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Therapeutic potential of otological formulations composed from *Triticum aestivum*, *Bixa orellana*, Tris-EDTA and N-acetylcysteine in the treatment of canine otitis externa

Potencial terapêutico de formulações otológicas compostas por *Triticum aestivum*, *Bixa orellana*, Tris-EDTA e N-acetilcisteína no tratamento de otite externa canina

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Abstract: The objective was to evaluate the efficiency of three otological solutions based on plant extracts and adjuvants in treating canine otitis externa. Three different ear solutions were developed, composed of ethanolic extracts of *Triticum aestivum*, *Bixa orellana*, and Tris-EDTA, ethanolic extracts of *Triticum aestivum*, *Bixa orellana*, and N-acetylcysteine, and a combination of *Triticum aestivum, Bixa orellana*, N-acetylcysteine, and Tris-EDTA. A commercial product was used as the control. The study included 64 dogs diagnosed with otitis externa, randomly divided into four groups, treated once daily for seven days. Clinical signs of otalgia, pruritus, odor, erythema, and cerumen amount were evaluated on days D0, D3, and D7. Samples for bacterial, fungal cultures, and cytology were collected before and after treatments. Clinical signs were scored from 0 to 3, with a sum generated per animal and group during each evaluation day. All evaluated compounds effectively reduced clinical signs and microorganisms involved in canine external otitis cases.

Keywords: adjuvants; plant extracts; otopathy; wheat; annatto

Resumo: Objetivou-se avaliar a eficiência de três soluções otológicas a base de extratos vegetais e adjuvantes no tratamento da otite externa canina. Foram desenvolvidas três diferentes soluções otológicas 1003 composta por extratos etanólicos de *Triticum aestivum, Bixa orellana* e Tris-EDTA,1004 composta por extratos etanólicos de *Triticum aestivum, Bixa orellana* e n-acetilcisteína, 1005 composta por *Triticum aestivum, Bixa orellana,* n-acetilcisteína e Tris-EDTA, como grupo controle foi utilizado produto comercial. Para o estudo foram selecionados 64 cães diagnosticados com otite externa, os quais foram divididos aleatoriamente em quatro grupos e todos os animais foram tratados durante sete dias. Durante o exame otológico realizado nos dias D0, D3 e D7, foram considerados os sinais clínicos de otalgia, prurido, odor, eritema e quantidade de cerúmen. Foram coletadas amostras para cultura bacteriana, fúngica e citologia antes e após os tratamentos. Após a avaliação, cada sinal clínico recebeu uma pontuação em escore de 0 a 3, o qual 0 era considerado ausente e 3 a forma mais

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grave de aparecimento, a partir desta pontuação foi gerado um somatório por animal e por grupo durante cada dia de avaliação. No final do tratamento, todos compostos avaliados foram eficazes para a redução dos sinais clínicos e microrganismos envolvidos em casos de otite externa canina.

Palavras-chave: adjuvantes; extratos vegetais; otopatia; trigo; urucum

1. Introduction

Canine otitis externa can present as chronic and recurrent. Often, the initial phase with milder clinical signs may go unnoticed by the owner, leading to disease progression. Topical therapy is the most recommended and used for otitis externa. It is challenging to find veterinary market products that are not a combination of antibiotics, antifungals, and glucocorticoids, complicating the choice based on cytological and clinical analyses, leading to unnecessary usage of these combinations, stimulating microbial resistance and the unnecessary use of glucocorticoids [(1)].

Frequent topical antibiotic treatment raises concerns due to the frequent bacterial resistance cases of various strains commonly isolated from canine otitis externa patients. Prudent antimicrobial use is crucial for public health, considering that bacteria or resistance genes can be transmitted to humans [(2)].

Canine otitis externa is a significant condition in veterinary dermatology, characterized by inflammation of the external auditory canal, causing acute clinical signs such as otalgia, odor, erythema, pruritus, and otorrhea, which can progress to chronic manifestations like hyperkeratosis, hyperpigmentation, and stenosis [(3)].

The condition has a multifactorial origin, presenting predisposing, primary, and perpetuating factors. Predisposing factors increase susceptibility, such as breed, presence of hair in the auditory canals, pendular morphology, and distribution of sebaceous glands. Primary factors effectively cause otitis, including allergic diseases like atopy, food allergies, and Flea Allergy Dermatitis (FAD), endocrine diseases, and immune-mediated diseases. Perpetuating factors contribute to the severity and therapy failure, with the most frequent being Malassezia pachydermatis yeast, gram-positive cocci bacteria like Staphylococcus intermedius, Staphylococcus aureus, and gram-negative bacteria like Pseudomonas spp. and Proteus spp. [(4)].

To prevent microbial resistance, alternatives are sought to avoid conventional antibiotic and antifungal use in mild and/or initial otitis externa cases. This study aimed to evaluate the effect of three otological solutions with plant extracts and adjuvants (Tris-EDTA and/or N-acetylcysteine) in treating naturally acquired canine otitis externa.

2. Material and methods

2.1. Extraction of plant extracts

Ethanolic extracts at a concentration of 50mg/mL from *Bixa orellana* (Annatto) and *Triticum aestivum* (Wheat) were used. Seeds were commercially obtained from Linea Verde

Alimentos Ltda, which has origin certification. The seeds were ground, weighed (25g), placed in an Erlenmeyer flask, and 500 ml of Ethanol PA was added. The mixture was filtered, sonicated for 30 minutes, and the solvent was removed using a rotary evaporator. The extract was stored in an amber bottle and refrigerated at 2ºC to 4ºC until use.

2.2. Formulation of otological solutions

Otological solutions were prepared using ethanolic extracts of annatto and wheat (50mg/mL). Solution 1003 included the extracts with Tris-EDTA, PEG 400, and PEG 4000. Solution 1004 contained the extracts with N-acetylcysteine, PEG 400, and PEG 4000. Solution 1005 combined the extracts with Tris-EDTA, N-acetylcysteine, PEG 400, and PEG 4000. These products are in the patent application process with INPI.

2.3. Study in dogs with naturally developed otitis

Approved by the Ethics Committee for Animal Experimentation (CEEA 23110.051174/2019.15), 64 rescued dogs were selected and housed in an NGO in Capão do Leão, RS. Inclusion criteria were the presence of at least three clinical signs compatible with canine otitis externa: erythema, exudate, pruritus, otalgia, edema, odor, and erosion/ulcer. Videoendoscopy was performed, excluding dogs with nodules or masses in the auditory canal and pregnant females.

The dogs were randomly distributed into four groups: Group 1003 (Annatto and Wheat Ethanolic Extracts with Tris-EDTA), Group 1004 (Annatto and Wheat Ethanolic Extracts with N-Acetylcysteine), Group 1005 (Annatto and Wheat Ethanolic Extracts with Tris-EDTA and N-acetylcysteine), and Positive Control Group (commercial product with clotrimazole, gentamicin sulfate, and betamethasone valerate). The evaluators were blinded to the treatments. Treatments were administered once every 24 hours for seven days. The ear and external auditory canal were cleaned with gauze before instilling the respective product: 4 drops for dogs under 15 kg and 8 drops for dogs over 15 kg.

Evaluations were conducted on days 0, 3, and 7 using otoscopic inspection and videoendoscopy (Vetcam device), assessing clinical signs (erythema, exudate, otalgia, odor, pruritus, edema, erosion/ulcer). Each clinical sign was scored from 0 to 3 (0: absent, 1: mild, 2: moderate, 3: intense), and erosion/ulcer was scored as absent (0) or present (2). Clinical sign scores were summed for group comparison. Swab samples were collected on days 0 and 7 for bacterial and fungal culture and cytology.

2.4. Cytological analysis

For cytological analysis, the swabs containing cerumen samples were rolled onto glass slides, stained with rapid panoptic, and subsequently examined under a microscope at 1000x magnification. Five microscopic fields were considered for the evaluation of these slides, and

the presence of cocci, bacilli, and M. pachydermatis yeast was quantified. After counting, the average of the five fields analyzed on each slide was calculated. A semiquantitative cytological scale, according to Budach & Mueller (2012), described in Table 1, was also used for the assessment of microorganisms and inflammatory cells present, being classified on a scale from 0 to 4 according to the amount observed.

Table 1. Semiquantitative Cytological Analysis Scale

Classificação	Descrição
0	No bacteria or inflammatory cells
1	1 to 2 bacteria per field
2	3 to 4 bacteria per field
3	5 to 6 bacteria per field
4	Over 6 bacteria per field

2.5. Antimicrobial activity

For fungal culture, samples were inoculated onto Sabouraud agar plates supplemented with chloramphenicol, stored in a microbiological incubator at 37°C for 48 hours, and then cytology was performed on the colonies that grew. For bacterial isolation, samples were inoculated onto blood agar and MacConkey agar, and stored in a microbiological incubator at 25°C. Finally, Gram staining was performed on the colonies that grew, along with catalase, coagulase, and peroxidase tests, as well as biochemical tests as necessary for the identification of each microorganism.

2.6. Statistical analysis

For statistical analyses, the SPSS ® 20.0 statistical package was used, considering a minimum significance level of 95%. All groups were compared using the Kruskal-Wallis test. For the comparison between collection days, the Friedman test was used. For microbiological cultures, the McNemar test was used to compare collections, and the Chi-square test was used to compare groups. Cytology was compared between collections using the Wilcoxon test and between groups using the Kruskal-Wallis test.

3. Results and discussion

The animals evaluated in this study were classified according to the score of clinical signs, as this is a clinical experiment with naturally affected animals, and otitis externa is multifactorial with different clinical manifestations and various agents. Therefore, it was not possible to standardize all these criteria within a single group.

The results indicated that product 1005 showed better efficacy than the others in reducing the clinical signs of otitis externa. In the Kruskal-Wallis test, all groups showed improvement in the intensity of clinical signs. However, the group treated with product 1005 was the one that most reduced clinical signs at the end of the treatment, showing a statistical difference ($p = 0.03$) from the other groups. Through the Friedman test, it was possible to evaluate that all groups showed a statistical difference ($p < 0.05$) between the beginning and the end of the experimental period, demonstrating that all treated animals improved during the treatment (Figure 1).

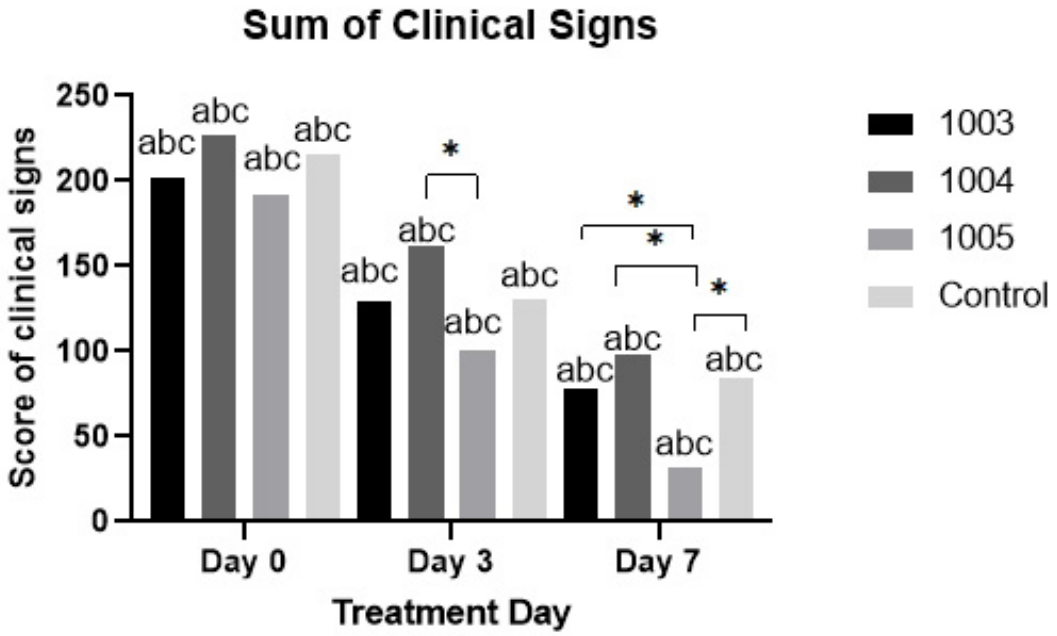

Figure 1 **Demonstration of the sum of clinical signs and statistical differences of clinical analyses performed on dogs treated with products 1003, 1004, 1005, and the control group. The symbol (*) represents the statistical difference between the evaluated groups.**

Regarding specific clinical signs, as well as the sum, they progressively decreased; however, it was observed that pruritus did not show a statistical difference between the groups. For odor ($p < 0.05$) and exudate ($p < 0.05$), a significant improvement was noted in group 1005 compared to the other tested groups (Figure 2). Clinical signs of edema, erosion, and ulcer, being less frequent and involved in more severe cases of otitis, were present in a small number of ears, which progressively reduced with all tested treatments, therefore, it was not possible to make a comparison between the groups.

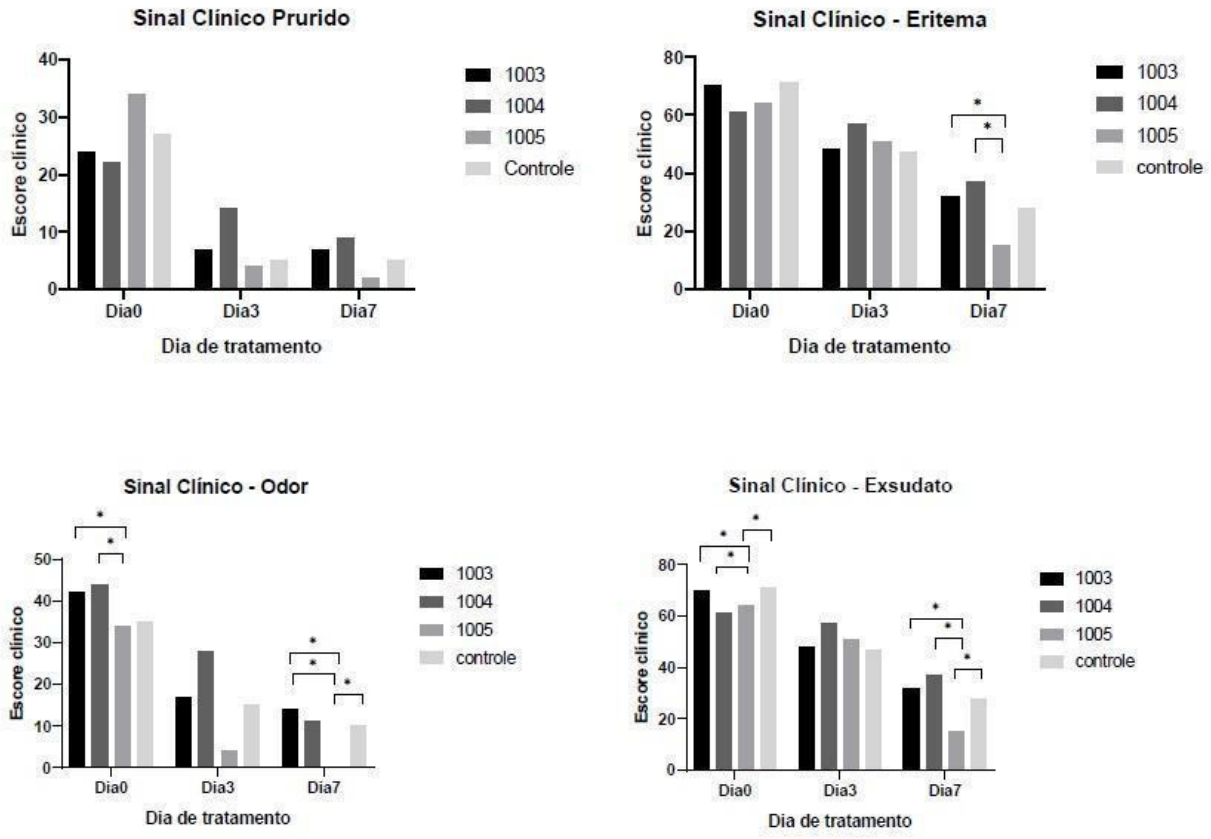

Figure 2 Demonstration of the sum of clinical signs a) Pruritus, b) Erythema, c) Odor, and d) Exudate, in groups treated with products 1003, 1004, 1005, and the control group. The letters represent the statistical differences between collections and the symbol (*) represents the statistical difference between groups.

The reduction in clinical signs may be related to the action of plant extracts, which are potentiated by the action of adjuvants. Extracts from annatto and wheat plants are known to have healing properties, thus demonstrating efficiency in wound healing. The action of the 25% aqueous wheat extract, in another study, was responsible for reducing the clinical and microbiological parameters of otitis externa, making it a promising option. Annatto (Bixa orellana), often used in popular medicine, has pro-inflammatory properties that recruit defense cells to the site of inflammation and consequently, through the body's response, improve clinical signs. Additionally, it is known to accelerate the formation of scabs and the healing process, possibly due to high concentrations of fatty acids, such as oleic and linoleic acids, which were also found in our chromatographic analysis. Other compositions using the combination of annatto and wheat extracts have already shown promise, being able to reduce clinical signs of canine otitis externa.

The antimicrobial potential was evaluated against isolates of the yeast *M. pachydermatis* and the bacteria *Staphylococcus epidermidis*, coagulase-negative *Staphylococcus*, *Proteus* sp., *Enterobacter* sp., *Bacillus* sp., *Streptococcus* sp., and polymicrobial infections (Figures 3 and 4). The yeast *M. pachydermatis* was isolated in all groups and showed susceptibility to all tested products, as demonstrated by the McNemar test. Through the Chi-square test, it was observed that there was no difference between collections and treatment groups, demonstrating that all treatments were effective without any difference between groups.

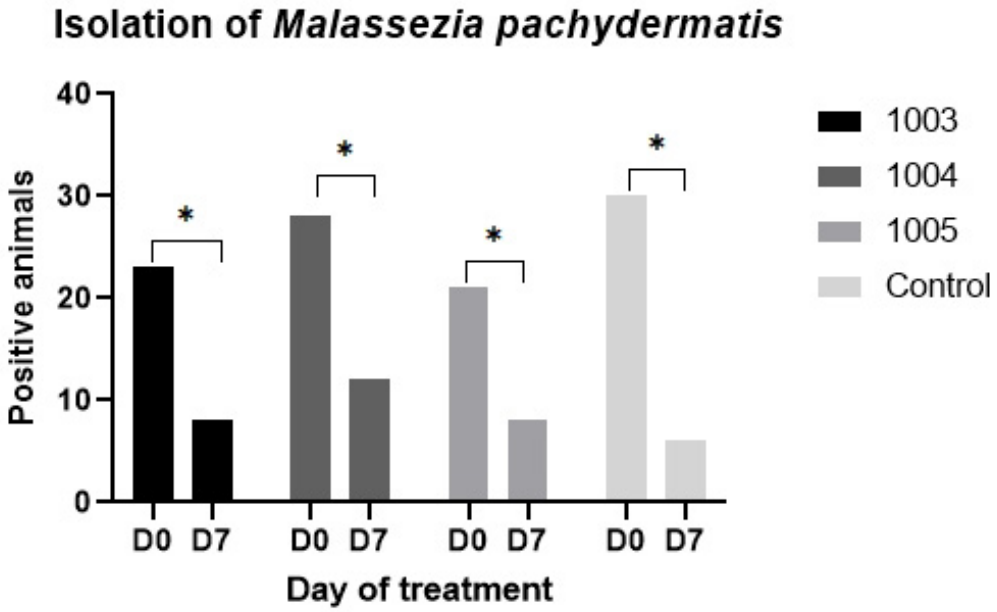

Figure 3 Demonstration of isolations and response to treatment of the different test and control groups, against the yeast *Malassezia pachydermatis* isolated from dogs with otitis externa. The symbol (*) demonstrates the statistical difference between the beginning (D0) and the end of treatment (D7).

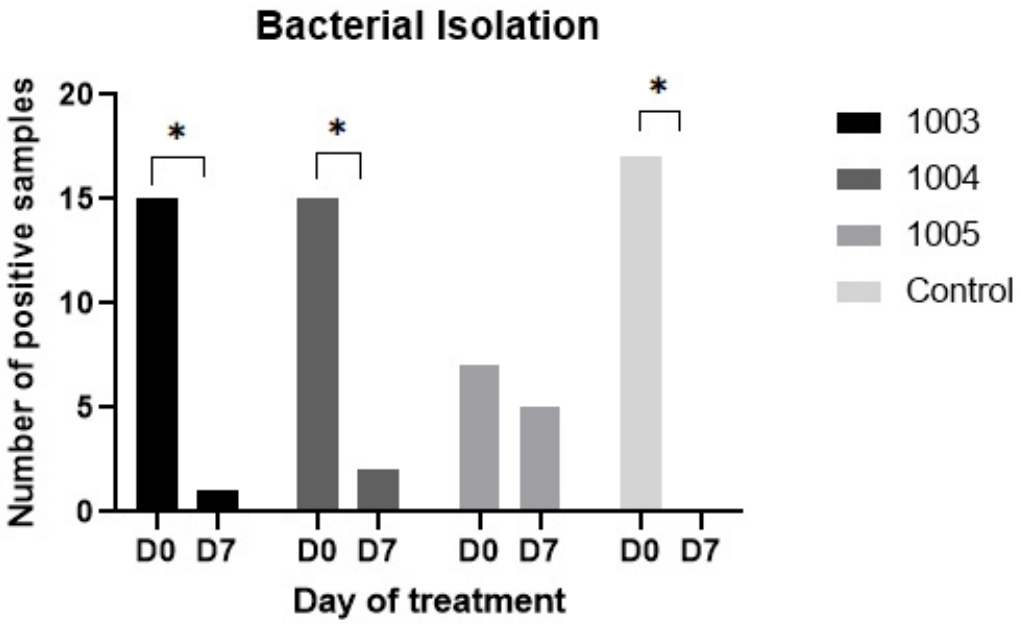

Figure 4 Demonstration of isolations and response to treatment of the different test and control groups, against bacteria isolated from dogs with otitis externa. The symbol (*) demonstrates the statistical difference between the beginning and end of treatment.

In bacterial isolations, groups 1003, 1004, and the control group showed a statistical difference between the period before and after the end of treatment, decreasing the number

of isolated bacteria. However, group 1005 did not show a significant difference in the number of isolates, possibly due to the smaller number of isolates obtained before the start of treatment, which could also justify the better reduction of clinical signs in this group (Figure 4).

For both cytological evaluations, the Wilcoxon test was used for comparison between collections, and the Kruskal-Wallis test was used for comparison between groups. According to Figure 5, it was observed that the predominant microbiota was composed of cocci-shaped bacteria, followed by the yeast *Malassezia* sp., and less predominantly by bacilli-shaped bacteria. All tested groups showed a statistical difference between the beginning and the end of treatment, demonstrating that the microbial load reduced in all groups (Figure 5). Regarding the difference between the tested groups, there was a statistical difference on Day 0 between group 1003 and 1005, and between 1005 and the control group; this same difference was maintained at the end of the treatment on Day 7, not demonstrating any difference in the efficiency of the tested treatments. Through the semi-quantitative scale, it was possible to evaluate that all tested groups decreased the quantity of microorganisms during the treatment, not differing among them (Figure 6).

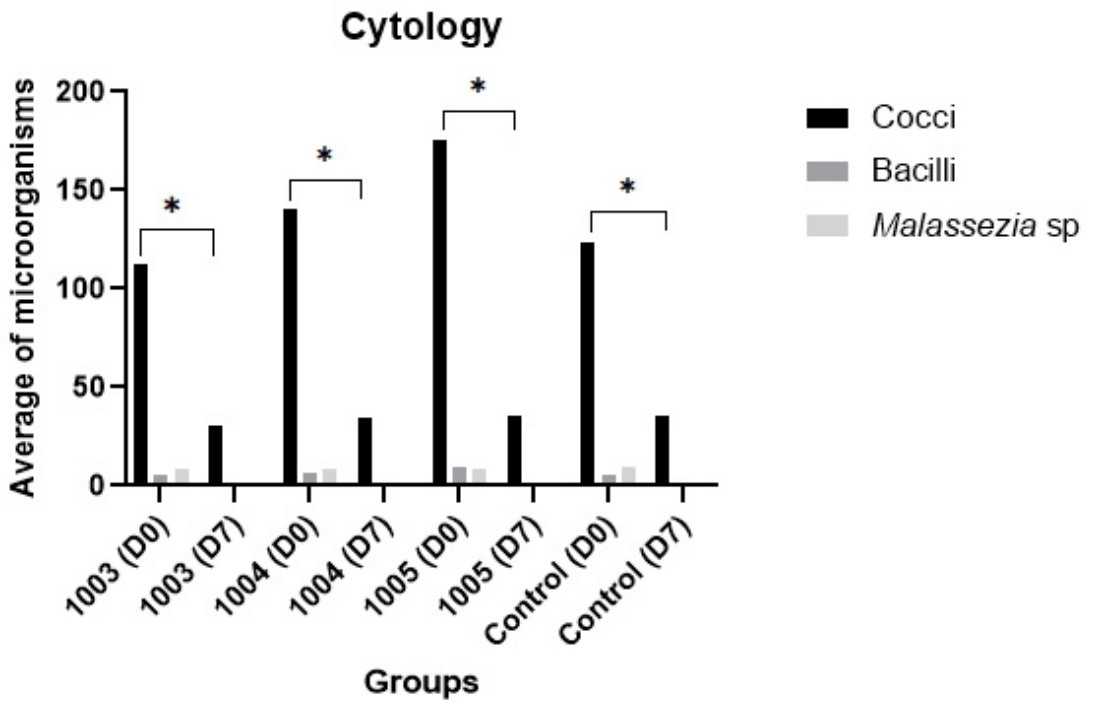

Figure 5 Demonstration of the average microorganisms (cocci, bacilli, and *Malassezia* sp.) evaluated in cytological samples from dogs with canine otitis externa, treated with the test products (LFCO 1003, 1004, 1005, and control), the symbol (*) demonstrates the statistical difference between collections.

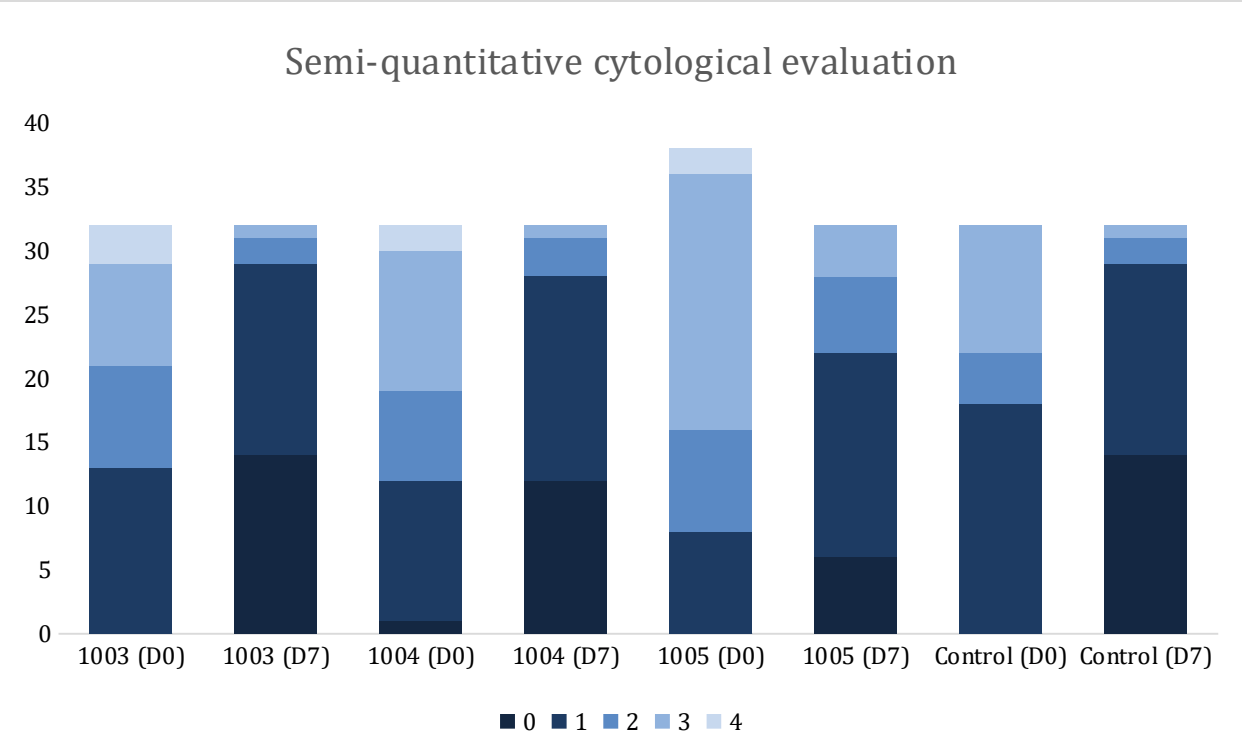

Figure 6 Demonstration of the semi-quantitative cytological analysis of dogs with otitis externa treated with the test groups and the control group. The stacked bars demonstrate the number of auditory canals with different scores.

Just as they have healing properties, the extracts that make up our products also showed antifungal activity against the yeast *Candida albicans* and antibacterial activity against bacteria *Pseudomonas aeruginosa*, *Escherichia coli*, *Streptococcus* spp., and *Staphylococcus aureus*.

In addition to the use of plant extracts from *Bixa orellana* and *Triticum aestivum*, our product contains adjuvants such as N-Acetylcysteine and Tris-EDTA in its formulation. Adjuvants are used to increase the efficiency of antimicrobial agents through different mechanisms, such as enzyme inhibitors that inactivate antibiotics, efflux pump inhibitors, bacterial membrane permeabilizers, biofilm dispersers, resistance element inhibitors, and bacterial cellular physiological pathway inhibitors. The use of adjuvants Tris-EDTA, disodium EDTA, and N-Acetylcysteine was effective against strains of otic pathogens such as *Malassezia pachydermatis*, *Staphylococcus pseudintermedius*, β-hemolytic, *Streptococcus* spp., *Pseudomonas aeruginosa*, and *Proteus mirabilis*.

Our composition differs from other otological products as it contains active principles that are effective in microbial inhibition and resolution of the inflammatory condition of otitis. The wheat and annatto extracts demonstrate antibacterial and antifungal potential, and the drugs N-acetylcysteine and Tris-EDTA present in the otological solution act as cerumen solvents, as well as facilitating the action of antimicrobials through different pathways. Thus, we believe that these drugs potentiate the action of the plant extracts from annatto and wheat.

As this is a clinical study, it was not possible to equate the groups in all criteria at the beginning of the treatment, presenting a bias in the statistical analysis of microbial reduction,

since not all groups presented the same microorganisms involved as these were naturally developed otitis cases.

4. Conclusion

Compounds 1003, 1004, and 1005 were effective in reducing clinical signs and the quantity of microorganisms involved in cases of canine otitis externa, demonstrating efficiency comparable to a commercial product. The solutions developed in this study are a viable treatment for mild or early cases of otitis.

Conflict of Interest Statement

The authors declare no conflicting interests.

Author Contributions

Conceptualization: R.S.A. Brito; Data curation: F.R.P. Bruhn; Investigation: A.G.A. Júnior and R.S.A. Brito; Methodology: R.A. Freitag, R. Vianna and R.S.A Brito; Project administration: M.O. Nobre and S. Jorge. Writing (original draft, proofreading and editing): GB Freitas and RSA Brito.

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