
# Articles & Studies

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Results

# A test on 150 clinical strains of canine origin indicates that Otodine® is active against all microorganisms associated with canine otitis in dogs

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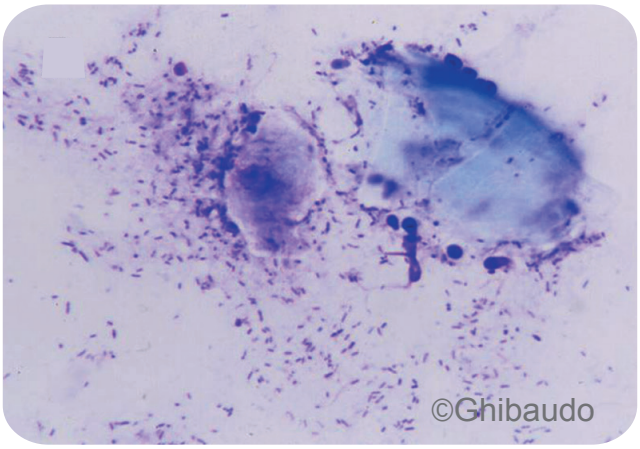
**Otodine®** is an ear antiseptic containing chlorhexidine digluconate and ethylene diamine tetra acetic acid-tromethamine (Tris-EDTA). Chlorhexidine digluconate exerts bactericidal activity by membrane disruption (1). Tris-EDTA increases permeability of the outer membrane in Gram-negative bacteria, thereby potentiating the effects of various antimicrobials, including antiseptics and systemic antibiotics (2-3). Combination of the two compounds results in a synergistic effect, allowing the use of low doses of chlorhexidine that are not ototoxic (4-6).
The antimicrobial activity of **Otodine®** was assessed by testing a comprehensive strain collection of pathogens associated with canine otitis.
The collection was composed by 150 clinical strains of canine origin belonging to *Corynebacterium auriscanis* (n=12), *Escherichia coli* (n=12), Malassezia pachydermatis (n=9), *Proteus mirabilis* (n=11), *Pseudomonas aeruginosa* (n=19), *Staphylococcus aureus* (n=22), *Staphylococcus pseudintermedius* (n=53) and *Streptococcus canis* (n=12).

**Otodine®** dilutions resulting in complete killing of the microorganisms tested were determined by the broth microdilution method (8) Briefly, each strain was incubated for 30 min at 37ºC in serial two-fold dilutions of **Otodine®** in Mueller-Hinton broth and strain survival was evaluated by transferring an aliquot of every dilution onto appropriate agar medium.

**Activity on Gram-negative bacteria**
All Gram-negative strains except one were completely killed at 1:8 dilutions of **Otodine®**. *P. mirabilis* was the most resistant species, whereas C. auriscanis was the most susceptible. *P. aeruginosa* and *E. coli* displayed intermediate levels of susceptibility. Strains belonging to these two species were generally eliminated by 1:16 dilution of the product (Table 1).

**Activity on Gram-positive bacteria**
Gram-positive strains were eliminated at 1:16 dilutions of the product. *S. pseudintermedius* and S. canis were more susceptible than *S. aureus* and 1:64 dilutions were generally sufficient to kill strains belonging to these two species. No significant difference was observed between meticillin-resistant and meticillin-susceptible staphylococci (Table 2).

**Activity on yeasts**
Surprisingly, in addition to the antibacterial activity against both Gram-negative and Gram-positive species, **Otodine®** showed an excellent fungicidal activity against *M. pachydermatis*. All strains tested were completely killed at 1:32 dilutions of the product (Table 3).




**Concluding remarks**
To my knowledge, this was the first study investigating the antimicrobial activity of a commercial ear antiseptic against a large collection of clinical strains isolated from dogs, including multi-resistant bacteria such as meticillin-resistant staphylococci. **Otodine®** was shown to be active *in vitro* against any pathogenic microorganisms involved in canine otitis, including multi-resistant Gram-positive and Gram-negative bacteria as well as *M. pachydermatis*. Such a broad spectrum of antimicrobial activity is particularly advantageous in the treatment of otitis externa as this disease condition of the dog often involves different microorganisms. Each of the 150 strains tested was completely killed after 30 min exposure to 1:4 or higher dilutions of **Otodine®** and comparable results were obtained by reducing the exposure time to 10 min. As similar or even higher concentrations are likely achieved in vivo following application of **Otodine®** in the infected ear canal, it is reasonable to conclude that this product can be successfully employed to treat any cases of ear infections by following the application time recommended by the manufacturer (i.e. at least 10 min).
Interestingly, meticillin-resistant and meticillin-susceptible staphylococci were equally affected by **Otodine®**. Combination of chlorhexidine digluconate and Tris-EDTA is efficacious against meticillin-resistant staphylococci, which notoriously are resistant to multiple antibiotics and represent a serious therapeutic problem(8), and does not seem to facilitate selection and spread of these harmful bacteria. The results of this study indicate that the formulation of **Otodine®** may be a valuable therapeutic tool to combat the recent emergence of meticillin-resistant staphylococci in veterinary dermatology.

# Results


**References**

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# Table

**Table 1.** Otodine® dilutions resulting in complete killing of Gram-negative bacteria

| Strain | Species | 1:2 | 1:4 | 1:8 | 1:16 | 1:32 | 1:64 | 1:128 | 1:256 |
|---|---|---|---|---|---|---|---|---|---|
| 1 | *Pseudomonas aeruginosa* | - | - | - | - | + | ++ | ++ | ++ |
| 2 | *Pseudomonas aeruginosa* | - | - | - | - | - | ++ | ++ | ++ |
| 3 | *Pseudomonas aeruginosa* | - | - | - | - | - | ++ | ++ | ++ |
| 4 | *Pseudomonas aeruginosa* | - | - | - | - | + | ++ | ++ | ++ |
| 5 | *Pseudomonas aeruginosa* | - | - | - | - | + | ++ | ++ | ++ |
| 6 | *Pseudomonas aeruginosa* | - | - | - | - | - | + | ++ | ++ |
| 7 | *Pseudomonas aeruginosa* | - | - | - | - | + | ++ | ++ | ++ |
| 8 | *Pseudomonas aeruginosa* | - | - | - | - | - | ++ | ++ | ++ |
| 9 | *Pseudomonas aeruginosa* | - | - | - | - | - | ++ | ++ | ++ |
| 10 | *Pseudomonas aeruginosa* | - | - | - | - | - | ++ | ++ | ++ |
| 11 | *Pseudomonas aeruginosa* | - | - | - | - | + | ++ | ++ | ++ |
| 12 | *Pseudomonas aeruginosa* | - | - | - | - | - | ++ | ++ | ++ |
| 13 | *Pseudomonas aeruginosa* | - | - | - | - | - | ++ | ++ | ++ |
| 14 | *Pseudomonas aeruginosa* | - | - | - | - | + | ++ | ++ | ++ |
| 15 | *Pseudomonas aeruginosa* | - | - | - | - | - | ++ | ++ | ++ |
| 16 | *Pseudomonas aeruginosa* | - | - | - | - | + | ++ | ++ | ++ |
| 17 | *Pseudomonas aeruginosa* | - | - | - | - | - | ++ | ++ | ++ |
| 18 | *Pseudomonas aeruginosa* | - | - | - | - | - | ++ | ++ | ++ |
| 19 | *Pseudomonas aeruginosa* | - | - | - | ++ | ++ | ++ | ++ | ++ |
| 20 | *Escherichia coli* | - | - | - | - | - | ++ | ++ | ++ |
| 21 | *Escherichia coli* | - | - | - | - | - | ++ | ++ | ++ |
| 22 | *Escherichia coli* | - | - | - | - | + | ++ | ++ | ++ |
| 23 | *Escherichia coli* | - | - | - | - | + | ++ | ++ | ++ |
| 24 | *Escherichia coli* | - | - | - | - | + | ++ | ++ | ++ |
| 25 | *Escherichia coli* | - | - | - | - | ++ | ++ | ++ | ++ |
| 26 | *Escherichia coli* | - | - | - | - | + | ++ | ++ | ++ |
| 27 | *Escherichia coli* | - | - | - | - | - | ++ | ++ | ++ |
| 28 | *Escherichia coli* | - | - | - | - | + | ++ | ++ | ++ |
| 29 | *Escherichia coli* | - | - | - | - | + | ++ | ++ | ++ |
| 30 | *Escherichia coli* | - | - | - | ++ | ++ | ++ | ++ | ++ |
| 31 | *Escherichia coli* | - | - | - | - | + | ++ | ++ | ++ |
| 32 | *Proteus mirabilis* | - | - | - | ++ | ++ | ++ | ++ | ++ |
| 33 | *Proteus mirabilis* | - | - | - | + | ++ | ++ | ++ | ++ |
| 34 | *Proteus mirabilis* | - | - | - | - | - | 17 | ++ | ++ |
| 35 | *Proteus mirabilis* | - | - | - | ++ | ++ | ++ | ++ | ++ |
| 36 | *Proteus mirabilis* | - | - | ++ | ++ | ++ | ++ | ++ | ++ |
| 37 | *Proteus mirabilis* | - | - | - | ++ | ++ | ++ | ++ | ++ |
| 38 | *Proteus mirabilis* | - | - | - | - | ++ | ++ | ++ | ++ |
| 39 | *Proteus mirabilis* | - | - | - | ++ | ++ | ++ | ++ | ++ |
| 40 | *Proteus mirabilis* | - | - | - | ++ | ++ | ++ | ++ | ++ |
| 41 | *Proteus mirabilis* | - | - | - | + | ++ | ++ | ++ | ++ |
| 42 | *Proteus mirabilis* | - | - | - | + | ++ | ++ | ++ | ++ |
| 43 | *Corynebacterium auriscanis* | - | - | - | - | - | - | - | - |
| 44 | *Corynebacterium auriscanis* | - | - | - | - | - | - | - | - |
| 45 | *Corynebacterium auriscanis* | - | - | - | - | - | - | - | - |
| 46 | *Corynebacterium auriscanis* | - | - | - | - | - | - | - | - |
| 47 | *Corynebacterium auriscanis* | - | - | - | - | - | - | - | - |
| 48 | *Corynebacterium auriscanis* | - | - | - | - | - | - | - | - |
| 49 | *Corynebacterium auriscanis* | - | - | - | - | - | - | - | - |
| 50 | *Corynebacterium auriscanis* | - | - | - | - | - | - | - | - |
| 51 | *Corynebacterium auriscanis* | - | - | - | - | - | - | + | + |
| 52 | *Corynebacterium auriscanis* | - | - | - | - | - | - | - | - |
| 53 | *Corynebacterium auriscanis* | - | - | - | - | - | - | - | - |
| 54 | *Corynebacterium auriscanis* | - | - | - | - | - | - | + | ++ |

| - complete killing (no growth) | + partial inhibition (1-100 colonies) | ++ no effect (confluent growth) |
|---|---|---|


Table

**Table 2.** Otodine® dilutions resulting in complete killing of Gram-positive bacteria

| Strain | Species[a] | 1:2 | 1:4 | 1:8 | 1:16 | 1:32 | 1:64 | 1:128 | 1:256 |
|---|---|---|---|---|---|---|---|---|---|
| 1 | *Streptococcus canis* | - | - | - | - | - | - | + | ++ |
| 2 | *Streptococcus canis* | - | - | - | - | - | - | + | ++ |
| 3 | *Streptococcus canis* | - | - | - | - | - | - | + | ++ |
| 4 | *Streptococcus canis* | - | - | - | - | - | - | + | ++ |
| 5 | *Streptococcus canis* | - | - | - | - | - | - | ++ | ++ |
| 6 | *Streptococcus canis* | - | - | - | - | - | - | + | ++ |
| 7 | *Streptococcus canis* | - | - | - | - | - | + | ++ | ++ |
| 8 | *Streptococcus canis* | - | - | - | - | - | - | + | ++ |
| 9 | *Streptococcus canis* | - | - | - | - | - | - | + | ++ |
| 10 | *Streptococcus canis* | - | - | - | - | - | - | + | ++ |
| 11 | *Streptococcus canis* | - | - | - | - | - | - | + | ++ |
| 12 | *Streptococcus canis* | - | - | - | - | - | - | + | ++ |
| 13 | *Staphylococcus pseudintermedius* – MS | - | - | - | - | - | - | + | ++ |
| 14 | *Staphylococcus pseudintermedius* – MS | - | - | - | - | - | - | + | ++ |
| 15 | *Staphylococcus pseudintermedius* – MS | - | - | - | - | - | - | - | + |
| 16 | *Staphylococcus pseudintermedius* – MS | - | - | - | - | - | - | + | ++ |
| 17 | *Staphylococcus pseudintermedius* – MS | - | - | - | - | - | - | + | ++ |
| 18 | *Staphylococcus pseudintermedius* – MS | - | - | - | - | - | - | + | ++ |
| 19 | *Staphylococcus pseudintermedius* – MS | - | - | - | - | - | - | + | ++ |
| 20 | *Staphylococcus pseudintermedius* – MS | - | - | - | - | - | - | - | + |
| 21 | *Staphylococcus pseudintermedius* – MS | - | - | - | - | - | - | + | ++ |
| 22 | *Staphylococcus pseudintermedius* – MS | - | - | - | - | - | - | + | ++ |
| 23 | *Staphylococcus pseudintermedius* – MS | - | - | - | - | - | - | + | ++ |
| 24 | *Staphylococcus pseudintermedius* – MS | - | - | - | - | - | - | + | ++ |
| 25 | *Staphylococcus pseudintermedius* – MS | - | - | - | - | - | - | + | ++ |
| 26 | *Staphylococcus pseudintermedius* – MS | - | - | - | - | - | - | + | ++ |
| 27 | *Staphylococcus pseudintermedius* – MS | - | - | - | - | - | - | + | ++ |
| 28 | *Staphylococcus pseudintermedius* – MS | - | - | - | - | - | - | + | ++ |
| 29 | *Staphylococcus pseudintermedius* – MS | - | - | - | - | - | - | + | + |
| 30 | *Staphylococcus pseudintermedius* – MS | - | - | - | - | - | - | + | ++ |
| 31 | *Staphylococcus pseudintermedius* – MS | - | - | - | - | - | - | + | ++ |
| 32 | *Staphylococcus pseudintermedius* – MS | - | - | - | - | - | - | + | ++ |
| 33 | *Staphylococcus pseudintermedius* – MS | - | - | - | - | - | - | + | ++ |
| 34 | *Staphylococcus pseudintermedius* – MS | - | - | - | - | - | - | + | ++ |
| 35 | *Staphylococcus pseudintermedius* – MS | - | - | - | - | - | - | + | + |
| 36 | *Staphylococcus pseudintermedius* – MS | - | - | - | - | - | - | + | ++ |
| 37 | *Staphylococcus pseudintermedius* – MS | - | - | - | - | - | - | + | ++ |
| 38 | *Staphylococcus pseudintermedius* – MS | - | - | - | - | - | - | + | ++ |
| 39 | *Staphylococcus pseudintermedius* – MS | - | - | - | - | - | - | + | ++ |
| 40 | *Staphylococcus pseudintermedius* – MS | - | - | - | - | - | - | - | ++ |
| 41 | *Staphylococcus pseudintermedius* – MS | - | - | - | - | - | - | - | ++ |
| 42 | *Staphylococcus pseudintermedius* – MS | - | - | - | - | - | - | + | + |
| 43 | *Staphylococcus pseudintermedius* – MS | - | - | - | - | - | - | - | ++ |
| 44 | *Staphylococcus pseudintermedius* – MS | - | - | - | - | - | - | - | ++ |
| 45 | *Staphylococcus pseudintermedius* – MR | - | - | - | - | - | - | + | ++ |
| 46 | *Staphylococcus pseudintermedius* – MR | - | - | - | - | - | - | + | ++ |
| 47 | *Staphylococcus pseudintermedius* – MR | - | - | - | - | - | - | + | ++ |
| 48 | *Staphylococcus pseudintermedius* – MR | - | - | - | - | - | - | + | ++ |
| 49 | *Staphylococcus pseudintermedius* – MR | - | - | - | - | - | - | + | ++ |
| 50 | *Staphylococcus pseudintermedius* – MR | - | - | - | - | - | - | + | ++ |
| 51 | *Staphylococcus pseudintermedius* – MR | - | - | - | - | - | - | + | ++ |
| 52 | *Staphylococcus pseudintermedius* – MR | - | - | - | - | - | - | + | ++ |
| 53 | *Staphylococcus pseudintermedius* – MR | - | - | - | - | - | - | + | ++ |
| 54 | *Staphylococcus pseudintermedius* – MR | - | - | - | - | - | - | + | ++ |
| 55 | *Staphylococcus pseudintermedius* – MR | - | - | - | - | - | - | + | ++ |
| 56 | *Staphylococcus pseudintermedius* – MR | - | - | - | - | - | - | + | ++ |
| 57 | *Staphylococcus pseudintermedius* – MR | - | - | - | - | - | - | + | ++ |
| 58 | *Staphylococcus pseudintermedius* – MR | - | - | - | - | - | - | + | ++ |
| 59 | *Staphylococcus pseudintermedius* – MR | - | - | - | - | - | - | + | ++ |
| 60 | *Staphylococcus pseudintermedius* – MR | - | - | - | - | - | - | + | ++ |
| 61 | *Staphylococcus pseudintermedius* – MR | - | - | - | - | - | - | + | ++ |
| 62 | *Staphylococcus pseudintermedius* – MR | - | - | - | - | - | - | + | ++ |
| 63 | *Staphylococcus pseudintermedius* – MR | - | - | - | - | - | - | + | ++ |
| 64 | *Staphylococcus pseudintermedius* – MR | - | - | - | - | - | - | + | ++ |
| 65 | *Staphylococcus pseudintermedius* – MR | - | - | - | - | - | - | + | ++ |
| 66 | *Staphylococcus aureus* - MS | - | - | - | - | - | - | + | ++ |
| 67 | *Staphylococcus aureus* - MS | - | - | - | - | - | + | ++ | ++ |
| 68 | *Staphylococcus aureus* - MS | - | - | - | - | - | - | + | ++ |
| 69 | *Staphylococcus aureus* - MS | - | - | - | - | - | + | + | ++ |
| 70 | *Staphylococcus aureus* - MS | - | - | - | - | + | + | ++ | ++ |
| 71 | *Staphylococcus aureus* - MS | - | - | - | - | + | + | ++ | ++ |
| 72 | *Staphylococcus aureus* - MS | - | - | - | - | - | + | ++ | ++ |
| 73 | *Staphylococcus aureus* - MS | - | - | - | - | + | + | ++ | ++ |
| 74 | *Staphylococcus aureus* - MS | - | - | - | - | ++ | ++ | ++ | ++ |
| 75 | *Staphylococcus aureus* - MS | - | - | - | - | ++ | ++ | ++ | ++ |
| 76 | *Staphylococcus aureus* - MS | - | - | - | - | - | + | ++ | ++ |
| 77 | *Staphylococcus aureus* - MS | - | - | - | - | - | + | ++ | ++ |
| 78 | *Staphylococcus aureus* - MS | - | - | - | - | + | + | ++ | ++ |
| 79 | *Staphylococcus aureus* - MR | - | - | - | - | - | - | + | + |
| 80 | *Staphylococcus aureus* - MR | - | - | - | - | - | + | ++ | ++ |
| 81 | *Staphylococcus aureus* - MR | - | - | - | - | + | ++ | ++ | ++ |
| 82 | *Staphylococcus aureus* - MR | - | - | - | - | + | ++ | ++ | ++ |
| 83 | *Staphylococcus aureus* - MR | - | - | - | - | + | ++ | ++ | ++ |
| 84 | *Staphylococcus aureus* - MR | - | - | - | - | ++ | ++ | ++ | ++ |
| 85 | *Staphylococcus aureus* - MR | - | - | - | - | + | ++ | ++ | ++ |
| 86 | *Staphylococcus aureus* - MR | - | - | - | - | + | ++ | ++ | ++ |
| 87 | *Staphylococcus aureus* - MR | - | - | - | - | + | ++ | ++ | ++ |

[a] MS, meticillin-susceptible; MR, meticillin-resistant.

| - complete killing (no growth) | + partial inhibition (1-100 colonies) | ++ no effect (confluent growth) |
|---|---|---|


## Table

**Table 3.** Otodine® dilutions resulting in complete killing of yeasts

| Strain | Species | 1:2 | 1:4 | 1:8 | 1:16 | 1:32 | 1:64 | 1:128 | 1:256 |
|---|---|---|---|---|---|---|---|---|---|
| 1 | *Malassezia pachydermatis* | - | - | - | - | - | + | ++ | ++ |
| 2 | *Malassezia pachydermatis* | - | - | - | - | - | - | ++ | ++ |
| 3 | *Malassezia pachydermatis* | - | - | - | - | - | - | ++ | ++ |
| 4 | *Malassezia pachydermatis* | - | - | - | - | - | - | + | ++ |
| 5 | *Malassezia pachydermatis* | - | - | - | - | - | - | ++ | ++ |
| 6 | *Malassezia pachydermatis* | - | - | - | - | - | - | + | ++ |
| 7 | *Malassezia pachydermatis* | - | - | - | - | - | - | + | ++ |
| 8 | *Malassezia pachydermatis* | - | - | - | - | - | - | ++ | ++ |
| 9 | *Malassezia pachydermatis* | - | - | - | - | - | - | + | ++ |

| - complete killing (no growth) | + partial inhibition (1-100 colonies) | ++ no effect (confluent growth) |
|---|---|---|

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# Biofilms

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## Introduction

Bacteria exist in two forms. The first is as solitary free floating "planktonic" organisms most commonly seen in acute infections. The second and most predominant form of microbial growth is in biofilms (Costerton et al., 1978). Biofilms can be defined as a microbially derived sessile community characterised by cells that are irreversibly attached to a surface or interface or to each other; are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription (Donlan and Costerton, 2002). Biofilms are everywhere and can form on virtually any body surface. They have been recognised in man in chronic sinusitis, chronic otitis media, chronic tonsillitis, dental plaque, chronic laryngitis, endocarditis, lung infections, biliary and urinary tract infections, osteomyelitis and chronic wounds (Bjarnsholt, 2013). Biofilms are now recognised as being clinically important in veterinary medicine and should be suspected in any chronic disease process (Gardner, 2011).

Not all bacterial species produce biofilm and the level of biofilm production can alter between different species. Bacteria found in canine diseases that have been shown to have biofilm producing properties include *Staphylococcus pseudintermedius* (Singh et al., 2013, Osland et al., 2012, Casagrande Proietti et al., 2015, Walker et al., 2016), *Pseudomonas aeruginosa* (Pye et al., 2013) and *Escherichia coli* (Nam et al., 2013, Oliveira et al., 2014, Vijay et al., 2015, Shimizu and Harada, 2017). Work by Han (2015) has shown that more than 90% of the isolates of both meticillin sensitive and resistant *Staphylococcus pseudintermedius* from healthy dogs are capable of producing biofilms (Han et al., 2015). A study by Pye (2013) showed that 40% of the isolates of *Pseudomonas aeruginosa* isolated from clinical cases of canine otitis externa were found to be capable of producing biofilms. In this study the biofilm minimum inhibitory concentrations (MICs) of *Pseudomonas spp.* for polymyxin B, neomycin, gentamicin and enrofloxacin were significantly higher than for the planktonic form, demonstrating the increased resistance of biofilm organisms at sites where they cause clinical disease (Pye et al., 2013). *Malassezia pachydermatis* has been shown to be capable of producing biofilms (Figueredo et al., 2012) particularly from cases of canine seborrheic dermatitis (Bumroongthai et al., 2016).

## Diagnosis of biofilm infections

In human medicine establishing that a biofilm infection is present can be challenging. A range of criteria can be useful these include, a history of a condition that predisposes to the development of biofilm formation such as an orthopaedic implant. The presence of cytological findings consistent with biofilm formation such as microbial aggregates. A culture revealing microbes known to be associated with biofilm formation; recurrence of infection despite appropriate antimicrobial therapy or therapeutic failure despite appropriate antibacterial therapy based on culture and susceptibility (Rowson and Townsend, 2016). Medics have access to more specialised diagnostic techniques including molecular methods such as polymerase chain reactions and 16S ribosomal probes.


Both of these techniques have been shown to have a higher degree of sensitivity than culture (Hall- Stoodley et al., 2006) but are not widely available or economically viable for veterinary clinicians. Veterinarians though can use the other diagnostic criteria to help make a diagnosis.

1) A history of a condition that predisposes to the development of biofilm formation
2) Clinical signs consistent with a biofilm infection
3) The presence of cytological findings consistent with biofilm formation
4) Therapeutic failure either due to lack of response or recurrence of infection despite appropriate therapy

### A history of a condition that predisposes to the development of biofilm formation

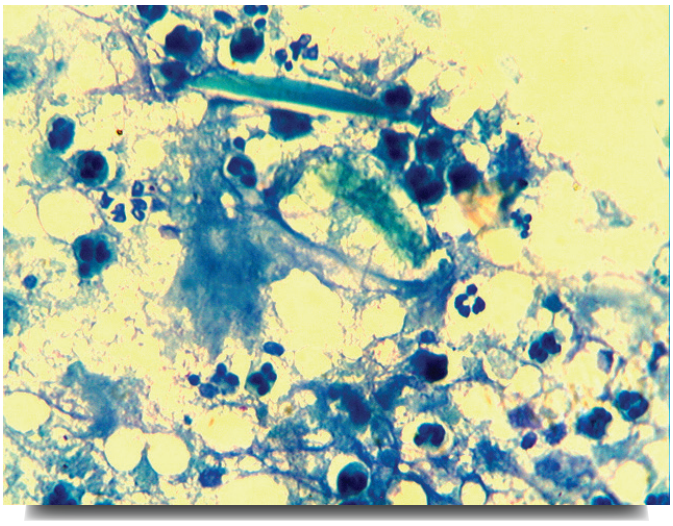
Almost any chronic disease process can lead to the formation of a biofilm infection. In dogs and cats biofilm infections have been associated with urine tract disease (Shimizu and Harada, 2017) , gastrointestinal disease (Silva et al., 2014, Reis et al., 2014), periodontal disease (Holcombe et al., 2014, Oliveira et al., 2016), otitis (Pye et al., 2013), dermatitis (Casagrande Proietti et al., 2015, Bumroongthai et al., 2016), chronic wounds (Swanson et al., 2014, Bayne, 2014) and implant infections (Thompson et al., 2011, Gallagher and Mertens, 2012, Savicky et al., 2013, Nicoll et al., 2014).

### Clinical signs consistent with a biofilm infection

Where biofilm infection is present the micro-organisms within the biofilm are embedded in a self- produced matrix of extracellular polymeric substance (EPS). This appears as a thick mucoid discharge (see George otitis case below).

### The presence of cytological findings consistent with biofilm formation

Cytology samples of discharge from an infection may be taken with a sterile swab. The swab should be gently rolled across a clean microscope slide, heat fixed and then stained with a Romanowsky stain such as Diff Quik. Microscopic examination will reveal signs of aggregates of infectious organisms often co-localised with inflammatory cells. This differs to the more distinct isolates found in cases where planktonic infection is present. Typically numbers of micro-organisms are sparse and found within the self-produced EPS matrix which usually takes up stain and appears as fine lacy background material (figure 1).

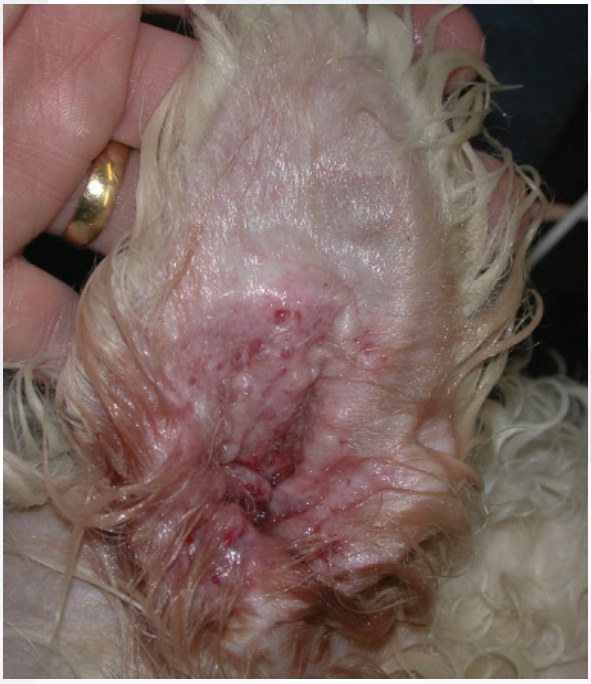


(figure 1)

**Therapeutic failure due to lack of response or recurrence of infection despite appropriate therapy**

Typical cases will fail to respond to antibiotic therapy that has been based on appropriate culture and susceptibility testing. Often the same pathogen is identified on numerous occasions with an unchanged susceptibility pattern (see figure 2 below). In other cases the infection appears to have resolved on antibiotic therapy but relapses once drugs are discontinued.

George is a 6 year old Clumber spaniel with a chronic history of otitis externa. His condition has shown a partial response to topical antibiotic therapy but has relapsed on cessation of treatment on several occasions. On this most recent occasion he has had a topical ear drop prescribed which contains marbofloxacin. This has been prescribed on the basis that *Pseudomonas spp.* has been cultured with a good sensitivity to marbofloxacin.



After 3 weeks of treatment his owner returned to complain the dog is no better. Repeat cultures grow *Pseudomonas spp.* again with the susceptibility below.

| Susceptibility | Antibiotic |
|---|---|
| Framycetin | R |
| Gentamicin | R |
| Polymyxin | S |
| Enrofloxacin | S |
| Marbofloxacin | S |
| Fusidic acid | R |
| Florfenicol | R |

The lack of response to therapy, the repeat cultures revealing the same pathogen with the same susceptibility pattern is consistent with a biofilm infection

(figure 2)


## Therapy of biofilm infections

Bacteria growing within a biofilm have the capacity to withstand and persist in the presence of higher levels of antibiotics than free living planktonic bacteria – this property is called recalcitrance (Rowson and Townsend, 2016). In clinical terms this means that biofilm bacteria can be from 100 to 1000 fold more resistant to antibiotics than their planktonic counterparts (Hoiby et al., 2010). Biofilm bacteria have several mechanisms that facilitate their resistance this includes restricted penetration of antibiotics; restricted growth at low oxygen tension; expression of biofilm specific genes and the presence of persisters (Ciofu et al., 2017). A range of different treatment options may need to be explored to effectively manage biofilm infections. Mechanical removal of the biofilm is the most effective method, however this is not always feasible to do. As well as the traditional use of antimicrobial therapy other more novel anti-biofilm strategies have been explored in both human and veterinary medicine. Often combinations of therapy that include antimicrobial drugs with other anti-biofilm agents prove the most successful. EDTA tris has long been recognised as a principle which supports the treatment of chronic Gram negative infections. One of the newest principle which has been found to have excellent activity against biofilms is N-acetyl cysteine.

## EDTA tris

**Ethylenediaminetetraacetic acid (EDTA)** has been demonstrated to be an excellent support in case of bacterial infections when combined with tromethamine (Tris). Studies have shown:

**1) Damages the cell walls of planktonic bacteria**
**2) Synergistic with some antibiotics**
**3) Has low ototoxic potential**
**4) Use adjunct therapy for biofilm infection**

EDTA tris has been shown to have the ability to **damage planktonic bacterial cell walls** to increase antimicrobial penetration (Wooley and Jones, 1983, Farca et al., 1997, Buckley et al., 2013). It is well tolerated and demonstrates **low ototoxicity** (Paterson, 2018). It has been shown to have **additive effects with a wide range of antibiotics** including gentamicin and fluoroquinolones (Buckley et al., 2013) as well as silver sulphadiazine and chlorhexidine (Guardabassi et al., 2010). More recently, *in vitro* work has shown that EDTA tris may be a **useful adjunctive treatment** for chronic cases of *Pseudomonas* otitis where biofilms have developed, if gentamicin or neomycin is to be used as a topical treatment (Pye et al., 2014). EDTA tris is probably best employed because of its ability to potentiate antibiotics, as the initial pre-treatment flush for acute (planktonic) Gram negative infections.

## N-acetyl cysteine

N acetyl cysteine is a mucolytic that has been shown to have a wide range of additional properties. Studies have shown :

**1) Support in case of bacterial infections**
**2) Synergy with some antibiotics**
**3) Decreased biofilm formation**
**4) Reduced production of EPS**
**5) Promotion of mature biofilm disruption**

N-acetyl cysteine (NAC) is a valid support in case of bacterial infections caused by different pathogens. *Pseudomonas aeruginosa* appears to be particularly susceptible with *in vitro* antibacterial activity being recorded at minimum inhibitory concentrations (MIC) as low as 2- 20 ug/ml (Parry and Neu, 1977). A more recent study by Zhao (2010) has shown the MIC of NAC for *Pseudomonas aeruginosa* isolates from respiratory human respiratory infections to be 10 – 40mg/ml. Work by May (2016) has demonstrated that NAC has an MIC of 5 – 20mg/ml for common otic isolates *Staphylococcus pseudintermedius*, *Pseudomonas aeruginosa*, *Corynebacterium spp.* and *Beta haemolytic Streptococcus* (May et al., 2016). **Synergism** has been demonstrated with some of the third

generations antibiotics such as carbenicillin and ticarcillin (Roberts and Cole, 1981) and although these antibiotics are restricted for veterinary use this early study demonstrated the potential for NAC's ability to enhance antibiotic activity. NAC has also been shown *in vitro* to be synergistic with ciprofloxacin (El-Feky et al., 2009) suggesting that a fluoroquinolone NAC combination may be useful in cases of *Pseudomonas spp.* infection. NAC **decreases biofilm formation** by a variety of bacteria notably *Staphylococcus epidermidis* (Perez-Giraldo et al., 1997); *E. coli* (Marchese et al., 2003) and *Pseudomonas aeruginosa* (Zhao and Liu, 2010). It appears to have the ability to reduce adherence and also reduce the **production of EPS**. The production of EPS by biofilm bacteria acts as a physical barrier to the penetrations of drugs which play a significant part in the increased resistance to antibiotic therapy. Work by Zhao (2010) has shown that EPS production by *Pseudomonas aeruginosa* is significantly decreased in the presence of NAC. EPS production was decreased *in vitro* by 27% and 45% in the presence of 0.5mg/ml and 1mg/ml NAC (Zhao and Liu, 2010). NAC also has a direct effect to **promotes the disruption** of mature biofilms and reduce sessile cell viability within the biofilm (Olofsson et al., 2003).

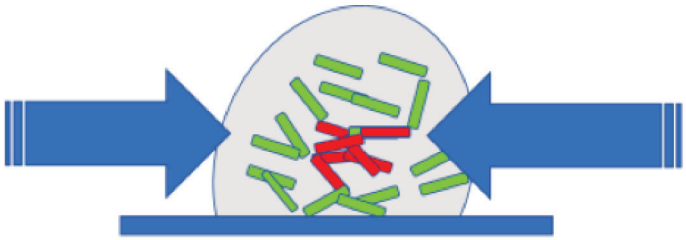
**Human studies** have shown that NAC is highly effective used with ciprofloxacin as a licensed otic (Ciprodex otic) to treat refractory otitis cases. In one *in vivo* study, Ciprodex otic was used with 0.5 or 2.0% NAC. Seven subjects with an average of 18.4 months of otorrhea despite therapy were included. Cessation of otorrhea was achieved in 6 of 7 subjects within 4 weeks of treatment. No subjects demonstrated ototoxicity via pre-treatment and post-treatment audiometry (Choe et al., 2007). A second *in vitro* study using the ciprofloxacin showed that where *P. aeruginosa* strains were resistant to ciprofloxacin, pre-treatment with NAC overcame the resistance leading to resolution of infection (Lea et al., 2014).

**Veterinary use** of NAC would seem based on human experiences to be suited to use with fluoroquinolones, selected on the basis of culture and sensitivity, in chronic otitis cases where first line antibiotics are ineffective. NAC also shows promise as an antibacterial agent for use in chronic infections in its own right (May et al., 2016) and may be useful with other topical drugs where biofilms are present, due to its vitro ability to reduce the production of EPS and reduce bacterial attachment, as a pre-treatment flush prior to antimicrobial therapy. A suitable protocol for use in *Pseudomonas spp.* otitis externa is outlined below.

## POSSIBLE MODE OF ACTION OF BIOFILM BUSTING DRUGS

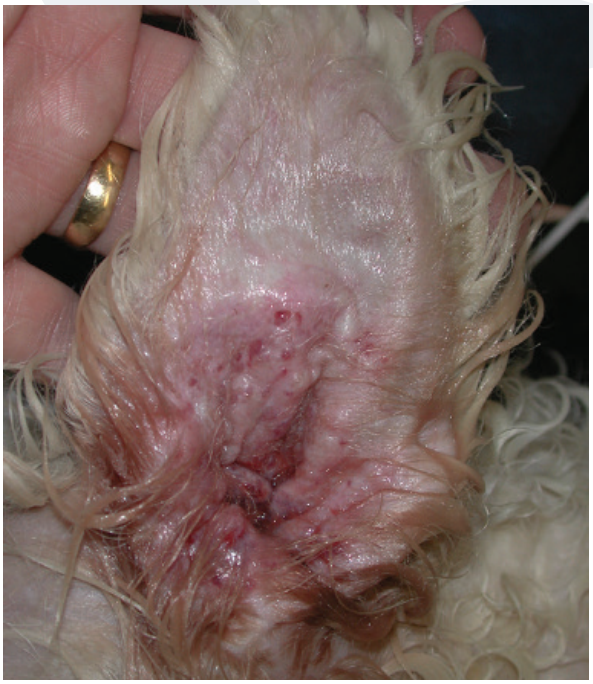
**NAC**

**Antibacterial properties**
**Reduced production of EPS**
**Promotion of mature biofilm disruption**



**Tris EDTA**

**Direct damage to bacteria cell wall to increase microbial penetration**
**May affect bacterial efflux pump**

Approach to George's otitis. George's clinical history, response to therapy and repeat culture results are consist with the presence of a biofilm infection. The combination of NAC as a pre-treatment flush is useful before application of antibiotics.

Step 1 Clean the ear thoroughly to remove as much of the discharge as possible. This is probably best achieved under heavy sedation or ideally under a full general anaesthetic. Appropriate analgesia is important to keep the dog comfortable during the procedure and post flush.

Step 2 Flood the ear canal with a solution of Tris-EDTA + N-acetyl (Tris-NAC®). This should remain in situ for a minimum of 5 minutes.

Step 3 Remove as much of the excessive liquid as possible using suction or absorbing the fluid onto a cotton wool pad. Instil 0.5ml of the marbofloxacin based ear drop into the ear. The dog may be sent home with an ear cleaning solution (Otodine®) to clean the ear effectively before application of the Tris-EDTA + NAC solution (Tris-NAC®). This should remain in place for 5 mins, before excessive fluid is removed using a cotton wool pad, before applying the antibiotic solution.

(figure 3)

## Conclusion

Bacterial biofilms are a major cause of infection in man and animals. They contribute to a wide range of veterinary diseases including urinary tract infections, periodontal disease, wound infection and otitis. Conventional antimicrobial therapy often fails to eliminate biofilm infection and new novel therapies to tackle these infections are currently being developed. N-acetyl cysteine appears to be a useful novel agent to help in the management of biofilm.

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# icf Bulletin

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**

nextmune

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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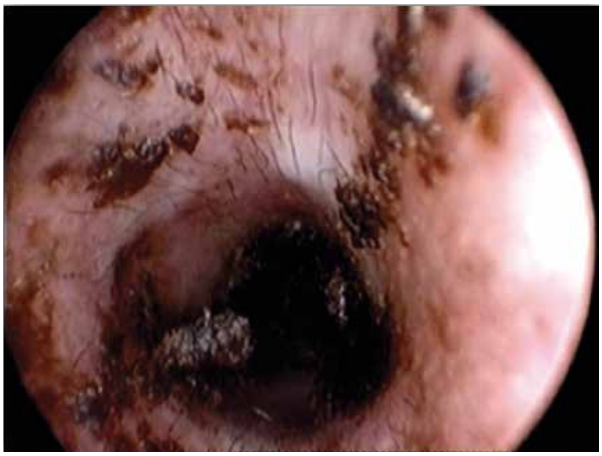
**Canine Cerumen** [1,2]

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, due to the inflammatory process, ceruminous glands show hyperplasia, increase secretory activity and, as a result, the amount of earwax produced.

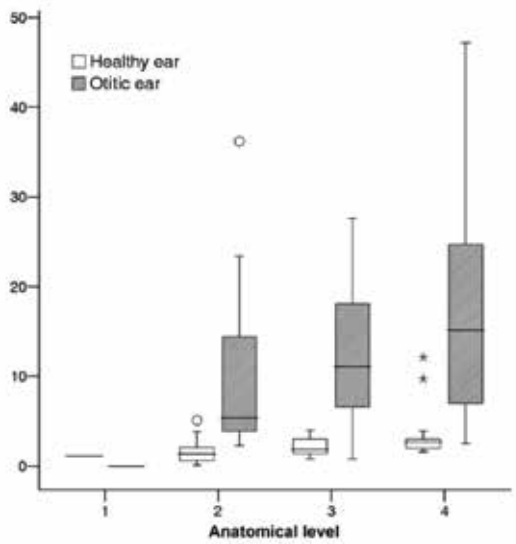



**Figure 5.** The mean proportion (%) of each section occupied by ceruminous glands in the aural skin at four anatomical levels in healthy ears ($n$ = 28) and ear with otitis externa ($n$ = 15). Outliers (circles) and extreme values (asterisks) are indicated.

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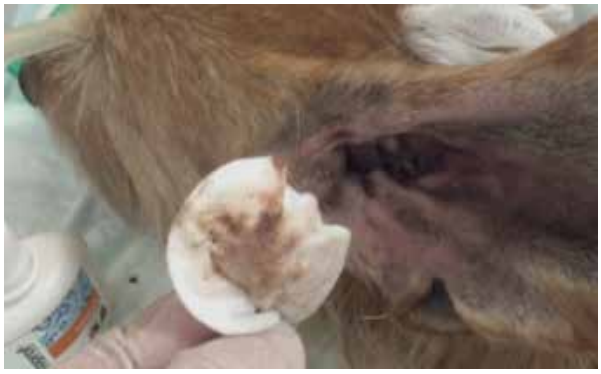
[3] **Histological changes in the external ear canal of dogs with otitis externa**
Hui-Pi Huang et al. Vet Dermatol. 2009

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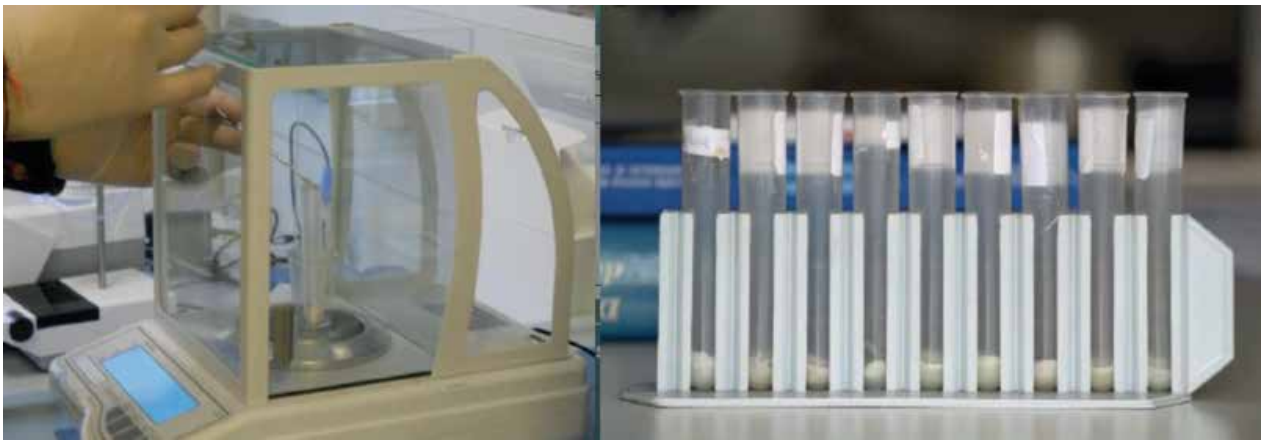
6 ***In vitro* investigation of ceruminolytic activity of various otic cleansers for veterinary use** Sánchez-Leal et al. Vet Dermatol. 2006

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% |

An analytical and experienced protocol was used
Standardized synthetic cerumen (SCC) was prepared, weighed in test tubes, and left to solidify



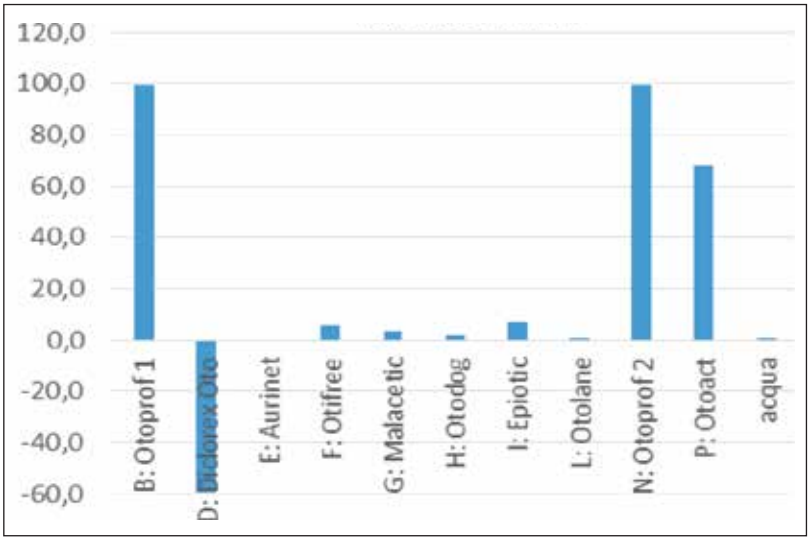
- An aliquot part of the otological product equal to two milliliters (ml) was added
- The tubes were shaken moderately at a temperature of 35 ° C for five minutes, and subsequently, were inverted
- After 24 hours each test tube was weighed, and another two ml of the product were added
- Five washes per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

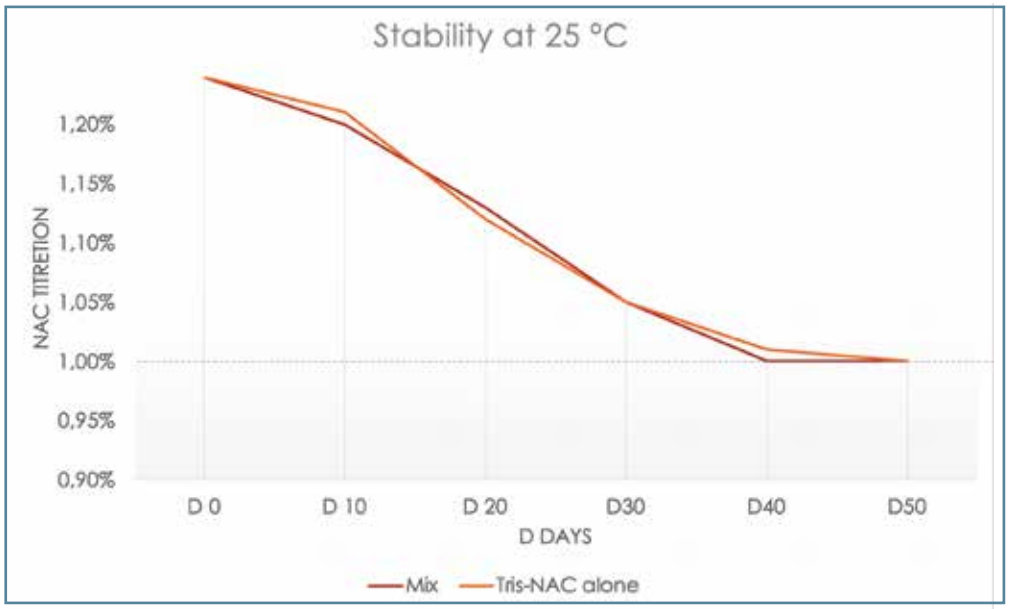
The data obtained – Stability - at 25 ° C

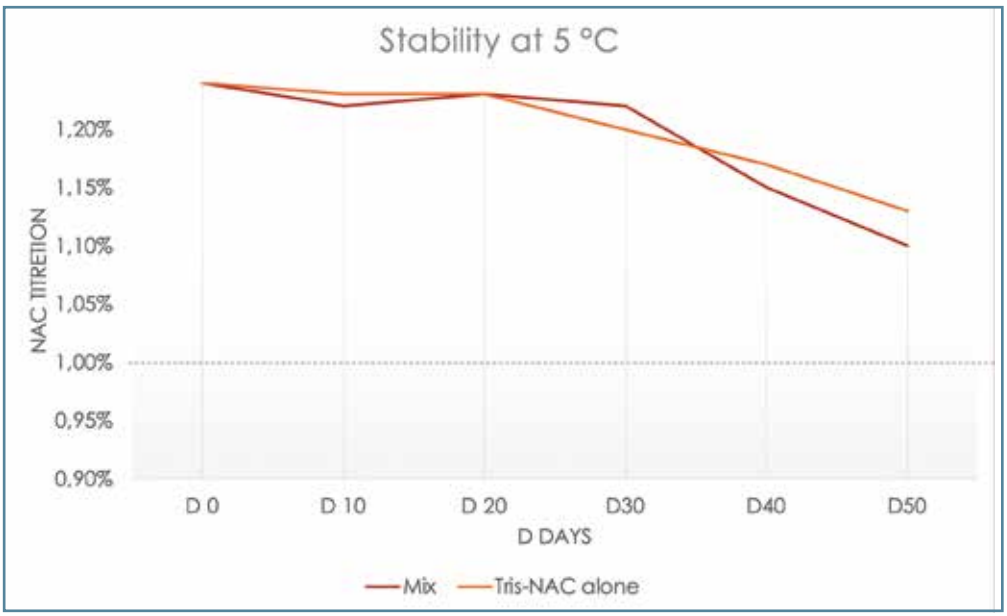
| | Time zero 05/05/20 | Day 10 15/05/20 | Day 20 25/05/20 | Day 30 05/06/20 | Day 40 15/06/20 | Day 50 26/06/20 |
|---|---|---|---|---|---|---|
| Appearance | Opaque liquid | Opaque liquid | Opaque liquid | Slightly opaque liquid | Slightly opaque liquid | Slightly opaque liquid |
| pH | 8.06 | 1: 8.46 | 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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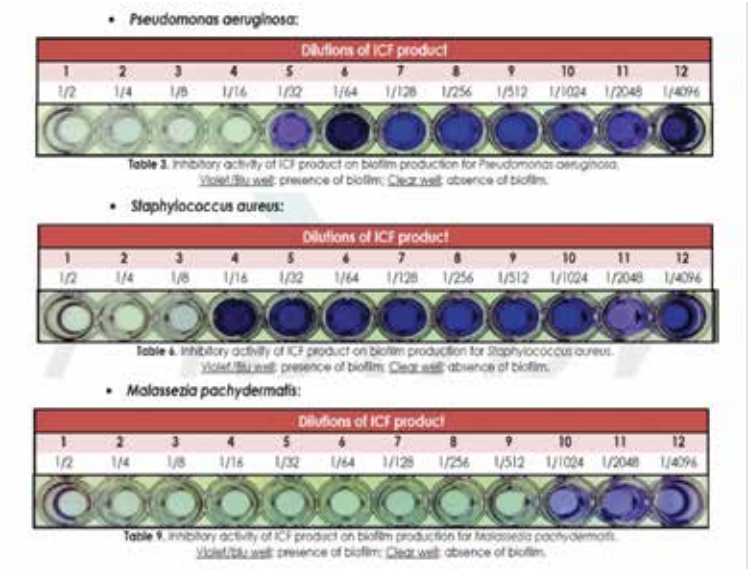
A biofilm is a structured consortium of bacteria embedded in a self produced polymer matrix consisting of polysaccharides, protein and DNA. Bacterial and yeast biofilms cause chronic infections because they show increased tolerance to antibiotics, antifungals and disinfectant chemicals, as well as resisting 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.*

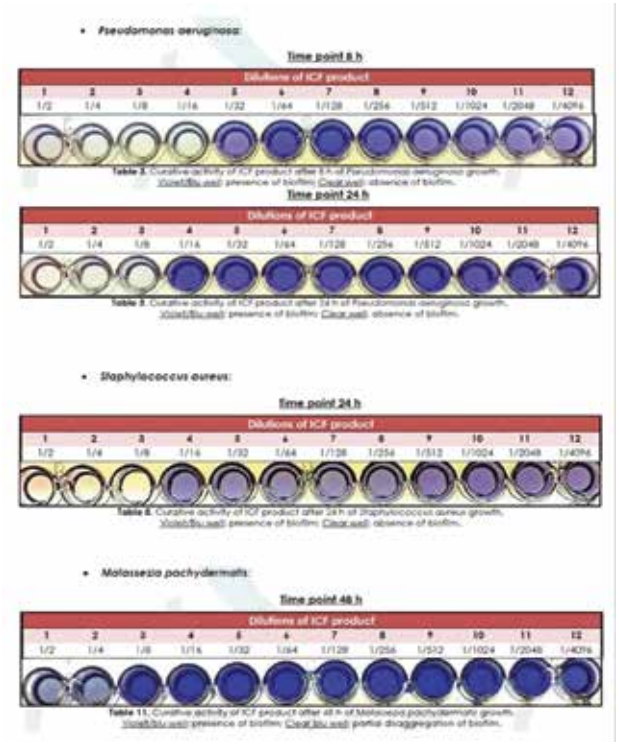
## MATERIALS AND METHODS

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.



## 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.





## 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.

Veterinary Dermatology

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# Clinical efficacy of spray-based heat-treated lactobacilli in canine atopic dermatitis: a preliminary, open-label, uncontrolled study

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**Background –** Canine atopic dermatitis (cAD) is a common inflammatory and pruritic skin disease, with various treatment options. The use of topical products containing natural ingredients has proven increasingly popular.

**Objective –** To evaluate the effects of a spray solution containing heat-killed *Lactobacillus rhamnosus* and *L. reuteri,* on the clinical signs and cutaneous microbiota of atopic dogs.

**Animals –** Ten privately owned, mildly affected, nonseasonally atopic dogs.

**Methods and materials –** The spray was applied to the ventrum every 24 h for 28 days. Clinical scores, skin barrier function and owner assessment were evaluated on day (D)0, D14, D28 and D42. The cutaneous microbiota was analysed on D0 and D28.

**Results –** A reduction in the total clinical score was seen at each time point (D14, $P = 0.03$; D28, $P = 0.04$; D42, $P = 0.001$). A reduction in the regional clinical scores was seen after D28 ($P = 0.01$) and D42 ($P = 0.003$). A significant reduction in the pruritus score was seen on D42 ($P = 0.01$). A lower hydration value was seen on D28 ($P = 0.02$) and D42 ($P = 0.02$) on the pinnae. A good-to-excellent response and an easy-to-use administration was reported by owners. There were no significant changes in the cutaneous microbiota after 28 days.

**Conclusions and clinical importance –** There was a significant and rapid decrease in the clinical signs associated with cAD after use of the spray. Future larger, randomized, controlled studies are needed to confirm these results and to assess the effects on the cutaneous immunity and microflora of atopic dogs.

## Introduction

Atopic dermatitis (AD) is one of the most common chronic inflammatory skin diseases in dogs. Genetic and immune system alterations along with defects of the skin barrier and modifications of the cutaneous microflora have been reported in atopic dogs.[1–4] Multiple treatment options are available;[5,6] there is a growing interest in the use of alternative products based on so-called natural ingredients.[7]

In human AD there are reports of the potential positive effects of topical applications of heat-killed beneficial bacteria.[8–10] In particular, the use of topical products containing heat-killed bacteria (*Vitreoscilla filiformis*, *Lactobacillus johnsonii* and *L. reuteri*) has been reported to show a significant decrease in clinical signs and pruritus in both atopic people and mice.[8–11] There was an overall reduction in the *Staphylococcus aureus* population reported in one study.[10] Some studies report that the use of rapidly killed beneficial bacteria (e.g. lactobacilli) can ameliorate the clinical signs of human AD and positively stimulate the local immunity, to the point of influencing the local microbiome.[12–14]

The objective of this preliminary, noncontrolled, prospective clinical trial was to assess the effects of a spray version of a veterinary product containing heat-killed lactobacilli (*L. rhamnosus* and *L. reuteri*) marketed as an adjuvant therapy for allergic dogs (LinkSkin spray, DRN Inc.; Cremona, Italy) on the clinical signs of canine (c)AD (primary outcome), the skin barrier and the cutaneous microbiota (secondary outcomes).

## Methods and materials

### Animal welfare documentation

All dogs were recruited at the authors' institution with previous approval from the University of Florida and a signed owner consent form.

### Canine population

Naturally affected, privately owned dogs with mild-to-moderate [Canine Atopic Dermatitis Extent and Severity Index, 4th iteration

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**Source of Funding:** This study was funded by DRN srl; this included provision of the product and financial support for the laboratory work; they had no influence on the study design and the preparation of this manuscript.

**Conflict of Interest:** Domenico Santoro has received reimbursements and consultation fees from DRN srl. None of the other authors has declared any conflicts of interest.

CADESI-04 with scores of 10 to 59][15] nonseasonal AD were enrolled in the study. Diagnosis of cAD was based on published guidelines.[16] All dogs received monthly flea prevention.

### Exclusion criteria

Dogs with clinical evidence or a history of malignant neoplasia, metabolic/endocrine, parasite or other allergic skin conditions (food/contact) were excluded. Dogs with superficial infections, diagnosed based on published guidelines,[17] were excluded. Owners withdrew drugs for their dog using the following periods: oral and topical antibiotics and antifungal agents (four weeks), corticosteroids (eight weeks for depo-injectable, four weeks for oral, three weeks for topical), antihistamines (two weeks), ciclosporin (four weeks), lokivetmab (eight weeks) and oclacitinib (one week). Dogs receiving allergen-specific immunotherapy for less than one year before enrolment were excluded from the study.

### Intervention

The treatment consisted of a water-based spray solution containing a mixture of lactobacilli (*L. reuteri* and *L. rhamnosus*) together with the culture media containing metabolites produced by the bacteria, tamarind extract and polyphenols. This solution had undergone Tyndallization, a process used to sterilize food from heat-resistant endospores and bacteria, based on the use of heat to reach boiling point (~100°C) for 15 min for three consecutive days. This methodology, although designed to allow endospores to germinate and be killed, allows minimal degradation of the bacteria walls with minimal alteration of the immunological properties of the bacteria.[12–14]

Other topical and/or systemic treatments including antimicrobial, humectant or antipruritic, were not allowed during the study. Owners sprayed the glabrous areas (axillae, ventral thorax, inguinal area and medial thighs) every 24 h for 28 days, irrespective of the distribution of the dog's skin disease. Licking and subsequent alteration of the cutaneous microbiota was prevented by distracting the dog and, when unsupervised, placing a T-shirt on the dog, throughout the 28 days of the study. The use of the T-shirt was preferred to the use of an E-collar for ethical reasons and tolerability. Each dog was clinically evaluated by the same investigator on Day (D)0, D14, D28 and D42.

### Clinical assessment

The primary outcome of this study was to assess the clinical efficacy of the spray for cAD. On D0, D14, D28 and D42, the severity of clinical signs was assessed using the CADESI-04 score system recording 20 body sites that are commonly affected in AD (total CADESI-04). These areas were scored on a scale between 0 and 3 (0,none;1, mild; (2, moderate; 3, severe) with a maximum score of 180.[15] A separate CADESI-04 score (regional) was calculated adding the score of only the treated areas (axillae, ventral thorax, inguinal area and medial thighs). At each visit, the pruritus was scored by the owners using a pruritus Visual Analog Scale (pVAS).[18,19] The percentage of dogs with a CADESI-04 N (<10) and with a pVAS10-N (<2) was calculated.[20] An owner global assessment of treatment efficacy (OGATE) score was recorded using a scale between 0 and 4 (0, no response; 1, poor; 2, fair; 3, good; 4, excellent). The percentage of dogs with an OGATE-G2E (>2) was calculated in a similar way to the CADESI-04 N.[20] An "ease to administer" score by the owners was recorded, based on asking the owners if the product was not easy, easy, or very easy to use.[21] Parameters considered by the owners included ease-of-spray, skin residues, stickiness and time of absorption.

### Skin barrier function evaluation

As secondary outcome, the skin barrier function was assessed in all dogs on D0, D14, D28 and D42; assessing skin hydration via corneometry and pH. These parameters were analysed on the inguinal, axillary, pedal and aural surfaces using a corneometer and expressed as microsiemens values [μS] (Corneometer CM825, Courage + Khazaka electronic GmbH; Cologne, Germany) and a pH meter (Skin-pH-meter PH 905, Courage + Khazaka electronic GmbH) as described previously.[22] Before each evaluation, if present, the T-shirt was removed and dogs were allowed to acclimatise to the testing room for 30 min. Temperature and humidity settings were maintained at 25 ± 5°C and 50 ± 10% for the entire duration of the study and as recommended by the manufacturer to optimize the performance of the instruments.

### Skin microbiota collection

Another secondary outcome was the assessment of the cutaneous microbiota before (D0) and after treatment (D28). Two skin swabs, one from the groin and one from both axillae, were collected using swab applicators (Isohelix DNA Buccal Swabs, Cell Projects Ltd; Harrietsham, Kent, UK) before the acclimatisation period. Each swab applicator was rubbed on the skin 20 times (10 strokes per swab side) per anatomical site, as reported previously.[23] In addition, an extra swab agitated for 20 s in the air was submitted as a negative control, to control for environmental contaminants. All samples were stored immediately at –80°C until processed.

### Library construction of microbial samples

For each extraction, 5 ng gDNA was used for 16S library construction using Quick-16S NGS Library Prep Kit (Zymo Research Corp.; Irvine, CA, US). PCR amplification was performed using V3–V4 primers (95°C for 10 min, 20 cycles of (95°C for 30 s, 55°C for 30 s and 72°C for 3 min). The PCR products were quantified by real-time PCR with a Bio-Rad CFX Real-Time PCR Detection System (Bio-Rad Laboratories; Hercules, CA, USA) and cleaned with the Zymo enzymatic cleanup kit. Each library was barcoded with five cycles of PCR amplification and cleaned with the Select-a-Size MagBeads (Zymo Research Corp.). All 48 libraries were pooled together for one MiSeq 2 x 250 cycles run.

### Statistical analyses

*Clinical and skin barrier assessment*. Data were analysed using the intention-to-treat analysis (ITT) with the last value carried forward. Data collection was tested for normality using the Shapiro–Wilk test (alpha level = 0.05). Then repeated measures ANOVA (skin hydration and pH) or Friedman's test (CADESI-04 and pVAS) followed by Dunnett's or Dunn's multiple comparison test were performed to evaluate the behaviour of each variable over time. A *P*-value of <0.05 was considered statistically significant. All statistical analyses were performed using PRISM 6.09 statistical software (GraphPad Software Inc.; La Jolla, CA, USA).

*Data analysis of microbial samples*. Reads of *16S RNA* (V3–V4 region) acquired from the Illumina MiSeq system were cleaned up with the CUTADAPT program (v2.8; https://cutadapt.readthedocs.io/en/stable/) [24] to trim off sequencing adaptors, low-quality bases and potential errors introduced during sequencing. Only sequences with length ≥60 bp were included in the analyses. The cleaned paired-end reads were merged and analysed using Quantitative Insight Into Microbial Ecology (QIIME, v1.9.1; http://qiime.org/).[25]

The merged *16S rRNA* sequences were grouped into operational taxonomic units (OTUs) (minimum OTU cluster size = 2; OTU similarity = 0.97) using both the open reference and closed reference OTU picking strategy and classified using the SILVA 16S/18S reference database.[26] The potential chimeric sequences and OTUs were detected and filtered at 97% cluster identity by VSEARCH (v2.13.4; https://manpages.debian.org/stretch/vsearch/vsearch.1).[27] Taxonomies were summarized at multiple taxonomic levels (L2–L6) as the OTU tables with the SAMTOOLS and R-based scripts developed at the Interdisciplinary Center for Biotechnology Research, University of Florida. The sequencing coverage was evaluated by rarefaction analysis and the estimated species richness and diversity indices were calculated with the alpha-diversity analysis. The differences of the microbial compositions between different samples were compared with the beta-diversity analysis, including the principal coordinate analysis (PCoA) based on the Bray–Curtis distance model in R/PHYLOSEQ. In addition, the quantitative comparison of taxa at different taxonomic levels between the different groups was performed based on the raw OTU tables using the DEB application.[28]

 

## Results

### Dogs

Ten dogs (five males, five females) were enrolled for this study. The average age was 5.3 ± 3.6 years. The average weight was 28.2 ± 24 kg. Breeds included great Dane (2), mixed-breed dogs (3), pit bull terrier (2), and one each of German shepherd dog, Jack Russell terrier and French bulldog. One mixed-breed dog was withdrawn from the study after D28 owing to relocation of the owner.

### Clinical assessment

Compared to D0, a significant reduction in the total CADESI-04 was seen at each time point ($P = 0.028$; $P = 0.036$; $P = 0.001$, respectively) (Figure S1). In particular, on D0, a median (range) total CADESI-04 score of 16.5 (13–32) was recorded. This score decreased to 8.5 (3–25) on D14, 9.5 (3–18) on D28 and 6.5 (0–14) on D42. A CADESI-04 N (<10) was achieved in 50% (five of 10), 50% and 90% (nine of 10) of dogs after D14, D28 and D42, respectively. Likewise, compared to D0, a reduction of the regional CADESI-04 was seen after 28 ($P = 0.013$) and 42 ($P = 0.003$) days (Figure 1). In particular, on D0, a median (range) regional CADESI-04 score of 6 (0–15) was recorded. This score decreased to 3 (0–6) on D14, 3 (0–4) on D28 and 0.5 (0–3) on D42.

Compared to D0, a significant reduction in the pVAS was seen on D42 ($P = 0.013$) (Figure S2). On D0, the pVAS was 4.9 ± 1.9, whereas on D14 it was 5.2 ± 2.2 and decreased to 4.1 ± 2.1 on D28 and to 2.5 ± 1.1 on D42. A pVAS10-N (<2) was achieved in 10% (one of 10), 30% (three of 10) and 30% of dogs on D14, D28 and D42, respectively.

On D14, OGATEs of a fair response by seven (70%) owners and a good response by two (20%) owners were recorded (Figure S3). On D28, there was an OGATE equivalent to a fair response by three (30%) owners and a good response by six (60%) owners (Figure S3). On D42, OGATEs of a fair response by one (10%) owner and a good response by six (60%) owners and an excellent response by two (20%) owners were recorded (Figure S3). An OGATE of a poor response was reported by one (10%) owner for the entire duration of the study. An OGATE-G2E (>2) was achieved in (two) 20%, (six) 60% and 60% of dogs on D14, D28 and D42, respectively.

On D14, two (20%) owners reported the product to be easy to use, whereas eight (80%) owners judged the product very easy to use (Figure S3). Likewise, on D28 and D42, one (10%) owner reported the product to be easy to use, whereas nine (90%) owners judged the product very easy to use (Figure S3).

During the study, major adverse effects were not observed in any dog. Only one dog had a noninfective, papular eruption after 14 days in the study. However, the eruption was not severe enough for the dog to be dropped from the study. The eruption went away after a series of topical shampoos containing oatmeal (not allowed during the study).

### Skin barrier function evaluation

A significant change in the skin hydration and pH of the inguinal, axillary and interdigital areas was not seen at any time point (Figure 1). A significant reduction in the skin hydration, not the pH, was seen on the pinnae on D28 ($P = 0.02$) and D42 ($P = 0.047$) when compared to D0 (Figure 1).

### Skin microbiota evaluation

Microbiome gDNA extractions were processed by using ZymoBIOMICS DNA kits following the user guide. Bacterial and archaeal microbial communities of 40 canine skin samples were characterized by sequencing the V3–V4 regions of PCR-amplified *16S rRNA* genes. An average of 160,137 paired-end reads for each sample and approximately 159,491 cleaned merged reads (average 440 bp) were obtained for metagenomics analysis. Rarefaction analysis and alpha diversity measures showed that the bacterial communities were sufficiently sampled and that further sequencing would have been unlikely to significantly increase the observed microbial diversity detected.

Altogether, an average of 3,322 unique OTUs, ranging from 963 to 10,850, were obtained from each sample corresponding to about 14 phyla, 28 classes, 57 orders, 95 families and 164 genera of prokaryotes (Figures S4–S12). All microbial communities analysed in both groups (before and after treatment) were dominated by a few microbial genera, including *Staphylococcus* (Firmicutes), *Conchiformibius* (Proteobacteria) and *Porphyromonas* (Bacteroidetes) (Figures S4–S12). The microbial community profiles of the different samples based on the OTU tables

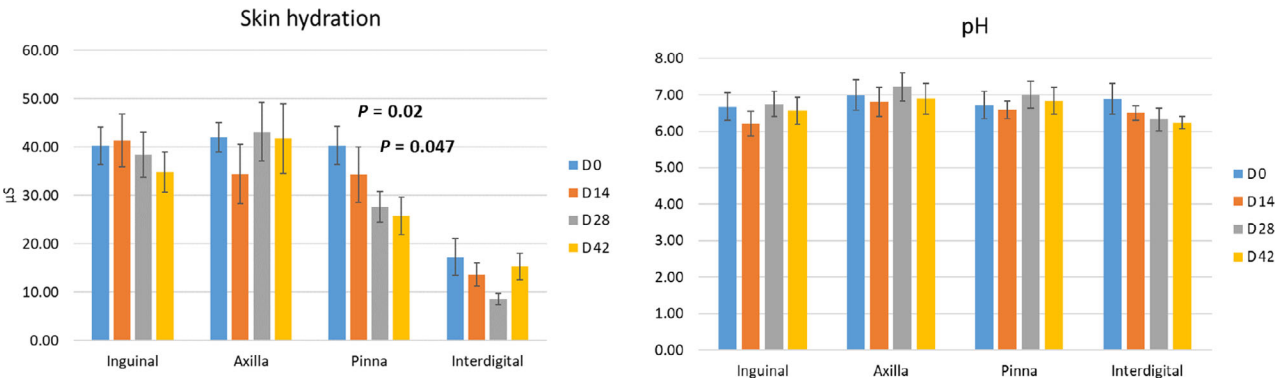


**Figure 1.** Average of the individual skin hydration and cutaneous pH values.
Hydration values are expressed in microsiemens (µS) as conductance units. Groups were compared using repeated measurements ANOVA with Dunnett's multiple comparison tests. Bars, SE.

 

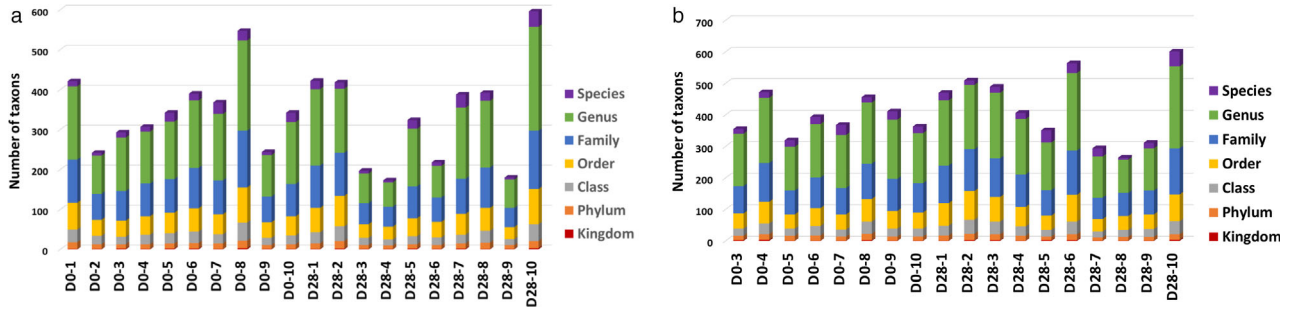


**Figure 2.** The distribution of operational taxonomic units at different taxonomic levels in the (a) axillae and (b) inguinal samples.

and the Bray–Curtis distance model showed some differences between the two groups (Figures S4–S12). However, these differences did not reach a statistically significant level (Figure 2).

## Discussion

To the best of the authors' knowledge, this is the first study to report a potentially beneficial effect from the topical use of heat-treated bacteria in the management of cAD. A significant reduction in the clinical score and pruritus was observed for two weeks after the spray was stopped, showing a residual beneficial effect. In addition, most of the owners deemed the product to be easy to very-easy to use and effectively to manage cAD. An overall improvement of the clinical signs was seen in areas not directly treated by the product (e.g. feet and head). Even so, no changes in the cutaneous microbiota were seen after 28 days of daily application of the compound.

In the present study, the daily application of the spray significantly and relatively rapidly decreased the clinical signs of dogs with mild-to-moderate AD without the use of any other therapeutic intervention. The only allowed "intervention" was the use of a T-shirt when the dog was unsupervised. However, this was not considered sufficient to influence the results because the T-shirt was not constantly worn and because the dogs were still able to scratch and rub. The amelioration of the disease was not limited only to the treated areas, but also to the rest of the body, suggesting a beneficial effect of the product on the systemic, not only local, immune response. These results are in agreement with previous studies showing an amelioration of the clinical signs of AD in people after oral and topical administration of heat-killed lactobacilli.[10,29,30] The present study also is in agreement with a previous human clinical trial that used a topical product containing *Vetrosciella filiformis* extract in atopic patients.[8] In that study, there was a a significant improvement reported in clinical signs, pruritus and quality of sleep, after only 28 days of daily application of heat-killed bacteria.[8] Altogether, these results suggest that a short-term daily application of heat-killed beneficial bacteria can be used as adjuvant therapy in AD.

Given that no significant changes in the microbiota were detected, it is possible to hypothesize that heat-treated lactobacilli do not directly interfere with the cutaneous microbiota. Rather, they may have a profound effect on the local and systemic immune response in atopic dogs. Several studies have shown the beneficial direct effect of heat-killed and treated (by Tyndallization) bacteria on the cutaneous and oral immune response without the potential drawbacks of administering live micro-organisms (e.g. overgrowth or passage of multidrug-resistant genes).[12–14]

This study had several limitations including the small number of dogs enrolled, wide variety of breeds and ages, as well as the short treatment period. A small number of dogs was chosen because the study was designed to collect preliminary data for future studies. The choice was arbitrary, although based on a hypothesized over-time success rate of 75% of the product against a placebo effect of 30%. Based on these criteria a minimum number of eight dogs would have been necessary to see a significant clinical difference, and consequently 10 dogs were selected. The wide variation in breed and ages could explain the lack of significant changes in the cutaneous microbiota after treatment; furthermore, it is well-recognised that the cutaneous microbiota can be highly variable between individuals. Thus, a reduction in interindividual variability would have been more beneficial for the microbiota evaluation. Furthermore, only two microbiological samples were taken (D0 and D28) during the study; it is unknown if microbiota changes would have been evident by D42 when a more significant clinical amelioration was seen. In addition, along with the metagenomic approach, it could have been interesting to simultaneously culture the skin microflora to evaluate for changes in the bacteria population.

Given the open-label nature of the study and the small number of dogs enrolled, a larger double-blinded, placebo-controlled, randomized clinical trial is desirable to confirm the clinical benefits showed in this study and to better evaluate the effects of the LinkSkin spray on the cutaneous microbiota.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article.

**Figure S1.** Median values for total and regional CADESI-04 scores and pVAS scores.
**Figure S2.** Median values for pVAS scores
**Figure S3.** OGATE and ease of administration of lactobacilli-based spray
**Figure S4.** The distribution of the top 20 operational taxonomic units at genus level in the axillae samples
**Figure S5.** PCoA at the genus level in the axillae samples based on Bray–Curtis dissimilarity matrices
**Figure S6.** The distribution of the top 20 OTUs at the family level in the axillae samples
**Figure S7.** PCoA at family level in the axillae samples based on Bray–Curtis dissimilarity matrices
**Figure S8.** The distribution of the top 20 OTUs at the genus level in the inguinal samples
**Figure S9.** PCoA at the genus level in the inguinal samples based on Bray–Curtis dissimilarity matrices
**Figure S10.** The distribution of the top 20 OTUs at the family level in the inguinal samples
**Figure S11.** Figure S11 PCoA at the family level in the inguinal samples based on Bray–Curtis dissimilarity matrices

**Résumé**
**Contexte –** La dermatite atopique canine (DAC) est une dermatose inflammatoire et prurigineuse fréquente avec de nombreuses options thérapeutiques. L'utilisation de topiques contenant des ingrédients naturels rencontre un succès grandissant.
**Objectif –** Evaluer les effets d'une solution en spray contenant *Lactobacillus rhamnosus* et *L. reuteri* tué par la chaleur, sur les signes cliniques et le microbiote cutané des chiens atopiques.
**Sujets –** Dix chiens de propriétaires, atteints de dermatite atopique non saisonnière modérée.

 

**Matériels et méthodes –** Le spray a été appliqué sur l'abdomen toutes les 24h pendant 28 jours. Les scores cliniques, la fonction barrière cutanée et l'estimation des propriétaires ont été évalué à jour (J) 0, J14, J28 et J42. Le microbiote cutané a été analysé à J0 et J28.
**Résultats –** Une diminution du score clinique total a été observé à chaque point de contrôle (J14, $P = 0.03$; J28, $P = 0.04$; J42, $P = 0.001$). Une diminution des scores cliniques régionaux a été vu après J28 ($P = 0.01$) et J42 ($P = 0.003$). Une diminution significative du score de prurit a été vu à J42 ($P = 0.01$). Une valeur d'hydratation plus faible a été vue à J28 ($P = 0.02$) et J42 ($P = 0.02$) sur le pavillon auriculaire. Une réponse bonne à excellente et une administration facile a été notée par les propriétaires. Il n'y avait pas de changement significatif dans le microbiote cutané après 28 jours.
**Conclusions et importance clinique –** Il y avait une diminution rapide et significative des signes cliniques associés à la DAC après utilisation du spray. D'autres études plus larges, randomisées, contrôlées sont nécessaires pour confirmer ces résultats et pour déterminer les effets sur l'immunité cutanée et la microflore des chiens atopiques.

**Resumen**
**Introducción –** la dermatitis atópica canina (cAD) es una enfermedad cutánea inflamatoria y pruriginosa común, con varias opciones de tratamiento. El uso de productos tópicos que contienen ingredientes naturales ha demostrado ser cada vez más popular.
**Objetivo –** evaluar los efectos de una solución en aerosol que contiene *Lactobacillus rhamnosus* y *L. reuteri* destruidos por calor, en los signos clínicos y la microbiota cutánea de perros atópicos.
**Animales –** diez perros de propietarios particulares, levemente afectados, atópicos no-estacionales.
**Métodos y materiales –** El aerosol se aplicó en la zona ventral cada 24 h durante 28 días. Las puntuaciones clínicas, la función de barrera cutánea y la valoración del propietario se evaluaron los días (D) 0, D14, D28 y D42. La microbiota cutánea se analizó en D0 y D28.
**Resultados –** se observó una reducción en la valoración clínica total en cada momento (D14, $P = 0{,}03$; D28, $P = 0{,}04$; D42, $P = 0{,}001$). Se observó una reducción en las valoraciones clínicas regionales después de D28 ($P = 0{,}01$) y D42 ($P = 0{,}003$). Se observó una reducción significativa en la valoración de prurito en D42 ($P = 0{,}01$). Se observó un valor de hidratación más bajo en D28 ($P = 0.02$) y D42 ($P = 0.02$) en el pabellón auricular. Los propietarios informaron de una respuesta buena a excelente y una administración fácil de usar. No hubo cambios significativos en la microbiota cutánea después de 28 días.
**Conclusiones e importancia clínica –** Hubo una disminución rápida y significativa de los signos clínicos asociados con la cAD después del uso del aerosol. Se necesitan estudios controlados, al azar y con mayor número de animales en el futuro para confirmar estos resultados y evaluar los efectos sobre la inmunidad cutánea y la microflora de los perros atópicos.

**Zusammenfassung**
**Hintergrund –** Die canine atopische Dermatitis (cAD) ist eine häufige entzündliche und juckende Hauterkrankung, für die es verschiedene Behandlungsmöglichkeiten gibt. Die Verwendung von topischen Produkten, die natürliche Wirkstoffe beinhalten, haben sich als zunehmend beliebt gezeigt.
**Ziel –** Eine Evaluierung der Wirksamkeit einer Spraylösung, welche durch Hitze-abgetötete *Lactobacillus rhamnosus* und *l. reuteri* beinhaltete, auf die klinischen Zeichen und die kutanen Mikrobiota der atopischen Hunde.
**Tiere –** Zehn private nicht-saisonal atopische Hunde , die nur mild betroffen waren.
**Methoden und Materialien –** Der Spray wurde 28 Tage lang alle 24 h auf dem Ventrum aufgetragen. Klinische Werte, die Funktion der Hautbarriere und eine Beurteilung der BesitzerInnen wurde am Tag (D)0, D14, D28 und D42 evaluiert. Die kutanen Mikrobiota wurden am D0 und D28 analysiert.
**Ergebnisse –** Eine Reduzierung des klinischen Gesamtwerts wurde zu jedem Zeitpunkt festgestellt (D14, P = 0,03; D28, P = 0,04; D42, P = 0,001). Eine Reduzierung der lokalen klinischen Werte konnte nach D28 (P = 0,01) und D42 (P = 0,003) festgestellt werden. Eine signifikante Verminderung der Juckreizwerte wurde am D42 (P = 0,01) festgestellt. Ein niedrigerer Wert wurde am D28 (P = 0,02) an den Ohren festgestellt. Die BesitzerInnen berichteten von einer guten bis ausgezeichneten Reaktion und einer leichte Methode der Anwendung. In den kutanen Mikrobiota bestanden nach 28 Tagen keine signifikanten Unterschiede.
**Schlussfolgerungen und klinische Bedeutung –** Nach Verwendung des Sprays gab es eine signifikante und rasche Abnahme der klinischen Zeichen der cAD. Es sind zukünftige, randomisierte, kontrollierte Studien nötig, um diese Ergebnisse zu bestätigen und die Auswirkungen auf die kutane Immunität und die Mikroflora atopischer Hunde zu bestätigen.

**概要**
**背景 –** 犬特□性皮炎(cAD)是一种常□的炎性和瘙痒性皮肤病，有多种治□□□。使用含有天然成分的外用□品已被□明越来越受□迎。

 

**目的 –** □价含有□□活鼠李糖乳杆菌和□伊氏乳杆菌的□□溶液□特□性患犬□床体征和皮肤微生物群的影响。
**□物 –** 10只□度□病、非季□性特□性私家犬。
**方法和材料 –** 每24h将□□□用于腹部, 持□28天。在第0天(D)、D14、D28和D42□行□床和皮肤屏障功能□分和主人□分。在D0和D28分析皮肤微生物群。
**□果 –** 在每个□□点均□察到□床□□分降低(D14,P = 0.03;D28,P = 0.04;D42,P = 0.001)。D28(P = 0.01)和D42(P = 0.003)后□察到外部□床□分降低。在D42□察到瘙痒□分□著降低(P = 0.01)。在D28(P = 0.02)和D42(P = 0.02)□察到耳廓上的水合□□低。犬主人□出了良好至极佳的□效和□□方便的反□。28天后皮肤菌群无明□□化。
**□□和□床重要性 –** 使用□□后, 与cAD相关的□床体征□著快速减少。未来需要更大□模、随机、□照研究来□□□些□果, 并□估□特□性犬皮肤免疫和微生物群落的影响。

**要約**
**背景 —** 犬アトピー性皮膚炎(cAD) は、一般的な炎症性および掻痒性皮膚疾患であり、さまざまな治療オプションがある。天然成分含有外用製品の使用はますます人気があることが証明されている。
**目的 —** 本研究の目的は、アトピー犬の臨床徴候と皮膚微生物叢に対する、熱殺菌したLactobacillus rhamnosusおよびL. reuteri含有スプレー液の効果を評価することであった。
**被験動物 —** 個人所有の、軽症、非季節性アトピー犬10頭。
**材料と方法 —** スプレーを24時間ごとに28日間腹側に塗布した。臨床スコア、皮膚バリア機能、および所有者の評価を、(D)0、D14、D28、およびD42日に評価した。皮膚微生物叢はD0およびD28に解析した。
**結果 —** 各時点で総臨床スコアの低下が見られた (D14、P = 0.03; D28、P = 0.04; D42、P = 0.001) 。 D28(P = 0.01) およびD42(P = 0.003) 後に、局部の臨床スコア低下が見られた。掻痒スコアの有意な低下がD42で見られた(P = 0.01) 。D28(P = 0.02)およびD42(P = 0.02) で、耳介のより低い水和値が見られた。所有者から良ー優れた反応および使いやすい管理が報告された。 28日後、皮膚微生物相に有意な変化は認められなかった。
**結論と臨床的重要性 —** スプレー使用後、cADに関連する臨床徴候が有意かつ急速に減少した。これらの結果を確認し、アトピー犬の皮膚免疫および微生物叢への影響を評価するには、将来のより大規模なランダム化比較試験が必要である。

**Resumo**
**Contexto –** A dermatite atópica canina (DAC) é uma doença cutânea inflamatória e pruriginosa comum, com várias opções de tratamento. O uso de produtos tópicos contendo ingredientes naturais tem se mostrado cada vez mais popular.
**Objetivo –** Avaliar os efeitos de uma solução em *spray* contendo *Lactobacillus rhamnosus* e *L. reuteri* inativados pelo calor, nos os sinais clínicos e na microbiota cutânea de cães atópicos.
**Animais –** Dez cães atópicos não sazonais de propriedade privada, levemente afetados.
**Métodos e materiais –** O *spray* foi aplicado no abdômen a cada 24 horas por 28 dias. Escores clínicos, função de barreira cutânea e avaliação do proprietário foram avaliadas nos dias (D) 0, D14, D28 e D42. A microbiota cutânea foi analisada em D0 e D28.
**Resultados –** Observou-se uma redução no escore clínico total em todos os momentos (D14, *P* = 0,03; D28, *P* = 0,04; D42, *P* = 0,001). Observou-se uma redução nos escores clínicos regionais após D28 (*P* = 0,01) e D42 (*P* = 0,003). Uma redução significativa no escore de prurido foi observada no D42 (*P* = 0,01). Observou-se um valor de hidratação inferior no D28 (*P* = 0,02) e no D42 (*P* = 0,02) no pavilhão auricular. Os proprietários relataram resposta boa a excelente e fácil administração. Não houve alterações significativas na microbiota cutânea após 28 dias.
**Conclusões e importância clínica –** Houve diminuição significativa e rápida dos sinais clínicos associados à DAC após o uso do spray. Futuros estudos maiores, randomizados e controlados são necessários para confirmar esses resultados e avaliar os efeitos sobre a imunidade cutânea e microflora de cães atópicos.

 

# Using Linkskin Spray to Treat Pyoderma in Companion Animals

## THE PRODUCT

Canine and feline skin is dominated by organisms including *Proteobacteria, Firmicutes, Fusobacteria, Bacteroides, Actinobacteria, Alternaria* and *Cladosporium.* Studies have demonstrated that cutaneous dysbiosis is associated with significant skin abnormalities. For example, the skin microbiota of clinically healthy dogs with a strong odor had increased numbers of *Malassezia* and staphylococci.

In addition, allergic dogs have increased *Staphylococcus* and *Corynebacterium* numbers compared with healthy controls, and the increases correlate with disease severity. Research studies conducted in 2010 demonstrated that oral probiotic therapy can prevent and improve canine atopic dermatitis clinical signs. A 2021 study published in *Veterinary Dermatology* concluded that Nextmune's Linkskin Spray produced a significant and rapid decrease in canine atopic dermatitis clinical signs.

Linkskin Spray is a solution that supports the skin barrier in dogs and cats by restoring the skin microbiome balance and improving skin homeostasis. Active ingredients include tyndallised lactobacilli (i.e., *Lactobacillus rhamnosus* and *Lactobacillus reuteri),* algal extracts, tamarind extract and vegetable polyphenols.

- **Tyndallised lactobacilli** — Tyndallization is primarily used to sterilize food from heat-resistant endospores and bacteria. The process involves heating the solution to boiling point for 15 minutes for three consecutive days, which allows minimal bacterial wall degradation and minimal alterations in the bacterial immunological properties, and ensures stability and efficacy in an aqueous solution.
- **Algal extracts** — Algal extracts provide nutrients to support diversity and balance in the skin microbiota.
- **Tamarind extract and vegetable polyphenols** — These substances provide antioxidant properties to normalize the skin microenvironment and prevent skin irritation and pruritus.


**FIGURE 1.** A 3-month old mixed-breed dog presented with multifocal erythematous and itchy areas of probable allergic origin (**BEFORE**), with improvement shown after Linkskin spray and tablets were provided for eight days (**AFTER**).

The following case example illustrates application of Linkskin Spray in a canine patient.

## PRESENTATION

Ade, a 9-year-old Boston terrier, had a history of severe allergies,

including a cutaneous food allergy and nonseasonal atopy.
He was previously treated with immunotherapy and cytopoint injections, which improved his clinical signs by about 70%. However, he experienced recurrent skin fold infections, especially in the neck and armpit areas, and flares about every month. Disinfectants caused topical reactions.

## TREATMENT

The author, a board-certified dermatologist, elected to use Linkskin spray based on positive results with similar cases.

The spray was applied aggressively on Ade twice daily. On the haired areas, the client helped by lifting the hair, gently moistening the area and leaving the skin to dry naturally. Treatment progressed as follows:

- **Three weeks** — After three weeks of treatment, Ade's owner reported dramatic improvement, with much less pruritus. At his dermatological recheck, he had minimal cocci bacteria or staph, which was in stark contrast to his usual cytology results that had typically revealed a bacterial overgrowth, despite no active pyoderma. He exhibited some lichenification and hyperpigmentation from chronic inflammation, but his hair was regrowing and his skin was no longer actinic. His skin was mostly normal and no longer dry and scaly.
- **Six weeks** — Ade continued to do well on the Linkskin spray, and after six weeks, his dermatologist discontinued the full-body therapy. She recommended twice-weekly applications to the problematic skin folds on Ade's neck and armpit areas.
- **14 months** — Ade has had no infection recurrence in 14 months, and his owner continues to treat his skin folds twice weekly with Linkskin spray.


**FIGURE 2.** Linkskin spray for topical use in dogs and cats

## DISCUSSION

Linkskin spray can be used to treat dermatologic conditions in dogs and cats, and is especially helpful to treat recurrent pyoderma and skin fold infections. Linkskin spray can be applied to a cotton ball and gently administered in the patient's skin folds with the goal of reestablishing normal flora. The product is also useful in elderly pets that are less mobile and prone to skin infections. In the author's experience, cytology performed on dogs and cats after treatment with Linkskin spray demonstrates less harmful bacteria and yeast, which helps to maintain normal skin flora. Linkskin is most effective in pyoderma cases when used all over the patient's body, rather than treating only active lesions. Treatment typically starts twice a day for four to six weeks, and then reduces in frequency to maintenance therapy two to three times a week.

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