ORIGINAL ARTICLE

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Use of antimicrobial photodynamic therapy in the treatment of otitis caused by *Malassezia* spp. in domestic dogs

Uso da terapia fotodinâmica antimicrobiana no tratamento das otites causadas por Malassezia spp. em cães domiciliados

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ABSTRACT: Several factors favor the recurrence of canine otitis due to chronic inflammation, the aim of this research was to evaluate the efficiency of antimicrobial Photodynamic Therapy (aPDT) as an alternative treatment for otitis externa in dogs, compared to conventional topical therapy. Fourteen domestic dogs of different breeds, with spontaneous otitis externa caused by *Malassezia* spp. were randomly allocated into two groups: conventional topical therapy (G1), with the application of cerumenolytic and an antimicrobial twice a day for seven days (in 14 ears); and aPDT (G2), using a diode laser with 0.1W power, wavelength (λ) of 660nm and irradiation every 48 hours, for seven days (in 13 ears). The therapeutic protocol was assessed every 48 h and the times 0 (T0), 48h (T48), 96h (T96) and 144h (T144) were chosen for statistical comparison. Both treatments were efficient in reducing clinical signs, with no significant difference (P>0.05) between them. In the cytological evaluation for *Malassezia* spp., the mean count at T0 was 25.80 ±5.012 cells/field of 100x in G1 and 40.35±5.216 cells/field of 100x in G2, with no significant difference between the groups before the beginning of treatments (p=1.000). At T144, both groups showed a significant reduction (P<0.05) with a mean count of 2.35 ±0.958 cells/field of 100x in G1 and 3.22±0.997 cells/field of 100x in G2. In conclusion, aPDT provided similar results to conventional therapy, but only required 3 interventions, making it an alternative therapy in cases of otitis with the presence of *Malassezia* spp.

KEYWORDS: Antibiotic; phototherapy; inflammation; low-power laser; topical treatment.

RESUMO: Vários fatores favorecem a recorrência da otite canina devido à inflamação crônica, o objetivo desta pesquisa foi avaliar a eficiência da terapia fotodinâmica antimicrobiana (PDTa) como tratamento alternativo para otite externa em cães, em comparação com a terapia tópica convencional. Catorze cães domésticos de diferentes raças, com otite externa espontânea causada por *Malassezia* spp. foram alocados aleatoriamente em dois grupos: terapia tópica convencional (G1), com aplicação de cerumenolítico e antimicrobiano duas vezes ao dia durante sete dias (14 orelhas); e PDTa (G2), utilizando laser de diodo com potência de 0,1W, comprimento de onda (λ) de 660nm e irradiação a cada 48 horas, durante sete dias (13 orelhas). O protocolo terapêutico foi avaliado a cada 48h e os tempos 0 (T0), 48h (T48), 96h (T96) e 144h (T144) foram escolhidos para comparação estatística. Ambos os tratamentos foram eficientes na redução dos sinais clínicos, não havendo diferença significativa (P>0,05) entre eles. Na avaliação citológica para *Malassezia* spp., a contagem média em T0 foi de 25,80 ±5,012 células/campo de 100x no G1 e 40,35±5,216 células/campo de 100x no G2, sem diferença significativa entre os grupos antes do início dos tratamentos (p=1,000). Em T144, ambos os grupos apresentaram redução significativa (P<0,05) com contagem média de 2,35 ±0,958 células/campo de 100x no G1 e 3,22±0,997 células/campo de 100x no G2. Conclui-se que a PDTa forneceu resultados semelhantes à terapia convencional, sendo uma terapia alternativa em casos de otite com presença de *Malassezia* spp.

PALAVRAS-CHAVE: Antibiótico; fototerapia; inflamação; laser de baixa potência; tratamento tópico.

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INTRODUCTION

Canine otitis externa is an inflammation of the external ear canal of multifactorial etiology, aggravated by bacterial and fungal infections, and classified as acute, chronic or recurrent. Otitis externa is the most common ear canal disorder in canine general practice, accounting for approximately 5-20% of major complaints (Cardoso *et al.*, 2011; Santos; Guimarães, 2020; Boone *et al.*, 2021).

The pathogenesis of otitis externa has not been fully explained, but is mediated by primary factors; and predisposing factors may be involved in its establishment. The primary factors are observed during allergic processes, endocrinopathies, parasites, foreign body, keratinization impairment, immune-mediated and auto-immune diseases, neoplasia. Secondary factors, such as bacterial and fungal overgrowth with the presence of *Malassezia* spp., may occur if these primary factors remain present (Saridomichelakis *et al.*, 2007, Paterson, 2016). Thus *Malassezia* spp. it is a commensal microorganism found in the ears of dogs and is often considered a secondary factor in otitis externa and, if left untreated, can lead to the perpetuation of the disease (Boone *et al.*, 2021).

The clinical signs associated with otitis externa caused by *Malassezia* spp. often include the presence of erythema, pruritus (which can vary from mild to intense), excessive cerumen production (usually blackish brown color), pain, hyperemia, fetid odor, lichenification, and hyperpigmentation of the pinna (Guillot; Bond, 2020; Santos; Guimarães, 2020). Severe cases can lead to hearing loss or impairment due to the accumulation of exudate, stenosis of the auditory canal or glandular hyperplasia (Bajwa, 2019).

The commercially available treatment in Brazil that is widely used for external otitis consists of the combination of antibiotics, antifungal agents and corticosteroids, preferably via topical application. However, persistence of the otitis externa is common due to failure to identify the pathogen, failure to identify and treat an underlying disease, or incorrect administration of the drug by the owner. In case of otitis externa not responding to treatment, resistant *Malassezia* spp. should be considered. Recent papers highlighted the emergence of resistant *Malassezia* yeast with an increased tolerance to azole, explaining the presence of an unresponsive or recurrent otitis externa (Brilhante *et al.*, 2018; Schlemmer *et al.*, 2019).

Photodynamic therapy (aPDT) has been used to treat localized wounds with bacterial infection. This involves the use of a photosensitizer, with specific wavelength light and molecular oxygen, which initiates a chemical reaction to form reactive oxygen species and singlet oxygen, producing structural changes that are fundamental for the survival of the microorganism (Aureliano *et al.*, 2014; Sellera *et al.*, 2019). The use of aPDT has grown in veterinary medicine, since it offers several advantages, such as absence of toxicity to the host organism, effectiveness in reducing microbial strains in a short period of time, less damage to adjacent tissues, absence of systemic effects, being a non-invasive method, lack of cumulative effects, high repeatability and not causing bacterial and viral resistance (Weiss *et al.*, 2012; Aureliano *et al.*, 2014; Sellera *et al.*, 2019).

It is known that aPDT satisfactorily controls bacterial infections (De Oliveira *et al.*, 2012; Weiss *et al.*, 2012; Alzamora Filho *et al.*, 2018), in addition to being considered promising in the treatment of fungal diseases such as canine dermatophytosis with clinical cure and absence of recurrence (Cabral *et al.*, 2021), cryptococcosis (DE OLIVEIRA *et al.*, 2012) and sporotrichosis (Nardoni; Corazza; Mancianti, 2008).

However, the effects of aPDT in the treatment of external otitis associated with *Malassezia* spp. are not yet known. The aim of this study was to evaluate the efficiency of antimicrobial Photodynamic Therapy (aPDT) as an alternative treatment for otitis externa in dogs, compared to conventional topical therapy.

MATERIAL AND METHODS

The study was carried out at Universidade Estadual de Santa Cruz's (UESC) Veterinary Hospital and at a private clinic located in the city of Itabuna, Bahia. It was approved by the Universidade Estadual de Santa Cruz's (UESC) Ethics Committee on the Use of Animals (CEUA) under protocol No. 011/19.

Fourteen domestic dogs of different breeds, aged between 9 months and 11 years were used and all of them presented excessive production of brownish cerumen, erythema in the external acoustic meatus, intense pruritus, and fetid odor at the time of consultation. Thirteen animals presented bilateral and one unilateral otitis externa. The dogs were randomly distributed among the different groups and the treatment was initiated by ear (G1: 14 and G2:13) and none of the patients from either group presented any other physical changes of note. After clinical otological examination of the animals, cytology of the cerumen from the external auditory canal was performed. The *Malassezia* spp. count was conducted using a 100x optical microscope (Zeiss®) and those animals with a count greater than 10 yeast cells/field were considered to have otitis perpetuated by *Malassezia* spp. (Leonard *et al.*, 2022). All animals wore an Elizabethan collar during the therapy period and auricular cytology was performed before each session.

The 7 animals in group 1 (G1), composed of 14 ears, were treated with conventional topical therapy. This consisted of the application of a ceruminolytic (salicylic acid 0.11g; 90% lactic acid 2.98g, herbal essence 0.01g), to the ear canal every 24 h, and an antimicrobial agent (gentamicin 300mg; betamethasone valerate 122mg; clotrimazole 1,000mg) every 12 h. In group 2 the 7 animals (G2), composed of 13 ears, antimicrobial photodynamic therapy (G2) was performed using a diode laser with 0.1W of power and spot area of 0.028cm², and radiance of 3,57 mW/cm². Firstly, the animals were manually restrained and then an aqueous solution of methylene

blue (300 μM) was applied to the ear canal for a pre-irradiation time of 3 minutes, followed by irradiation with red laser (λ = 660 nm), using 9J energy per application point, fluence/point of 321J/cm^2 and exposure time/point of 90 seconds, as shown in figure 1. This procedure was repeated every 48 h, for a total of three sessions (De Oliveira; Aguiar; Câmara, 2014).

The ears of both groups were assessed every 48 h and, before the therapeutic procedure, material was collected for ear cytology. The experiment was double-blind, so that all slides were analyzed by the same pathologist, without giving them any information about clinical changes and the therapy used. A rapid panoptic commercial kit (Laborclin®) was used to stain the slides, which were analyzed at 40x magnification to identify *Malassezia* spp. and then 100x magnification was used for yeast counting (10 fields per slide) and the mean of the values obtained was considered as the final value for each ear. Clinical changes were evaluated on a visual analogue scale, considering zero (0) as an absence of any sign, one (1) for mild change; two (2) for moderate change and three (3) for intense change. The data evaluated were erythema, pruritus, odor, secretion, edema and lichenification.

Figure 1. Locations of diode laser irradiation points (ʎ= 660nm, 100mW) in the ear canal of the dogs.

A statistical test similar to analysis of variance (non-parametric), part of the nparLD2 package, was performed to assess the types of treatment, progress over time, and to differentiate between the ears (right and left). This considered the influence of the "animal" variable, due to the particularities of each animal (due to the evaluation of right and left ears). To assess each treatment and the significance between the times, the Friedman test was performed followed by the Wilcoxon post-hoc test, with Bonferroni correction, considering significance when $p<0.05$. This analysis was performed in the R program, rstatix package.

RESULTS

During the physical examination, it was observed that all of the dogs had pendulous ears and nine had a history of recurrent otitis (G1: 10 ears and G2: 8 ears). The clinical signs observed in the animals prior to conventional treatment (G1) and aPDT (G2) are shown in Table 1.

At T0, inflammation of the skin and external auditory canal, clinical signs observed in otitis externa, were observed in animals of both groups. None of the animals displayed painful sensitivity to external palpation in the region corresponding to the tympanic membrane. No tympanic membrane damage or other comorbidity was observed during otoscopy of the external ear canal.

The animals in both groups showed clinical improvement 48 h after the start of treatment. A reduction in edema, decrease in the amount of cerumen, and less erythematous areas were visually observed, and the owners reported a decrease in pruritus. There was no change in the intensity of lichenification in this evaluation period. It is noteworthy that there was a progressive improvement in the clinical variables evaluated, so that at T144 there was total regression of the changes of interest (erythema, pruritus, foul odor, edema and lichenification) in both groups (Figures 2 and 3).

Evaluation of the mean quantity of *Malassezia* spp. in the conventional treatment group (G1) and aPDT (G2) (Table 2) showed that there was a decrease in both over the treatment period, demonstrating significant efficacy ($p< 0.0001$) when considering all times from T48 onwards in relation to T0. The decreases were all significant, with the exception of laser treatment between T48 and T96 ($p= 0.674$), and the most

Table 1. Description of clinical signs evaluated in the ears of animals in group 1 and group 2 before starting treatment.

	Clinical Signs					
Changes	**Erythema**	**Pruritus**	**Foul odor**	**Secretion**	**Edema**	**Lichenification**
G1	14 ears (100%)	14 ears (100%)	14 ears (100%)	14 ears (100%)	14 ears (100%)	12 ears (85,71%)
G2	12 ears (85,71%)	13 ears (100%)	13 ears (100%)	13 ears (100%)	7 ears (53,85)	4 ears (30,77%)
Total	26 ears (96,30%)	27 ears (100%)	27 ears (100%)	27 ears (100%)	21 ears (77,78%)	16 ears (59,26%)

G1: Conventional Treatment, G2: aPDT Treatment

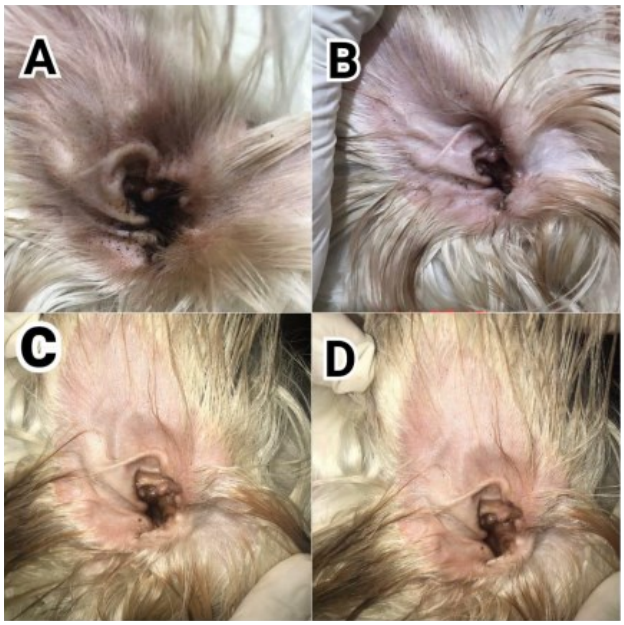

Figure 2. Assessment of clinical evolution with conventional treatment, G1. (A: before starting treatment; B: after 48h of treatment; C: after 96h of treatment; D: after 144h of treatment)

Figure 3. Assessment of clinical evolution with aPDT treatment, G2. (A: before starting treatment; B: after 48h of treatment; C: after 96h of treatment; D: after 144h of treatment).

representative was observed at T48, with a decrease of 71.5% in G1 (from 25.8 to 7.3, p = 0.015) and 69.2% in G2 (from 40.4 to 12.4, p= 0.001) (figure 4). Although the type of treatment did not make any difference, the group in which there was the greatest decrease in the infectious agent over the complete treatment period (until T144) was G2, with a 92.0% reduction between the initial time and the final time (from 40.4 to 3.2, with significance, p= 0.001), while in G1 it was 90.9% (from 25.8 to 2.4, with significance, p= 0.005) (Table 3).

Table 2. Mean *Malassezia* spp. count for the conventional (Group 1) and laser (Group 2) treatments as a function of times 0, 48, 96 and 144 hours.

Treatment/Time	Mean	Standard Error
Conventional T0 (n=14)	25.80±10.37	5.01
Conventional T48 (n=14)	7.34±3,63	1.75
Conventional T96 (n=14)	3.74±2,02	1.24
Conventional T144 (n=14)	2.35±1,98	0.96
aPDT T0 (n=13)	40.35±10,79	5.22
aPDT T48 (n=13)	12.41±3,78	1.83
aPDT 96 (n=13)	7.48±2,17	1.29
aPDT 144 (n=13)	3.22±2,06	0.99

Regarding the effects of treatments on the left and right ears, analysis similar to ANOVA (non-parametric ANOVA) of the mean *Malassezia* spp. count did not show a significant difference between G1 and G2 regardless of time (p= 0.156). The mean for yeast cells in the left ears was 13.3 and in the right ears was 12.3, considering both groups and all evaluation times.

DISCUSSION

This study shows similar efficacy between aPDT and conventional therapy on otitis externa caused by *Malassezia spp.* In the present study, dogs aged between 9 months and 11 years were diagnosed with otitis externa caused by *Malassezia* spp. These data corroborate the study by De Oliveira; Aguiar; Câmara (2014), who in 2012 observed a high occurrence of otitis externa in animals up to 10 years of age, with a gradual decline in the number of cases from the second year of life. However, some authors do not characterize age as a risk factor for infection (ALZAMORA FILHO *et al.*, 2018).

All the animals had the characteristic of pendular ears. This is a predisposing factor in dogs with this anatomical characteristic because the auditory canal provides a favorable environment for multiplication of *Malassezia* spp. (Brito *et al.*, 2019; Boone *et al.*, 2021). The present study also corroborates data presented by O'Neill *et al.* (2021), who pointed out the same anatomical characteristic as a risk factor for the development of canine otitis externa.

Furthermore, it is highlighted that *Malassezia* spp. is a perpetuating agent, therefore its pathological potential is associated with primary conditions such as atopy, inflammation of bacterial origin and endocrine disorders, mainly hypothyroidism (Bajwa,

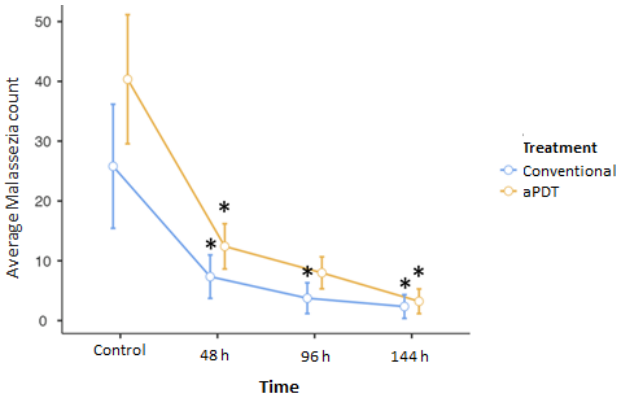

Figure 4. Mean Malassezia spp. count per ear. Note the significant (*) progressive decrease in the mean yeast count per ear over the different treatment times, with a more significant reduction between T0 and T48 in both treatments (conventional: p= 0.015, laser: p= 0.001).

2017). Given this, it is suggested that reducing the *Malassezia* ssp. enables the reduction of recurrences, described in the present study in nine animals, and increases the chances of successful treatment of primary factors associated with otitis externa.

The most frequent clinical signs observed in the 14 dogs evaluated were similar to those reported by Magalhães *et al.* (2017), who observed increased auricular secretion (68.2%), odor (45.4%), pruritus (40.9%), and erythema (40.9%) in 23 animals evaluated. Otalgia is a characteristic clinical sign of otitis, mainly when the middle portion of the ear is involved (Boone *et al.*, 2021). None of the animals evaluated in this study presented painful sensitivity to external palpation in a region compatible with the tympanic bulla.

Cytological examination is considered as the diagnostic method of choice to identify and counting *Malassezia* spp. in

Table 3. Comparisons between mean *Malassezia* spp. counts for the conventional (Group 1) and laser (Group 2) treatments as a function of times 0, 48, 96 and 144 hours.

Comparisons					
Treatment	**Time**	**Treatment**	**DM**	**T**	**pBonferroni**
T0 Conventional	T0	aPDT	-14,552	-2,012	1,000
	T48	Conventional	18,457	4,012	0,0015*
	T48	aPDT	13,390	2,510	0,547
	T96	Conventional	22,057	4,428	0,005*
	T96	aPDT	17,817	3,442	0,062
	T144	Conventional	23,450	4,481	0,005*
	T144	aPDT	22,579	4,418	0,006*
T0 aPDT	T48	Conventional	33,010	5,988	< 0,001*
	T48	aPDT	27,943	5,836	< 0,001*
	T96	Conventional	36,610	6,827	< 0,001*
	T96	aPDT	32,369	6,244	< 0,001*
	T144	Conventional	38,002	7,165	< 0,001*
	T144	aPDT	37,131	6,818	< 0,001*
T48 Conventional	T48	aPDT	-5,067	-2,001	1,000
	T96	Conventional	3,600	2,050	1,000
	T96	aPDT	-0,640	-0,294	1,000
	T144	Conventional	4,993	2,656	0,396
	T144	aPDT	4,121	2,043	1,000
T48 aPDT	T96	Conventional	8,667	3,924	0,019
	T96	aPDT	4,426	2,415	0,674
	T144	Conventional	10,060	4,879	0,002*
	T144	PDTa	9,188	4,695	0,003*
T96 Conventional	T96	aPDT	-4,240	-2,364	0,754
	T144	Conventional	1,393	1,652	1,000
	T144	aPDT	0,521	0,327	1,000
T96 aPDT	T144	Conventional	5,633	3,499	0,054
	T144	aPDT	4,762	5,428	< 0,001*
T144 Conventional	T144	aPDT	-0,871	-0,630	1,000

Mean difference (MD); t (Wilcoxon post-hoc test value)
Significant when p< 0.05 (significant values with *).

the exudate present in the pinna (Choi *et al.*, 2018). It is used for therapeutic assessment until the otitis is resolved (Gotthelf, 2004) and is a practical and low-cost examination (Hnilica, 2012). The yeast cell count in cytological smears from the canine ear is the main evaluation criterion, and the presence of more than 10 yeast cells per field suggests ear pathology (Nobre *et al.*, 2001; Magalhães *et al.*, 2017). Therefore, the cytological evaluation allowed the diagnosis and monitoring of otitis in the animals that composed the different groups in the present study.

In this study, conventional treatment was considered to be effective in treating clinical signs related to otitis externa and in reducing the amount of *Malassezia* spp. in the evaluated ears. Generally, in Brazil, the commercially available pharmacological treatment for canine otitis externa caused by fungal infections is based on the administration of topical otological solutions containing antifungals, antibiotics and glucocorticoids (Peano; Gallo, 2008). However, there is a concern about the emergence of antimicrobial resistance, reiterating the need to use alternative therapies such as aPDT to reduce the possibility of microbial resistance to drugs (Alves; De Oliveira; Negri, 2018).

The association of laser light with the photosensitizer has led to excellent results in combating localized infections without the use of antibiotics (Alzamora Filho *et al.*, 2018). This was also observed in the present study, since both conventional treatment and aPDT were considered effective at reducing the quantity of yeast in the evaluated ears. Similarly, Bevilacqua; Brugnera Junior; Nicolau (2007) described this as an attractive approach to the treatment of fungal infections, such as those caused by *Candida albicans*.

Regarding the choice of photosensitizer, although different classes have been used in the treatment of canine otitis with aPDT, such as porphyrins (Seeger *et al.*, 2020) and Indocyanine Green (Guidi, 2021), little is known about the effects of these molecules on the production of oxygen species reactive and singlet oxygen. The methylene blue was used considering that its effects are already known in this context, in addition to being a widely used photosensitizer due to its low cost. and easy acquisition (Kwiatkowski *et al.*, 2018).

Regarding treatment time, Tambella *et al.,* (2020), observed a progressive reduction in the amount of yeasts over the time of application of topical gel illuminated by LED in the treatment of external otitis associated with *Malassezia* spp. This corroborates the findings of the present study, since although the technique used in this study is different, the photodynamic effects of both are similar. It is worth noting that, although the most significant decrease in *Malassezia* spp. was observed 48 h after the beginning of treatment, the reduction continued until the seventh day of treatment. This shows the importance of continuing treatment for the success of the established protocol.

According to Rosser Jr. (2004), otitis can also result from iatrogenic causes, such as trauma at the time of cleaning, or during the application of medication, due to the inability of owners to perform the procedures correctly. Thus, the antimicrobial photodynamic therapy used in this study is a technique that allows access to anatomical areas that are difficult to access and it can be used repeatedly without causing microorganism resistance. Furthermore, this form of therapy does not cause deleterious effects on the tissues surrounding the lesion (Youf *et al.*, 2021). The technique should be performed by a trained professional, enabling the monitoring and correct application of the therapy (Torezan; Niwa; Festa Neto, 2009). This avoids occasional changes to the protocol resulting from the behavior of the owner. It is also noteworthy that, due to the fact that it is not an invasive technique, the animals do not develop resistance to the treatment.

In recent years, the use of aPDT has become more common in Veterinary Medicine and, as a result, there is a greater tendency for professionals to use this technique. Furthermore, the growing technological advances favor the improvement of the technique and enable its use in the treatment of different types of diseases (Sellera *et al.*, 2017). Given this, it is expected that aPDT will become an increasingly accessible alternative with increasingly lower costs.

CONCLUSION

In this research, it was observed that both photodynamic therapy and the therapy considered commercially available (conventional) were effective for the treatment of *Malassezia* spp. As such, aPDT reduced *Malassezia* spp. in the ear canal and resulted in the disappearance of clinical signs with a lower frequency of treatment, promoting the animal's wellbeing. In addition, the use of aPDT can prevent or reduce the use of antibiotics and topical anti-inflammatory drugs. Therefore, photodynamic therapy can be considered an excellent option for the resolution of secondary factors of otitis in dogs, such as malasseziosis, when it comes to acute otitis externa.

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