75 rpm), the OD was read (λ = 590 nm) in a microplate spectrophotometer (BMG Labtech CLARIOStar Plus).

Statistical Analysis of Antibiofilm Assay

The OD values were normalised within each plate, and the values were analysed using one-way ANOVA with multiple comparisons conducted using the Fisher's LSD test against the negative controls. Analysis

was performed using GraphPad Prism (v9.1.0).

Minimum inhibitory concentration (MIC) Determination

MIC determinations of the ear cleanser products were carried out using a broth micro-dilution assay against the test species of bacteria. Bacterial suspensions at OD_{600} of 0.1 were prepared from pelleted (3500 rpm for 10 min at 20°C) broth cultures. Serial 1 in 2 dilutions of test substances were first made in a total broth volume of 90µL per well of the micro titre plate. Bacterial suspension (90µL) and medium (90uL) were then added to each well and then incubated at 37°C for 24 hours. Optical density at 600nm was read before and after incubation using a microplate spectrophotometer (BMG Labtech CLARIOStar Plus). MICs were recorded as the lowest concentration of product inhibiting growth as measured by optical density after subtracting from the baseline measurement. Experiments were repeated twice.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data generated or analyzed during this study are included in this article and are available from the corresponding author on reasonable request.

Competing interests

Céline Nicolas, Vanessa Chala and Pierre Jasmin are employees of Virbac. Abish Stephen and Robert Allaker received funding from Virbac to conduct this study.

Funding

This study was fully funded by Virbac, France.

Authors' contributions

ASS, VC, CSN, PJ and RPA: Planned, designed the study, provided resources, and revised the manuscript. ASS and RPA: Conducted the experiments, analysed the data and prepared the manuscript. All authors have read and approved the final manuscript.

Acknowledgements

None.

References

1. Bradley CW, Lee FF, Rankin SC, Kalan LR, Horwinski J, Morris DO, et al. The otic microbiota and mycobiota in a referral population of dogs in eastern USA with otitis externa. Vet Dermatol. 2020;31(3):225-e49.
2. Chermprapai S, Ederveen THA, Broere F, Broens EM, Schlotter YM, van Schalkwijk S, et al. The bacterial and fungal microbiome of the skin of healthy dogs and dogs with atopic dermatitis and the impact of topical antimicrobial therapy, an exploratory study. Vet Microbiol [Internet]. 2019;229(September 2018):90–9. Available from: https://doi.org/10.1016/j.vetmic.2018.12.022.
3. Favrot C, Steffan J, Seewald W, Picco F. A prospective study on the clinical features of chronic canine atopic dermatitis and its diagnosis. Vet Dermatol. 2010;21(1):23–31.
4. O'Neill DG, Church DB, McGreevy PD, Thomson PC, Brodbelt DC. Prevalence of disorders recorded in dogs attending primary-care veterinary practices in England. PLoS One. 2014;9(3).
5. Hensel P, Santoro D, Favrot C, Hill P, Griffin C. Canine atopic dermatitis: Detailed guidelines for diagnosis and allergen identification. BMC Vet Res [Internet]. 2015;11(1):1–13. Available from: http://dx.doi.org/10.1186/s12917-015-0515-5.
6. Santoro D, Marsella R, Pucheu-Haston CM, Eisenschenk MNC, Nuttall T, Bizikova P. Review. Pathogenesis of canine atopic dermatitis: Skin barrier and host-micro-organism interaction. Vet Dermatol. 2015;26(2):84-e25.
7. Roland PS, Stroman DW. Microbiology of acute otitis externa. Laryngoscope. 2002;112(7):1166–77.
8. Petersen AD, Walker RD, Bowman MM, Schott HC, Rosser EJ. Frequency of isolation and antimicrobial susceptibility patterns of Staphylococcus intermedius and Pseudomonas aeruginosa isolates from canine skin and ear samples over a 6-year period (1992–1997). J Am Anim Hosp Assoc. 2002;38(5):407–13.
9. Bugden DL. Identification and antibiotic susceptibility of bacterial isolates from dogs with otitis externa in Australia. Aust Vet J. 2013;91(1–2):43–6.
10. Clark SM, Loeffler A, Bond R. Susceptibility in vitro of canine methicillin-resistant and-susceptible staphylococcal isolates to fusidic acid, chlorhexidine and miconazole: Opportunities for topical therapy of canine superficial pyoderma. J Antimicrob Chemother. 2015;70(7):2048–52.
11. Robinson VH, Paterson S, Bennett C, Steen SI. Biofilm production of Pseudomonas spp. isolates from canine otitis in three different enrichment broths. Vet Dermatol. 2019;30(3):218-e67.
12. Santoro D, Ahrens K, Vesny R, Navarro C, Gatto H, Marsella R. Evaluation of the in vitro effect of Boldo and Meadowsweet plant extracts on the expression of antimicrobial peptides and inflammatory markers in canine keratinocytes. Res Vet Sci [Internet]. 2017;115(May):255–62. Available from: http://dx.doi.org/10.1016/j.rvsc.2017.05.021.
13. Santoro D, Bohannon M, Ahrens K, Navarro C, Gatto H, Marsella R. Evaluation on the effects of 0.1% Peumus boldus leaf and Spiraea ulmaria plant extract combination on bacterial colonization in canine atopic dermatitis: A preliminary randomized, placebo controlled, double-blinded study. Res Vet

Sci [Internet]. 2018;118(November 2017):164–70. Available from: https://doi.org/10.1016/j.rvsc.2018.02.006.

14. Swinney A, Fazakerley J, McEwan N, Nuttall T. Comparative in vitro antimicrobial efficacy of commercial ear cleaners. Vet Dermatol. 2008;19(6):373–9.
15. Rème CA, Pin D, Collinot C, Cadiergues MC, Joyce JA, Fontaine J. The efficacy of an antiseptic and microbial anti-adhesive ear cleanser in dogs with otitis externa. Vet Ther. 2006;7(1):15–26.
16. Pye CC, Yu AA, Weese JS. Evaluation of biofilm production by Pseudomonas aeruginosa from canine ears and the impact of biofilm on antimicrobial susceptibility in vitro. Vet Dermatol. 2013;24(4).
17. Sanchez CJ, Mende K, Beckius ML, Akers KS, Romano DR, Wenke JC, et al. Biofilm formation by clinical isolates and the implications in chronic infections. BMC Infect Dis. 2013;13(1):1–12.
18. Kwon AS, Park GC, Ryu SY, Lim DH, Lim DY, Choi CH, et al. Higher biofilm formation in multidrug-resistant clinical isolates of Staphylococcus aureus. Int J Antimicrob Agents. 2008;32(1):68–72.
19. Reiter KC, Da Silva Paim TG, De Oliveira CF, D'Azevedo PA. High biofilm production by invasive multiresistant staphylococci. Apmis. 2011;119(11):776–81.
20. Borriello G, Paradiso R, Catozzi C, Brunetti R, Roccabianca P, Riccardi MG, et al. Cerumen microbial community shifts between healthy and otitis affected dogs. PLoS One [Internet]. 2020;15(11 November):1–18. Available from: http://dx.doi.org/10.1371/journal.pone.0241447.
21. Tang S, Prem A, Tjokrosurjo J, Sary M, Van Bel MA, Rodrigues-Hoffmann A, et al. The canine skin and ear microbiome: A comprehensive survey of pathogens implicated in canine skin and ear infections using a novel next-generation-sequencing-based assay. Vet Microbiol. 2020;247(May).
22. Korbelik J, Singh A, Rousseau J, Weese JS. Analysis of the otic mycobiota in dogs with otitis externa compared to healthy individuals. Vet Dermatol. 2018;29(5):417-e138.
23. Bradley C, Morris D, Rankin S, Cain C, Misic A, Houser T, et al. Longitudinal evaluation of the skin microbiome and association with microenvironment and treatment in cancine atopic dermatitis. J Invest Dermatol. 2016;136(6):1182–90.

Tables

Table 1

Organism	Isolate name	Notes
Methicillin sensitive *Staphylococcus pseudintermedius* (MSSP)	GL001B	Infection isolate, genotyped using thermonuclease gene.
Methicillin resistant *Staphylococcus pseudintermedius* (MRSP)	GL119A	Infection isolates, genotyped using thermonuclease gene. Presence of methicillin resistance confirmed with *mecA* detection.
Methicillin resistant *Staphylococcus aureus* (MRSA)	A004	
Methicillin sensitive *Staphylococcus aureus* (MSSA)	B027	Infection isolate. Genotyped using thermonuclease gene.
Pseudomonas aeruginosa	288230/476168	Canine otitis isolate

Figures

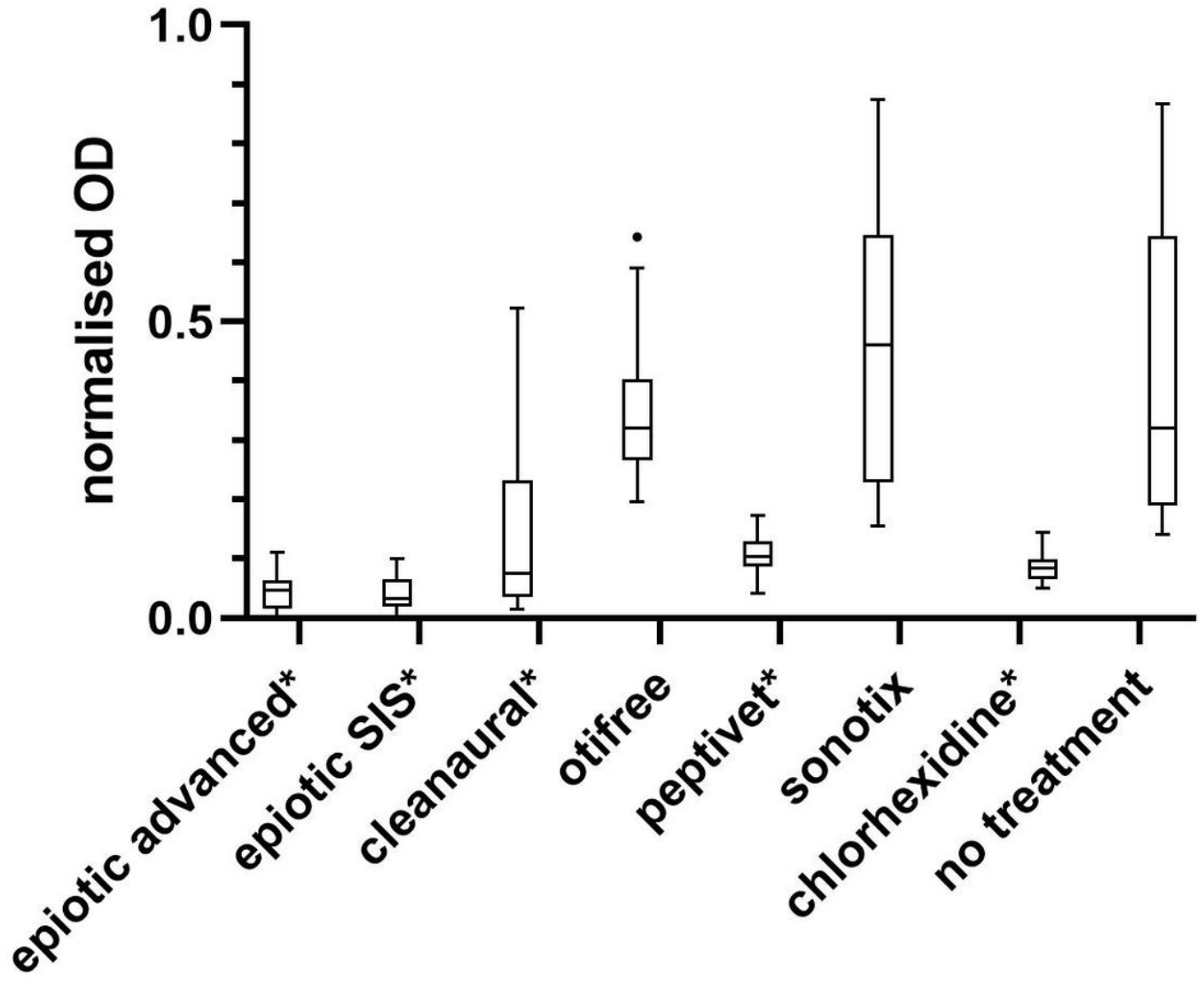

Figure 1

Box plot showing normalised optical density of Pseudomonas aeruginosa biofilms treated with undiluted ear cleanser products. Outliers are denoted by dots and determined by the Tukey method. *indicates statistically significant comparisons with the no treatment control.

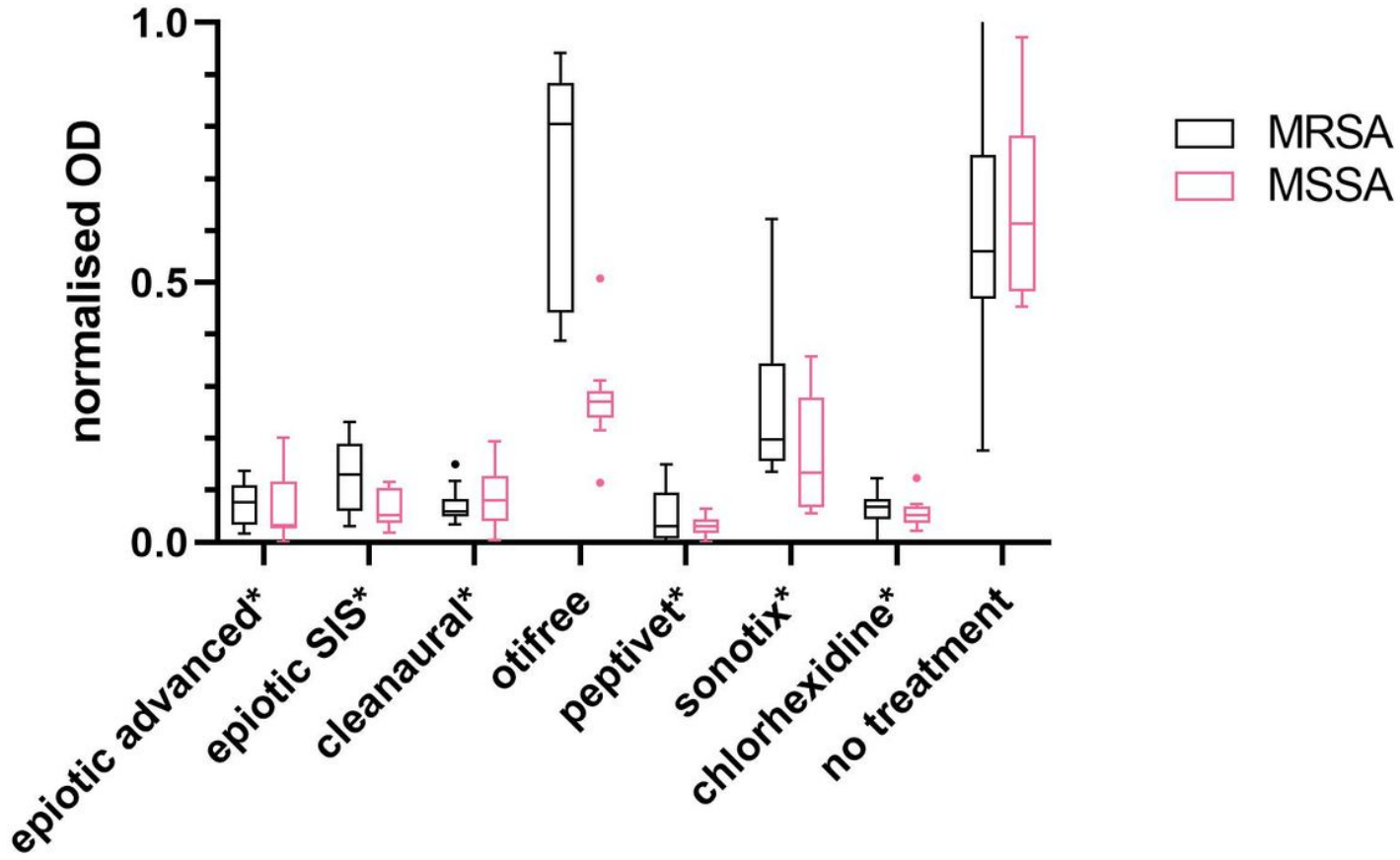

Figure 2

Box plot showing normalised optical density of methicillin resistant (MRSA) and sensitive (MSSA) Staphylococcus aureus biofilms treated with undiluted ear cleanser products. Outliers are denoted by dots and determined by the Tukey method. *indicates statistically significant comparisons with the no treatment control.

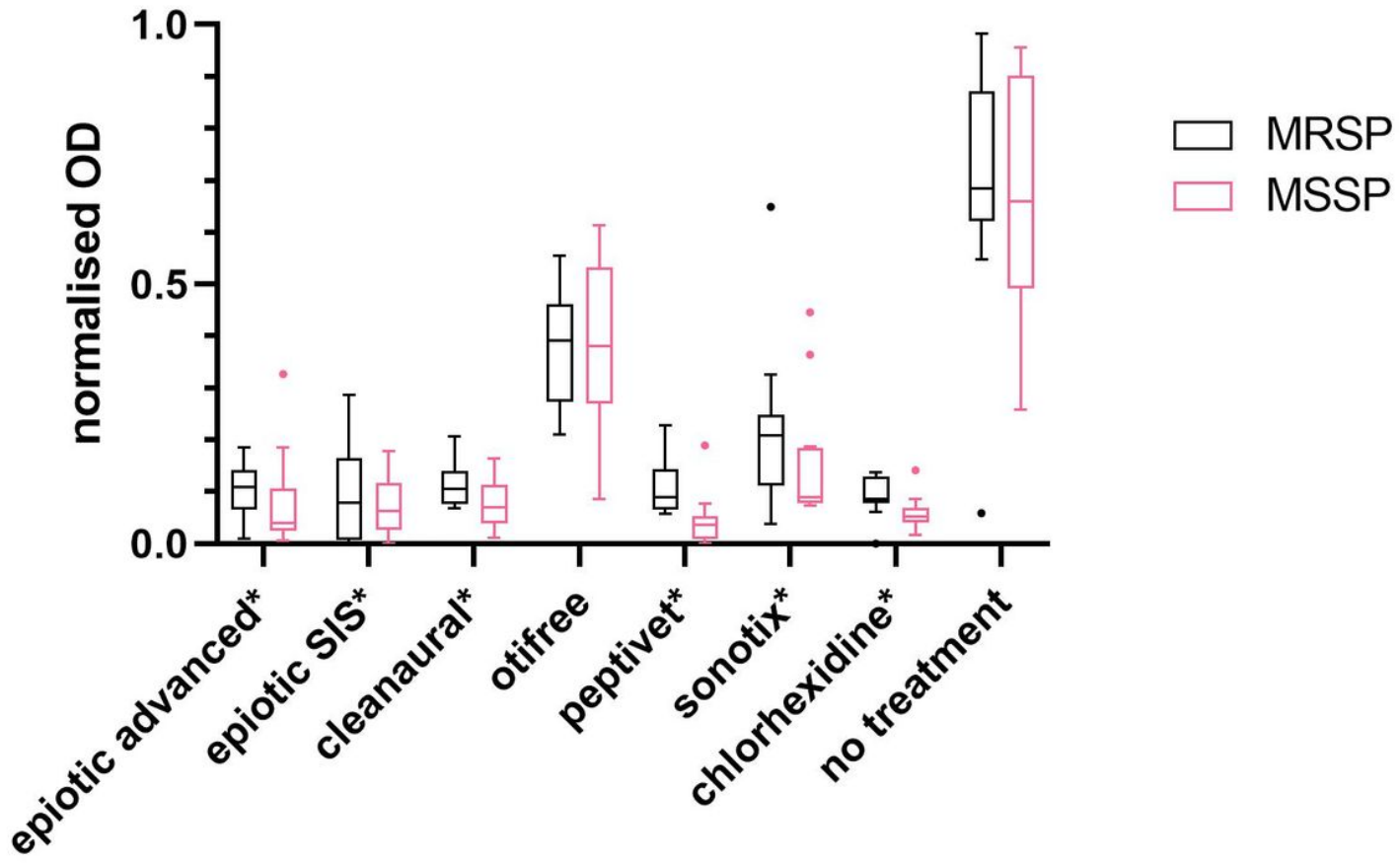

Figure 3

Box plot showing normalised optical density of methicillin resistant (MRSP) and sensitive (MSSP) *Staphylococcus pseudintermedius* biofilms treated with undiluted ear cleanser products. Outliers are denoted by dots and determined by the Tukey method. *indicates statistically significant comparisons with the no treatment control.

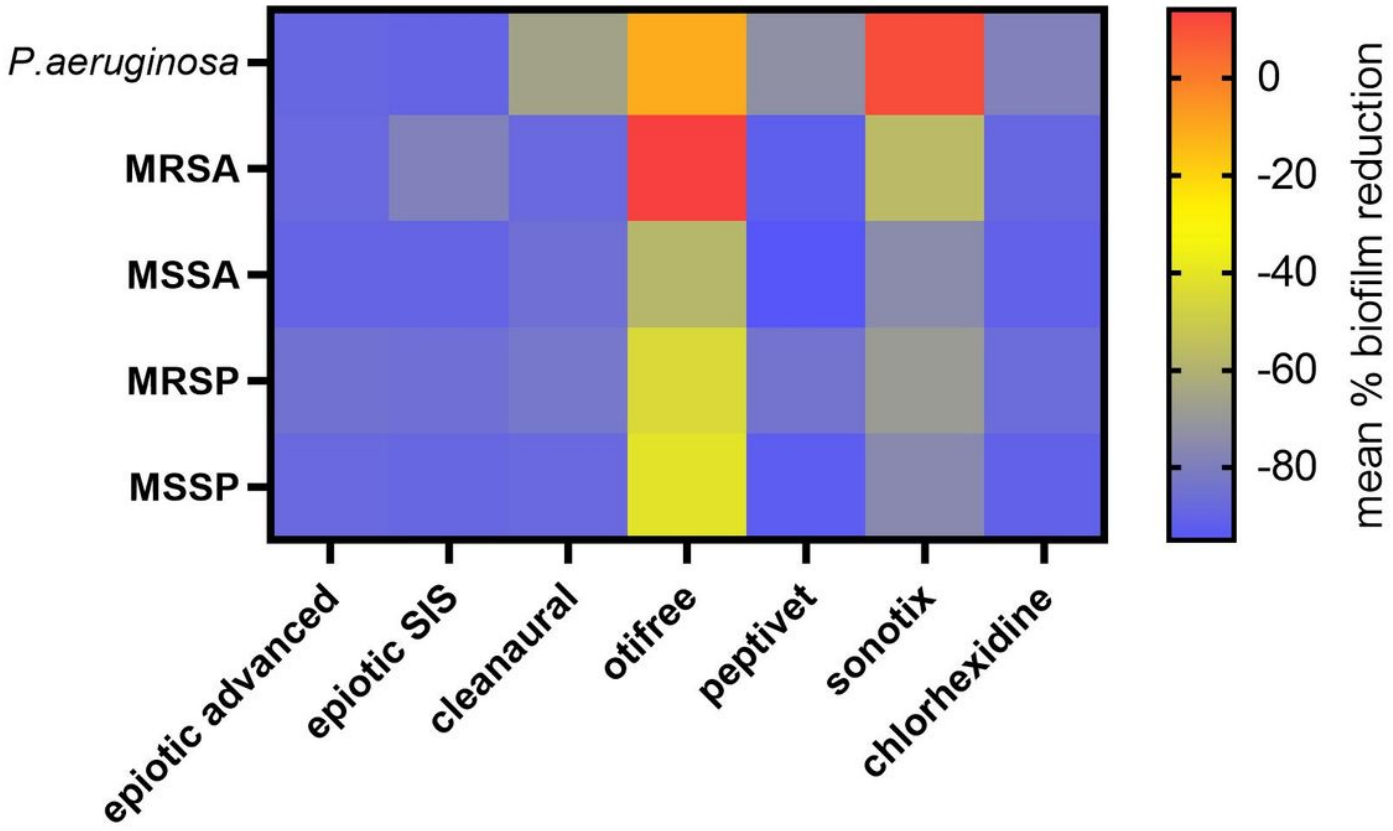

Figure 4

Heatmap showing mean % reductions in the biofilms relative to the no treatment controls for all strains and all ear cleansers studied.

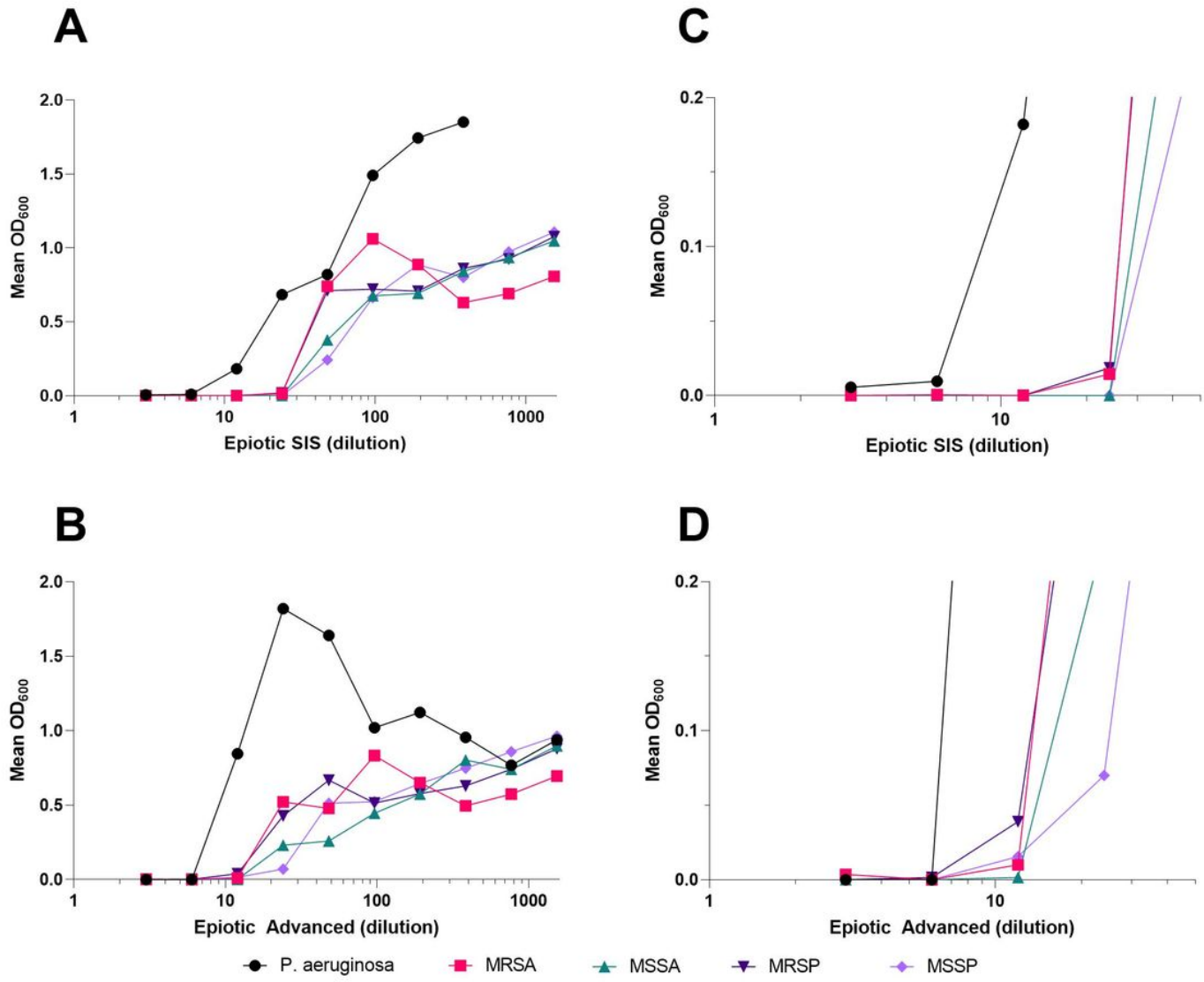

Figure 5

Line plot of the Epiotic SIS (A) and Epiotic Advanced (B) dilutions against mean optical density for the different strains from duplicate MIC experiments; (C) and (D) plots show the lower dilutions only, to point to the MIC for the different strains.