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

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Abstract

Background *Staphylococcus* spp., and *Pseudomonas* spp., including multidrug resistant staphylococci are frequent isolates from canine otitis externa and atopic dermatitis. The ability of these bacteria to form biofilms significantly contributes to the chronic nature of otitis. To manage microbial overgrowth, ear cleanser products are commonly used. It is important therefore, to measure their antibiofilm effects. In this study, six ear cleansers (Epiotic® SIS, Epiotic® Advanced, Cleanaural®, Otifree®, Peptivet® and Sonotix®) were evaluated against clinical isolates of *Pseudomonas aeruginosa*, methicillin resistant and sensitive *Staphylococcus aureus* and *Staphylococcus pseudintermedius*. Antibiofilm activity was measured using a colorimetric assay that detects viable cells through the reduction of thiazolyl blue tetrazolium bromide (MTT). Additionally, minimum inhibitory concentration (MIC) of Epiotic SIS and Epiotic Advanced were determined using a broth micro-dilution assay to assess their ability to inhibit bacteria in the planktonic state.

Results Epiotic (SIS and Advanced), Cleanaural and Peptivet showed high antibiofilm activity, with Otifree and Sonotix showing moderate to low antibiofilm activity. Notably, Otifree was significantly less effective at inhibiting methicillin-resistant *S. aureus* compared to methicillin-sensitive strains. *P. aeruginosa* biofilms were less effectively disrupted by some ear cleansers, and the MIC results indicated that less diluted solutions were required to inhibit this isolate compared to the staphylococcal species. 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 variable activity against multidrug resistant and sensitive *Staphylococcus* spp. and *P. aeruginosa* isolates in both biofilm and planktonic phases. The observed differences between bacterial strains and cleanser formulations highlight the importance of selecting appropriate products for targeted microbial control, which can lead to more effective management of chronic otitis externa and atopic dermatitis in dogs.

Keywords Canine, Otitis, Biofilm, Antimicrobial, Cleanser, MRSA, *Staphylococcus*, *Pseudomonas*

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Background

Atopic dermatitis and the commonly associated otitis externa are among the most prevalent primary inflammatory skin conditions in dogs, often leading to secondary bacterial infections [1–5]. Besides hypersensitivity and the resulting inflammation, additional host factors such as keratinisation and abnormal cerumen production are thought to contribute to the pathogenesis of these conditions. Yeasts, staphylococci and *Pseudomonas* spp, are commonly isolated from infected canine skin and ears [6–9]. These infections are typically polymicrobial in nature, with a proportion of these cases involving multidrug resistant staphylococci and/or biofilm forming *Pseudomonas* species [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 include 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 which can be 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 aimed to compare the in vitro antibiofilm activity of two widely used ear cleansers, Epiotic® SIS and Epiotic® Advanced, with four other commercially available ear cleanser products. Additionally, when used on dogs or cats with clinical conditions, ear cleansers can be diluted by wax and secretions. To address this, we determined the minimum inhibitory concentration (MIC) for Epiotic® SIS and Epiotic® Advanced to help ascertain the dilution levels at which the cleansers remain effective.

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 negative control, Figs. 1, 2 and 3). Mean biofilm removal compared to negative control was 79% for both Epiotic formulations and at least 66% and 73% with Cleanaural and Peptivet respectively (Figs. 1, 2, 3 and 4). There were no significant differences in antibiofilm effectiveness among Epiotic SIS, Epiotic Advanced, Peptivet, and Cleanaural for any of the strains tested. The antibiofilm effect of the two other products (Otifree® and Sonotix) was not as strong and varied between strains. In contrast, the antibiofilm effects of Otifree and Sonotix were less consistent and varied depending on the bacterial strain. Otifree was ineffective against *Pseudomonas aeruginosa* (Fig. 1) and methicillin-resistant *Staphylococcus aureus* (MRSA; Fig. 2). However, it showed significant antibiofilm effects against other strains, reducing biofilm by 41–58% compared to the negative control ($p < 0.001$; Figs. 2, 3 and 4). Similarly, Sonotix was ineffective against *P. aeruginosa* (Fig. 1) but effectively reduced biofilms of other strains by 57–76% compared to the negative control ($p < 0.001$; Figs. 2, 3 and 4). When comparing the efficacy of all products, Otifree exhibited significantly lower antibiofilm activity ($p < 0.05$) compared to Epiotic SIS, Epiotic Advanced, Peptivet, and Cleanaural across all tested strains. Sonotix also showed significantly lower efficacy ($p < 0.05$) compared to the same four ear cleansers specifically for *P. aeruginosa*, MRSA, and methicillin-resistant *Staphylococcus pseudintermedius* (MRSP).

Minimum inhibitory concentration of epiotic ear cleansers

Significant growth inhibition was observed for staphylococcal strains with up to a 24-fold dilution of Epiotic SIS and a 12-fold dilution of Epiotic Advanced. In contrast, *P. aeruginosa* was susceptible to up to a 6-fold dilution of the ear cleansers (Fig. 5). There were no significant differences in MIC values between methicillin-sensitive and methicillin-resistant staphylococcal strains for either Epiotic SIS or Epiotic Advanced. However, Epiotic SIS was effective at higher dilutions compared to Epiotic Advanced, indicating that formulation differences may influence antimicrobial efficacy (Fig. 5).

Discussion

Staphylococci are among the most abundant organisms present in the canine skin microbiota, with strains belonging to the genera *Staphylococcus* spp., and *Pseudomonas* spp. frequently isolated from canine atopic dermatitis and otitis externa [20–23]. Notably, a high proportion of *Pseudomonas* spp, isolates from these infections can form biofilms and thus contribute to its virulence and persistence, underlining the need to characterise the antibiofilm activities of commercially available ear cleansers [10, 11, 16–19].

Variation in antibiofilm efficacy among ear cleansers

Our study has found significant variations in the antibiofilm effects of different ear cleansers across various bacterial strains (Figs. 1, 2, 3 and 4). Specifically, 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. Generally, all products showed slightly reduced efficacy against resistant staphylococcal

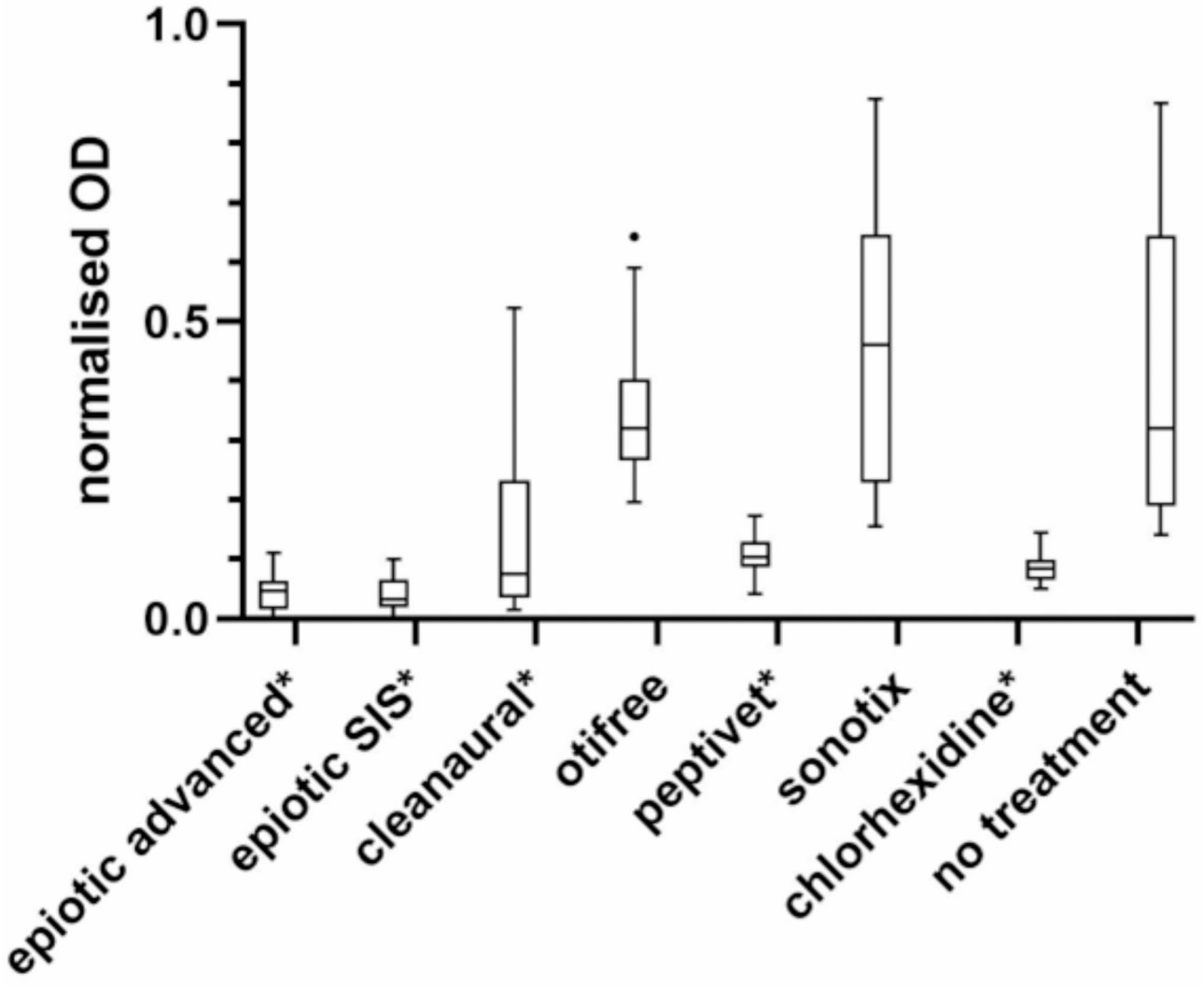

Fig. 1 Normalized optical density of *Pseudomonas aeruginosa* biofilms treated with undiluted ear cleanser products. Each box represents the interquartile range (25th to 75th percentile) with the median value indicated by the horizontal line within the box. Whiskers extend to the minimum and maximum values within 1.5 times the interquartile range. Outliers are displayed as individual dots, identified using the Tukey method. Asterisks denote treatments that are statistically significantly different from the no-treatment control ($p < 0.05$)*

strains compared to their sensitive counterparts. Previous research has established a link between multidrug resistance and biofilm-forming ability in *S. aureus* [18, 19]. It is possible that MRSA and MRSP strains form biofilms with higher biomass or have different extracellular matrix (ECM) compositions that are harder to disrupt. However, our experiments indicated that both sensitive and resistant strains exhibited similar growth in the no-treatment controls, suggesting comparable cell numbers in biofilms. This implies that the ECM composition, rather than the number of cells, may differ in resistant strains, affecting their susceptibility to disruption.

Considerations for in vivo effectiveness

A critical aspect of our study is understanding how the antibiofilm assay evaluates the effectiveness of ear cleansers. This assay measures the bactericidal activity against bacteria within biofilms and assesses the cleanser's ability to disrupt the biofilm structure itself. Specifically, after applying the ear cleansers, non-adherent cells are removed. This step allows us to evaluate two key actions of the cleansers: the ability to break down the biofilm's extracellular matrix (ECM), which holds the bacterial community together, and the capacity to kill the more resilient, adherent bacterial populations that remain after biofilm disruption. By removing non-adherent cells, the antibiofilm assay provides a comprehensive assessment of both biofilm integrity and bacterial viability within the biofilm. This is a crucial consideration for accurately interpreting the cleansers' antibiofilm efficacy, as it reflects their potential effectiveness in clinical settings

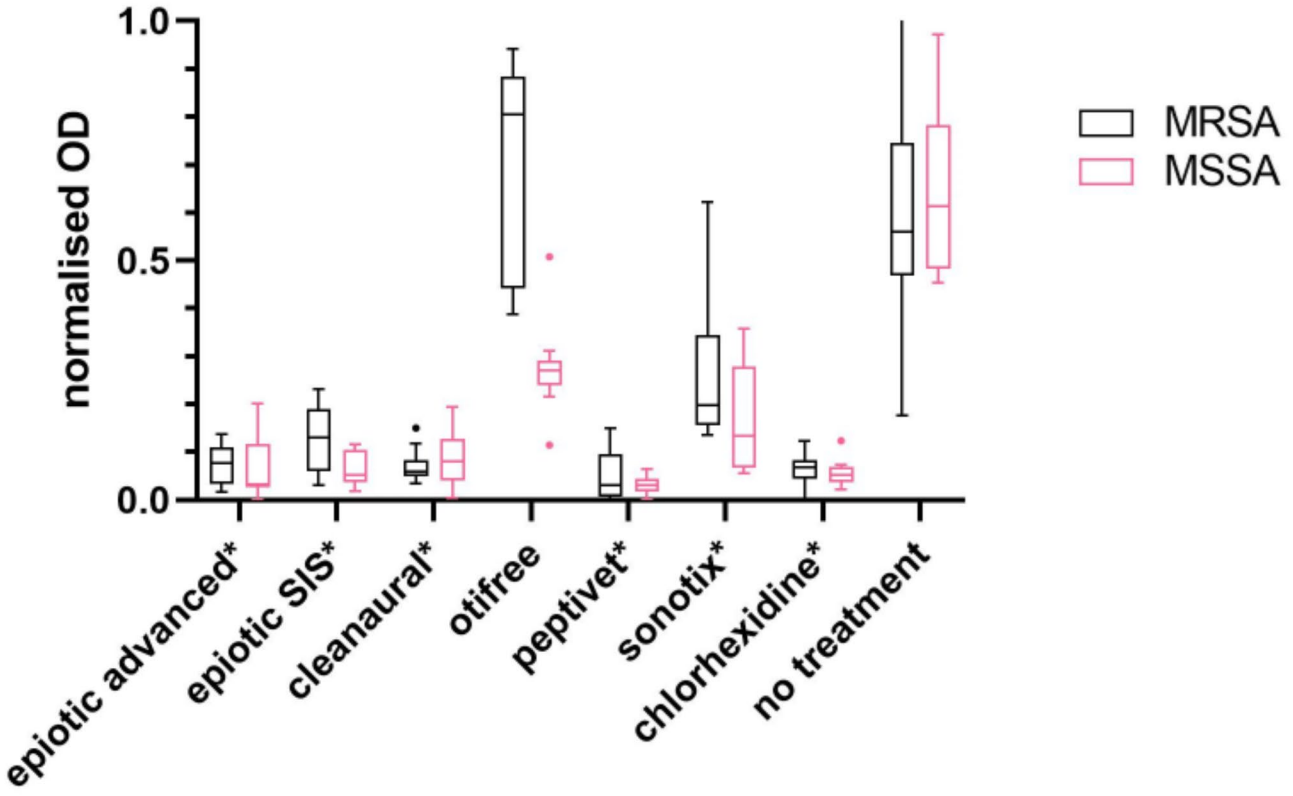

Fig. 2 Normalized optical density of methicillin-resistant (MRSA) and methicillin-sensitive (MSSA) *Staphylococcus aureus* biofilms treated with undiluted ear cleanser products. Each box represents the interquartile range (25th to 75th percentile) with the median value indicated by the horizontal line within the box. Whiskers extend to the minimum and maximum values within 1.5 times the interquartile range. Outliers are displayed as individual dots, identified using the Tukey method. Asterisks denote treatments that are statistically significantly different from the no-treatment control ($p < 0.05$)

where both biofilm disruption and bacterial killing are necessary for successful treatment.

When evaluating treatment effectiveness in vivo, it is also essential to consider various factors that influence the contact time between the cleanser and the pathogen. Factors such as the anatomical structure of individual dogs' ears, the specific area of the ear being treated and the proximity to the eardrum, along with the pet owner's approach to cleaning and the techniques employed, can all influence treatment outcomes. To simulate practical conditions, our experiments utilized a 15-minute treatment duration for the biofilm assays. However, in real-life scenarios, the sustained inhibition of biofilm growth may also depend on how long the antimicrobial agents remain on the affected skin surface. Therefore, the duration of antimicrobial activity should be considered when developing treatment plans.

Minimum inhibitory concentration (MIC) and its relationship to antibiofilm activity

In the Minimum Inhibitory Concentration (MIC) assays, we observed that methicillin-resistant strains required more concentrated ear cleanser solutions for effective inhibition compared to methicillin-sensitive strains, indicating that resistant strains are less susceptible to the cleansers, necessitating higher concentrations for inhibition (Fig. 5). The MIC findings complement the antibiofilm assay results by providing insight into the cleansers' effectiveness against planktonic bacterial cells. While the MIC assays focus solely on inhibiting bacterial growth in the planktonic phase, the antibiofilm assays assess both biofilm disruption and bactericidal activity within the biofilm as discussed above. Our results demonstrated that methicillin-sensitive staphylococcal strains were more susceptible to both antibiofilm disruption and MIC inhibition than their resistant counterparts. Although there were no significant differences in the antibiofilm activity between Epiotic SIS and Epiotic Advanced across all strains, the MIC assays revealed that Epiotic SIS exhibited higher activity than Epiotic Advanced against all *Staphylococcus* spp. strains tested (Fig. 5). This suggests that formulation differences between Epiotic SIS and Epiotic Advanced can influence their antibiofilm and bactericidal efficacies.

Efficacy against Pseudomonas aeruginosa

The Epiotic SIS and Epiotic Advanced ear cleansers showed high antibiofilm efficacy against the

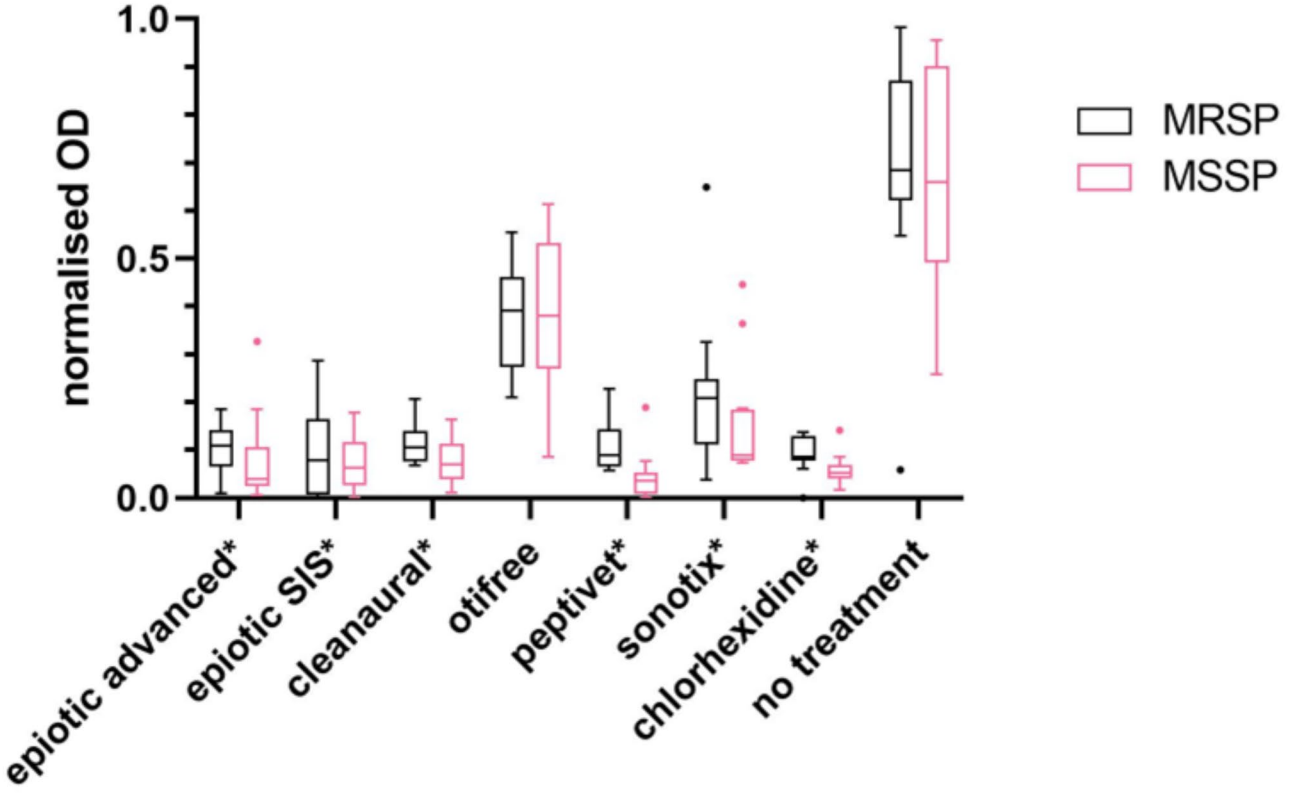

Fig. 3 Normalized optical density of methicillin-resistant (MRSP) and methicillin-sensitive (MSSP) *Staphylococcus pseudintermedius* biofilms treated with undiluted ear cleanser products. Each box represents the interquartile range (25th to 75th percentile) with the median value indicated by the horizontal line within the box. Whiskers extend to the minimum and maximum values within 1.5 times the interquartile range. Outliers are displayed as individual dots, identified using the Tukey method. Asterisks denote treatments that are statistically significantly different from the no-treatment control ($p < 0.05$)

gram-negative *P. aeruginosa* strain, comparable to the *Staphylococcus* spp. strains, whereas the other ear cleansers showed reduced efficacy against *P. aeruginosa* when compared 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 in clinical settings.

While the antimicrobial effects observed may be specific to the strains tested, the key finding of our study is the substantial differences in antibiofilm effects between products and the relatively minor differences in activity between resistant and sensitive strains of the same *Staphylococcus* species. This indicates that the antibiofilm efficacy of a product is generally consistent within a bacterial species, even though bactericidal effects may vary depending on the specific strain.

Conclusion

Several commercially available products exhibit antimicrobial and/or wax elimination properties, however, this study has highlighted variation in antibiofilm activities between products. Further, observed differences between antibiotic resistant and sensitive clinical isolates suggest that both antibiofilm activity of a product and identification of the causative organism and its antimicrobial resistance profile should be integral criteria in treatment considerations. For recurrent and/or chronic infections, an adapted treatment approach that considers the presence of biofilms on canine skin and ears may also be necessary.

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 clinical isolates kindly supplied by the Royal Veterinary College, UK. *Staphylococcus*

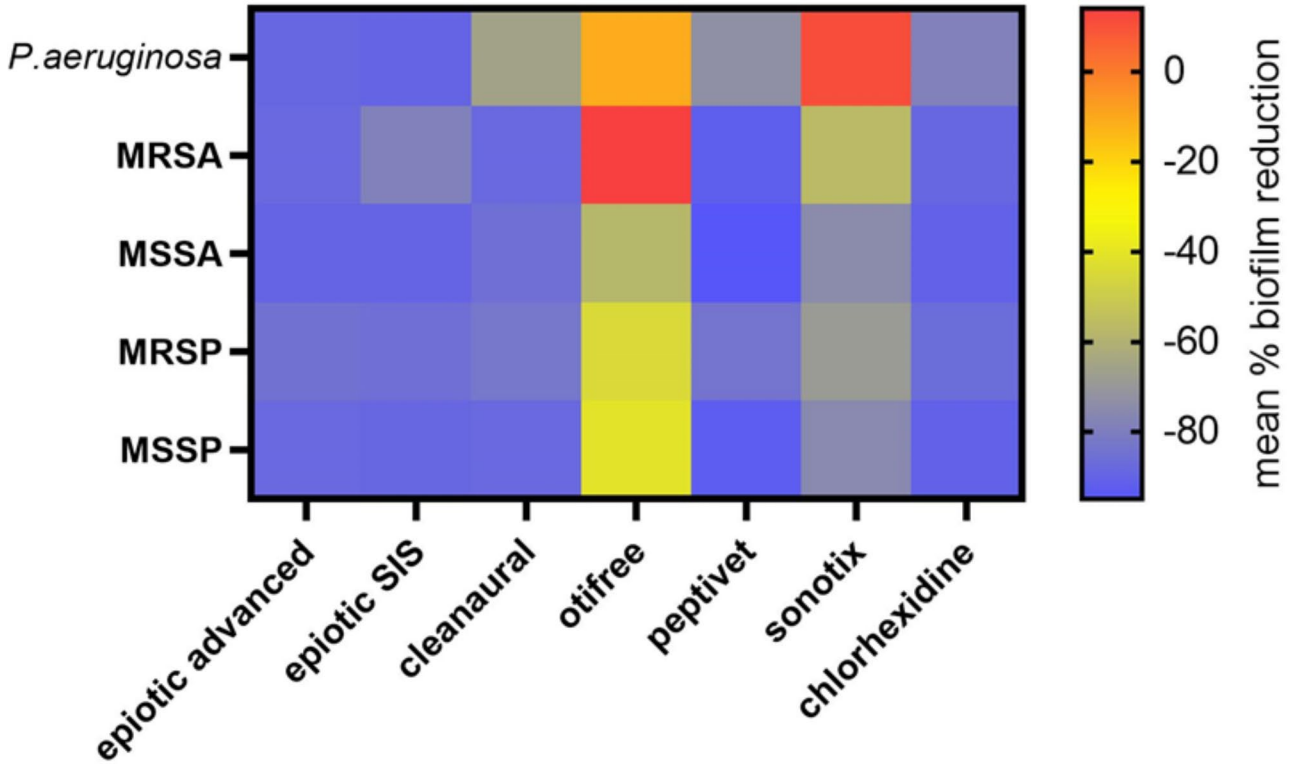

Fig. 4 Heatmap showing the mean percentage reductions in biofilm density relative to no-treatment controls for all bacterial strains and ear cleanser products tested. Each cell represents the average biofilm reduction achieved by a specific ear cleanser against a particular bacterial strain

pseudintermedius isolates were collected as part of a study in Germany, whereas the *Staphylococcus aureus* isolates were from a study conducted in the United Kingdom [24, 25]. 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 h 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 600 nm (OD_{600}). Standardization was performed according to the standard curve constructed over a range of OD values with viable counts performed at each OD. Biofilms were then formed in wells of micro titre plates by adding 100µL of standardized inoculum for a period of 90 min at 37 °C under shaking (75 rpm) to obtain pre-adhesion [26]. Following this period, the supernatant was discarded, and 200µL broth for biofilm growth added and incubated aerobically for 24 h. The biofilms were then separately exposed to the 100µL of test substances, chlorhexidine (0.2% w/v; positive control), and negative control (deionised water) for 15 min 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 [27]. Briefly, test solutions were removed by pipetting from the wells ensuring non-adherent cells were removed from the wells, then 50µL of MTT solution and 50µL of medium was added and the plate incubated (at 37 °C for 1 h) 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 min of incubation at 37 °C with shaking in an orbital shaker at approx. 75 rpm), the OD was read ($\lambda = 590$ nm) in a microplate spectrophotometer (BMG Labtech CLARIOStar Plus).

Statistical analysis of antibiofilm Assay

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

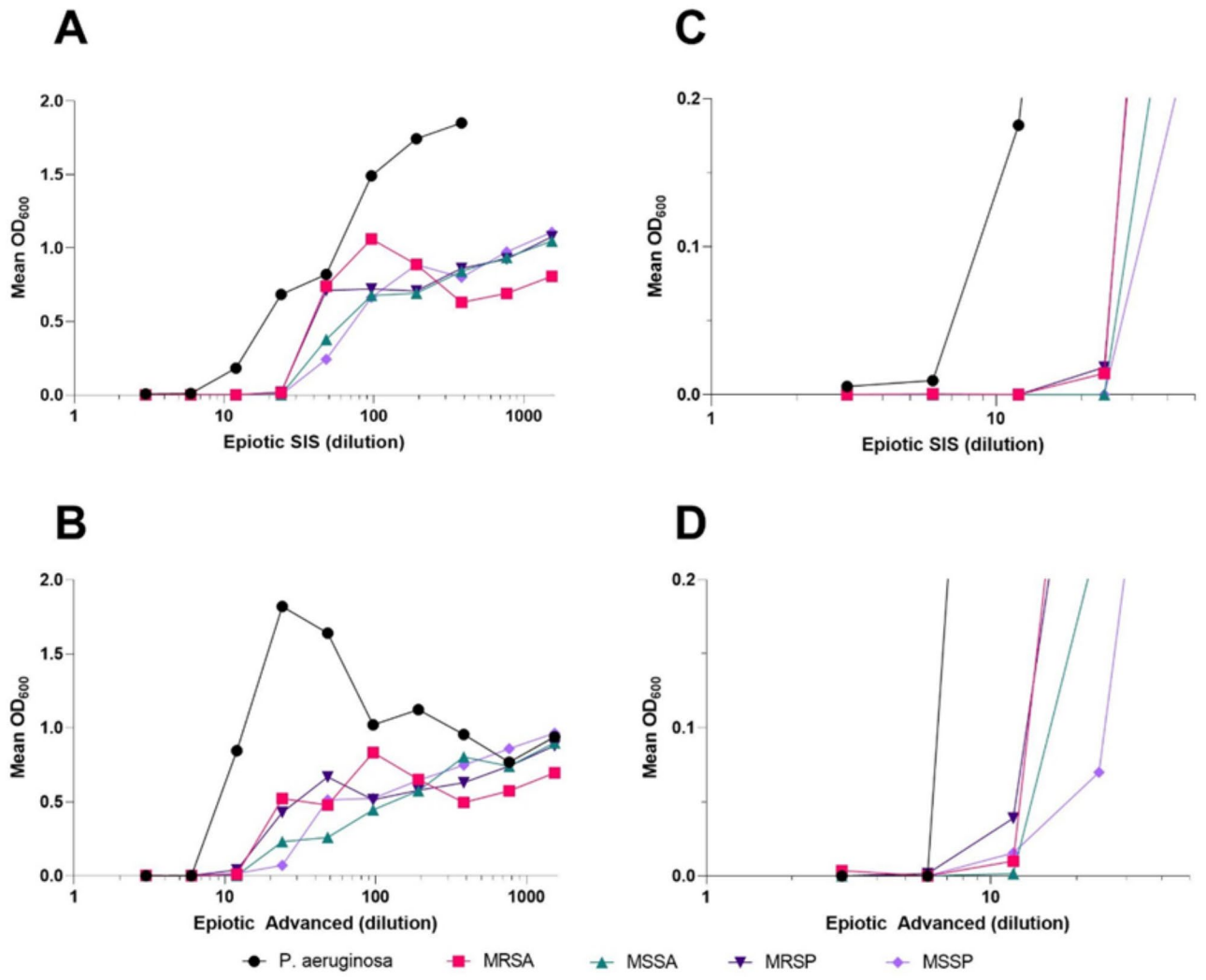

Fig. 5 Minimum Inhibitory Concentration (MIC) of Epiotic® SIS (**A**) and Epiotic® Advanced (**B**) ear cleansers against various bacterial strains. Panels (**A**) and (**B**) display line plots illustrating the relationship between different dilutions of Epiotic® SIS and Epiotic® Advanced, respectively, and the mean optical density at 600 nm (OD600) for each bacterial strain from duplicate MIC experiments. Panels (**C**) and (**D**) focus on the lower dilution ranges of Epiotic® SIS and Epiotic® Advanced, respectively, to clearly indicate the MIC values for each strain. In all experiments, the growth control wells without antibacterial agents showed approximately 1 OD600, confirming bacterial growth

Table 1 List of all strains used in this study

Organism	Isolate name	Notes
Methicillin sensitive *Staphylococcus pseudintermedius* (MSSP)	GL001B	Ear Infection isolate, genotyped using thermonuclease gene.
Methicillin resistant *Staphylococcus pseudintermedius* (MRSP)	GL119A	Skin infection isolate, genotype: thermonuclease gene, *mecA* positive.
Methicillin resistant *Staphylococcus aureus* (MRSA)	A004	Skin Infection isolate, genotype: thermonuclease gene, *mecA* positive.
Methicillin sensitive *Staphylococcus aureus* (MSSA)	B027	Skin infection isolate. Genotyped using thermonuclease gene.
Pseudomonas aeruginosa	288,230/476,168	Canine otitis isolate

A p-value < 0.05 was considered significant. 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 [28]. 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 h. Optical density at 600 nm 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, with growth control wells containing no antimicrobial agent, included in each experiment. Plates also contained chlorhexidine (0.2% w/v) as positive control.

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None.

Author 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.

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

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

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

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