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SCIENTIFIC RESEARCH AND VETERINARY INFORMATION

News from 32nd ESVD 2021

The comparative cerumenolytic activity of otic preparations, an *in vitro* study

Stability of the N-acetylcysteine (NAC) with Tris-EDTA solution and in combination with dexamethasone sodium phosphate in aqueous solution for 50 days

Tris-NAC ® (Tris EDTA + N acetylcysteine) activity against biofilm production, an *in vitro* study

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ICF Evolution of Knowledge

The comparative cerumenolytic activity of otic preparations, an *in vitro* study

Milanesi N. et al. Proceedings ESVD 32nd 2021

The comparative cerumenolytic activity of otic preparations, an *in vitro* study

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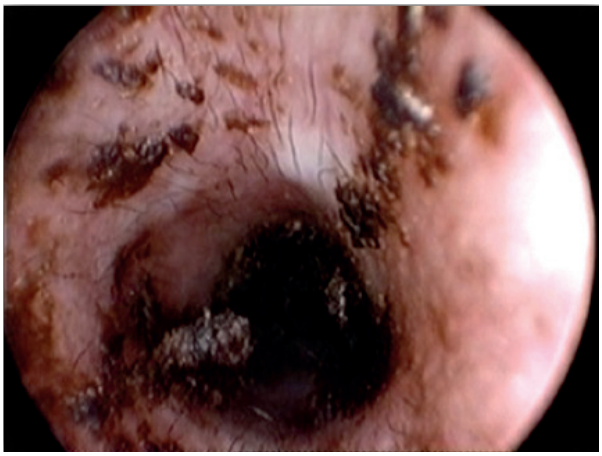
Canine Cerumen [1,2]

It covers the epithelial surface of the external ear canal

- Cerumen is formed of the secretions of sebaceous and ceruminous glands together with exfoliated cells

The lipid component of earwax can vary widely (18.2-92.6%)

Canine cerumen is mainly composed of ***exfoliated cells, waxes, oils, free fatty acids (margaric, stearic, oleic, linoleic acid), esters, immunoglobins and proteins***



Actions:

1. Facilitates the capture and excretion of debris
2. It maintains the moisture content through a repulsive effect on water
3. Antimicrobial activity (oleic and linoleic acid)

[1] **Anatomy and physiology of the canine ear**
Lynette K. Cole; Vet Derm, 2009

[2] **Ceruminal diffusion activities and ceruminolytic characteristics of otic preparations - an *in vitro* study**
Stahl J et al. BMC Veterinary Research. 2013

Histological changes in the external ear canal of dogs with otitis externa [3]

- Canine otitis externa (OE) is one of the most common diseases in small animal practice
- It is a syndrome with a multifactorial aetiology
- In chronic OE, the quantity of ceruminous glands increases significantly, caused by the inflammatory process and gland hyperplasia can be observed

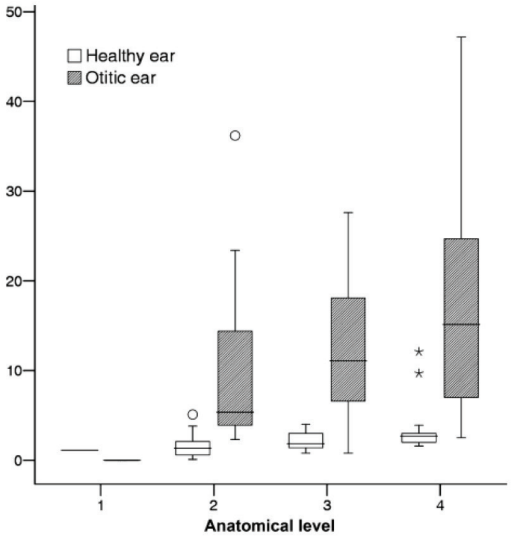
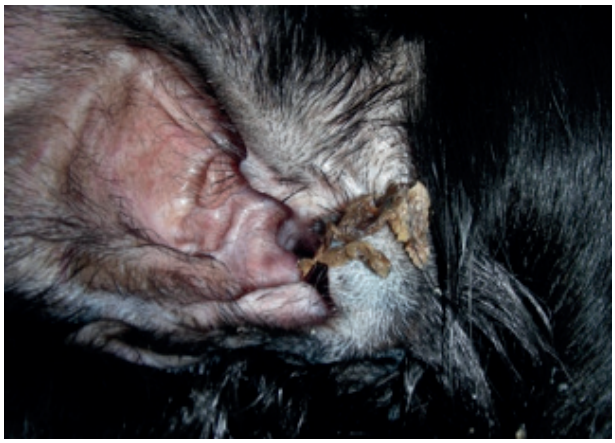


Figure 5. The mean proportion (%) of each section occupied by ceruminous glands in the aural skin at four anatomical levels in healthy ears (n = 28) and ear with otitis externa (n = 15). Outliers (circles) and extreme values (asterisks) are indicated.

Pathological changes in the external ear canal of dogs with COE [4]

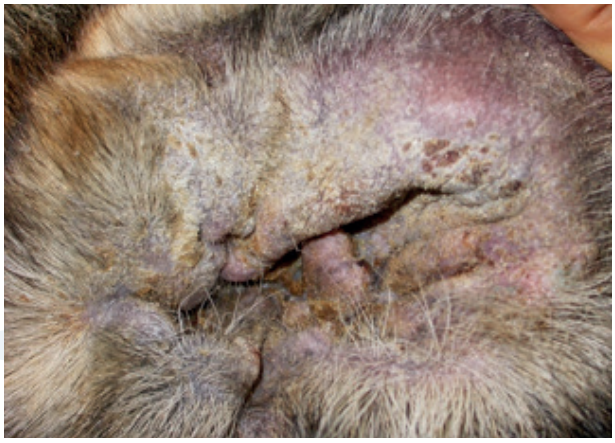
Earwax changes:

1. Reduction of lipid content
2. Increased humidity
3. Decreased antibacterial activity
4. Epithelial migration (EM) absent
5. Excess of wax material
6. Production of earwax plugs



Cerumen and ceruminous canine otitis [5]

An excess of cerumen may be irritating, prevent medications from contact with the ear canal wall and create a favourable environment for microorganisms to proliferate leading to ceruminous otitis externa and secondary yeast (*Malassezia*) and bacterial infections



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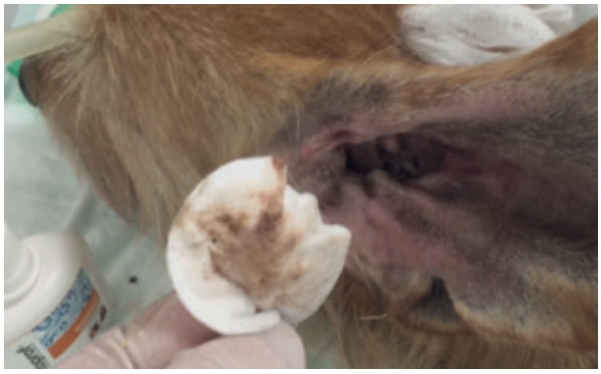
[3] **Histological changes in the external ear canal of dogs with otitis externa**
Hui-Pi Huang et al. Vet Dermatol. 2009

[4] **Breed variations in histopathologic features of chronic severe otitis externa in dogs: 80 cases (1995-2001)**
Angus JC et al. J Am Vet Med Assoc. 2002

[5] ***In vitro* investigation of ceruminolytic activity of various otic cleansers for veterinary use**
Sánchez-Leal et al. Vet Dermatol. 2006

Reasons for the use of ceruminolytic agents [6]

- Ear cleaning can be beneficial in maintaining the normal otic environment, especially in 'seborrhoeic' or allergic dogs with excessive cerumen in their ears
- Cerumenolytic activity and diffusion through cerumen residues helps to make the disintegration of earwax and its detachment from the ear epithelium faster and more effective



Ceruminolytic ear cleaners [7]

• The ability to effective clean ears to remove wax is an important part of the treatment and then ongoing maintenance therapy for otitis
• Diocytl sodium sulphosuccinate (DOSS), calcium sulphosuccinate, urea or carbamide peroxide are potent ceruminolytics. Urea and carbamide peroxide are foaming agents that release oxygen in situ to help break up debris
• Cerumino-solvent ear cleaners are organic oils and solvents that soften and loosen cerumen. Examples of these are butylated hydroxytoluene, cocamidopropyl betaine, glycerine, lanolin, propylene glycol and squalene

The comparative cerumenolytic activity of otic preparations, an *in vitro* study
• The cerumenolytic activity of some otic preparations represents a valuable aid for the management of this condition
• The study aimed to evaluate the wax removal efficacy, *in vitro*, of various veterinary otic preparations used in Europe

Materials and Methods
• evaluate the effectiveness of the otic preparations:
Otoprof ®, Otoact ®, Epiotic ®, Diclorex oto ®, Otifree ®, Aurinet ® Malacetic ®, Otodog ®, Otolane ®

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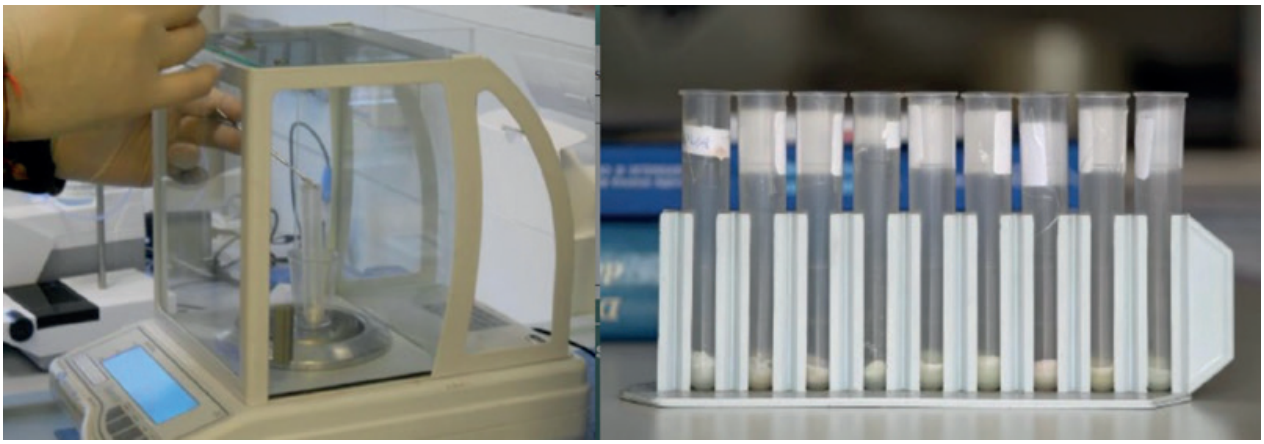
6 ***In vitro* investigation of ceruminolytic activity of various otic cleansers for veterinary use**
Sánchez-Leal et al. Vet Dermatol. 2006

7 **Paterson S. Topical ear treatment—options, indications and limitations of current therapy.** J Small Anim Pract 2016

Products	Agents
Otoprof ®	Aqua, Dioctyl Sodium Sulphosuccinate, Carbamide peroxide, Propylene Glycol
Otoact ®	Squalene, Salicylic acid, Butyl extract of chamomile, Tannic acid
Epiotic ®	Aqua, Disodium EDTA, PCMX, Diethylhexyl sodium sulfosuccinate, Salicylic acid, Glycotechnology (Rhamnose, Galactose, Mannose), Defensin technology (Peumus boldus leaf extract, Spiraea ulmaria extract).
Diclorex oto ®	Glycerin, Dipropylene glycol, Isopropyl myristate, Cetearyl alcohol, Oleic acid, Cetrimonium chloride, Hydroxyethylcellulose, Glycyrrhetinic acid, Chlorhexidine digluconate 0,3%, BHT, Disodium, EDTA, water
Otifree ®	Calendula 1%, 40% propylene glycol, water, emulsifiers, basil oil. Neutral pH solution
Aurinet ®	Aqua, paraffinum liquidum, polysorbate 60, ethylhexyl palmitate, sorbitan stearate, polyacrylamide, benzyl alcohol, c13-14 isoparaffin, cetearyl stearate, cetearyl alcohol, thymus zygis herb oil, zinc oxide, laureth-7, eucalyptus globulus leaf/twig oil, dehydroacetic acid, peg-20 stearate, salicylic acid, eugenia caryophyllus bud oil, eugenol, linalool, limonene, sodium hydroxide, citral, citric acid
Malacetic ®	Aqua, 2% acetic acid, 2% boric acid
Otodog ®	Aqua; fermented vegetable extract; sodium laureth sulfate; citric acid; sodium benzoate; cupric sulfate; parfum; methylchloroisothiazolinone
Otolane ®	Acetic acid: 0.5 %, Chlorhexidine digluconate: 0.2%

Standardized synthetic cerumen (SCC) was prepared, weighed in test tubes, and left to solidify



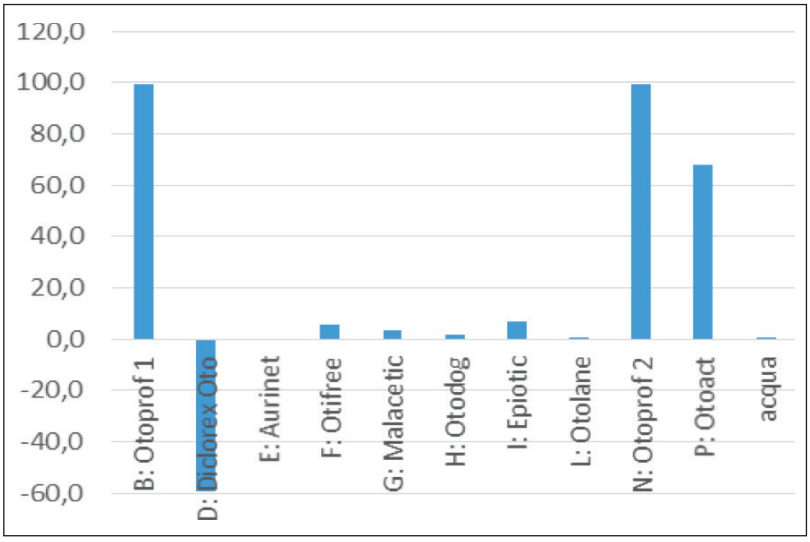
- An aliquot part of the otological product equal to two milliliters (ml) was added
- The tubes were shaken moderately at a temperature of 35 ° C for five minutes, and subsequently, were inverted
- After 24 hours each test tube was weighed, and another two ml of the product were added
- Five washes per tube were repeated and the test was performed in triplicate

Results

Standard artificial ear wax removal rate for each test; expressed as a ± confidence limit at the 80th percentile, according to a normal distribution

Sample	test 1	test 2	test 3	test 4	test 5
B: Otoprof 1	19,7 ± 2,1	33,6 ± 10,5	52,8 ± 12,2	82,8 ± 12,0	99,4 ± 0,3
D: Diclorex Oto	-54,9 ± 2,1	-59,3 ± 11,0	-56,2 ± 6,6	-56,1 ± 9,1	-59,6 ± 5,0
E: Aurinet	-2,2 ± 1,5	-1,0 ± 1,3	-8,4 ± 3,1	-6,1 ± 1,7	-0,1 ± 5,9
F: Otifree	-0,8 ± 0,9	1,7 ± 1,6	1,9 ± 1,8	6,4 ± 1,5	5,8 ± 1,6
G: Malacetic	-0,2 ± 0,3	1,3 ± 0,5	1,0 ± 0,5	3,0 ± 1,9	3,7 ± 0,5
H: Otodog	0,3 ± 0,1	1,1 ± 0,2	1,2 ± 0,1	1,8 ± 0,1	1,7 ± 0,2
I: Epiotic	1,4 ± 0,9	-0,9 ± 6,5	4,0 ± 1,3	6,8 ± 1,2	7,0 ± 1,2
L: Otolane	0,0 ± 0,5	1,1 ± 0,7	-0,4 ± 1,3	0,4 ± 1,2	0,2 ± 1,4
N: Otoprof 2	20,2 ± 1,6	43,6 ± 2,6	63,3 ± 3,7	95,8 ± 4,0	99,7 ± 0,4
P: Otoact	-18,3 ± 1,4	-5,7 ± 1,4	13,3 ± 1,5	31,5 ± 2,1	68,0 ± 3,2
acqua	-1,4 ± 0,2	0,0 ± 0,2	-0,1 ± 0,4	1,4 ± 0,1	0,4 ± 0,1

Standard artificial earwax percentage removal histogram after the fifth wash. All tested products are listed



• 99.4% of removed cerumen was obtained only with the product containing carbamide peroxide and DOSS (Otoprof ® ICF Srl, Palazzo Pignano, Italy)
• A good cerumenolytic property, 65%, was obtained from the product containing squalene (Otoact ® ICF Srl, Palazzo Pignano, Italy)
• Other products containing Disodium EDTA, PCMX, diethylhexyl sodium sulfosuccinate (like Epiotic ® Virbac Srl, Milano, Italy) showed less cerumenolytic activity with 7% and the remaining ones with a range from 5 to 1% of activity

Conclusions
This *in vitro* comparison study demonstrated that the molecules with the most cerumenolytic activity are *carbamide peroxid*e and DOSS (**Otoprof ®**) and *squalene* (**Otoact ®**).
Clinical studies in dogs with ceruminous otitis are required to confirm the *in vivo* efficacy of these formulations.

Discussion
Investigators used synthetic cerumen based on the average results of human and canine cerumen studies:
- Bortz JT et al. *Composition of cerumen lipids.* J Am Acad Dermatol. 1990
- Masuda A. et al. *Study of lipid in the ear canal in canine otitis externa with Malassezia pachydermatis*. J Vet Med Sci. 2000
- Sánchez-Leal J. *In vitro investigation of ceruminolytic activity of various otic cleansers for veterinary use*. Vet Dermatol. 2006
- Stahl J et al. *Ceruminal diffusion activities and ceruminolytic characteristics of otic preparations - an in-vitro study.* BMC Vet Res. 2013

No DOSS, urea or carbamide peroxide cleaners were tested in these *in vitro* and *in vivo* studies.

Our *in vitro* study was the first that demonstrates the DOSS and carbamide peroxide cerumenolytic efficacy and confirm that squalene has a good activity too.

Stability of the N-acetylcysteine (NAC) with Tris-EDTA solution and in combination with dexamethasone sodium phosphate in aqueous solution for 50 days

Milanesi N. et al. Proceedings ESVD 32nd 2021

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Reasons for use ear "clinical made" mixtures

1. Where we encounter multiply resistant organisms, and these is no other licensed alternative
2. When we are treating otitis media and there is no licensed drug
3. Where we want to avoid polypharmacy and there is no licensed alternative

Factors to be considered when mixing drugs

1. Knowledge of ototoxicity
2. Microbial contamination – bacteria, fungi, yeast
3. Temperature – high temperature accelerates drug degradation
4. pH – most drugs stable pH 4 to 8
5. Light – increases oxidation
6. Drug incompatibility within mixtures

Stability of the N-acetylcysteine (NAC) with Tris-EDTA solution and in combination with dexamethasone sodium phosphate in aqueous solution for 50 days

• The objective of this study was to evaluate
1. the stability of the formulation containing N-acetylcysteine (NAC) + Tris-EDTA (Tris-NAC®, ICF Srl, Palazzo Pignano, Italy)
2. the compatibility between Tris-NAC® and dexamethasone sodium phosphate in aqueous solution, 2 mg/ml injectable formulation (Dexadreson ®, MSD Italia S.r.l.) for a period of 50 days

Materials and Methods

• To evaluate the compatibility between dexamethasone and Tris-NAC, redox titrations were performed to evaluate the NAC content in the formulations
Two tests were performed:
1) stability assessment of Tris-NAC mixing 100 ml of the formulation with 5 ml of a solution of dexamethasone sodium phosphate (2mg/ml); and
2) stability assessment of the Tris-NAC formulation without any additions
- The two formulations were stored for **50 days** at **25 °C** and **5 °C** and titrations were performed every 10 days to evaluate the **NAC content** in the formulation and pH variations
- In original bottles of Tris-NAC (solutions protected from light)

Results

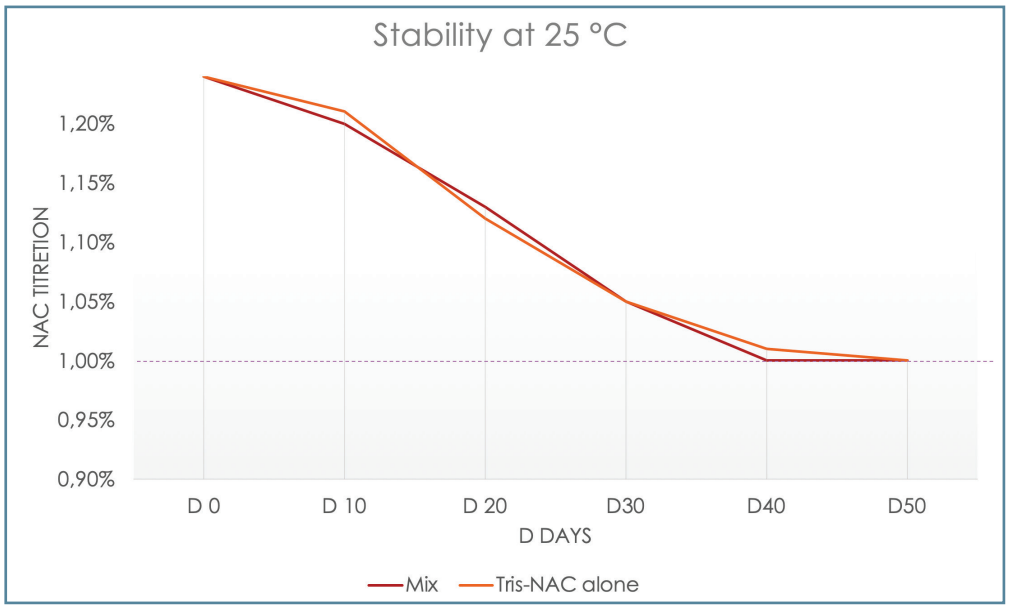
The data obtained – Stability - at 25 ° C

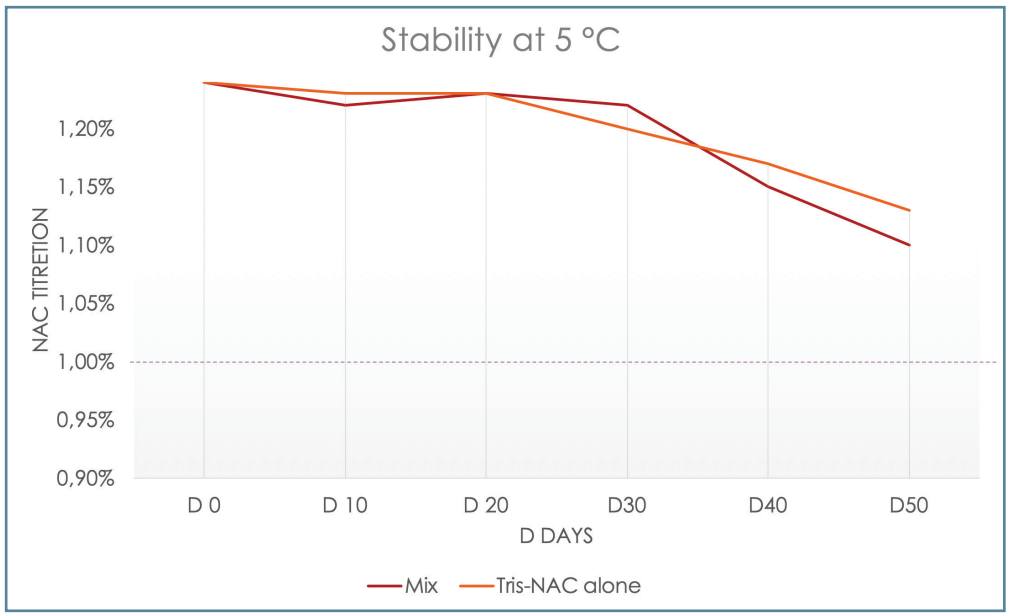
	Time zero 05/05/20	Day 10 15/05/20	Day 20 25/05/20	Day 30 05/06/20	Day 40 15/06/20	Day 50 26/06/20
Appearance	Opaque liquid	Opaque liquid	Opaque liquid	Slightly opaque liquid	Slightly opaque liquid	Slightly opaque liquid
pH	8.06	1: 8.46	1: 8.14	1: 7.99	1: 7.91	1: 7.94
		2: 8.40	2: 8.15	2: 7.98	2: 7.97	2: 7.95
NAC titration	1.24%	1: 1.205%	1: 1.13%	1: 1.05%	1: 1.00%	1: 1.00%
		2: 1.21%	2: 1.12%	2: 1.05%	2: 1.01%	2: 1.00%
Loss of NAC	Tris-NAC+DEX	1: 2.82%	1: 8.87%	1: 15.32%	1: 19.35%	1: 20.97%
	Tris-NAC	2: 2.42%	2: 9.68%	2: 15.32%	2: 18.55%	2: 19.35%

The data obtained – Stability - at 5 ° C

	Time zero 05/05/20	Day 10 15/05/20	Day 20 25/05/20	Day 30 05/06/20	Day 40 15/06/20	Day 50 26/06/20
Appearance	Opaque liquid	Opaque liquid	Opaque liquid	Slightly opaque liquid	Slightly opaque liquid	Slightly opaque liquid
pH	8.06	1: 8.42	1: 8.41	1: 8.19	1: 8.03	1: 8.00
		2: 8.46	2: 8.37	2: 8.10	2: 8.10	2: 8.07
NAC titration	1.24%	1: 1.22%	1: 1.23%	1: 1.22%	1: 1.15%	1: 1.10%
		2: 1.23%	2: 1.23%	2: 1.20%	2: 1.17%	2: 1.13%
Loss of NAC	Tris-NAC+DEX	1: 1.61%	1: 0.81%	1: 1.61%	1: 7.26%	1: 11.29%
	Tris-NAC	2: 0.81%	2: 0.81%	2: 3.23%	2: 5.65%	2: 8.87%

The data obtained – Stability – at 25°C





1. The data obtained, experimentally, regarding the NAC titre in the two formulations were found to be comparable
2. NAC titre remain above 1,00%* in all groups even after 50 days
3. No significant pH variation was observed in any groups

* =/> 1,00% is consider NAC titre with antibiofilm activity

Conclusions

In conclusion both formulations, after 50 days at 25 °C and 5 °C, maintain the NAC titration almost unchanged compared to time zero

Discussion

Some studies have investigated the stability of antibiotics and glucocorticoids mixed with ear cleaners:

- Metry C.A. et al. Determination of enrofloxacin stability and *in vitro* efficacy against *Staphylococcus pseudintermedius* and *Pseudomonas aeruginosa* in four ear cleaner solutions over a 28-day period. Vet Dermatol. 2011
- Emery C.B. et al. *Preliminary study of the stability of dexamethasone when added to commercial veterinary ear cleaners over a 90 day period.* Vet Dermatol. 2021

Two concentrations (0.1 and 0.25 mg/mL) of dexamethasone were formulated for each cleaner solution from a 2 mg/mL solution and stored in the original manufacturers' bottles at two temperatures: room (22 ˚C) and refrigerated (4 ˚C)

Samples were evaluated in triplicate, using liquid chromatography-tandem mass spectrometry at 10 time points over 90 days

A solution was considered stable if the dexamethasone value remained >90% of the target concentration.

All dexamethasone solution values were stable to 90 days except two solutions for ecA (0.15% ketoconazole, TrisEDTA); the 0.25 mg/mL dexamethasone concentration was only stable to 14 (4 ˚C) and 21 days (22 ˚C)

[1] Emery C.B. et al. Preliminary study of the stability of dexamethasone when added to commercial veterinary ear cleaners over a 90 day period. Vet Dermatol. 2021

Tris-NAC ® (Tris EDTA + N acetylcysteine) activity against biofilm production, an *in vitro* study

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A biofilm is a structured consortium of bacteria embedded in a self produced polymer matrix consisting of polysaccharides, protein and DNA Bacterial and yeast. Biofilms cause chronic infections because they show increased tolerance to antibiotics, antifungals and disinfectant chemicals, as well as resisting phagocytosis and other components of the body's defense system.
The aim of the study was to assess the **efficacy** of a product containing N-acetylcysteine + Tris EDTA (**Tris-NAC** ® ICF Srl, Palazzo Pignano, Italy) in *preventing the formation of biofilm and aiding its disintegration.*

MATERIALS AND METHODS

The study of biofilm growth inhibition, conducted by the Clever Bioscience Srl laboratory in Pavia (Italy) was evaluated using the microplate biofilm formation test against three microorganism strains *Pseudomonas aeruginosa* (ATCC 27853) *Staphylococcus aureus* (ATCC 25923) *Malassezia pachydermatis* (DSM 6172). The same three microorganisms were used to study the formulation's action on the biofilm breakdown for this study, the microorganisms were grown in microtiter plates, and the method to evaluate the growth of the biofilm is called Microtiter Dish Biofilm Formation Assay Biofilm growth and production were assessed at two different times (8 hours and 24 h) for bacteria and at three different times for yeasts (8 h, 24 h, and 48 h) Furthermore, the formulation can inhibit the biofilm formation of all three microorganisms.

- *Pseudomonas aeruginosa:*

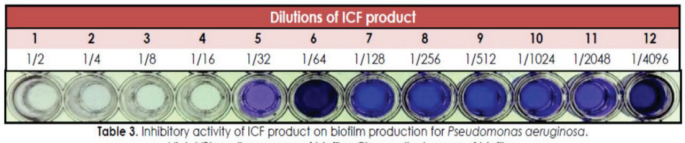

Table 3. Inhibitory activity of ICF product on biofilm production for *Pseudomonas aeruginosa*.
Violet/Blu well: presence of biofilm; Clear well: absence of biofilm.

- *Staphylococcus aureus:*

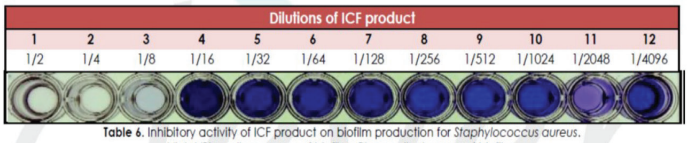

Table 6. Inhibitory activity of ICF product on biofilm production for *Staphylococcus aureus*.
Violet/Blu well: presence of biofilm; Clear well: absence of biofilm.

- *Malassezia pachydermatis:*

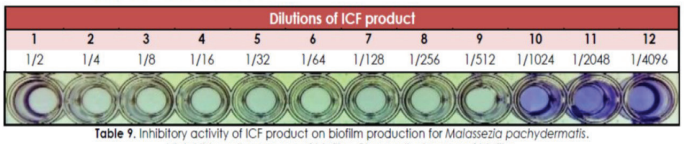

Table 9. Inhibitory activity of ICF product on biofilm production for *Malassezia pachydermatis*.
Violet/blu well: presence of biofilm; Clear well: absence of biofilm.

RESULTS

The data show that Tris-NAC ® can disaggregate *Pseudomonas aeruginosa* biofilm both at 8 hours and at 24 hours *Staphylococcus aureus* biofilm at 24 hours (in this case, at the 8 th hour, the biofilm was still absent), and it was not able to completely disaggregate the *Malassezia pachydermatis* biofilm at 48 hours (at the 8th hour and at the 24th hour the biofilm was absent). Furthermore, the formulation can *inhibit the biofilm formation* of all three microorganisms.

- ***Pseudomonas aeruginosa:***

Time point 8 h

Dilutions of ICF product											
1	2	3	4	5	6	7	8	9	10	11	12
1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048	1/4096

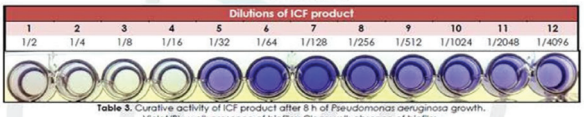

Table 3. Curative activity of ICF product after 8 h of *Pseudomonas aeruginosa* growth.
Violet/Blu well: presence of biofilm; Clear well: absence of biofilm.

Time point 24 h

Dilutions of ICF product											
1	2	3	4	5	6	7	8	9	10	11	12
1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048	1/4096

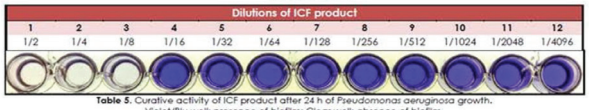

Table 5. Curative activity of ICF product after 24 h of *Pseudomonas aeruginosa* growth.
Violet/Blu well: presence of biofilm; Clear well: absence of biofilm.

- ***Staphylococcus aureus:***

Time point 24 h

Dilutions of ICF product											
1	2	3	4	5	6	7	8	9	10	11	12
1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048	1/4096

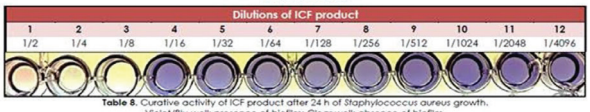

Table 8. Curative activity of ICF product after 24 h of *Staphylococcus aureus* growth.
Violet/Blu well: presence of biofilm; Clear well: absence of biofilm.

- ***Malassezia pachydermatis:***

Time point 48 h

Dilutions of ICF product											
1	2	3	4	5	6	7	8	9	10	11	12
1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048	1/4096

Table 11. Curative activity of ICF product after 48 h of *Malassezia pachydermatis* growth.
Violet/Blu well: presence of biofilm; Clear blu well: partial disaggregation of biofilm.

CONCLUSIONS

The use of topical products such as Tris-NAC ® to prevent microbial biofilm formation is primary prevention in the management of skin and ear infections in dogs and cats. And the biofilm's breaking capacity makes the product useful as a therapy combined with other topical antimicrobials to treat diseases and avoid their chronicity and relapses. Subsequently, at different dilutions, the Tris-NAC ® formulation was added to the same plates to evaluate the ability to desegregate the biomass during the 24 hours. The test was carried out in triplicate to obtain reliable results.

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