

In vitro evaluation of the antimicrobial activity and diffusion capacity of solutions used for canine ear cleaning

Avaliação in vitro da atividade antimicrobiana e capacidade de difusão de soluções utilizadas para limpeza de ouvido em cães

Evaluación in vitro de la actividad antimicrobiana y la capacidad de difusión de las soluciones utilizadas para la limpieza de oídos caninos

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Abstract

Otitis is a common condition among dogs and requires appropriate treatment. Given the importance of the otitis externa and the use of products to combat this condition, this study evaluated the in vitro antimicrobial abilities of six commercial ear cleaners solutions (EC1 to EC6) and solutions of 3% boric acid, 3% lactic acid, 0.11% salicylic acid, 0.5% chlorhexidine, and 3% propylene glycol against *Staphylococcus* spp., *Pseudomonas* spp., *Proteus* spp., and *Malassezia* spp. The in vitro diffusion capacity in synthetic cerumen was also assessed. Dogs with clinical signs of otitis externa were selected and samples were collected from the external ear canals. The microbiological study was performed to identify the microorganisms from samples collected and the microorganisms isolated were used in the study. One ceruminolytic for human use and five commercial solutions used for canine ear cleaning were selected based on the diversity of the components used in each formula. The results show a variation of antimicrobial activity and diffusion capacity ear cleaners and its compounds. Lactic acid, chlorhexidine, EC1, EC2, EC4, and EC5 showed the best results for microbiological growth inhibition; boric acid, salicylic acid, propylene glycol and EC6 had little or no effect on microorganism growth. The ECs tested demonstrated diffusion capacity using the SSC. EC1 was the solution with the most significant responses, both as an antimicrobial agent and with regards to diffusion capacity. Among the commercial veterinary products tested, EC4 was found to have the best results.

Keywords: Antimicrobial activity; Canine ear cleanser; Ceruminolytic agents; Otitis externa.

Resumo

A otite é uma condição comum entre os cães e requer tratamento adequado. Dada a importância da otite externa e do uso de produtos para combater essa condição, este estudo avaliou a atividade antimicrobiana in vitro de seis soluções de limpeza de ouvido comerciais (EC1 a EC6) e soluções de ácido bórico 3%, ácido láctico 3%, 0,11 % de ácido salicílico, 0,5% de clorexidina e 3% de propilenoglicol contra *Staphylococcus* spp., *Pseudomonas* spp., *Proteus* spp. e *Malassezia* spp. A capacidade de difusão in vitro em cerúmen sintético também foi avaliada. Cães com sinais clínicos

de otite externa foram selecionados e amostras foram coletadas dos canais auditivos externos. O estudo microbiológico foi realizado para identificar os microrganismos das amostras coletadas e os microrganismos isolados foram utilizados no estudo. Um ceruminolítico para uso humano e cinco soluções comerciais para limpeza auricular canina foram selecionadas com base na diversidade dos componentes utilizados em cada fórmula. Os resultados mostram uma variação na atividade antimicrobiana e na capacidade de difusão dos limpadores de ouvido e seus compostos. Ácido láctico, clorexidina, EC1, EC2, EC4 e EC5 mostraram os melhores resultados para inibição do crescimento microbiano; ácido bórico, ácido salicílico, propilenglicol e EC6 tiveram pouco ou nenhum efeito no crescimento do microrganismo. As soluções testadas demonstraram capacidade de difusão usando o SSC. EC1 foi a solução com as respostas mais significativas, tanto como agente antimicrobiano quanto em relação à capacidade de difusão. Entre os produtos veterinários comerciais testados, o EC4 apresentou os melhores resultados.

Palavras-chave: Atividade antimicrobiana; Agentes ceruminolíticos; Limpador de ouvido canino; Otite externa.

Resumen

La otitis es una afección común entre los perros y requiere un tratamiento adecuado. Dada la importancia de la otitis externa y el uso de productos para combatir esta condición, este estudio evaluó las capacidades antimicrobianas in vitro de seis soluciones comerciales de limpiadores de oídos (EC1 a EC6) y soluciones de ácido bórico 3%, ácido láctico 3%, 0.11 % de ácido salicílico, 0,5% de clorhexidina y 3% de propilenglicol contra *Staphylococcus* spp., *Pseudomonas* spp., *Proteus* spp. y *Malassezia* spp. También se evaluó la capacidad de difusión in vitro en cerumen sintético. Se seleccionaron perros con signos clínicos de otitis externa y se recolectaron muestras de los conductos auditivos externos. El estudio microbiológico se realizó para identificar los microorganismos que se utilizaron en el estudio. Se seleccionaron un ceruminolítico para uso humano y cinco soluciones comerciales utilizadas para la limpieza de oídos caninos en función de la diversidad de los componentes. Los resultados muestran una variación de la actividad antimicrobiana y la capacidad de difusión. El ácido láctico, la clorhexidina, EC1, EC2, EC4 y EC5 mostraron los mejores resultados para la inhibición del crecimiento microbiológico; El ácido bórico, el ácido salicílico, el propilenglicol y el EC6 tuvieron poco o ningún efecto sobre el crecimiento de microorganismos. Las CE probadas demostraron capacidad de difusión utilizando el SSC. EC1 fue la solución con las respuestas más significativas, tanto como agente antimicrobiano como en cuanto a capacidad de difusión. Entre los productos veterinarios comerciales probados, se encontró que EC4 tenía los mejores resultados.

Palabras clave: Actividad antimicrobiana; Limpiador de oídos canino; Agentes ceruminolíticos; Otitis externa.

1. Introduction

Management of otitis require appropriate topical ear cleaners and prescription of ear drops. Diagnose and manage primary skin diseases, treat secondary infections and recognize any perpetuating or predisposing factors and manage them are the key to successful therapy of otitis (Paterson, 2016). The use of systemic medications to treat otitis is often associated with a low efficacy due to the inadequate penetration of these drugs into the lumen of the ear canal. Most of the topical medications comprise antimicrobial and antifungals agents, and glucocorticoids in their formulations, increasing the selective pressure and facilitating the emergence of multidrug-resistant bacterial strains (Guardabassi et al., 2004; Guardabassi et al., 2010).

There are different cleaning products with a range of active ingredients including ceruminolytics, surfactants, astringents, antimicrobials and anti-inflammatories. Ceruminolytics dissolve cerumen and dried debris in the ear canal, while surfactants emulsify debris and keep it in solution, and astringents dry the surface of the ear canal. Anti-inflammatories are often to inhibit inflammation and pruritus and antimicrobial activity limits the proliferation of bacteria and yeast (Nuttall & Cole, 2004; Swinney et al., 2008). Moreover, topical treatment should be performed after chemical removal of cerumen, but it may remain in the external ear canal, affecting the treatment successful. Therefore, otic preparations with active ingredients should persist inside the external ear canal for adequate effects and ceruminolytic activity and diffusion through cerumen residues are beneficial for high efficacy (Stahl et al., 2013).

Antimicrobial resistance is a growing concern in human and veterinary healthcare and effective use of antiseptics to reduce antibiotic use is often studied (Rafferty et al., 2019). The use of antiseptics as an alternative therapy in the control of multidrug-resistant bacteria has gained importance in veterinary medicine, provided there is no evidence of resistance to these agents. This approach would lead to a lesser selection of resistant strains of commensal and pathogenic microbiota (Banovic et al., 2013). Another advantage of using cleaning solutions is the absence of substances with potential to cause adverse reactions,

such as glucocorticoids. Thus, the use of these antiseptic ear solutions may be considered as an important tool in the treatment of otitis, as they could reduce the use of antibiotics (Mason et al., 2013).

There are few reports of antimicrobial efficacy for most ear cleaners (Swinney et al., 2008), therefore studies on ear cleaners efficacy is important to improve treatment of canine otitis. Stahl et al. (2013) performed a detailed study of the composition of canine cerumen and developed a standardized synthetic cerumen (SSC), which allowed the assessment of the diffusion activities of eight commercial solutions in ear cleaning and the treatment of otitis. Therefore, the objectives of this study were to evaluate the in vitro antimicrobial activity of six commercial ear cleaners and five compounded products against main five microorganisms involved in canine otitis externa and to assess the in vitro diffusion capacity of these 11 solutions using the SSC.

2. Methodology

2.1 Selection of samples.

The microorganisms were selected based on the studies of Bugden (Bugden, 2013) and Lyskova et al. (Lyskova et al., 2007). Dogs with clinical signs of otitis externa presented to the Department of Dermatology of the Veterinary Hospital of the UFMG from April 2013 to November 2014 were selected. All animals were evaluated by veterinarians experienced in dermatology and the inclusion criteria for the study were the following: (i) presence of two or more clinical signs of otitis; and (ii) presence of bacteria and/or yeasts in the cytological examination of the cerumen, performed at the exam room, following sample collection. The study design was reviewed and approved by the Animal Care and Use Committee at the Federal University of Minas Gerais (CEUA/UFMG, protocol 246/2013).

The samples were collected from the external ear canals with a sterile swab and stored in Stuart transport media for up to 48 hours. The samples were forwarded to the Applied Microbiology Laboratory of the School of Veterinary Medicine of UFMG, where they were inoculated onto Petri plates containing blood agar culture medium, and then incubated at 37°C for 24 to 48 hours, in order to obtain isolated colonies. After incubation, the isolated colonies were subjected to Gram staining for the identification of two main groups, Gram-positive and Gram-negative bacteria.

Gram-positive bacteria with an arrangement similar to grape bunches were then inoculated onto Petri plates containing Mueller-Hinton agar culture medium and incubated at 37°C for 24 to 48 hours. Bacteria of the genus *Staphylococcus* were identified by biochemical tests for phenotypic identification as proposed by Bannoehr & Guardabassi (Bannoehr & Guardabassi, 2012) and Quinn et al. (Quinn et al., 2011). After phenotypic identification, all *Staphylococcus* spp. were identified using molecular methods, with specific primers for *S. pseudintermedius*, as previously described Sasaki et al. (Sasaki et al., 2010) and then subjected to polymerase chain reaction (PCR) analysis for detecting the presence of the *mecA* gene, according to Mehrotra et al. (Mehrotra et al., 2000).

From the PCR results, 10 samples of *S. pseudintermedius* were selected for the in vitro study, five methicillin-resistant *S. pseudintermedius* (MRSP), and five methicillin-susceptible *S. pseudintermedius* (MSSP). Five additional samples of *S. schleiferi coagulans* (SC) were also selected for analysis.

The isolates characterized as Gram-negative rods were seeded onto MacConkey agar Petri plates and incubated at 37°C for 24 to 48 hours. The isolated colonies were characterized by biochemical tests for the phenotypic identification of bacterial species from the *Pseudomonas* and *Proteus* genera, according to the methods described by Oplustil et al. (Oplustil et al., 2000); five samples each of *Proteus* spp. (PRT) and *Pseudomonas* spp. (PSM) were selected for the in vitro studies.

The samples that showed oval formations or “bottle-shaped” unipolar budding, characteristic of *Malassezia* spp., upon cytological analysis were seeded onto Sabouraud agar Petri plates without a lipid source for the selection of the species

Malassezia pachydermatis (MLZ) and incubated at 25°C for up to 14 days (Quinn et al., 2011); a total of five samples were selected.

2.2 Selection of ear cleaners (ECs).

One ceruminolytic for human use (EC1), and five commercial solutions (EC2-EC5) used for canine ear cleaning were selected based on the diversity of the components used in each formula. The composition of the commercial formulations is detailed in Table 1. Knowledge of the components of the ear cleaning solutions used in the present study is important to evaluate the results obtained and compare with literature. Five single substances used in canine ear cleaning were also selected: 2% boric acid, 3% lactic acid, 0.11% salicylic acid, 0.5% chlorhexidine, and 3% propylene glycol, compounded in aqueous solutions.

Table 1. Composition of commercial ear cleaning solutions used in the study and their percentage values according to the manufacturer information.

Solution	Components	Concentration (mg/mL or %)
EC1	Carbamide peroxide	10.0
EC2	Lactic acid	29.0
	Docusate sodium	19.5
	<i>Aloe vera</i>	41.03
	Allantoin	10.0
	Melaleuca	10.0
EC3	Propylene glycol	30.0
	Extract of chamomile	1%
	Cocamidopropyl betaine	6.0
EC4	Salicylic Acid	1.1
	Lactic Acid	28.8
EC5	Salicylic Acid	1.1
	Lactic Acid	29.8
	Boric Acid	20.0
	<i>Aloe vera</i>	10.0
EC6	Polysorbate 20	305.0
	Triethanolamine	0.07 %
	Preserval PE	40.0

Source: Authors according to the manufacturer information.

2.3 Evaluation of the antimicrobial activity of the ECs.

Samples properly identified as five methicillin-resistant *S. pseudintermedius* (MRSP), methicillin-susceptible *S. pseudintermedius* (MSSP), *S. schleiferi coagulans* (SC), *Pseudomonas* spp. (PSM), and *Proteus* spp. (PRT) were transferred to a tube containing 3mL of Mueller-Hinton broth and incubated at 37°C until they reached the standard turbidity of 0.5 of the McFarland scale (CLSI, 2012, 2017). To prepare the disc-diffusion test plates, 4mm of Mueller-Hinton and Sabouraud agar, were added to 150x15mm Petri dishes, according to the recommendations of the CLSI (CLSI, 2017) for antibacterial and antifungal susceptibility testing, respectively. Due to the limited size of the plates, each test was performed in two plates. Using

an 8mm biopsy punch, six equidistant wells were punched in one culture plate and seven in the second culture plate, yielding 13 wells: one positive control, one negative control, and the other 11 for one of the treatments each (Mason et al., 2013). Then those plates were seeded, in quadruplicate, using a sterile swab with the samples of MRSP, MSSP, SSC, PSM, and PTR grown in the Mueller-Hinton broth.

The samples of *M. pachydermatis* (MLZ) were transferred to tubes containing 3 mL of saline and incubated at 37°C until they reached the standard turbidity of 0.5 of the McFarland scale (CLSI, 2009). Using a sterile swab, cultures were seeded, in quadruplicate, onto the plates containing Sabouraud agar.

For each susceptibility test, using a micropipette, 200µL of the each of the commercial ECs, 200µL of each manipulated aqueous solutions, 200µL of the positive control [0.3% Ciprofloxacin for bacteria (Humphries et al., 2016) and 1% miconazole for *M. pachydermatis* (Rojas et al., 2016) and 200µL of the negative control (water base) were added to the wells. After incubation for 24 hours, the sizes of the halos formed around the wells were measured using a millimeter ruler.

2.4 Production of standardized synthetic cerumen (SSC) and evaluation of the diffusion capacity of EC

The production of the SSC was performed according to the method described by Sánchez-Leal et al. (Sánchez-Leal et al., 2006); the final composition of the prepared SSC is described in Table 2. One of the objectives of the present study was to determine the diffusion activity of otic preparations through cerumen. Therefore, to analyze this, we used an in vitro method using synthetic canine cerumen. Because an in vivo study to test different products simultaneously would be too expensive, an in vitro method was developed based on the average composition of natural canine cerumen as described in the literature. (Sánchez-Leal et al., 2006).

In order to evaluate the EC diffusion capacity, 4mm of the SSC produced was added to 60×15-mm Petri plates and, in each plate, a well was made using an 8mm biopsy punch. Using a micropipette, 200µL of the different ECs or pure aqueous base, plus a marker (Oil Red O, ref:O-0625, Sigma Aldrich) were added to each well in each plate, to observe the distance travelled by the ECs in the SSC. Four replicates of the test were performed. Using a millimeter ruler, the size of the halos formed around the wells after 12 and 24 hours was measured, and this was characterized as the diffusion of the ECs in the SSC.

Table 2. Standardized synthetic cerumen (SSC) composition.

Component	%
Myristic acid (ref: M-3128, Sigma-Aldrich)	33.6
Oleic acid (ref: O-1008, Sigma-Aldrich)	9.4
Palmitic acid (ref: P-0500, Sigma-Aldrich)	33.6
Cholesterol (ref: C-8503, Sigma-Aldrich)	10.9
Squalene (ref: S-3626, Sigma-Aldrich)	12.5

Source: Authors based on Sánchez-Leal et al. (2006).

2.5 Statistical analysis

The present study is an descriptive and experimental research with a qualitative and quantitative approach, through the analysis of numerical data and interpretation of the data obtained (Pereira et al., 2018). Data were analyzed using SAS (version 9.2). Descriptive analyses were performed, and principal component analysis was based on a correlation matrix and was calculated using a fitted mean input, and a biplot was obtained to illustrate the relationships between the ECs, microorganisms, and diffusion in SSC after 12 and 24 hours. The biplot was generated by tracing the entries according to their scores of the first

and second major components. For the variable halo size in the SSC, where the EC × time interaction was significant, unfolding was performed, and the means for the ECs and time factors were compared by the Scott-Knott test. Statistical significance was set at $P \leq 0.05$.

3. Results

The results of the inhibition of microorganism growth in the presence of the substances tested in the study are shown in Table 3, where the mean size of the inhibition halos measured in shown, together with the results of negative and positive controls. The largest halos of growth inhibition were formed by the action of EC1 on all microorganisms, followed by EC2, EC4, chlorhexidine and lactic acid. Salicylic acid had no action on all microorganisms, boric acid slightly inhibited the growth of only SC, and EC6 and propylene glycol had low inhibition on all microorganisms.

Table 3. Mean size (mm) of inhibition growth halos of microorganisms in the presence of the ear cleaners tested.

Ear-cleaner (EC)	MOO (microorganism)					
	MRSP	MSSP	SC	PRT	PSM	MLZ
Control +	46.2	38	36.6	47.6	50.2	36.2
Control -	0	0	0	0	0	0
Boric acid	0	0	3.2	0	0	0
Lactic acid	22.2	24.4	19.4	15.4	15.8	37.2
Salicylic Acid	0	0	0	0	0	0
Chlorhexidine	28	29.6	28.2	21	21.2	29.6
Propylene glycol	2.4	12.4	8.8	0	0	0
EC1	45.2	49.6	31.2	34	24.8	58.4
EC2	21.6	24.2	29	20.6	16.4	42.2
EC3	14	16.2	23.8	0	0	20.2
EC4	20.6	20.6	20.4	19.8	17.8	41.8
EC5	12.2	16.8	13	8.6	14.8	39.8
EC6	3.4	5.2	3.4	2.8	0	0

MRSP: Methicillin-resistant *Staphylococcus pseudintermedius*; MSSP: Methicillin susceptible *S. pseudintermedius*; SC: *S. schleiferi coagulans*; PRT: *Proteus* spp.; PSM: *Pseudomonas* spp.; MLZ: *Malassezia pachydermatis*. Source: Authors.

The halo diffusion of oil red supplemented with the ear cleaners tested through the SCC after 12 and 24 hours are shown in table 4, in which the mean size of the halos measured for each ear cleaner tested are shown and compared. All ear cleaners tested showed diffusion capacity in the SSC used. In the EC×time interaction, the most significant diffusion activities after 12 hours were observed with boric acid, salicylic acid, chlorhexidine, propylene glycol, EC1, EC4, EC5, and EC6. After 24 hours, the same ECs, except for propylene glycol and EC5, displayed the most significant activities (Table 4).

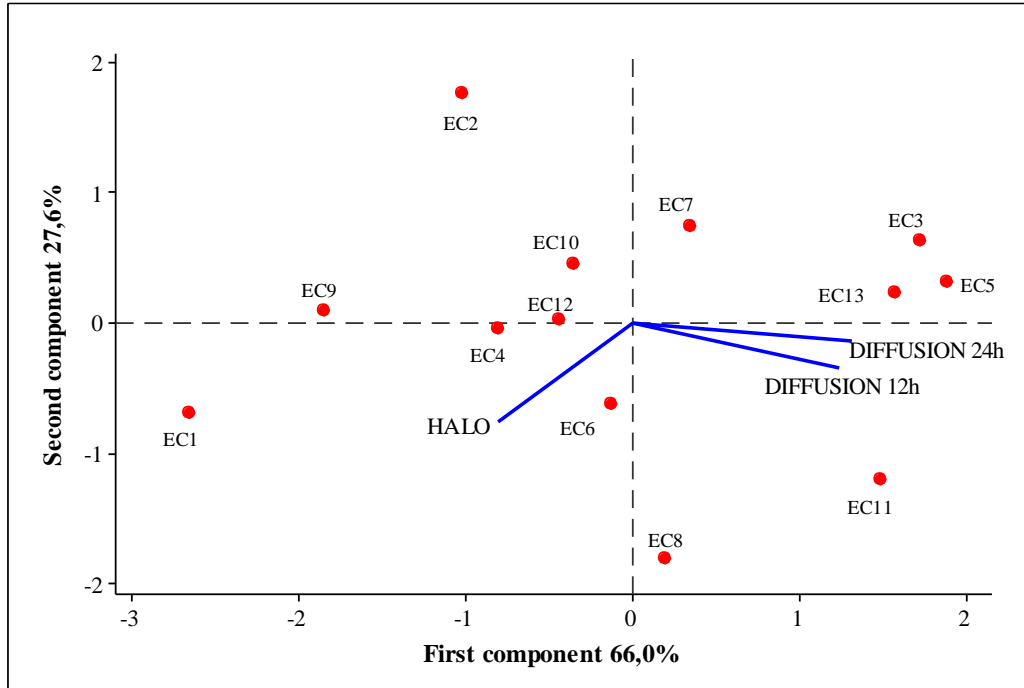
Table 4. Mean sizes (mm) of the halos formed by the ear cleaners in SSC after 12 and 24 h

Ear cleaner	Time			SEM	P-value		
	12 hours	24 hours	Mean		12 hours	24 hours	Mean
Boric acid	18.8 a	24.2 a	21.5 a				
Lactic acid	17.2 b	19.4 b	18.3 b				
Salicylic Acid	21.2 a	22.4 a	21.8 a				
Chlorhexidine	18.6 a	20.6 a	19.6 b				
Propylene glycol	18.6 a	20.2 b	19.4 b				
EC1	19.8 a	21.8 a	20.8 a	1.883	< 0.0121	< 0.0150	< 0.0104
EC2	16.4 b	17.0 b	16.7 b				
EC3	17.8 b	19.4 b	18.6 b				
EC4	21.6 a	22.8 a	22.2 a				
EC5	18.6 a	18.8 b	18.7 b				
EC6	21.2 a	21.6 a	21.4 a				

Means followed by the same letter did not differ statistically among themselves, by the Scott-Knott test with 5% significance. SEM: Standard error of the mean. Source: Authors.

Using principal component analysis (PCA) it was found that the first component explains 66% of the vector behavior, while the second component explains 27.6% of the vector behavior and the results of the PCA are demonstrated in Figure 1. Halo is a strong negative vector in component 2 and therefore this component focuses on halo inhibition growth of the microorganisms, or antimicrobial activity. A strong positive vector is demonstrated in component 1 for diffusion in 12 and 24 hours, so this component focuses on a diffusion capacity. Salicylic acid, boric acid, EC4 and EC6 had the greatest positive vector on the first component, and are positive correlated with diffusion, but are negative correlated with chlorhexidine, lactic acid EC5, these correlated positively with halo (Figure 1).

Figure 1. Biplot of main components. Vectors indicate the variables; symbols mark the 13 different ECs. EC1. Positive control; EC2. Negative control; EC3. Boric acid; EC4. Lactic acid; EC5. Salicylic acid; EC6. Chlorhexidine; EC7. Propylene glycol; EC8. Ear cleaner 1 (EC1); EC9. Ear cleaner 2 (EC2); EC10. Ear cleaner 3 (EC3); EC11. Ear cleaner 4 (EC4); EC12. Ear cleaner 5 (EC5); EC13. Ear cleaner 6 (EC6).



Source: Authors.

4. Discussion

The present study assessed if an ECs, by itself or as a part of a commercial solution, can inhibit the growth of specific microorganisms, verified by the formation of growth inhibition halos, and also if these solutions have the ability to diffuse in a medium that simulates canine cerumen, visualized by the diffusion of oil red supplemented with ear cleaners tested. The solution with highest activity against all microorganisms was EC1, which also showed diffusion capacity in the SCC. The active compound of EC1 is carbamide peroxide, a ceruminolytic that release oxygen in situ to help break up debris (Paterson, 2016). Carbamide peroxide decomposes into hydrogen peroxide and urea when in contact with cerumen (Thickett & Cobourne, 2009). Urea acts as an emollient (Sant'Anna Addor et al., 2009) and an antimicrobial (Grether-Beck et al., 2012), while hydrogen peroxide decomposes to water and oxygen with generation of heat, and its oxidant activity acts as an antimicrobial agent and disinfectant (Marrero et al., 2017). Marrero et al. (2017) evaluated the in vitro activity of 1.5% hydrogen peroxide, a decomposition product of carbamide peroxide, and also found activity against microorganism growth. According to Marrero et al. (2017), although hydrogen peroxide does not produce reactions in the ear at concentrations of up to 5%, its ototoxicity is controversial, being recommended only when there is no rupture of the tympanic membrane. Therefore, EC1 demonstrated good activity against common microorganisms that cause otitis and had a good diffusion in the SCC, but it is for human use only.

EC2, EC4, chlorhexidine and lactic acid also showed inhibition for the microorganisms tested and good diffusion capacity, although lactic acid and EC2 apparently do not have good diffusion compared to all ear cleaners tested. Moreover, EC4 demonstrated positive factor correlated with diffusion, and chlorhexidine and lactic acid correlated positively with

inhibition halo. Organic acids, such as boric acid, lactic acid and salicylic acid are astringents, help buffer the ear canal environment, and have a moderate antimicrobial effects (Marrero et al., 2017; Swinney et al., 2008). In a study conducted by Swinney et al. (2008), the presence of specific organic acids was not associated with antimicrobial efficacy, and the authors highlighted that although they have some antimicrobial effect, dilution and interaction with other ingredients in commercial ear cleaners may reduce the antimicrobial efficacy. Chlorhexidine is an antimicrobial compounds frequently incorporated in ear cleaners to retard microbial proliferation (Nuttall & Cole, 2004) and is an antiseptic commonly used in the dermatological treatment of companion animals with superficial pyoderma caused by *S. pseudintermedius* (Borio et al., 2015).

Salicylic acid had no growth inhibition on microorganisms tested, since no halo formation was observed, although diffusion was observed in the SSC. Salicylic acid and EC4 had positive factor on diffusion principal component analysis and lactic acid with inhibition halo formation. Salicylic acid is a bacteriostatic agent that is keratoplastic at low concentrations and keratolytic at higher concentrations (above 2%) (Nuttall & Cole, 2004). These results indicate that salicylic acid might have no activity when used alone, since EC4 has salicylic acid and lactic acid, and had significant activity and might justify the use of salicylic acid in association with lactic acid. Considering the activity of lactic acid on the microorganism's growth and the diffusing capacity of the salicylic acid, solutions that combine these two components can possibly act more effectively if we consider a synergistic effect. If salicylic acid penetrates the cerumen; consequently, lactic acid can increase its area of activity. This is a common association used in commercial ECs such as EC4 and EC5.

The commercial ear cleaner EC6 had no inhibition halo formation on growth for PSM and MLZ and no inhibition growth halo was observed against PTR and PSM for EC3. EC6 displayed good activity when its diffusion capacity in SSC was analyzed and also was positive correlated with diffusion in PCA. In a previous study, ear cleaners showed variable antimicrobial activity against different isolates of *Pseudomonas* and EC4 resulted in variable and inconsistent inhibition of the *Pseudomonas* isolates, while an ear cleaner with 0.15% chlorhexidine showed excellent activity against *Pseudomonas*, suggesting that chlorhexidine at this concentration is a safe choice as a flush in otitis (Steen & Paterson, 2012). In case of *M. pachydermatis*, EC1 generated a larger growth inhibitory halo, as did lactic acid and solutions containing lactic acid. These findings the activity of chlorhexidine, lactic acid and EC2 for *Pseudomonas* spp. and *M. pachydermatis*.

Boric acid did not show activity against all microorganisms tested. A study on in vitro antimicrobial activity of acetic acid/boric acid impregnated cleansing wipes found no antimicrobial activity, although they are marketed as antiseptic, antibacterial and/or antifungal cleansing wipes (Rafferty et al., 2019). Propylene glycol showed no activity against PRT, PSM and MLZ, despite reports of its antimicrobial (Nalawade et al., 2015) and antifungal actions (Lloyd et al., 1998). Moreover, its diffusion capacity is lesser than that of other ECs; in addition, there are reports of its ototoxicity (Vassalli et al., 1988). A study with a commercial product with propylene glycol in its composition, demonstrated poor antimicrobial action of this compound (Marrero et al., 2017). Propylene glycol is a ceruminolytic commonly included in ear cleaners to soften and dissolve cerumen (Nuttall & Cole, 2004). Altogether, our study indicates that boric acid, salicylic acid, propylene glycol are not indicated for use alone and commercial solutions EC6 and EC3 should be used with caution.

Our study has some limitations. Antimicrobial efficacy of available commercial ear cleaners is especially variable and it is very difficult to draw conclusions about the their importance, as the cleaners contain a great variety of ingredients in different combinations and concentrations (Swinney et al., 2008). It is not possible to evaluate the diffusion of the ear cleaners into the surrounding medium for antimicrobial and diffusion studies, therefore, the study is more descriptive than analytical. Also, many factors affect the response of in vivo therapy, therefore, in vitro studies may not represent in vivo responses, rather, indicate the use of certain products. We selected the strains for the study very carefully, but different strains may show different biological characteristics. But studies like ours provide knowledge of ear cleaners properties and help to choose the best product for specific clinical situations (Marignac et al., 2019).

5. Conclusion

The current study demonstrates the inhibitory activity of ECs on the growth of important microorganisms associated with canine otitis, in conjunction with their diffusion capacities, and indicates that EC1 presented the best results. The commercial solution EC4 was the best veterinary solution for combating microorganisms involved in otitis, followed by EC2, chlorhexidine and lactic acid. Salicylic acid had no halo formation, while and boric acid, EC3, EC6 and propylene glycol showed low inhibition on all microorganisms. The results show a variation of antimicrobial activity and diffusion capacity ear cleaners and its compounds. Further studies are necessary to establish clinical efficacy and if the results found can be replicated with larger bacterial/fungal samples and different synthetic cerumen, with the addition of other components of natural cerumen like keratinocytes or proteins.

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