Vol 29, Issue 3, June 2015

VETERINARIA

Use of lactoferrin, verbascoside and glycerophosphoinositol emulsion in the treatment of otitis externa: a pilot study in ten dogs

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Otitis externa is a common clinical problem that often requires complex pharmacological therapies. The aim of this study was to evaluate the efficacy of a topical aural emulsion containing lactoferricin, verbascoside and glycerophosphoinositol lysine in the treatment of erythematous otitis externa with microbial overgrowth. Ten dogs with erythematous otitis and bacterial and/or fungal overgrowth were included according to good general practice guidelines. The animals were evaluated at the time of inclusion in the study (V1), day 7 (V7), day 14 (V14) and 7 days after the end of treatment (V21). At each control, clinical parameters were evaluated (by a VAS and modified CADESI) and cytological smears of ear wax were examined. Every sample was quantitatively assessed for epithelial cells, cocci, rods and *Malassezia* spp. Statistical analysis was performed with Shapiro-Wilk and Kruskal-Wallis tests for normally distributed data; Friedman's test was used for non-parametric data and repeated analyses. A p-value <0.05 was considered statistically significant. There were statistically significant improvements of all parameters after 7 days of treatment and these improvements persisted for at least 1 week after cessation of topical treatment. This pilot study suggests that daily use of an emulsion containing lactoferricin, verbascoside and glycerophosphoinositol lysine for 2 weeks is effective in reducing clinical signs and microbial overgrowth in recurrent otitis externa.

Keywords - otitis externa, dog, lactoferricin, verbascoside, glycerophosphoinositol lysine.

INTRODUCTION

Otitis externa is a common clinical condition in veterinary medicine and has been estimated to affect as many as 15% of dogs.[1] It is a multifactorial condition and it is important to identify all the factors involved.[1] In recurrent or chronic otitis, besides the primary causes, there are often secondary factors involved, such as bacterial or fungal infections, as well as the perpetuating and predisposing factors.[1] Topical treatment, with antimicrobial agents and detergents, is fundamental in the

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Received: 04/02/15 - Accepted: 20/05/15

Work presented as a poster at the congress: "Antibiotic Alternatives for the new Millennium", EuroSciCon, London, UK, 5th-7th November, 2014.

management of these cases and contributes to limiting the use of topical and systemic antibiotics.[2] Considering the increase in the phenomenon of antibiotic resistance that is occurring in veterinary medicine,[3] the development of alternative agents, such as antimicrobial peptides, is becoming ever more important. LAS™ is a patented mixture of three compounds of natural origin: lactoferricin, verbascoside and glycerophosphoinositol lysine (GPI).

Lactoferricin is a peptide obtained from the hydrolysis of lactoferrin: it has demonstrated antibacterial properties *in vitro*, and also has anti-inflammatory, antiviral, anti-oxidant and antitumor activities.[4,5,6,7,8] It can penetrate the bacterial cell membrane and interact electrostatically with the glycosaminoglycans of the matrix and the negatively-charged cell surface.[4] Lactoferricin also has bactericidal activity against a broad spectrum of Gram-positive and Gram-negative bacteria, including *Staphylococcus aureus*[9,10] and is able to prevent the formation of the biofilm by some species of bacteria[11] and inhibit the *in vitro* growth of *Candida albicans*.[11]

Lactoferricin, verbascoside and glycerophosphoinositol lysine are naturally occurring compounds with antimicrobial, anti-inflammatory and/or antioxidant effects, respectively

Verbascoside is a water-soluble phenol derivative that occurs naturally in the vegetable kingdom.[12] This molecule promotes epithelial repair and reduces inflammation thanks to its anti-oxidant and iron-chelating effects and its property of inducing glutathione-S-transferase.[12] Some *in vivo* studies have shown that it is an effective anti-oxidant and anti-inflammatory agent in murine models of colitis, periodontitis and spinal injury.[13,14,15]

GPI is a patented semi-synthetic derivative[16] obtained starting from the lecithins present in sunflowers; it has an anti-inflammatory effect. Through negative feedback, GPI is able to inhibit the activity of cytosolic phospholipase A2 (cPLA2), an enzyme that causes the release of arachidonic acid which, in its turn, is metabolised to synthesise mediators of inflammation, such as prostaglandins and leucotrienes.[17] Various *in vitro* and *in vivo* studies have demonstrated that GPI has an anti-inflammatory effect on the skin and that it also reduces both pruritus and trans-epidermal water loss (TEWL).[17,18,19]

The aim of this study was to evaluate the efficacy of an emulsion containing LAS™ administered via the auditory canal for the treatment of otitis externa caused by bacterial or fungal overgrowth in the dog and its effect on the microbial flora of the ear.

MATERIALS AND METHODS

This clinical study was carried out in accordance with Good Clinical Practice [Italian Official Gazette (G.U.) n. 289; December 10, 1996; 47–53] and with the consent of the animals' owners.

Animals

The animals selected for this study were owned dogs with a history of recurrent otitis, defined as more than three episodes of otitis in the preceding 18 months with evidence of unilateral or bilateral erythematous, ceruminous otitis on clinical examination. The dogs included had bacterial, fungal or mixed overgrowth, diagnosed by cytological examination of the waxy discharge.

Dogs were excluded if they had otitis caused by foreign bodies or parasites (e.g. otoacariasis, auricular mange), malignancies of the auditory canal, chronic idiopathic hyperplastic polypoid otitis, purulent otitis, evidence of otitis media (head rotation, rupture of the tympanum, radiological evidence of an effusion in the tympanic bulla). They were also excluded if they had been treated with systemic or topical antibiotics or antifungal agents, including auditory canal detergents, in the preceding 7 days.

In the case of intense pain or difficulty in manipulating the animal, prednisone could be given orally at the dose of 1 mg/kg/day for 3 days, provided that the treatment was stopped at least 2 days before the inclusion examination. Other pharmacological treatments, already being taken at the time of the patients' inclusion evaluation, are reported in Table 1.

The owners were free to withdraw their animals from the study at any time. Any deviation from the study protocol or adverse reaction to the drug led to the dog being excluded from the study.

Clinical evaluations and sampling

At the time of the selection assessment the dogs considered had unilateral or bilateral erythematous, ceruminous otitis. Using an otoscope, both auditory canals were examined and cytological examination of the ear wax was conducted. The material for this cytological examination was collected with a cotton swab introduced in the auditory canal, smeared onto a glass slide and stained with a modified Romanowsky stain (Diff-Quik® Baxter Diagnostic AG, Dubingen, Switzerland). Each sample was examined for the average number of keratinocytes, nucleated keratinocytes, neutrophil granulocytes and macrophages in ten fields at a 40X magnification of areas of material. The findings were then reported semi-quantitatively in a chart as follows: a score of 1 if <5 cells/field; a score of 2 for 5-10 cells/field; a score of 3 for 11-20 cells/field; and a score of 4 if >20 cells/field. If neutrophil granulocytes

Table 1 - Pharmacological treatments that the animals were receiving at the time of their study inclusion evaluation		
Case	**Treatment**	**Concomitant disorders**
1	Oclacitinib	Atopic-like dermatitis
2	Oclacitinib	Atopic dermatitis
3	Oclacitinib	Atopic dermatitis
4	Milteforan	Leishmaniosis
5	Oclacitinib	Atopic dermatitis
6	Oclacitinb	Atopic-like dermatitis
7	No treatment	Atopic dermatitis and AFR
8	Cyclosporine	Atopic dermatitis
9	Asit (specific immunotherapy)	Atopic dermatitis
10	Asit (specific immunotherapy)	Atopic dermatitis

or macrophages were present in the ten fields examined, the patient was excluded from the study.
The microbial count was determined in the same way, but evaluating the presence of coccoid, and rod-like bacteria and yeasts of the *Malassezia spp*. The microbial counts were also reported in a semi-quantitative way, with scores of 1, 2, 3 and 4 for counts of <10-20/field, 21-30/field; 31-40/field and >40/field, respectively.
If the otoscopic and cytological examinations were compatible with infection by bacteria and/or *Malassezia* spp. (more than 10 bacteria or 10 yeasts per field at a magnification of 40X, presence of keratinocytes and the absence of neutrophil granulocytes and macrophages) the animal was included in the study (inclusion evaluation, V1). At the time of the inclusion evaluation, the conditions of the ear and cutaneous lesions of the pinna were evaluated. The owner was also asked to evaluate his or her own dog's pruritus using a visual analogue scale (VAS).[20]

Evaluation of the auditory canal
The external auditory canals were evaluated by inspection of the vertical and horizontal parts with an otoscope. The parameters assessed were: amount of wax, erythema, erosions and ulcers. The scores attributed for each parameter were: 0, no lesion present; 1, mild; 2 and 3, moderate; 4 and 5, severe. The final score corresponds to the sum of each parameter (Figure 1).

EVALUATION			**erythema**	**ulcers**	**erosions**	**cerumen**
EXTERNAL AUDITORY CANAL	left	vertical horizontal				
	right	vertical horizontal				
grading (each lesion and site): absent: 0; 1: mild; 2,3: moderate; 4,5: severe				TOTAL Score		

MODIFIED CADESI			**erithema**	**lichenification**	**excoriations**	**self-induced alopecia**	**TOTAL**
Face	Pre-auricular						
	Peri-ocular						
Head	Dorsal						
Pinna	left	convex					
		concave					
	right	convex					
		concave					
grading (each lesion and site): absent: 0; 1: mild; 2,3: moderate; 4,5: severe				TOTAL Score			

Figure 1 - Scheme used for the clinical evaluation of the external auditory canal and the pinna using the modified CADESI.

Evaluation of the cutaneous lesions via the modified CADESI
The cutaneous lesions were evaluated by using a modification of the Canine Atopic Dermatitis Extent Severity Index (CADESI)[21] considering exclusively the score and parameters concerning the head and pinnae (Figure 1). The cutaneous changes evaluated were: erythema, lichenification, alopecia and excoriations. As for the auditory canal, the assessor attributed scores of: 0 for no lesion present; 1 for mild; 2 and 3 for moderate; 4 and 5 for severe. The final score is the sum of the scores for each parameter (Figure 1).

At inclusion into the study, a clinical evaluation was made of the auditory canal and pinna using the CADESI, pruritus was judged using a VAS and the cytology of the ear wax was studied.

Evaluation of pruritus by the visual analogue score
The owner was asked to express his or her opinion on the animal's degree of pruritus by placing a mark on a 10 cm line with descriptors at precise intervals. The value of the pruritus corresponded to the distance of the mark from zero. The value, corresponding to a number from 0 to 10, was then noted on a chart.

Treatment
At the end of the inclusion assessment (V1), the first treatment was given by the investigator, who introduced 4-8 drops of the product (Actea Oto®, Candioli, Turin, Italy) into the auditory canal or canals with otitis without any additional lavage. The base of the ear was massaged gently to promote adequate distribution of the product within the ear. The drug was then given to the owner with instructions to repeat the treatment every 12 hours for 14 days. The control evaluations were fixed for 7 (V7) and 14 (V14) days. The final visit (V21) was carried out at 21 days. During every evaluation the ear was examined with an otoscope, a cytological examination was performed, the cutaneous lesions were assessed using the modified CADESI and pruritus was evaluated by the VAS.
At the last evaluation (V21), the owner was asked to express an opinion on the response to treatment and the ease of administering the therapy, choosing from between poor, modest, good and excellent.

STATISTICAL ANALYSIS
The statistical analysis was performed using SPSS software (version 19; SPSS Inc., Chicago, IL, USA). The data were analysed using the Shapiro-Wilk and Kruskal-Wallis tests if the data were distributed normally, whereas when the data were not normally distributed, Friedman's test for one-way analysis of repeated measures was used. p values ≤ 0.05 were considered statistically significant.

RESULTS
Ten dogs entered the study; all the animals completed the study and none had adverse reactions to the drug.

Assessment of the auditory canal
After the first 7 days of treatment, there was a 48% decrease in the mean score, corresponding to improvement of auditory canal lesions ($p<0.0001$ (Table 2, Figure 2). In four of the ten dogs there were reductions of 50% or more of the score. In only one dog (case 3) was the score at V7 higher than that at V1. The scores decreased further between V1, V7, V14 and V21 with a statistically significant difference. On day 21 the mean score was 65% lower than the score at the inclusion evaluation ($p<0.0001$).

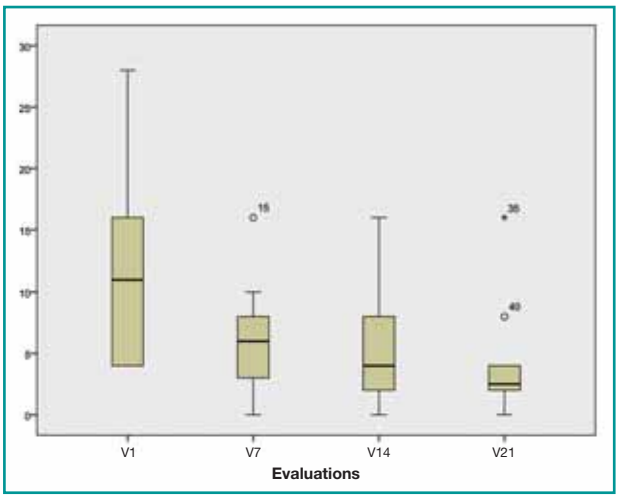

Figure 2 - Evaluation of the decrease in lesion score for the auditory canal score at V1, V7, V14 and V21.

Table 2 - Scores for the lesions and clinical parameters of all the dogs at V1, V7, V14 and V21

Evaluation	Auditory canal	Modified CADESI	VAS
V1	11.9 ± 2.378	13.30 ± 3.636	5.0 ± 0.601
V7	6.20 ± 1.436	6.70 ± 2.191	3.18 ± 0.536
V14	5.10 ± 1.449	5.80 ± 2.225	2.90 ± 0.640
V21	4.10 ± 1.509	4.60 ± 1.945	3.30 ± 0.578

Evaluation of the pinna through the modified CADESI

After the first 7 days of treatment the mean reduction in the CADESI score was 49%, which corresponds to an improvement in the lesions of the pinna. In five of the ten dogs there was an improvement of 50% or more. The improvement continued until day 21 with statistically significant differences between V1 and V7, V14 and V21 ($p<0.0001$) (Table 2, Figure 3). There was not a statistically significant difference between V14 and V21. At V21 the mean score was 65% lower than that at V1.

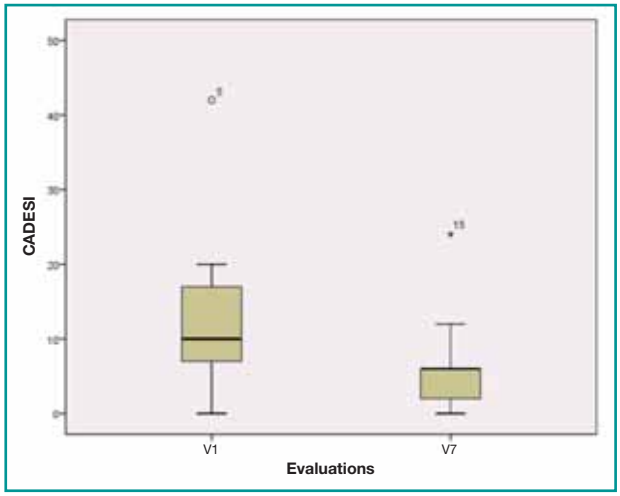

Figure 3 - Decrease in the modified CADESI score between V1 and V7, V14 and V21.

Evaluation of pruritus using a visual analogue scale

There was a statistically significant reduction of 36% in the mean score between V1 and V7 ($p=0.008$) (Table 2, Figure 4). In contrast, there were not statistically significant differences between the values at V14 and V21 compared to those at V7, while there were statistically significant differences with respect to V1 (data with a non-normal distribution, Friedman's test, $p<0.0001$).

The clinical and cytological parameters were improved at the control evaluation after 14 days of topical treatment.

Cytological examination of the ear wax

Keratinocytes

Between V1, V7, V14 and V21 there was a statistically significant reduction of 68% in the number of cells ($p<0.0001$) (Table 3, Figure 5). The difference between V14 and V21 was not statistically significant.

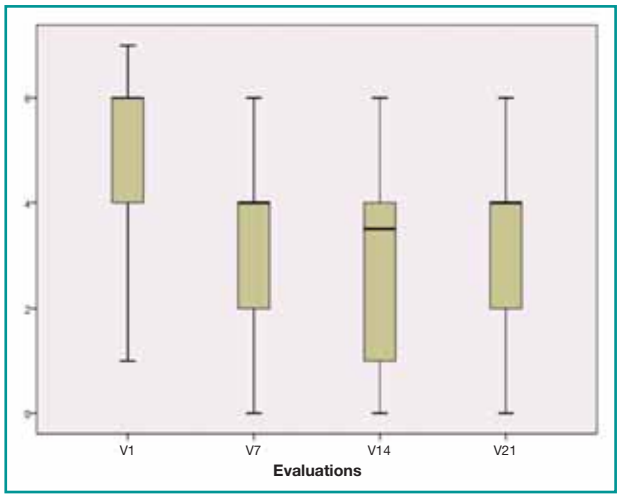

Figure 4 - Evaluation of the VAS between V1, V7, V14 and V21. There is a significant decrease in pruritus between V1 and V21.

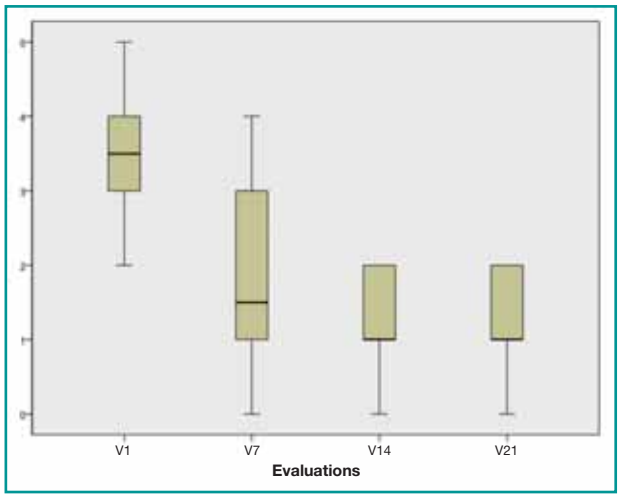

Figure 5 - Evaluation of epithelial cells (keratinocytes). There is a statistically significant decrease between V1 and V7, whereas values remain constant between V14 and V21.

Table 3 - Scores of the cytological evaluations carried out at V1, V7, V14 and V21

Evaluation	Keratinocytes	Bacteria	*Malassezia* spp.
V1	3.50 ± 0.342	2.50 ± 0.342	0.6 ± 0.221
V7	1.80 ± 0.389	1.3 ± 0.260	0.3 ± 0.153
V14	1.10± 0.233	0.7 ± 0.213	0
V21	1.20 ± 0.249	0.7 ± 0.213	0

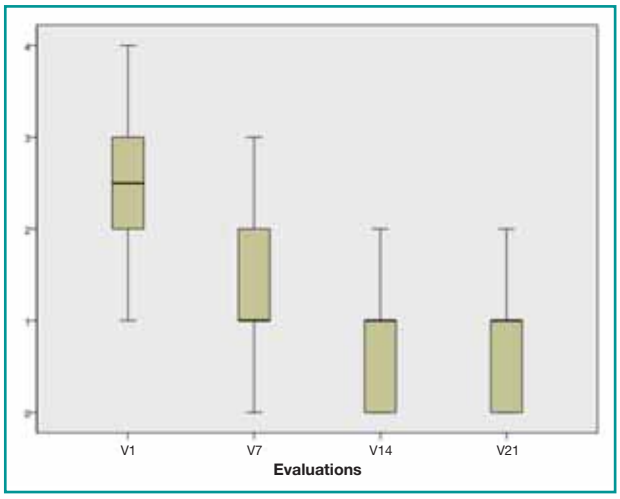

Figure 6 - Evaluation of bacterial load. Also in this case there is a statistically significant difference between V1 and V7, but not between V14 and V21.

Cocci and rod-like bacteria

There was a statistically significant reduction of 48% in the number of bacteria between V1, V7, V14 and V21 ($p<0.0001$) (Table 3, Figure 6), whereas there was not a statistically significant difference between V14 and V21.

Malassezia spp.

A 50% reduction in the number of yeasts was seen between V1 and V7; this difference was statistically significant ($p<0.0001$) (Table 3, Figure 7). *Malassezia* spp. were not identified in the cytological examinations at V14 and V21.

Owners' satisfaction

Response to treatment

One owner did not express an evaluation; of the remaining nine, seven judged the response to treatment as 'good', one considered it 'excellent' and the other considered the response 'modest'.

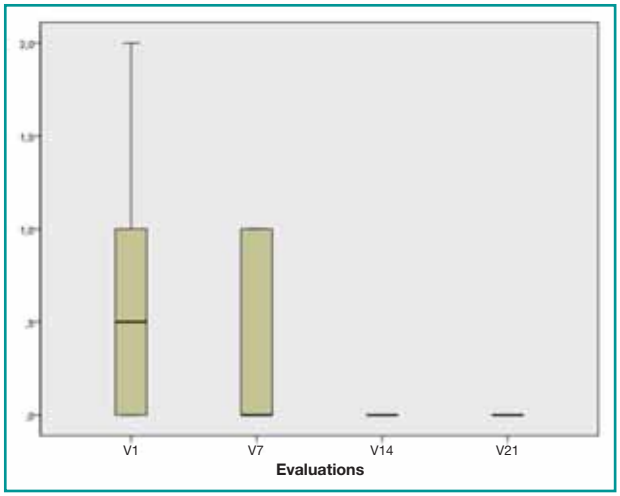

Figure 7 - Growth of *Malassezia* spp. at each evaluation.

Ease of administration

One owner did not express an evaluation; eight of the other nine considered the ease of administration 'good', while one judged it 'excellent'.

DISCUSSION

On the basis of the data obtained, the aural product under investigation had good efficacy in reducing the clinical signs of erythematous ceruminous otitis externa and in treating yeast and bacterial overgrowth.

Lactoferricin can interact with the cell membrane of both Gram-positive and Gram-negative micro-organisms[8,9] through a permeabilising effect that leads to rupture of the membrane and that initially inhibits the biosynthesis of macromolecules and ultimately leads to cell death. The mechanism of action differs depending on whether Gram-negative or Gram-positive bacteria are involved: in the former case the peptide acts on lipopolysaccharides, while in the latter it acts on lipoteichoic and teichoic acids.[8]

The improvements of the clinical and cytological parameters are probably due to the antimicrobial and anti-inflammatory properties of lactoferricin and verbascoside, respectively.

In detail, lactoferricin is able to inhibit the growth of numerous bacteria, including *Staphylococcus aureus*, at concentrations of 2-8 μM.[9,10] Furthermore, it can inhibit the growth of some yeasts *in vitro*[12] and prevent the formation of the biofilm in some species of bacteria (*Porphyromonas gingivalis* and *Prevotella intermedia*).[11] The biofilm is a structured community of micro-organisms that adhere to a surface and produce a polymeric matrix that envelops and protects them.[22] This structure favours the persistence of infections and is one of the mechanisms of resistance to antibiotics.[23] Various *in vitro* and *in vivo* studies have demonstrated that both lactoferrin and lactoferricin counteract the formation of the biofilm and promote its breakdown; they have a dose-dependent action that is synergistic with some antibiotic treatments.[24] The mechanism of action is partially unknown, but it is thought that they act in several ways, for example by stimulating bacterial mobility, blocking lecithin-dependent bacterial adhesion,[24] or by down-regulating the production of cellulose, one of the main constituents of the matrix.[23] In this pilot study, the antimicrobial characteristics of lattoferricin were probably responsible for the improvement in the cytological parameters after the treatment. In particular, it was noted that in the first

7 days there were decreases in the loads of cocci and bacilli, as well as in *Malassezia* spp., and that these reductions were maintained until day 21. It is interesting to note that after 2 weeks of treatment the *Malassezia* spp. load was reduced and this state continued for at least 1 week after suspension of treatment. Since the animals in this study were owned dogs, it was not possible to compare the data from this clinical study with those from a control group and, therefore, evaluate any activity of a placebo (a product without active principles). Previous studies with aural products showed that the use of vehicle alone, in the complete absence of pharmacologically active principles, led to clinical improvements in some cases. These improvements were probably due to the effect of cleaning the ear and removing wax and exudate.[25] However, since the product investigated in the current study was not used for auricular lavage but as a topical emulsion product, it seems unlikely that the antimicrobial effect was associated only with mechanical removal of matter from the ear.

Glycerophosphoinositol lysine contributes to reducing TEWL in humans.

Verbascoside is a phenol that occurs naturally in the vegetable kingdom and has marked anti-inflammatory and anti-oxidant properties.[13] Its efficacy in lowering the levels of pro-inflammatory cytokines and free radicals was demonstrated in some *in vivo* murine models of colitis, periodontitis or spinal trauma.[14,15,16] Studies on the activity of this molecule in the dog have not been published so far.

GPI is a derivative of sunflower lecithins; it has an anti-inflammatory action from its effect on the physiological system of auto-regulation of the inflammatory cascade, with a mechanism similar to that of the glucocorticoids,[26] but without the side effects of these latter drugs. GPI has been tested in humans with allergic and inflammatory dermatitis with good results as far as regards reducing erythema, pruritus, excess sebum and dandruff.[27] There are no corresponding veterinary studies. The anti-inflammatory properties of GPI and verbascoside can reasonably be considered responsible for the improvement in the conditions of the pinna and auditory canal observed during treatment with LAS™; there was a fast, progressive improvement in the state of the auditory canal with reductions in erythema, erosions and ulcers, when present. The parameters used to assess the condition of the pinna also improved considerably, with decreases in erythema, alopecia, excoriations and lichenification. In human medicine, GPI can contribute to normalising the value of TEWL. It was not possible to measure the TEWL in this study to evaluate whether the same results can be achieved in dogs. Furthermore, there are no veterinary studies on the use of this compound as a cutaneous anti-inflammatory agent. For this reason, it would be interesting to collect more data from dogs on cutaneous and auricular erythematous lesions with bacterial and fungal overgrowth, and also on any changes in TEWL.

The persistence of some lesions, although less severe, at the end of the treatment was probably related to the incomplete control of the underlying diseases, primarily atopic dermatitis. As far as regards pruritus, there was an improvement in the first week of use of the product, which was maintained in the subsequent weeks of topical treatment. In this case too, the residual pruritus was very probably related to the underlying disorders.

This study has various limitations. The foremost is the small number of animals included. This is essentially because of the difficulty in selecting owners willing to cooperate with the frequent clinical controls. Furthermore, as mentioned previously, a placebo was not used for ethical reasons. In the future it would be interesting to perform a multicentre, double-blind study to evaluate better and confirm the effect of the product investigated and to assess whether there is any improvement in lesions related to administration of vehicle only.

Further studies are necessary to confirm the efficacy of the product.

Other limitations are the assessment methods used in this study, which have not been validated so far. A semi-quantitative method for the evaluation of skin cytology has been described and validated; this evaluation is based on scores from 0 to 4+ and has criteria similar to those used in this study but does not define precise numbers of cells for each score.[28] For this reason we preferred to use a system which, although not validated, gives a more precise evaluation of the number of micro-organisms present. As far as concerns the evaluation of the clinical conditions of the auditory canal and pinna, we chose to use a five-point scale and a modified version of CADESI, respectively, because at the time there were no validated methods. Recently an objective clinical score was proposed to evaluate otitis externa in the dog.[29] It could be helpful to use this score in future studies.

In conclusion, from the data collected the product under investigation can be considered an effective treatment for the control of mixed microbial overgrowth during erythematous, ceruminous otitis externa.

KEY POINTS

- Lactoferricin is an antimicrobial agent active against numerous species of bacteria, including Staphylococcus aureus, and is able to reduce the formation of the biofilm.
- Verbascoside is a phenol derivative with anti-oxidant and anti-inflammatory properties; its activity has been demonstrated in models of colitis, periodontitis and spinal trauma.
- Glycerophosphoinositol lysine has an anti-inflammatory effect and is able to improve transepidermal water loss (TEWL) and cutaneous characteristics in human patients with allergic dermatitis.

ACKNOWLEDGEMENTS

We thank the Istituto Farmaceutico Candioli Spa for having provided the product used in the study.

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