Malassezia *overgrowth in dogs in Northern Italy: frequency, body distribution, clinical signs and effects of pharmacologic treatments*

Michele Berlanda[1], Carlotta Valente[1], Carlo Guglielmini[1], Patrizia Danesi[2], Barbara Contiero[1] and Helen Poser[1]

[1]*University of Padova, Via 8 Febbraio 2, 35122 Padova, Italy.*
[2]*Istituto Zooprofilattico Sperimentale delle Venezie, Viale dell'Università 10, 35020 Legnaro (PD), Italy.*

[*]*Corresponding author at: University of Padova, Via 8 Febbraio 2, 35122 Padova, Italy.*
E-mail: michele.berlanda@unipd.it.

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Summary
The present study describes *Malassezia* populations in clinically healthy dogs (HD) and dogs with *Malassezia* overgrowth (MO), and evaluates the correlation with clinical signs and previous treatments. Thirteen clinically HD and 84 dogs with MO were enrolled. Clinical history and previous treatments were recorded. After a complete physical and dermatological examination, Canine Atopic Dermatitis Extent and Severity Index_03 scores were calculated. Samples for cytology and mycological cultures were obtained from four body regions and from skin lesions. *Malassezia* overgrowth was diagnosed by cytology. A global score (GS) for quantitative evaluation of the population of *Malassezia* was calculated. In dogs with MO, the highest frequency of yeast detection was found in skin lesions (82%, $P < 0.001$). Sum of GS (GSs) obtained from dogs with MO (68, 0-621) was significantly higher compared to those of HD (3, 0-48, $P < 0.001$). GSs in dogs previously treated with antibiotics (312.5, 30-975) was significantly higher compared to those of dogs that not have received antibiotics (80, 0-975, $P = 0.015$). No difference was found between dogs treated and those not treated with steroids.

Introduction

Malassezia sp. yeasts are normal inhabitants of the mammalian skin surface and are usually considered opportunistic pathogens (Guillot and Bond 1999). The Taxonomic classification divided the genus *Malassezia* into 18 species (Gupta *et al.* 2004, Batra *et al.* 2005, Cabañes *et al.* 2007, Castellà *et al.* 2014, Theelen *et al.* 2018, Lorch *et al.* 2018). All species within the genus are non-mycelial, unipolar, and budding. Furthermore, all species except *Malassezia pachydermatis* are lipid-dependent due to their inability to synthesize some fatty acids and their requirements for lipid supplementation for *in vitro* growth. *M. pachydermatis* is the most common species of this genus in dogs and is frequently isolated from ear canals, skin, and mucosal surfaces (oral and anal), and less commonly from the anal sacs and vagina (Guillot and Bond 1999, Bond *et al.* 1995). *Malassezia* dermatitis is a term used to describe skin diseases associated with *Malassezia* overgrowth (MO) in affected regions that show a good clinical and cytological response to appropriate antifungal treatment (Bond and Loyd 1997). Pruritus is a major clinical sign of *Malassezia* dermatitis. Other symptoms include erythema, alopecia, greasy exudation, and scaling. Hyperpigmentation and lichenification are usually observed in chronic cases. Frequently, affected body regions include lips, ear canals, ventral neck, axillae, groin, interdigital webs, perianal area, and intertriginous regions (Miller *et al.* 2012).

The exact pathogenesis of skin inflammation caused by *M. pachydermatis* is still unclear. A key role seems to be played by phospholipase production (Coutinho and Paula 2000, Teramoto *et al.* 2015) and by hypersensitivity against yeast antigens (Bond *et al.* 2002, Bond *et al.* 2006a, Bond *et al.* 2006b, Khosravi *et al.* 2007, Layne and Deboer 2016). Many skin disorders, especially atopic dermatitis, keratinization defects, recurrent bacterial pyoderma, and endocrine diseases alter sebum production and cause a disruption of the epidermal barrier, favouring yeast proliferation (Bond and Ferguson 1996). It has also been proposed by some authors that long-term

administration of glucocorticoids or antibiotics may increase *Malassezia* populations (Bond and Ferguson 1996, Ihrke *et al.* 1993, Mauldin *et al.* 1997). A significant effect from prednisone and cyclosporine on cutaneous *Malassezia* populations in dogs with atopic dermatitis has recently been excluded (Widmer *et al.* 2018). Lastly, studies providing evidence on the effect of antibiotic treatments on the *Malassezia* population are lacking in dogs.

The present study describes the frequency of detection and body distribution of *Malassezia* yeasts in dogs in Italy. We also examined the clinical signs, grade of lesions, and predisposing factors of *Malassezia* dermatitis with particular attention on the effects of previous pharmacological treatments.

Materials and methods

Study design

Dogs with skin and ear diseases and cytological evidence of MO were prospectively enrolled in a veterinary teaching hospital (VTH) in northern Italy for a period of three years.

A control group of healthy dogs (HD) was also recruited, including dogs in good general health that were presented for routine visits or vaccinations. These animals were free of clinical signs and had no history of skin and ear diseases. Moreover, these dogs had not received any medication during the previous three months.

Clinical examination

Clinical history was collected from all dogs with a special focus on therapies in the previous three months. In addition, to complete physical and dermatological examination, the Canine Atopic Dermatitis Extent and Severity Index-03 score (CADESI-03) was calculated in dogs with MO (Olivry *et al.* 2007, Machado *et al.* 2011). Client's informed consent was obtained for each dog before examination.

Sampling procedures

Samples were obtained from all dogs from axilla, ventral interdigital webs of the forelimb, ear pinna, and ear canal of the right side. Furthermore, in animals with dermatitis or otitis, additional samples were collected from specific lesion. Samples for cytologic examination were collected from the skin with a tape-strip technique (Bond *et al.* 1994) and from the ear canal using cotton swabs rolled on a glass slide. Samples for mycological cultures were collected by rubbing a sterile swab moistened in sterile saline (0.9%) solution on a skin area of approximately 1 cm^2 in all the above-mentioned cutaneous sites.

Cytology

Each tape-strip was stained with modified Diff-Quick® method skipping the first step of alcohol fixation, and examined microscopically at 40x magnification. Ear samples were fixed and stained with the Diff-Quick® method and examined as skin specimens. Yeasts were identified according to their typical morphology (cell shape, size, and budding pattern). The number of yeasts was assessed in 5 random fields at 40x magnification (Cafarchia *et al.* 2005), and number of yeasts per field was graded as follows: 0, no yeasts found; A, 1-5 yeasts; B, 6-10 yeasts; C, 11-50 yeasts; D, 51-100 yeasts; and E, > 100 yeasts. *Malassezia* overgrowth was diagnosed when more than 2 and 10 yeasts were counted in 5 random fields at 40x magnification in skin and ear cytology, respectively (Mauldin *et al.* 1997, Bond *et al.* 1993).

Mycological culture

Swabs were preserved not more than 24 hours at 4 °C before mycological cultures were performed. Swabs were plated directly onto Petri dishes containing Sabouraud Dextrose Agar (SDA) and modified Dixon's Agar (DA) medium and incubated at 32 °C for 15 days. The positivity threshold was set at 70 UFC.

Identification

Colonies were identified as *Malassezia* based on microscopic and macroscopic morphology and were suspected to belong to the non-lipid dependent *M. pachydermatis* species when grown on the SDA medium. Identification was confirmed by PCR and sequencing of the large-subunit (26S) ribosomal DNA gene as previously reported (Kurtzman and Robnett 1997).

Statistical analysis

The statistical analyses were carried out using the Statistical Analysis System version 9.0 (SAS Inst. Inc., Cary, NC, USA). Distribution of data was assessed by use of the Shapiro-Wilk's normality test. Normally and non-normally distributed data were reported as mean ± SD and median (range), respectively. Frequencies of the detection from the considered body regions were reported as a percentage and compared using the chi-square test. Due to the non-normal distribution of the yeast population sizes, a Global Score (GS) was calculated by summing

the corresponding scores of various sampled regions. In particular, the score of the sampled regions was calculated using the following formula: Score = 3*number of microscopic fields A + 8* number of microscopic fields B + 30*number of microscopic fields C + 75*n° microscopic fields D + 100*number of microscopic fields E. The coefficients were determined considering the median of the number of yeasts per field per each grade, except for the E grade, as all fields were considered having 100 yeasts per field. The association between the the sum of GS (GSs) of *Malassezia* and the severity of skin lesions (CADESI-03 scores) among the dogs with MO was also evaluated by use of the Spearman's correlation index. The effect of pharmacological therapies (systemic antibiotics and steroids in the previous three months of examination) on non-normally distributed data was tested with the Mann-Whitney Test.

Normally distributed data were analysed using an analysis of variance (ANOVA). The statistical models included the fixed effects of group of dogs (healthy dogs vs dogs with MO), sex, clinical problem (pruritus, multifocal alopecia, diffuse alopecia, and otitis externa), and treatments (topical and systemic corticosteroid, topical, and systemic antibiotic). A value of $P \leq 0.05$ was considered to be statistically significant.

Results

Population and clinical data

A total number of 240 dogs with skin problems was presented at the VTH in the enrolment period. Among these, 97 dogs fitted the inclusion criteria and were enrolled in the study. They were grouped as follows.

1. Healthy dogs (HD) (n = 13).

2. Dogs with MO (n = 84).

The population included 52 females (2 HD and 50 with MO) and 45 males (11 HD and 34 with MO). All animals lived in Northern Italy. The median age at first examination of HD and dogs with MO was 58 months (3-132 months) and 71 months (4-192 months), respectively. In dogs with MO, 31 received systemic antibiotics (11 cefalexin, 8 amoxicillin + clavulanic acid, 3 benzylpenicillin + dihydrostreptomycin, 1 enrofloxacin, 1 clindamycin and 1 enrofloxacin + metronidazole, 6 not specified) in the previous three months. Table I summarizes the list of presenting complaint in dogs with MO.

Frequency of cytological detection

Table II summarizes the results of detection of *Malassezia* spp. in four sampling sites in HD (n = 13) and in five sampling sites in dogs with MO (n = 84). In the HD group, *Malassezia* yeasts were detected by cytology in 8/13 dogs (62%) without any significant difference (chi-square = 4.42, P = 0.22) in the frequency of yeasts among the four anatomical areas.

In dogs with MO, the greatest frequency of detection of *Malassezia* spp. was recorded from the cutaneous lesions (82%, $P < 0.001$). The frequency of yeasts was significantly higher on the ear canal compared to the axilla (43% vs 23%, $P < 0.001$), while interdigital webs and ear pinna showed intermediate values (33% and 26%, respectively; $P < 0.001$). The comparison of the frequencies of cytological detection of *Malassezia* species in different affected regions among MO and HD showed a tendency for significant differences regarding ear canal and ear pinna (43% vs 15%, chi-square = 3.6 P = 0.05; and 26% vs 54% chi-square = 4.1 P = 0.05, respectively) (Table II).

Table I. *Main presenting complaints (%) in 84 dogs with* Malassezia *overgrowth.*

Problem	% of cases*
Pruritus	73%[a]
Otitis externa	18%[b]
Diffuse alopecia	5%[b]
Multifocal alopecia	5%[b]

*Values with different letters along column are significantly different (Chi square = 80.0; $P < 0.001$).

Global score (GS)

Although the GS was not available in 53.6% (45/84) of dogs with MO, the GSs obtained from four sampled anatomical regions in HD (3, 0-48) were significantly lower compared to those of 39/84 dogs with MO (68, 0-612, $P < 0.001$) (Table III). In dogs with MO, the GS (312.5, 30-975) of the 12 dogs treated with systemic antibiotics in the previous three months was significantly higher compared to that of the 27 not treated dogs (80, 0-975, P = 0.015) (Figure 1). The GS was not significantly affected by treatment with systemic or topical steroids (P = 0.35) (Figure 2).

Table II. *Cytological detection of* Malassezia *spp. yeasts in four and five sampling sites in 13 clinically healthy dogs (HD) and 84 dogs with* Malassezia *overgrowth (MO), respectively.*

Sampling site	HD°	Dogs with MO*	P
Ear canal (%)	15%	43%[b]	0.05
Interdigital webs (%)	38%	33%[bc]	0.72
Ear pinna (%)	54%	26%[bc]	0.05
Axilla (%)	31%	23%[c]	0.52
Skin lesions (%)	-	82%[a]	

°Chi square = 4.42, P = 0.22; *Values with different letters along column are significantly different (Chi square = 80.0; $P < 0.001$).

Table III. *Global scores (GS) of the four and five sampling sites in 13 clinically healthy dogs (HD) and 39 dogs with* Malassezia *overgrowth (MO), respectively.* Data are reported as Median (range).

Sampling site	HD	MO	P
Ear canal	0 (0-30)	0 (0-475)	0.132
Interdigital webs	0 (0-3)	0 (0-500)	0.196
Ear pinna	0 (0-39)	0 (0-500)	0.748
Axilla	0 (0-39)	0 (0-35)	0.525
Skin lesions	-	12 (0-475)	-
Sum of GS*	3 (0-48)	68 (0-621)	< 0.001

*Without GS of lesions.

Lesion scores

In 39/84 dogs with MO, the CADESI-03 score was 18 (0-43). No significant difference was found in CADESI-03 score between male and female (P = 0.163). Neither antibiotics nor corticosteroids significantly influenced the CADESI-03 score. No correlation (R = 0.48; P = 0.0021) was found between CADESI-03 scores and modified GSs. Since the CADESI-03 score does not consider ear canals, the GS of this area was not considered in this single analysis.

Culture and identification of the Malassezia yeasts

From 142 swabs taken from 52 animals (39 with MO and 13 HD), 26 samples (18.3%) were considered positive. All isolates were able to grow on SDA (at 32 °C) and were identified as non-lipid dependent *Malassezia* yeasts. Sequencing of the 26S rDNA gene confirmed *Malassezia pachydermatis* in all isolates. GenBank® (http://www.ncbi.nlm.nih.gov/genbank/) accession numbers were MN198166, MN198167, MN198171, MN198176, MN198172, MN198173, MN198174, MN198175, MN198177, MN198179, MN198170, MN198168, MN198178 and MN198169.

Discussion

The main result of the present study is the observed increased number of *Malassezia* yeast in a canine population treated with systemic antibiotic therapy.

Therefore, this is the first time that the effect of antibiotic treatments on the number of cutaneous *Malassezia* yeasts is evidenced by a clinical study.

The GS and then the number of *Malassezia* yeasts in dogs that had received antibiotics were indeed significantly higher compared to those of non-treated dogs. In fact, the association between *Malassezia* dermatitis and previous antibiotic therapy has been reported in the canine literature without clinical evidence (Miller *et al.* 2012). A recent study evidenced that the skin microbiota can be influenced by topical antimicrobial therapy, but the effect of systemic antibiotic treatment was not considered (Chermaprai *et al.* 2019). There are at least two possible explanations for our results. First, the changes in the skin microbiota following antibiotic treatment may predispose it to *Malassezia* overgrowth giving rise to the thought that *Staphylococcus* and *Malassezia* species might have a symbiotic relationship.

Second, there may be some common predisposing factors that can favour both the secondary bacterial

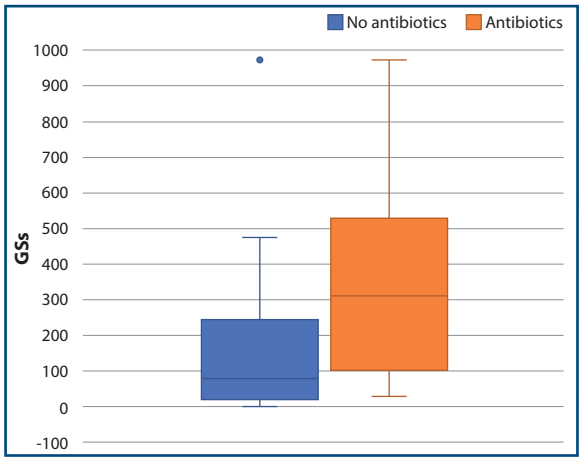

Figure 1. *Box plot showing the sum of global score (GSs) of 12 and 27 dogs that received (Antibiotics) and did not receive (No antibiotics) systemic antibiotic therapy in the previous three months before examination.* Boxes represent the interquartile range (25th to 75th percentile). The horizontal line in each box represents the median. Whiskers represent the 5th and 95th percentiles. Outliers are plotted separately as circles. Values are significantly different, P = 0.015.

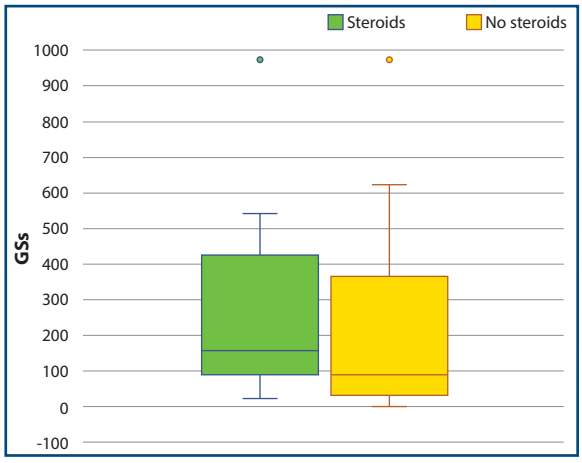

Figure 2. *Box plot shows the sum of global score (GSs) of 10 and 27 dogs that receive (Steroids) and did not receive (No steroids) systemic steroid therapy during the previous three months before examination.* The horizontal line in each box represents the median. Boxes represent the interquartile range (25th to 75th percentile). Whiskers represent the 5th and 95th percentiles. Outliers are plotted separately as circles. Values are not significantly different, P = 0.35.

infections, which justify the previous antibiotic treatments, and the overgrowth of *Malassezia*. Some changes in the skin barrier and immunological status have demonstrated a predisposition to bacterial and yeast overgrowth in dogs with atopic dermatitis (Santoro *et al.* 2015). In the present study, 61 (73%) dogs with *Malassezia* overgrowth were examined for pruritus with a presumptive diagnosis of allergic dermatitis, confirming the findings of other investigations concerning this association (Machado *et al.* 2011). Therefore, these subjects could have been predisposed to skin bacterial secondary infections and to *Malassezia* overgrowth.

Many patients of the present study were referred by other practices and so the lack of information about prior bacterial infections was consequently a limitation of the present study. Further studies are necessary to understand whether there may be an altered competition with the resident microbial flora or if other mechanisms, not considered in the present study, can explain the presence of a greater number of *Malassezia* in dogs treated with antibiotics (Plant *et al.* 1992, Bond and Ferguson 1996). In contrast to the effect of antibiotic therapy, glucocorticoids treatment showed no significant influence on GS. Recently, Widmer and colleagues (Widmer *et al.* 2018) have shown no significant impact of prednisone on canine cutaneous microbiota. Thus, previous steroid therapy does not seem to affect the composition of the canine skin flora.

The cytological examination revealed that the number of yeasts on five random fields can differ considerably in areas within the same slide. For this reason, we studied the Global Score (GS), which permits to overtake the effect of this variability and enhance the importance of fields with a high number of yeasts. As expected and reported in previous studies, the yeast population size (expressed by the GS) differed significantly between the healthy and diseased animals (Crespo *et al.* 2002, Nardoni *et al.* 2004, Yurayart *et al.* 2011, Cafarchia *et al.* 2005). Furthermore, in the present study, the frequency of yeast detection from dogs with skin disease was significantly higher when compared to that of clinically HD; this was also evident from areas not interested by lesions, although the frequency of yeast detection was higher in areas with skin lesions, as previously reported (Bond and Lloyd 1997, Maachado *et al.* 2011, Nardoni *et al.* 2004, Yurayart *et al.* 2011). Despite the differences in GS and in the frequency of yeast detection, no significant differences or correlations were found in the evaluation of CADESI-03 scores (Olivry *et al.* 2007, Olivry *et al.* 2008). However, it has to be said that CADESI-03 scores were developed and validated only for canine atopic dermatitis and may not be valid for *Malassezia* dermatitis. GS also has not been validated. This could represent a weakness of the present study.

It is generally accepted that MO is secondary to hypersensitivity disorders, endocrine diseases, and defects of keratinization (Miller *et al.* 2012). Therefore, in the present study only a part of the dogs could have had hypersensitivity disorder and, in particular, atopic dermatitis. This could also represent a study limitation, as already highlighted in a previously published study that uses the CADESI-03 scores for evaluating the difference between dogs with *Malassezia* dermatitis and dogs with cutaneous lesions but without evidence of *Malassezia* dermatitis (Machado *et al.* 2011). Moreover, the present study is based on the count of yeasts, which is probably a relevant factor, but does not consider that some strains could express different virulence factors such as phospholipase productions (Coutinho *et al.* 2000, Teramoto *et al.* 2015, Buommino *et al.* 2016, Cafarchia and Otranto 2004), or that the susceptibility to this organism could differ between hosts (e.g. *Malassezia* hypersensitivity) (Bond *et al.* 2006a, Bond *et al.* 2006b). In both cases, clinical signs of *Malassezia* dermatitis would likely develop also with low numbers of yeasts. Finally, secondary bacterial infections that may have influenced the CADESI score should have been considered. This represents, as mentioned before, another study limitation.

In conclusion, the present study provides helpful insights into the frequency of detection and body distribution of *Malassezia* in healthy dogs and in dogs with skin disease. In particular, our results highlight the importance of the number of yeasts resulting from the skin cytology for the diagnosis of MO, even though the different pathogenicity of the involved yeast strains needs also to be considered. Previous antibiotic treatments may represent a predisposing factor for the increased number of *Malassezia* yeast supporting what has previously been assumed but without clinical evidence. However, further studies are necessary to confirm this hypothesis, also considering the potential effect of other factors not evaluated in the present study such as the concurrent predisposition to both MO and bacterial infections.

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