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Importance of *Malassezia pachydermatis* in dogs

L'importanza di *Malassezia pachydermatis* nel cane

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Abstract

The genus *Malassezia* belongs to *Basidiomycota* and currently includes 16 species, of which *M. pachydermatis* is the most commonly isolated from dogs. Actually, *M. pachydermatis* is a member of the normal microbiota of the skin and mucosal sites of dogs. Under certain conditions, these yeasts can act as opportunistic pathogens causing skin and ear infections of these animals.

Topical and oral antifungal agents are frequently used for the therapy of *Malassezia* dermatitis and otitis, which are among the most frequently reported skin disorders in dogs. However, with the expanding use of antifungal agents, resistant strains of *Malassezia* are being increasingly detected. The development of resistance to these antifungals and other antimicrobials among veterinary pathogens also poses a potential threat to human health, particularly among zoonotic multidrug-resistant strains with potential to cause severe, life-threatening infections, which may be the case of *M. pachydermatis*. Restricting the use of critically important antimicrobials to safeguard their future effectiveness, a fundamental element of antimicrobial stewardship, is essential and it is driving a search for alternative treatments for these infections.

The emergence of antimicrobial resistance represents a serious human and animal health risk. Therefore, good antimicrobial stewardship is essential to prolong the lifespan of existing therapies and new strategies are required to fight the infections caused by *Malassezia* yeasts in humans and animals.

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1. Introduction

Microbes coexist with mammals and create complex ecosystems on body surfaces, which contribute to health and disease. The constant exposure of the host to microbes shapes the its immune reactivity locally and at distant sites (Belkaid and Tamoutounour, 2016). On the other hand, perturbations that change the equilibrium of the microbiota may lead to the overgrowth of species with pathogenic potential. In fact, microorganisms that typically display a commensal lifestyle, have also been associated with the development of disease. This is the case, for example, of the genus *Malassezia*, which is represented by a group of lipophilic yeasts that have evolved as skin commensals and opportunistic cutaneous pathogens of a variety of mammals and birds (Guého-Kellerman et al., 2010). Actually, >90% of the fungi inhabiting the mammalian skin belong to the genus *Malassezia* (Findley et al., 2013).

The transition from the commensal to the pathogenic lifestyle is particularly frequent in *Malassezia* strains associated to dogs, such that cases of *Malassezia* otitis externa and dermatitis. For example, the prevalence of otitis externa amongst dogs attended in primary care practices is around 10% (O'Neill et al., 2014) and *M.pachydermatis* is responsable for up to 70% of such (Forster et al., 2018).
Once otitis is resolved, pet owners should be aware that relapses may occur (recurrent/chronic diseases) with the simultaneous development of resistance to various agents, so it has become a problem difficult to manage. Empirical evidence further suggests that chronic otitis may be a major reason for premature euthanasia, as sometimes euthanasia is the only available option.
We know otitis in humans is very painful and causes irritability, the same is true for pets. Perhaps, we should investigate further if ear infections play a major role in behavioural changes in pets and how this disease may affect the bond between the pet owner and the pet itself.
Indeed, it is no secret that otitis is one of the most common diseases treated by vet practitioners and as the disease is often caused by several factors, it can easily become chronic, leading to frustrated pet owners and miserable looking pets.

As for dermatologic infections caused by *M. pachydermatis* often exhibit a chronic (recurrent) course, which has a severe and significant impact on the life quality of affected animals. Unfortunately, cases of chronic ear infection of rather complex etiology and unresponsive to prolonged pharmacological treatment are common in small animal practice (Angus, 2005). Furthermore, the treatment of these conditions can be complicated due to the adverse effects of existing antifungal drugs, changes in susceptibility or even development of antifungal resistance

of the yeast strains and the ability of *Malassezia* yeasts to form biofilms. As consequence, current approaches to treat these infections are suboptimal and should clearly be improved.

Various findings support the capacity of *M. pachydermatis* for developing resistance. These include some reports of treatment failure in dogs, the reduced antifungal activity found against yeast isolates sampled from dogs with exposure to antifungal drugs and strains exposed to antifungal agents *in vitro*. At the same time, it is possible that the prevalence of antifungal resistance is underestimated in the literature, mostly due to the difficulty of obtaining laboratory confirmation of the resistance given that a standard procedure for susceptibility testing of *M. pachydermatis* is still unavailable (but see Cafarchia et al. (2012a) and Álvarez-Pérez et al. (2014) for some proposals of testing protocols).
These considerations highlight the need for maintaining surveillance and vigilance for the possible emergence of clinically relevant resistance and, additionally, the urgent need for finding an efficient treatment for these skin and ear diseases in our pets. Standard reference methods of antifungal susceptibility testing of *M. pachydermatis* are required to assist veterinary practitioner in the management of chronic cases. It would also be really interesting to get a deeper understanding of the link between *Malassezia* colonization and the development of skin and ear disorders, but this requires a better knowledge of the mechanism by which the immune system interacts with the fungus. In this regard, there have been significant recent advances in understanding of the mechanisms of interaction between *Malassezia* yeasts and dogs (see, for example, Bond et al., 2020).

In any case, the outcome of *Malassezia* growth on the skin (commensal existence or inflammation and disease) is dependent upon the metabolic activities of the yeasts (expression of cell wall and secreted virulence attributes) and the host's innate and adaptive immune defensive responses. Interactions with other skin commensals (especially the staphylococci) may also play a role in determining the outcome of colonization in animals, although this area is largely unexplored (Ianiri et al., 2018), especially in dogs and cats. All these processes should ideally result in a delicately balanced homeostatic relationship. The presence of *Malassezia* yeasts within the stratum corneum exposes the host to an array of chemicals, immunogens and allergens of fungal origin, comprising fungal cell wall-associated carbohydrates, proteins and lipids; secreted enzymes that generate both substrates for nutrition, and an array of irritant metabolic by-products (Ashbee et al., 2010a; Sparber and Leibundgut-Landmann, 2017).

I chose this topic for my dissertation as it is a current and constantly evolving theme in veterinary medicine and given that in recent years there have been an increasing number of cases of dermatitis and otitis found in pets. In this context, the objective of my research is to review the current information on the ecology and pathophysiology of *Malassezia pachydermatis*, the diagnosis and new therapeutic candidates for the treatment of the ear diseases that this yeast species causes in dogs.

Furthermore, during the time I spent at Universidad Complutense de Madrid (Madrid, Spain) as part of the Erasmus Exchange Programme, I had the opportunity to take part to several clinical cases of skin and ear disease caused by the genus *Malassezia.* As well as during my internship I undertook different activities, for example, infectious diseases and pathological anatomy practicum in which I spent few days in the laboratory, in particular also in the mycology department, where I saw different strains of *Malassezia*. Overall, I thought it would have been interesting to investigate above this issue which will unfortunately be increasingly present in future days. Moreover, this topic caught my attention due to the potential zoonotic transmission of *Malassezia* yeasts from animals to humans. The zoonotic potential of *Malassezia* yeasts was first proposed in the context of a neonatal intensive care unit, where a group of low birth weight neonatal patients receiving lipid emulsions were infected by *M. pachydermatis* strains which were likely transmitted by contact with healtcare workers whose hands were colonized by the same strains found in pet dogs (Chang et al., 1998). Note that hand contamination by *M. pachydermatis* is common among dog owners, especially in owners of allergic dogs with *Malassezia* overgrowth (Morris, 2005). *Malassezia* yeasts can also cause sporadic bloodstream infections in adults, especially in immune-compromised patients.

2. *Malassezia*

2.1 The genus *Malassezia*. General characteristics, phylogeny and species of relevance to veterinary medicine

Malassezia yeasts form a well-defined and unique cluster of lipophilic fungi living almost exclusively on the skin and mucosal sites of warm-blooded vertebrates (Lorch et al., 2018). The genus *Malassezia* (Baillon 1889) is usually considered as a monophyletic taxon within the phylum *Basidiomycota* and subphylum *Ustilaginomycotina*, a highly diversified group of fungi comprising more than 1,500 species and including some relevant plant pathogens (Begerow, 2006).

As a consequence, it has been proposed that the genus should be assigned as its own class, namely the *Malasseziomycetes* (Wang et al., 2014). In 2015, a study reported the sequences, assemblage and annotations for the genomes of 14 *Malassezia* species, including multiple strains of the most relevant species in medical dermatology: *M. globosa, M. sympodialis, M. restricta* and *M. furfur* (Wu et al., 2015).

The first remarkable feature of *Malassezia* genomes is their small size (~10 Mb), which is about half of the size of other known basidiomycetous fungi, with some species having less than 4,000 predicted genes (Boekhout et al., 1998; Xu et al., 2007; Wu et al., 2015).

Furthermore, functional analyses indicate that *Malassezia* yeasts display unique characteristics comprising (i) a low carbohydrate-degrading capacity due to reduction of glycosyl hydrolase-encoding genes; (ii) a lipid dependence for growth due to the lack of a fatty acid synthase gene; and (iii) a concomitant expansion of lipid hydrolysing enzymes, that allow *Malassezia* yeasts to collect and use fatty acids from the skin or mucosal surfaces of their hosts (Bond et al., 2020). It has been suggested that if there is an extant sexual cycle for some *Malassezia spp*., it is more likely to be bipolar or pseudo-bipolar with two mating types (Wu et al., 2015; Coelho, 2010).

On lipid-enriched media such as modified Dixon's agar, *Malassezia* colonies are cream to yellowish, smooth or lightly wrinkled, glistening or dull, with the margin being either entire or lobate. Microscopically, *Malassezia* yeasts appear as small ovoid, ellipsoidal or cylindrical cells (1.5 to 6.0 µm by 3.5 to 8.0 µm). Reproduction is by budding on a broad base and from the same site at one pole (Bond et al., 2020) (Figure 2). *Malassezia* yeasts have a thick cell wall (~0.12 µm) morphology (inner spiralling/corrugation) whose innermost layer shows a characteristic serrated structure (Figure 1) (Guillot et al., 1995; Swift et al., 1965; David et al., 2007).

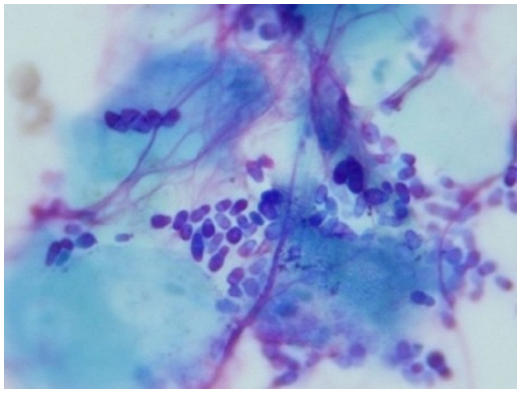


***Figure 1**. Malassezia pachydermatis and keratinocytes. Allergiepoli V.H., 2014*

During the last decade, the analysis of the genome of *Malassezia* yeasts has allowed a better understanding of how these fungi, whose ancestors were most probably plant or soil residents,

manage to survive and thrive in the skin of animals (Xu et al., 2007). Actually, the genus *Malassezia* was created in 1889 to accommodate a single species, *M. furfur*, detected in cutaneous lesions in humans (Baillon,1889). Weidman (1925) was the first scientist to recover *Malassezia* yeasts from the skin of an animal, an Indian rhinoceros (*Rhinoceros unicornis*) with a generalized exfoliative dermatitis.

The taxonomy of genus *Malassezia* is evolving, in fact, molecular biology techniques have allowed the identification of new species, from the two species identified in 1989 to the current 18 species. Besides, the number of currently described *Malassezia* species is likely underestimated due to a sampling bias toward humans and domestic animals, and it is probable that such number will keep increasing when the skin microbiota of wild animals is investigated.

In 1989, the genus *Malassezia*, also known under the generic name *Pityrosporum* proposed by Sabouraud in 1904, comprised only two taxa: *M. furfur* (syn. *P. ovale, P. orbiculare*), a lipid-dependent species found on human skin, and *M. pachydermatis* (syn. *P. pachydermatis, P. canis*), a species isolated from the skin of animals, especially dogs.

In 1996, Guého et al. collected and examined multiple isolates from humans and animals, which resulted in the complete revision of the genus *Malassezia* and the description of four new species (namely, *M. obtusa, M. globosa, M. slooffiae* and *M. restricta*). Eleven more species were described subsequently by different groups and from diverse hosts: *M. dermatis, M. japonica* and *M. yamatoensis* from humans in Japan; *M. nanafrom* cases of otitis externa in cats and cattle; *M. caprae* from goats; *M. equina* from horses; *M. cuniculi* from rabbits; *M. arunalokei* from humans in India; *M. brasiliensis* and *M. psittaci* from domesticated parrots in Brazil and *M. vespertilionis* from bats in the USA. Several *Malassezia* specific names were proposed according to the latin names of the animals from which the yeasts were initially isolated.

To date, the following species have been isolated from animals and are therefore relevant in veterinary dermatology (Table 1) (Bond et al., 2020): *M. pachydermatis*, initially isolated from a rhinoceros within Pachydermata, an obsolete nineteenth-century taxonomic order of mammals (Weidman, 1925); *M. furfur, M. sympodialis, M. globosa, M. slooffiae, M. nana, M. caprae* from goats (Cabañes et al., 2007); *M. equina* from horses (Cabañes et al., 2007); *M. cuniculi* from rabbits (Cabañes et al., 2011); *M. brasiliensis* and *M. psittaci* from parrots (Cabañes et al., 2016); and, more recently, *M. verspertilionis* from bats (Lorch et al., 2018).

Table 1. *Summary of the taxonomy of the genus. Bond et al. (2020)*

Malassezia species	Presence in animals	Presence in humans
M. furfur	HS (dogs, cats, others)	HS, PV, fungaemia
M. pachydermatis	HS+ LS (dogs, cats, many others, mostly canids)	HS (dog contact), fungaemia
M. sympodialis	HS, OT (cats)	HS, AD, SD
M. globosa	HS, OT (cats)	HS, PV, SD, AD
M. obtusa	--	HS, LS
M. slooffiae	HS (pigs, cat claws)	HS, LS
M. restricta	--	HS, SD
M. dermatis	--	HS, AD
M. japonica	--	HS, AD, SD
M. nana	HS (cats, horses), OT (cats, cattle)	--
M. yamatoensis	--	HS, SD
M. caprae	HS (goats)	--
M. equina	HS, LS (horses)	--
M. cuniculi	HS (rabbits)	--
M. arunalokei	--	HS, SD
M. brasiliensis	HS (parrots)	--
M. psittaci	HS (parrots)	--
M. vespertilionis	HS (bats)	--

-- not reported, AD atopic dermatitis, HS healthy skin, LS lesional skin, PV pityriasis versicolor, SD seborrheic dermatitis, OT otitis

Malassezia species are lipid-dependent due to an inability to synthesize long-chained (C14 or C16) fatty acids de novo (Shifrine and Marr, 1963). Nevertheless, there are some differences in lipid dependence among the species and this variability has been used for the development of specific tests for their identification (Guillot et al, 1996). For example, historically, *M. pachydermatis* has been regarded as a "lipophilic but not lipid-dependent" species because it was the only member of the genus able to grow on Sabouraud's dextrose agar without lipid supplementation (Guillot and Bond, 1999).Recently, genome sequencing has confirmed that *M. pachydermatis* lacks a fatty acid synthase gene like the other members of the genus (Wu et al., 2015), but it is uniquely capable of utilising the lipid fractions of the peptone component of

Sabouraud's dextrose agar for growth (Puig et al., 2017). These observations explain its failure to grow on lipid-free defined media and thus *M. pachydermatis* should now also be regarded as being "lipid-dependent" (Puig et al, 2017).

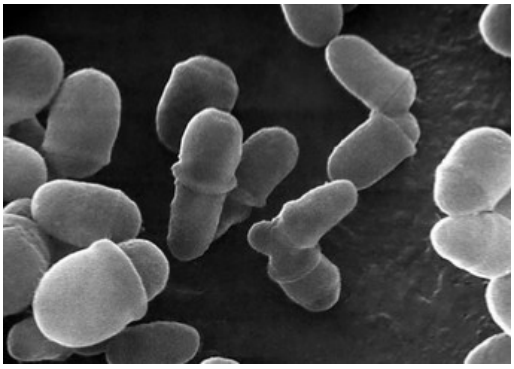


Figure 2. *Reproduction of Malassezia by budding. Angiolella et al., 2017*

2.2 Ecology of *Malassezia* yeasts in dogs

M. pachydermatis remains by far the most important and prevalent *Malassezia* species in dogs, whereas the other lipid-dependent species of the genus are detected more frequently in other animal species (like cats) or body sites. In fact, culture-based studies clearly demonstrate that *M. pachydermatis* is a normal inhabitant of the healthy canine skin and mucosae, and it is the predominant cutaneous yeast in both healthy dogs and dogs with *Malassezia* dermatitis or otitis (Gustafson, 1955; Dufait, 1983). *Malassezia* yeasts have been isolated from almost all domestic animals, different wild animals in captivity and also from wildlife (Bond et al., 2020). In addition, the presence of *Malassezia*-like organisms has been reported in a wide range of environmental sources (e.g. deep-sea sediments, hydrothermal vents and arctic soils) and invertebrates such as marine sponges, stony corals, lobster larvae and nematodes (Amend, 2014; Renker et al., 2003).

Colonisation of canine skin by *M. pachydermatis* probably occurs in the very first days of life. How this occurs is not completely understood, but it likely involves the transfer of yeast cells from the bitch's microbiota following removal of the amniotic membrane, licking and nursing in the same manner as it occurs with the staphylococci (Saijonmaa-Koulumies et al., 2002). In a study performed in 22 newborn Rottweiler puppies, *Malassezia* yeasts were recovered from around 40% of samples collected from the lips, nail beds and ears, at three, seven and 35 days of age (Wagner et al., 2000). It was seen that the sites most frequently colonised by *M. pachydermatis* in healthy pet dogs of various breeds were the peri-oral/ lip region and interdigital

skin (up to 80% of samples tested positive for yeast presence). In contrast, the yeast was less often detected (<25% of samples) in the skin of the axilla, groin and dorsum.
Moreover, the perianal skin and anal mucosa are frequent carriage sites whereas nasal and oral carriage is less frequent. So, population sizes of the genus *Malassezia* vary markedly between anatomical sites, and between different breeds of dogs that are identified to be at increased risk of *Malassezia* dermatitis and otitis. Oral carriage of *Malassezia* may also have relevance as a source of transfer to the skin (Brito et al., 2009; Santin et al., 2013). In this regard, another study pointed out the possible transfer of *Malassezia* yeasts between the perioral area and pruritic skin lesions of the inguinal area as a consequence of frequent licking, and between undamaged interdigital regions as a result of persistent scratching (Cafarchia et al., 2005b). Therefore, studies of oral carriage of *Malassezia* may have relevance to track possible sources of skin colonization (Brito et al., 2009; Satin et al., 2013).

The identification of lipid-dependent yeasts has been traditionally based only on morphological and physiological characteristics (Cafarchia et al., 2005b; Nardoni et al., 2004), so the prevalence of non-pachydermatis *Malassezia* in clinical samples of canine origin might have been underestimated. More recently, methods based on next generation sequencing (NGS) have allowed a better characterization of the complex microbial communities occurring on animal skin and made it possible to detect *Malassezia* species that would otherwise be missed using culture-based approaches (Meason-Smith et al., 2017; Older et al., 2019). Few phenotypic tests are currently available for species-level differentiation of *Malassezia* species, which means that DNA sequencing is key for reliable species-specific identification.

So far, only a very few studies have examined the skin microbiota of dogs and cats (Meason-Smith et al., 2017; Cusco et al., 2017). The cutaneous mycobiota in dogs seems to be influenced by various factors, including environmental exposure to fungi, cohabitation with other pets, and skin health status. Surprising, *Malassezia* yeasts were not identified as the most abundant fungal organisms on healthy canine skin. Furthermore, Meason-Smith et al. (2015) were unable to detect any significant differences in the relative abundance of *Malassezia* yeasts between healthy and allergic dogs. The discrepancy between the results obtained by NGS and culture-dependent approaches, which demonstrate increased incidences of *M. pachydermatis* in allergic dogs (White et al., 1998), may be related to methodological differences. Another explanation would be that dysbiosis is present at *Malassezia* species level in allergic dogs (Meason-Smith et al., 2015). This latter hypothesis was recently investigated by Meason-Smith et al. (2019), who

collected skin samples from healthy, naturally affected allergic, and experimentally sensitized atopic dogs. Using NGS and *Malassezia* species-specific quantitative real-time PCR (qPCR), these authors demonstrated that *M. globosa* was significantly more abundant on healthy canine skin, *M. restricta* was significantly more abundant on healthy skin, whereas *M. pachydermatis* was significantly more abundant on naturally-affected allergic skin and on allergen-induced atopic skin lesions (Guillot et al., 2020). NGS methods were also recently applied to the analysis of the mycobiota present in the external ear canal of dogs (Korbelik et al., 2018). Samples were collected from six dogs with otitis externa and five healthy dogs. In cases of otitis externa, the mycobiota was largely dominated by *Malassezia* yeasts. These commensal *Malassezia* populations provide a reservoir of yeasts that might proliferate and or induce an inflammatory response under the influence of various host predisposing factors.

Finally, it is important to point out that, although accurate species-level identification is essential for unravelling the epidemiology of *Malassezia* species in animal medicine. Actually, when presented with a clinical case, it may not be strictly necessary for the attending veterinarian to know the species identity of the *Malassezia* strain(s) causing the disease, as inter- and intra-species variation in drug susceptibility is limited among these yeasts (Tragiannidis et al., 2010; Álvarez-Pérez et al., 2016). In any case, further developments in both diagnostic and antifungal drug susceptibility testing are urgently required to address these aspects.

2.3 Development of skin and ear sampling techniques for quantification of cutaneous *Malassezia* populations

2.3.1 Skin and ear sampling techniques

Methods for microbiological assessment of skin microbial colonization have traditionally included impression (cytology using slides or tape; culture) and dispersal (primarily cup-scrub or swab-wash) methods (Noble et al., 1974). Early studies carried out when dermatitis emerged as an important inflammatory disease of dogs primarily used cytological techniques. These techniques included (i) scraping methods (Dufait, 1983), (ii) direct impression with glass slides (Larsson et al., 1988), or (iii) slide preparations prepared by rolling swabs previously rubbed on the lesional skin (Mason et al., 1991).One study elegantly described the utility of the direct application of vinyl adhesive tape in the assessment of cutaneous bacterial and fungal populations in human skin (Keddie et al., 1961). In our knowledge, a systematic comparison of the effectiveness of staining methods for detecting *Malassezia* cells in specimens from animals is still pending, perhaps reflecting the widespread satisfaction with the use of a modified Wright-

Giemsa stain ("Diff-Quik") (Figure 3) that is likely to be available in the clinical area (Bensignor et al., 2002), although cotton-blue lactophenol (Larsoon et al., 1988) and May-Grunwald Giemsa (Cafarchia et al., 2005a) stains have also been used in some studies.

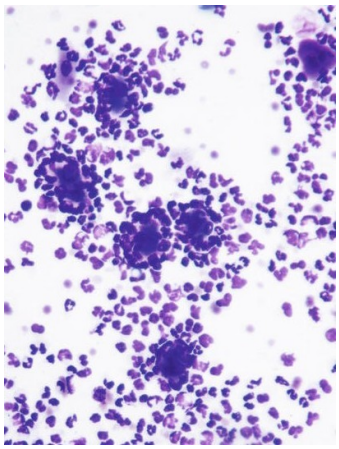


Figure 3. *Malassezia pachydermats (yeasts). Vroom, 2005*

Tape-stripping has gained wide acceptance in veterinary clinical practice as a rapid and versatile method for recovering stratum corneum cells and the microbes attached to them (Maynard set al., 2011). The adhesive tape can be applied to deeply folded or recessed areas that are not readily accessible for direct slide application, often with minimal animal restraint. The adhesive properties aid removal of skin surface material especially in erythematous lesions with limited exudation. By counting yeast cells in a certain number of microscopical fields in the tape-strip sample, a known area of skin surface is examined (Bond et al., 1993).

In cases of canine otitis, after clinical examination and physical examination of the animal, sterile swab is used to collect samples from the affected ears.
These samples are then smeared onto sterile glass slides and stained to examine under microscope after proper staining.
Moreover, an additional swab can be transferred to nutrient broth and/or used to inoculate solid mycological culture media, such as Sabouraud dextrose agar (SDA) (Figure 4) with chloramphenicol (0.05%) and Potato dextrose agar (PDA).
Inoculated SDA and PDA plates are then incubated for 48-96 h at 37°C to observe the growth and to study the colony and microscopic morphology of the yeast. Because yeasts colonize the surface of the ear canal, they are most easily found adhered to clumps of exfoliated squamous epithelial cells. There is no specific number that indicates yeast overgrowth (Karlapudi, 2017).

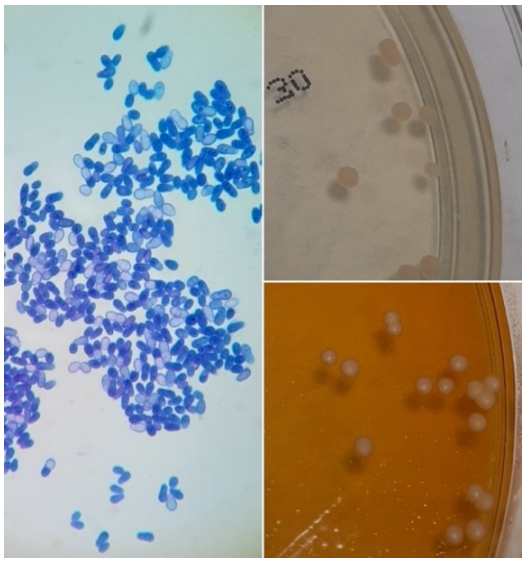


***Figure 4**. M. pachydermatis from a clinical case of canine bilateral otitis. On the left we can observe M. pachydermatis cells stained with methylene blue; the right panels show the colonies of Malassezia in agar Sabouraud y agar Dixon (above and below, respectively). Photo courtesy of Sergio Álvarez-Pérez, Universidad Complutende de Madrid, July 2020*

2.3.2 Growth factors of *Malassezia* species

In epidemiological studies and also for diagnostic purposes, it is important the selection of essential ingredients in the culture media for *Malassezia*.

Malassezia has been classified into 7 species by molecular analysis of nuclear ribosomal DNA/RNA (Guillot et al., 1995; Guého et al., 1996). Two additional species were reported a few years ago (Sugita et al., 2002). Unfortunately, it is difficult to cultivate some of the new species of *Malassezia* on potato dextrose agar with olive oil (Oil-PDA). Therefore, the development of an optimized culture medium capable of supporting the growth of all *Malassezia* species would be of great assistance in the diagnostic laboratory.

Kaneko et al. (2005) investigated during the growth abilities in different culture media of the following reference *Malassezia* strains: *M. furfur, M. sympodialis, M. globosa, M. obtusa, M. slooffiae*, *M. restricta* and *M. pachydermatis*. Tested culture media included Leeming and Notman Agar (LNA), several different variations of LNA, potato dextrose agar with olive oil (Oil-PDA) and modified Dixon agar (mDIX).

In addition, the growth of these same species was investigated on a modified CHROMagar Candida (LN-CHROM). All strains were transferred onto fresh LNA either at monthly (*M. furfur, M. sympodialis, M. obtusa, M. slooffiae*) or weekly (*M. restricta, M. globosa*) intervals and then washed once with sterilized saline solution. *M. pachydermatis* was transfered onto fresh PDA weekly.

Single colonies from 7 days old PDA cultures were directly streaked on to LN-CHROM then incubated for 4 days at 30°C. At the end of the study, six of the 7 *Malassezia* species grew better on LNA and LNA (-) than on Oil-PDA and mDIX (Table 2).

***Table 2**. Growth ability score of Malassezia species on different culture media after 4 days incubation at 30°C, as determined by Kaneko et al. (2005)*

	Oil-PDA	mDIX	LNA	LNA (-)
M. furfur	A	A	A	A
M. sympodialis	C	A	A	A
M. globosa	E	E	B	B
M. obtusa	E	D	B	B
M. slooffiae	C	A	A	A
M. restricta	E	D	D	D
M. pachydermatis	A	A	A	A

Oil-PDA= potato dextrose agar with olive oil; mDIX=modified Dixon agar; LNA= Leeming and Notman agar; LNA(-)= Leeming and Notman agar (without milk); A= growth at 10^2 inoculation levels; B= growth at 10^1 inoculation levels; C= growth at 10^0 inoculation levels; D= growth only in direct plating; E= no growth.

The exception was *M. restricta* which grew very slowly and formed small colonies on these two media. Furthermore, *M. globosa* and *M. restricta* did not grow in the absence of glycerol monosterate and developed poorly on media lacking Tween 60. Six of the 7 *Malassezia* species grew well on LN-CHROM agar (Table 3).

***Table 3**. Growth ability score on LNA and LN-CHROM after 4 days incubation at 30°C as determined by Kaneko et al. (2005)*

	LNA	LN-CHROM
M. furfur	A	A
M. sympodialis	A	A
M. globosa	B	B
M. obtusa	B	B
M. sloffiae	A	A
M. restricta	D	D
M. pachydermatis	A	A

LNA= Leeming and Notman agar; LN-CHROM= CHROMagar Candida (containing ox bile, glycerol monostearate, glycerol and Tween 60); A= growth at 10^2 inoculation levels; B=growth at 10^1 inoculation levels; C=/growth at 10^0 inoculation levels; D=/growth only in direct plating; E= no growth

Therefore, it was found that LNA and LNA (-)/ best supported the growth of *Malassezia* species. Furthermore, ox bile, glycerol monostearate, glycerol and Tween 60 would appear to be essential components of LNA required for the development of these yeasts. Besides, by adding these four essential ingredients to CHRO- Magar Candida (LN-CHROM), this medium was found to support the growth of all 7 *Malassezia* species.

While an overlay of olive oil on a solid nutrient medium has been widely used to promote the

growth of *Malassezia*, several of the recently recognized species do not grow well under these conditions. Based upon these initial studies, it would appear that LN-CHROM can be used for the simultaneous initial isolation of clinically important species of *Malassezia* (Kaneko, 2005). Nevertheless, modified Dixon agar is widely accepted as alternative culture media for *Malassezia* in most mycology laboratories.

2.4 *Malassezia* yeasts as zoonotic agents

2.4.1 Background

The concomitant presence of the same *Malassezia* species and genotypes in humans and animals could suggest that there is a link between animal and human infection. So far, there is no an absolute proof of the zoonotic transmission of *Malassezia*, but there are several lines of evidence in this regard, as explained below.

Several species of *Malassezia* are part of the commensal microbiome of healthy human skin (Gaitanis et al., 2012; Prohic et al., 2016). Spatial distributions of the species most commonly identified (*M. globosa, M. sympodialis, M. restricta*) may vary according to the age, body site and geographical location of the subjects studied (Nakabayashi et al., 2000). In addition, *M. pachydermatis* may also be isolated from healthy human skin by culture or detected by molecular-based techniques from the face and hands (Nakabayashi et al., 2000). Skin colonization by *Malassezia* species in full term healthy newborns has been also investigated (Bernier et al., 2002). *Malassezia pachydermatis* was not isolated from the skin of human neonates, while *M. sympodialis* and *M. globosa* colonisation begins at birth and increases in the first weeks of life. Pathogenic roles for various *Malassezia* species have been described in association with several human skin diseases including atopic dermatitis, seborrheic dermatitis, folliculitis, psoriasis and pityriasis versicolor (Sugita et al., 2010). Among these diseases, *M. pachydermatis* has been most commonly isolated from human patients with seborrheic dermatitis (Nakabayashi et al., 2000). However, it is difficult to assign a truly pathogenic role to *M. pachydermatis* since its identification from surface samples of human skin is typically due to contact with dogs. One exception has been the report of a facial granuloma in a dog owner, where *M. pachydermatis* was identified by electron microscopy in affected tissues, the yeast was grown and identified by standard microbiological methods and the patient responded to antifungal therapy (Fan et al., 2006).

2.4.2 *Malassezia* is specialized to live on human skin

Malassezia yeasts are associated with a variety of skin conditions in humans, including dandruff, atopic eczema (AE)/dermatitis, pityriasis versicolor, seborrheic dermatitis and folliculitis. Of the 14 currently recognized species of *Malassezia*, eight have been associated with humans (Hort et al., 2011).

Since *Malassezia* species are members of the skin commensal flora, the host immune system will be regularly exposed to these fungi. Accordingly, IgG and IgM specific to *Malassezia* can be detected in healthy individuals (Ashbee et al., 2010b). The defective skin barrier in AE patients, both in lesional and non-lesional skin, fails to provide sufficient protection against microbes and allergens, facilitating the interaction of *Malassezia* with the host's immune system.

Approximately 50% of adult patients with AE are sensitized to *Malassezia*, as reflected by the presence of allergen-specific IgE and T cell reactivity and/or positive atopy patch test reactions to the yeast (Scheynius et al., 2010). Furthermore, *Malassezia* cell wall carbohydrates have been long-recognized as IgE binding epitopes in humans with atopic dermatitis (AD) (Doekes et al., 1993), while other studies have highlighted their importance in fungal cell recognition by host phagocytic cells (Tada et al., 2006).

Moreover, the host interaction with *Malassezia* yeasts can stimulate the production of allergens. In this regard, the release of *Malassezia* allergens is significantly higher at a pH of 6, which is the skin pH of patients with AE, than at pH 5.5, which is the normal skin pH (Selander et al., 2006).

2.4.3 Zoonotic aspects

From a zoonotic perspective, the pathogenic role best documented for *M. pachydermatis* is a syndrome of life-threatening fungaemia that occurs in pre-term neonates while receiving lipid-rich nutritional infusions via catheter (Ilahi et al., 2018).

For example, an epidemiological investigation of an outbreak occurring in a neonatal intensive care unit (NICU) identified a single strain of *M. pachydermatis* – as determined by pulsed-field gel electrophoresis (PFGE) – which was isolated from 15 infants with sepsis, nine colonized infants, the hands of a nurse and three dogs owned by other health care workers in the NICU (Chang et al., 1998). No particular subtype was associated with a collection site or a particular time-period, but multiple genotypes might have colonized the same neonatal patient.

The outbreak resolved upon implementation of infection control measures, including withdrawal of lipid-rich hand moisturisers for staff. In addition to this neonatal syndrome, *M. pachydermatis*

has been implicated in severe systemic infections of immunocompromised adult patients (Choudhury et al., 2014).
The lesson learnt from these outbreaks is that there is a clear need for rigorous hand hygiene by health care professionals in contact with pet dogs and cats potentially harbouring *Malassezia* yeasts, especially when there is contact with immunocompromised individuals (Bond et al., 2020). Additionally, it is known that, in general, both chlorhexidine and polyhexanide have excellent *in vitro* activity against *M. pachydermatis* (Banovic et al., 2013), but it could be possible that some strains can be resistant to these sanitizing compounds.

2.5 Pathogenesis of *Malassezia*

The microbiota plays an integral role in shaping physical and functional aspects of the skin. While a healthy microbiota contributes to the maintenance of immune homeostasis, dysbiosis can result in the development of diverse skin pathologies (Sparber et al., 2020).
Commensal fungi of the mammalian skin, such as those of the genus *Malassezia*, are associated with common inflammatory skin and ear disorders, in particular dermatitis and otitis externa in dogs. Nevertheless, understanding of the causative relationship between fungal commensalism and disease manifestation remains incomplete. In particular, the mechanisms of fungal recognition and the immune pathways that maintain commensalism and/or promote pathology remain to be defined. Because of the constant presence of *Malassezia* on the host, the immune system is continuously exposed to the fungus, which shapes the immune reactivity of the host locally and at distant sites, as reflected by *Malassezia*-responsive T cells and antibodies in the blood of healthy individuals (Ashbee et al., 2010a).

2.5.1 *Malassezia* colonization of the mouse skin

To study the interaction of *Malassezia spp*. with the mammalian skin *in vivo*, Sparber et al. (2019) established an infection model in C57BL/6 mice. Epicutaneous application of *Malassezia ssp*. onto the dorsal ear skin resulted in robust colonization of the skin on day 2 post infection. *M. pachydermatis* colonization of the epidermis in the mouse skin was accompanied by the immune response against the yeast. In fact, these authors found infiltration of leukocytes to the site of infection. In particular, consistent with the massive infiltration of neutrophils and monocytes, they detected a strong rise in the local expression of neutrophil and monocyte-recruiting chemokines and growth factors in the *Malassezia*-exposed skin. Moreover, when

analysing cytokine expression in the *Malassezia*-exposed skin, the authors observed a pronounced and rapid induction of IL-17 in response to *M. pachydermatis* (Figure 5).

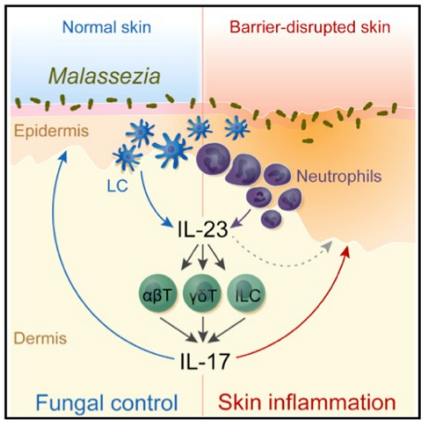


Figure 5. *Graphical abstract of the skin commensal yeast Malassezia that drives type 17 immunity in the skin. Sparber et al., 2019*

Another important cytokine is IL-23, which is key in preventing fungal overgrowth on the skin. These results demonstrate a critical role of the IL-23 and IL-17 axis in cutaneous immunity against *Malassezia*, as this axis promotes the inflammatory response in the barrier-disrupted skin, which mimics atopic dermatitis-like conditions (Sparber et al., 2019). In addition, the establishment of an animal model for *Malassezia* infection represents a major advancement in the field, as it opens the door to the future fungal-host interactions studies, which may allow a better understanding of the balance between cell host and microbes.

On the other hand, Merkel et al. (2018) have developed a minihost (invertebrate) experimental model wherein the pathogenicity of *M. pachydermatis* was evaluated in wild-type (WT) and Toll- deficient *Drosophila melanogaster*. WT flies were resistant to the infection, whereas Toll deficient flies showed inoculum-dependent mortality rates. Experimental models may provide valuable information on yeast virulence and host immune factors that are important in disease processes in various species.

2.5.2 Sensing of *Malassezia spp*. by the host

The interactions between *Malassezia* yeasts and the skin of their hosts, and the factors which influence transition from commensal to pathogenic microorganism, are the subject of intensive scientific endeavour (Gaitains et al., 2012). The presence of a nutritionally absorptive fungus within the stratum corneum exposes the host to an array of chemicals, immunogens and

allergens, comprising fungal cell wall-associated carbohydrates, proteins and lipids. Interaction with other commensal microbes might also influence pathogenicity and expression of virulence factors (Peleg et al., 2010). Thus, these commensal yeasts are likely highly regulated by continuous interactions with the host immune system and these interactions ultimately determine whether the outcome is inflammation (i.e. fungal disease) or not (Bond, 2019).

The specific attachment of the microbe to host cells, is a key step in colonisation and infection by commensal and pathogenic fungi. *Malassezia* cells adhering to keratinocytes have the potential to modulate the expression of an array of cytokines, chemokines and antimicrobial peptides, the outcome of which may be immune-stimulatory or immune-suppressive (Kistowska et al., 2014). A change in host immunity (i), altered skin microclimate (ii) or disruption in epidermal physiology (iii) associated with concomitant diseases may predispose animals to clinical disease. For example, co-proliferation of staphylococci in the same lesions may exacerbate clinical signs and necessitates concurrent antibacterial therapy in some cases (Rosales et al., 2005). *Malassezia pachydermatis* is thought to have a symbiotic relationship with commensal staphylococci, which produce mutually beneficial growth factors and micro-environmental alterations (Miller et al., 2013).
Therefore, the outcome of *Malassezia* growth in the stratum corneum is dependent upon the metabolic activities of the yeasts (expression of cell wall components and secreted virulence attributes), the host's innate and adaptive immune defensive responses and the interactions with other skin commensals (especially staphylococci) (Bond et al., 2019).

The evolution of genus *Malassezia* to lipid-dependency seems to be associated with a wide expansion of lipase and phospholipase encoding genes, and the loss of genes involved in carbohydrate metabolism (Schuster et al., 2017).Phospholipase activity in *M. pachydermatis* is stimulated by the endogenous opioid peptide ß endorphin present in the skin of dogs with dermatoses (Cafarchia et al., 2010). Such activity was found to be significantly higher among *M. pachydermatis* isolates derived from the dogs with otitis externa or skin lesions than among isolates obtained from dogs with healthy external ears, or non-lesional skin (Teramoto et al., 2015).
Laboratory data indicated that phospholipase production might act in synergism with biofilm formation to induce or exacerbate skin lesions in dogs (Figueredo et al., 2012).

2.5.3 *Malassezia*-induced immunity and immunopathology in the skin

The presence of *Malassezia* on the skin, both in normal and excessive numbers, is known to activate the skin immune system (Grice et al., 2017). *Malassezia* antigens can stimulate innate, antibody and cell mediated immune responses and triggering hypersensitivity reactions (Glatz et al., 2015a). In animals in which yeast overgrowth of organisms has occurred, or in individuals that are predisposed to allergic sensitization, the ensuing inflammatory response can lead to clinical signs such as dermatitis and pruritus.

Khantavee et al. (2019) investigated levels of allergen-specific IgE, IgG1, and IgG2 directed against *M. pachydermatis*, with total IgG levels, and correlated them with lesion severity in dogs with atopic dermatitis. They reported that specific IgE and total IgG against yeasts were significantly increased in atopic dogs of all ages. However, no significant relationships were found between the clinical score and any specific immunoglobulin levels for both microbe types (Guillot et al., 2020). Enhanced understanding of host-*Malassezia* interactions may contribute to improved diagnostic and therapeutic options for symptomatic dogs.

2.5.4 Activation of keratinocytes and recognition of *Malassezia spp*. by surface-bound receptors

The initial interplay between *Malassezia* and the skin immune system is likely to take place in the epidermis (Sparber et al., 2017). It has been demonstrated that application of *M. pachydermatis* suspensions on healthy dog skin can induce skin lesions similar to those observed in naturally occurring *Malassezia* dermatitis (Bond et al., 2004). This indicates that *Malassezia* cell surface markers or metabolic products derived from the yeast may be able to directly damage the skin or induce pathogenic effects by activating the skin immune system (Grice et al., 2017). In order for this to happen, antigens or allergens produced or expressed by *Malassezia* spp. would need to penetrate the stratum corneum, so that they can be recognised by Langerhans cells/tissue-resident dendritic cells (DCs), keratinocytes and macrophages, as well as by myeloid cells that are recruited to the skin under inflammatory conditions. These would then act as antigen presenting cells able to sensitise and then activate the T lymphocyte population. Moreover, an impaired barrier, which is the case in classic canine atopic dermatitis, is likely to facilitate transepidermal allergen penetration.

Keratinocytes recognise *Malassezia* antigens via Toll-like receptors (TLR) (Glatz et al., 2015b) and, in particular, TLR2 also contributes to fungal recognition by the host. TLR2 is implicated in sensing of *Malassezia spp*. and inducing a pro-inflammatory response characterized by the

release of cytokines, chemokines and antimicrobial peptides by keratinocytes (Baroni et al., 2006). Once activated, keratinocytes can alter their cytokine expression, with up-regulation of the immunosuppressive cytokines IL-10 and TGF-b and down-regulation of the inflammatory cytokine IL-1a (Ashbee, 2006). Keratinocytes activated by *Malassezia* antigens also produce antimicrobial substances (Donnarumma et al., 2004). In fact, the fungus is recognized by the host either directly through interaction of fungal cell wall components with membrane bound pattern recognition receptors (PRRs) or indirectly through soluble metabolites that are released by *Malassezia spp*.

The fungal cell wall is rich in carbohydrates, glycoproteins and it is surrounded by a lipid-rich outer layer (Mittag H., 1995) that are recognized by PRRs of the family of Syk-coupled C-type lectin receptor (CLR), which are expressed primarily by myeloid cells (Underhill et al., 2015). Binding to these receptors results in ligand internalization and activation of multiple signalling pathways, including the inflammasome (Sparber et al., 2017). Besides, activation of myeloid cells by *Malassezia spp.* induces the secretion of proinflammatory cytokines.
However, the relative contribution of individual receptors to fungal control during commensalism and in infectious setting remains to be clarified. Regarding the mature dendritic cells, which are key for the induction of adaptive immunity, they are mature antigen presenting cells which are capable of presenting peptides on MHC molecules to T cells (Banchereau et al., 1998).In addition, dendritic cells seem to be activated by interaction between Malassezia antigens and various members of the C type lectin class of receptors such as Mincle, Dectins 1 & 2, and Langerin. This results in the production of pro-inflammatory cytokines such as IL-1, IL-6, IL-8 and TNF-alfa (Figure 6) (Sparber et al., 2017).

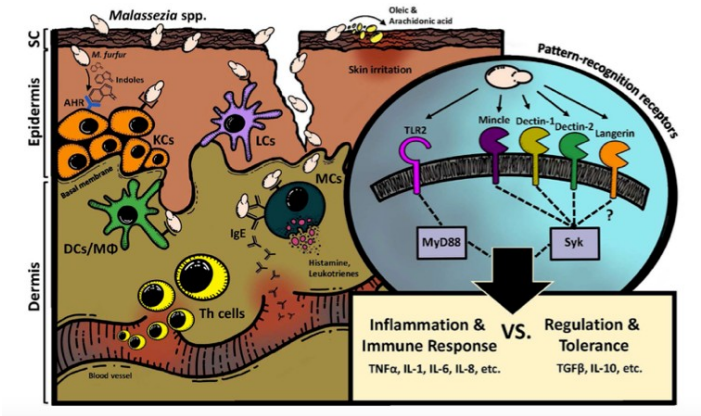


***Figure 6**. Interaction of Malassezia spp. with the mammalian skin. Sparber et al., 2017.*

Interestingly, dendritic cells that have been stimulated by *Malassezia* antigens appear to be resistant to lysis by natural killer cells, a mechanism that likely favours survival of the yeast in order to maintain antigen presentation (Buentke et al., 2002). Mincle binds to two distinct glycolipids in *Malassezia*, Dectin-2 recognizes the fungus through α-1,2-linked mannose. High-mannose binding is a general feature of Dectin-2, which is reported to recognize a variety of fungi (Brown et al., 2016).
Specific products of *Malassezia* metabolic pathways are thought to act as virulence factors promoting inflammation and pathology, while others downregulate the production of inflammatory mediators and thereby contribute to immune regulation. Fungal strains with altered production of such factors have been linked to *Malassezia*-associated skin disorders (Magiatis et al., 2013).

2.5.5 Innate immunity to *Malassezia spp.*

The majority of what is currently known about the host response to *Malassezia spp.* is based on *in vitro* studies with isolated myeloid cells or keratinocyte cell lines. Stimulation of these cells with *Malassezia* yeast leads to the induction of mainly pro-inflammatory cytokines, chemokines, and antimicrobial peptides (Ishikawa et al., 2013). Only few studies have examined regulatory cytokines such as IL-10 and TGF-β by the yeast (Yamasaki et al., 2009).
Given the association of *Malassezia spp*. with inflammatory skin disorders and allergic responses, the fungus may also interact with mast cells. Progenitor cell-derived mast cells from atopic patients show increased release of pro-inflammatory cytokines upon stimulation with *Malassezia* (Ribbing et al., 2011). Mast cells are directly activated by the fungus in a TLR2-dependent manner and release inflammatory mediators and cytokines. Moreover, the crosslinking of the high-affinity IgE receptor (FcεRI) by antigen-bound IgE can induce mast cell degranulation (Selander et al., 2009). Therefore, mast cells contribute to amplify the inflammatory response.

2.5.6 Adaptive immunity to *Malassezia spp.*

As a commensal, *Malassezia* interacts continuously with the immune system. Generally, the adaptive immune responses are heightened and qualitatively distinct in patients with *Malassezia*-associated diseases (Sparber et al., 2017).

2.5.6.1 Humoral responses to *Malassezia spp.*

As for humoral responses, *Malassezia*-specific antibodies are predominantly of the IgG and IgM isotypes (Ashbee et al., 2010a). In contrast, although *Malassezia*-specific IgE is not usually detected in healthy individuals, it is common in atopic patients (Johansson et al., 2003). A positive correlation was found between the sensitization to *Malassezia*-specific IgE and the severity of atopic dermatitis (Glatz et al., 2015b). However, whether the IgE response plays a pathogenic role in atopic and other *Malassezia*-associated inflammatory disorders or rather serves as a marker for the severity of disease remains unclear. In fact, as it would be expected in a typical immune response against an infectious agent, antibodies directed against antigens from *Malassezia* yeasts are produced in healthy dogs throughout life. IgM, IgG and IgA antibodies against Malassezia species are present in young and old dogs, but the amount of IgG and IgM tends to tail off with age corresponding with declining numbers of commensal yeasts (Cunningham et al., 1992). Using Western immunoblotting to detect IgG responses in dogs to extracts of *M. pachydermatis*, four proteins of 219, 110, 71 and 42 kDa were shown to be recognised mainly by dogs with *Malassezia* dermatitis when compared to healthy dogs (Bond et al., 2002).

In atopic dogs with or without cytological evidence of *M. pachydermatis* overgrowth, there are significantly higher serum titres of *Malassezia*-specific IgG than those seen in healthy dogs (Nuttall et al., 2001). However, no significant differences in IgG levels were found between atopic dogs with or without Malassezia overgrowth. By comparing the IgG response to *M. pachydermatis* antigens using Western immunoblotting, a protein of 25 kDa was identified in the majority of atopic dogs with *Malassezia* dermatitis, but only a few atopic dogs without *Malassezia* overgrowth and none of the normal dogs, suggesting that this protein may have some clinical relevance in the pathogenesis of *Malassezia* hypersensitivity (Chen et al., 2002). However, enhanced IgG responses can be seen in dogs with *Malassezia* dermatitis and in humans and dogs with atopic dermatitis. The role of this IgG response in the pathogenesis of skin disease is currently unclear in dogs. Anyway, these antibodies seem to be protective, in fact, IgG antibodies could activate the complement system, as it has been demonstrated with *M. furfur* and *M. globosa* and exacerbate the inflammatory response (Belew et al., 1980). Therefore, these antibodies could potentially provide a degree of protective immunity against *Malassezia* yeasts Alternatively, they might activate the complement system causing epidermal damage and inflammation.

Increased concentrations of *Malassezia*-specific IgE are frequently present in atopic dogs. The

development of allergen-specific IgE antibodies could lead to sensitization of cutaneous mast cells. Subsequent exposure to *Malassezia* allergens could trigger the release of inflammatory mediators, resulting in a type I hypersensitivity rection (Bond et al., 2019). IgE-mediated, or immediate-type hypersensitivity (ITH), to skin-associated microorganisms is recognized as an important factor in severity of atopic dermatitis (Santoro et al., 2015). In particular, immediate-type-hypersensitivity to *Malassezia* allergens is increasingly recognized in some dogs with atopic dermatitis (Farver et al., 2005). Moreover, dogs affected with atopic dermatitis are also frequently affected with recurrent otitis externa (Favrot et al., 2010).

However, dogs with recurrent *Malassezia* otitis had similar concentrations of allergen-specific IgE than those with healthy ears, suggesting that hypersensitivity is not always involved in such infections (Layne et al., 2016). Using Western immunoblotting to characterise individual antigen responses, proteins with molecular weights of 45, 52, 56 and 63 kDa from *M. pachydermatis* have been demonstrated to be major allergens in atopic dogs with *Malassezia* overgrowth. (Chen et al., 2002). The presence of *Malassezia*-specific IgE in dogs affected with a clinical problem commonly associated with atopic disease might be an indication that immediate-type hypersensitivity to *Malassezia* is present and might contribute to recurrence of otitis externa in IgE-positive affected dogs (Layne et al., 2016). These studies provide convincing evidence that proteins from *Malassezia* yeasts can act as allergens in dogs predisposed to the development of atopic dermatitis (Bond et al., 2019).

2.5.6.2 T Cell responses

T cell-mediated immunity responsiveness to *Malassezia* in patients with atopic dermatitis is associated with a Th2 response (Johansson et al, 2009), in line with the classical paradigm of Th2-polarized allergic T cells.

Therefore, a deficiency in cell-mediated responses could predispose to the overgrowth of *Malassezia*. *Malassezia*-derived antigens that permeate into and through the living epidermis are captured by epidermal Langerhan's cells and/or dermal dendritic antigen-presenting cells. These cells then migrate to regional lymph nodes and present the antigen to a T lymphocyte via a major histocompatibility complex (MHC) class II molecule

In cooperation with different cytokines, T helper (Th) 0 precursor cells differentiate into Th1 cells and/or Th2 cells.

A cytokine environment dominated by IL-12 would favour Th1 cell development, whereas IL-4 and IL-13 from Th2 cells. T helper cells activate B lymphocytes and stimulate them to

differentiate into antibody-forming plasma cells.

By secreting IL-2 and IFN-gamma, Th1 cells promote IgG production, whereas IL-4 and IL-13 from Th2 cells promote immunoglobulin class switching to IgE (Figure 7) (Bond et al., 2019).

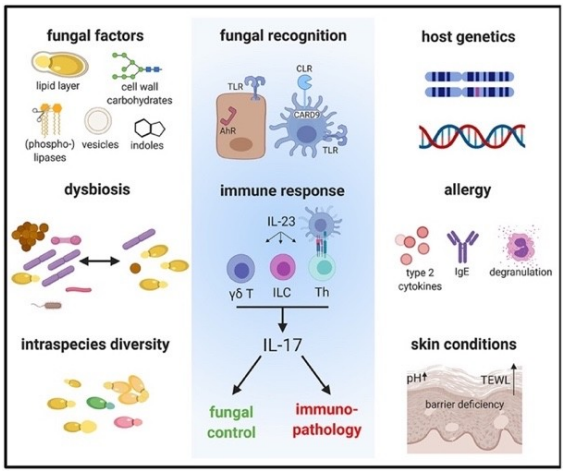


***Figure** 7. Overview of the immune response to Malassezia. Sparber et al., 2020.*

More recently, other T helper cell subsets such as Th17 and Th22 cells have been found enriched in allergic individuals (Cavani et al., 2012). Consistent with this notion, *Malassezia*-reactive skin harboring T cells from *Malassezia*- sensitized atopic dermatitis patients comprise not only Th1 and Th2 subsets but also IL-17- and IL-22-secreting cells (Balaji et al., 2011). Importantly, Th17 differentiation is a hallmark of T cell responses induced by CLR signalling (LeibundGut-Landmann et al., 2007). It is also unknown to which subset *Malassezia*-specific T cells belong in healthy individuals and to what extent T cell plasticity contributes to sensitization. Despite the possible role of T lymphocytes in protective immunity, there is evidence that these cells are involved in sensitization of humans and dogs that become allergic to the yeasts. Taken together, these findings provide compelling evidence that T lymphocytes play a pivotal role in the generation of hypersensitivity reactions to *Malassezia* species in genetically susceptible individuals (Bond et al., 2019).

3. Clinical disease: *Malassezia* dermatitis and otitis in dogs

3.1 Predisposing factors

Overgrowth of commensal *Malassezia* yeasts may occur due to alterations of the skin's surface microclimate, leading to inflammatory skin disease (Santoro et al., 2015). *Malassezia* dermatitis

in dogs is usually a secondary problem due to an underlying skin disease such as allergic disease, recurrent bacterial pyoderma and endocrine disease (Mauldin et al., 1997).
Many predisposing factors may result in the commensal *M. pachydermatis* becoming a pathogen. These factors increase a pet's risk of developing dermatitis and they include: increased moisture, altered surface lipids, and/or disruption of stratum corneum barrier function, aberrant immune responses, presence of skin folds and prolonged corticosteroid therapy (Hnilca, 2011).

Otitis is one of the most common and frustrating problems encountered in small animal practice, and *Malassezia* ranks among the most common fungal causes of otitis externa in dogs (Crespo et al., 2002). Actually, *Malassezia* is considered as a perpetuating factor of otitis externa that exacerbates the inflammatory process. It can induce permanent pathologic changes to the ear canal, which is the main reason for treatment failure in otitis externa (Kamaljyoti et al., 2017). Predisposing factors for development of *Malassezia* ear infection include excessive production of sebum and/or decreased quality of the sebum, high environmental temperature and accumulation of moisture, damage of epidermis, concurrent dermatoses, atopy and bacterial skin infections, anatomic breed characteristics, excessive hair in the ear canal and previous episodes of otitis (Patterson et al., 2002). All these predisposing factors increase the risk of developing otitis, usually by altering the ear canal environment in some way and making the ear more susceptible to primary cases (Clark, 2005). *Malassezia* ear infections could be secondary to other primary diseases. Griffin (2010) has proposed a new classification of otitis externa that divides the aetiology of the disease into primary causes, which are diseases or infections that directly cause inflammation in the ear, and secondary causes, which are predisposing or perpetuating factors (Tables 4 and 5) that contribute to ear disease (Griffin 2010).
Primary conditions, which often produce signs in addition to those of otitis, must be identified and treated successfully.These kinds of problems could include:
1. Immunologic dysfunctions (especially canine atopic disease, allergic disease). In particular, allergic disease is the most common primary cause of otitis. Otitis is reported as a sign in 55% of atopic and 80% of food-allergic dogs. Also, otitis may be the only sign in 5% of atopic and 25% of food-allergic dogs (Rosser et al., 2004). When such dysfunctions are present, the primary allergic disease should be managed as part of otitis therapy before the problem becomes chronic.
2. Parasites that can affect the ear, including ticks, fleas and mites. Common mites are Otodectes cynotis, Sarcoptes scabiei, Notoedres cati and Demodex canis. A careful otoscopic exam is necessary to identify the larger of these parasites.
3. Foreign bodies (for example plant awns).

4. Keratinisation disorders associated with otitis, such as hypothyroidism, sex hormone disorders, hyperadrenocorticism and idiopathic seborrhea in Cocker Spaniels.
5. Endocrinopathies (hypothyroidism, hyperadrenocorticism and diabetes mellitus).
6. Skin neoplasias.
7. Autoimmune diseases, such as pemphigus, which can produce signs in addition to those of otitis.

Predisposing Factors
Anatomic contributors
a. Pendulous ears
b. Stenotic canals
c. Increased ceruminous glands
d. Excessive hair
Environmental triggers
a. Increased humidity of ear canal
b. High environmental temperature and humidity
Swimming
Previous otitis
a. Abnormal epithelium
b. Stenotic canals
c. Failure of epithelian migration
Tumors or growths
Primary Factors
a. Allergies
b. Parasites
c. Foreign bodies
d. Keratinization disorders
e. Autoimmune diseases
Perpetuating Factors
a. Chronic changes of the ear canal
b. Otitis media
c. Chronic Infection

***Table 4**. Causes of otitis. Clark (2005)*

Anatomic breed features such as pendulous ears and narrow (Cocker Spaniels, Labrador Retrievers, Basset Hounds, Golden Retrievers, Irish Setters) or stenotic ear canals (Shar- Peis) can be predisposing factors. Long ears fold and cover the ear canal, preventing air from entering and drying it. So, the result is a moist, warm ear canal that is the perfect environment for microbial growth (Burnham McQuillan, 2005). In addition, Labrador Retrivers and Cocker Spaniels have higher numbers of ceruminous glands which produce more earwax. Some breeds grow excessive hair inside ear canal (Poodles and Lhasa Apsos) which is sometimes listed as a predisposing cause since increased moisture in the ear canal and, in some cases, slower drying. Some researchers have reported higher predisposition of male than female dogs to Malassezia otitis (Chaudhary and Mirakhur, 2002). Besides, *Malassezia* infection is more prevalent in dogs

of 1-3 years age. Season wise, the prevalence of *Malassezia* infection is highest during the most humid, sultry and warm months that are those from spring to autumn season. Anyway, an effective medical approach to this complicated problem starts with a thorough understanding of its causes (Figure 8).

As regards dermatitis, gender and age do not appear to be consistently correlated with the presence of *Malassezia* dermatitis, but breed predilections in dogs have been described in several studies. As for breeds, most studies have identified the following breeds as particularly susceptible to *Malassezia* dermatitis: West Highland white terriers, English setters, Shih tzus, Basset hounds, American cocker spaniels, Boxers, Dachshunds, Poodles and Australian silky terriers (Mauldin et al., 1997). Breeds with conformations that favour skin folds are also prone to infections at intertriginous anatomical sites. This likely reflects local climatic differences involving factors such as reduced air movement, increased skin temperature and humidity, retained secretions and surface frictional trauma (Jenkinson, 1992).
Although not specifically studied in the dog, it is also generally recognized that *Malassezia* dermatitis is more common in tropical climates, and during warm, humid months in more temperate latitudes, reflecting external environmental effects on the skin microbiota (Theelen et al., 2018).

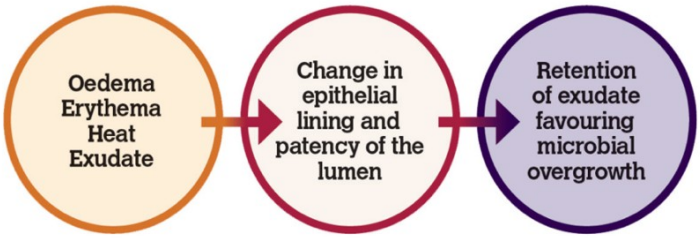


***Figure 8**. Pathophysiological changes lead to otitis externa. Patel A., 2020*

3.2 Perpetuating factors

Perpetuating factors typically come into play after predisposing and primary factors have resulted in otitis, and they can cause the disease to continue even after the primary problems are resolved (Table 5).
These perpetuating factors, such as chronic infection, otitis media and chronic anatomic changes in the ear canal, must be identified and resolved. A severely hyperplastic, stenotic and calcified ear is no longer a candidate for medical therapy.

Otitis media may be present in up to 80 % of dogs with chronic otitis, and the tympanic membrane may or may not be intact in these cases (Gotthelf, 2004). Chronic infection by other microorganisms is a common perpetuating factor. In addition, bacteria and yeast can be involved, either alone or in combination in the same animal.

Table 5. *Important perpetuating factors in otitis. Paterson (2016)*

Pathological changes in the external ear canal	Inflammation in walls leading to loss of epithelial migration Inflammation of glandular tissue leading to narrowing of the canal and increased cerumen production
Otitis media	Ruptured ear drum Mucopurulent discharge due to inflammation of mucoperiosteum with bulla Biofilm formation within middle ear

When we talk about yeasts infections, and in particular of those caused by *Malassezia*, the main predisposing disorders are skin and ear skin diseases. *Malassezia* dermatitis (Figure 9) is common in dogs and affected sites include lip margins, ear canals, axillae, groin, ventral neck, interdigital skin, facial folds or tail folds, perivulvar skin, and perianal skin (Pattersonet al., 2002). Lesions may be localized or generalised. Pruritus, a major sign, is usually severe and is accompanied by unpleasant smell. Skin lesions may present in various forms, which can be affected by chronicity of disease, primary underlying disease, previous therapy, and concurrent bacterial infection (Bajwa, 2017).

Figure 9*. Erythema and greasy seborrhea due to Malassezia (canine). Photo taken by me*

As said above, the sites most frequently colonized by this yeast in dogs of various breeds are the peri-oral/ lip region and interdigital skin (Figure 10). Common presentations of *Malassezia* dermatitis include:

1. Regional or generalized alopecia with erythema (exfoliative erythroderma).
2. Scaly, waxy, or greasy seborrhea (yellow or slate gray).
3. Crusts or papulocrustous lesions resembling superficial staphylococcal infection.
4. Lichenification and/or hyperpigmentation (leathery or elephant-like skin).
5. Paronychia with dark brown nail bed discoloration, with or without obsessive paw chewing.
6. Lip margin hypotrichosis and/or crusting.
7. Intertrigo.
8. Lesions consistent with self-trauma due to pruritus.
9. Malodor.

In chronic cases, hyperpigmentation and other secondary lesions due to scratching and licking have been reported (Dorogi, 2002).

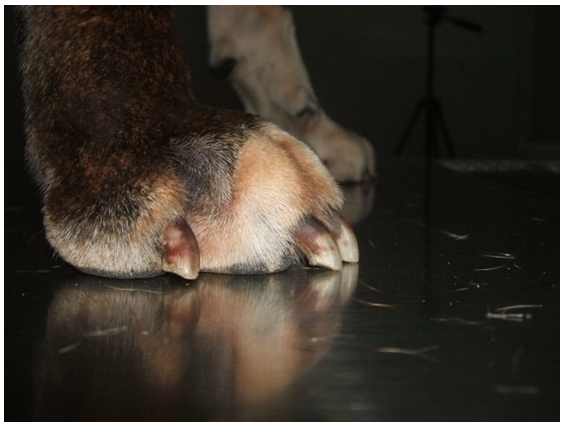


***Figure 10**. Onychomycosis caused by Malassezia pachydermatis in a dog. Chiappani, 2018.*

The external ear canal is similar in structure to the interfollicular epidermis of the skin. It is a stratified cornifying epithelium with adnexal organs, such as hair follicles and their associated sebaceous and ceruminous glands. Therefore, any disease that affects the skin can also affect the external ear canals (Figure 11) (Zamankhan et al., 2010).

Otitis is one of the most common conditions among dogs and the condition can be caused by multifactorial etiology as bacteria, fungi and/or parasites. Otitis caused by *Malassezia spp.* are particularly challenging as they cannot respond to routine medical procedures for bacterial and fungal infections (Zamankhan et al., 2010).

Infection with either yeast and/or bacteria does not occur in a normal ear, as the environment inside the external ear canals of most dogs is sterile. Infection develops because of inflammation produced by primary factors, usually in combination with perpetuating and predisposing factors. As otitis progresses, the inflammation created by primary factors leads to changes in the ear canal leading to modification of the micro-nvironment and changes in the microbial populations (Petrov et al., 2013).

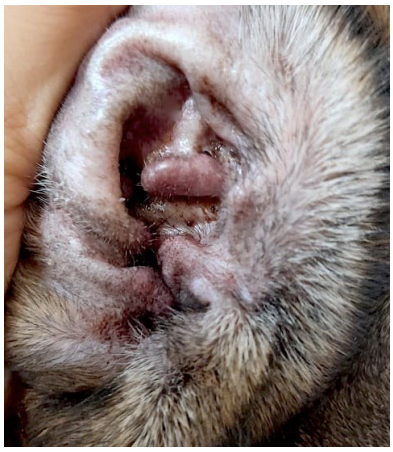


***Figure 11**. Malassezia otitis externa, secondary to atopic dermatitis (dogs). Photo taken by me*

Malassezia spp. are normal commensals and occasional pathogens of the cutaneous flora of dog and cat and a prevalence of canine otitis cases was up to 40% and with a higher prevalence (67.74%) of M. pachydermatis (Bugden, 2013).

The prevalence of *Malassezia* otitis changes widely across studies. For example, Karlapudi et al. (2017), during a specific study with 62 dogs that presented signs suggestive of ear infection, identified by microscopic and culture-based techniques 18 cases where *M. pachydermatis* was the primary etiological agent. Canine otitis is the inflammation of a dog's ear. Otitis externa is a chronic inflammation of external ear canal and it is the most common ear disease of dogs, affecting up to 20% of the dog population. In particular, *M. pachydermatis* is the most common fungal etiologic agent of chronic otitis externa in dogs.
Whereas, otitis media is an inflammation of middle ear. With otitis media, exudates can fill the middle ear cavity and must be flushed out manually. When necessary, the appropriate myringotomy incision is made, avoiding the malleus attachment. If the tympanic membrane is ruptured, ototoxic substances and drugs can enter the middle ear and affect the sensitive structures of the inner ear (Figure 12).

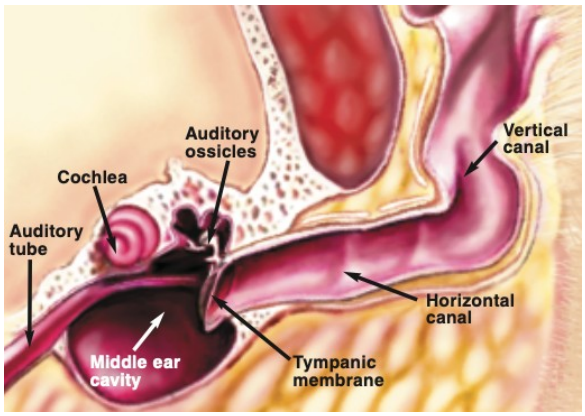


***Figure 12**. Inside the ear, The anatomic structure of the ear canal. McQuillan B.B., 2005*

True primary pathogens are rare, and the vast majority of infections are secondary to pre-existing inflammation, foreign bodies, obstruction or other primary problems (Zamankhan et al., 2010).

Clinically, ear infections among dogs can be divided into erythroceruminous or suppurative otitis. Erythroceruminous otitis is most commonly associated with *Staphylococcus* or *Malassezia spp*. and is characterised by erythema, pruritus and a ceruminous to seborrhoeic discharge (blackish waxy ear discharge). In contrast, the most frequent manifestations associated with suppurative otitis are erythema, ulceration, pain and a purulent discharge, which are most commonly caused by *Pseudomonas* species (Griffin, 2010).
The main important clinical presentations canine otitis are characterised by (Figure 13):
1. Localized or generalized erythema and edema of ear pinna with foul odour. Sometimes, the ear canal is filled up with dirt and thickened wax, and it can also present signs of dermatitis, pruritus, alopecia and dry scabby lesions.
2. Frequent itching.
3. Discharge of different colours and varied viscosity along with strong odour of decomposing fat.
4. Signs of scratching of ears/ unilateral dropping pinna.
5. Shacking of head with occasional tilting for varied period.
6. Signs of going in circles.
Moreover, the production of cerumen is severely increased in all cases of otitis externa, and *Malassezia* is more likely to be cultured from ears with increased cerumen.

Figure 13. *Malassezia otitis in English Bulldog. Photo taken by me*

3.3. Examples of clinical cases of *Malassezia spp.*

As reported above, otitis externa (OE) is one of the most common disorders to affect dogs. A 1995 prevalence study involving over 31,000 dogs from 52 general veterinary practices in the United States concluded that canine OE was the third most common disorder with a prevalence of 13% (Lund et al., 1999).

Álvarez-Pérez et al. (2016) reported a list of numerous clinic cases of canine otitis attended between 2013 to 2014 at the Clinical Veterinary Hospital of Complutense University of Madrid (Spain). Below, there are some examples of clinical cases included in that paper:

Case 1.

Date: March 2013

Species, breed: Dog, Golden Retriever

Sex and age (years): Female, 11

Diagnosis: Chronic bilateral otitis (Figure 14)

Microbiological analysis: *Malassezia pachydermatis* (right ear) and *Staphylococcus spp*. (right and left ear)

Previous treatment: Antimicrobials;

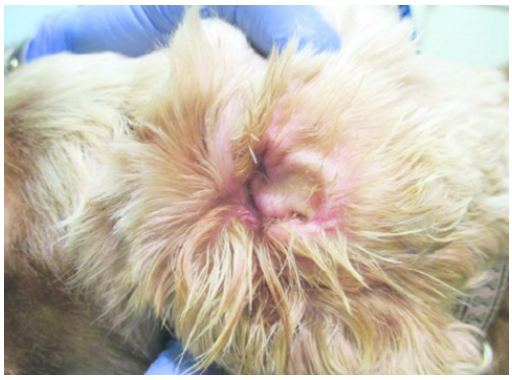


Figure 14. *Chronic otitis in Golden Retriever. Barnard et al., 2017*

Case 2.

Date: May 2013

Species, breed: Dog, Cocker Spaniel

Sex and age (years): Male, 2

Diagnosis: Chronic bilateral otitis (Figure 15)

Microbiological analysis: *Malassezia pachydermatis* (right ear)

Previous treatment: Prednisone, ear drops (marbofloxacin, clotrimazole and dexamethasone)

Other concomitant conditions: Allergy;

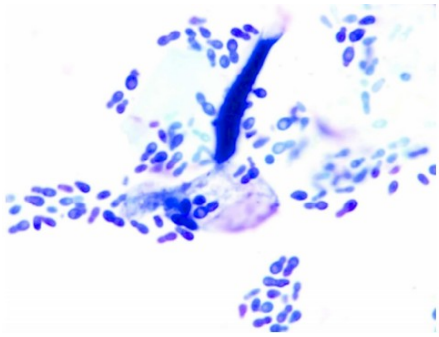


Figure 15*. Chronic otitis in Cocker Spaniel, Ear Citology Yeast. Barnard et al., 2017*

Case 3.

Date: September 2013

Species, breed: Dog, West Highland White Terrier

Sex and age (years): Male, 9

Diagnosis: Bilateral suppurative otitis (Figure 16)

Microbiological analysis: *Malassezia pachydermatis* (right and left ear)

Previous treatment: Cortisone;

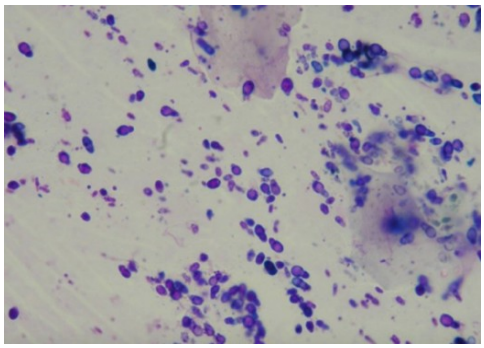


***Figure 16**. Yeast organisms revealed on cytology of purulent otic exudate. Chiapatti, 2018.*

Case 4.

Date: October 2013

Species, breed: Dog, Basset Hound

Sex and age (years): Female, 4

Diagnosis: Unilateral otitis

Microbiological analysis: *Malassezia pachydermatis* (left ear) (Figure 17)

Previous treatment: Systemic antibiotics, ear drops (marbofloxacin, clotrimazole and dexamethasone), otic cleansers;

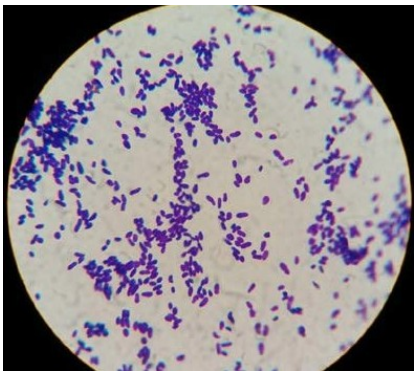


***Figure 17**. Microscopic morphology of Malassezia pachydermatis (Gram staining). Metiner et al., 2016*

Case 5.

Date: May 2013

Species, breed: Dog, Beagle

Sex and age (years): Female, 8

Diagnosis: Chronic bilateral otitis

Microbiological analysis: *Malassezia pachydermatis* (right ear) and *Staphylococcus spp.,* Streptococcus spp. (left ear)

The same animal in the case 5, after one year was still presenting ear problems:

Date: March 2014
Species, breed: Dog, Beagle
Sex and age (years): Female, 9
Diagnosis: Bilateral otitis
Microbiological analysis: *Malassezia pachydermatis* (left ear) and *Streptococcus spp*. (left ear)

4. Diagnostic approach in the veterinary clinic

Malassezia dermatitis and otitis in dogs have evolved from an obscure and controversial disease, to now being included in the routine diagnosis of most general veterinary clinics. Clinical signs are well recognised and diagnostic approaches are well developed (Bond et al., 2020), as shown below. Anyway, the first step in diagnosing and treating ear disease is to correctly perform the clinical examination.

4.1 Diagnosis of *Malassezia* dermatitis

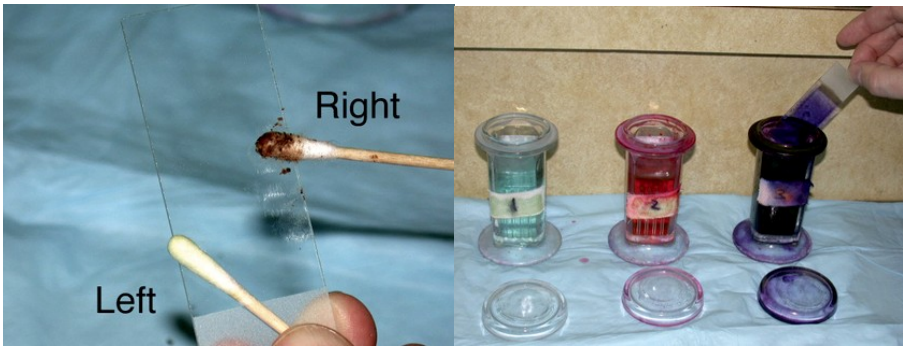
The assessment of the presence and number of *Malassezia* species is an important step in the characterisation of the cutaneous ecosystem of healthy and symptomatic dogs and cats. Studies utilising traditional cytological and cultural methods have clearly demonstrated that *Malassezia* yeasts are normal inhabitants of healthy canine skin and mucosae (Gustafson, 1955).

Researchers and clinicians have developed a range of semi-quantitative and quantitative methods for enumeration of yeasts in skin, some of which have important applications in veterinary clinical practice for routine diagnosis, assessment of response to therapy, and in research and development of novel therapeutic agents and formulations.
A diagnosis of *Malassezia* dermatitis cannot be made by histopathology analysis of the stratum corneum alone. Molecular techniques are pivotal in the accurate identification of many of the currently recognised *Malassezia* species, with the usual exception of *M. pachydermatis* (which is readily distinguished from the other species by growth on conventional Sabouraud's dextrose agar without lipid supplementation).

Following the original elegant description of tape-stripping method in human dermatology (Keddie et al., 1961), this method has gained wide acceptance in veterinary clinical practice as a rapid and versatile procedure for recovering stratum corneum cells and the microbes associated to them (Maynard et al., 2011). Light microscopical examination (40–50 or 100x oil objectives)

of tape- strips (Figure 18), or dry scrapes, stained with modified Wright Giemsa stain ("Diff-Quik" or generic equivalents) is rapid and convenient for assessment of the presence and numbers of *Malassezia* yeasts (Moraru et al., 2019). Regardless the procedure used, it is important to establish whether *Malassezia* yeasts can be identified cytologically in lesional areas.

***Figure 18**. Swab use to collect sample and preparation with Diff-Quik. Angus, 2016.*



Delayed-type hypersensitivity (type IV) against yeast allergens is also known to exist in dogs with *Malassezia* dermatitis (Darabi et al., 2009). Other options for assessing the role of immediate-type hypersensitivity to *Malassezia*, and other allergens, in recurrent *Malassezia* otitis externa include intradermal test reactivity and response to allergen-specific immunotherapy.
In any case, the most useful and practical method of diagnosis of *Malassezia* dermatitis is cytologic examination (Mauldin et al., 1997). Samples collected using glass slide impression, acetate tape impression, superficial skin scraping, or cotton swab method are evaluated under the microscope. If present, yeast organisms are often observed in clusters or adhered to keratinocytes (Miller et al., 2013). Ultimately the diagnosis of *Malassezia* dermatitis should rely on the combination of clinical presentation and cutaneous cytology.

4.2 Diagnostic of *Malassezia* otitis

The normal environment of the ear canal is important in maintaining ear health. Layne et al. (2016), during a study aimed to determinate how frequently *Malassezia*-specific IgE could be detected in the sera of dogs with recurrent *Malassezia* otitis externa, demonstrated that *Malassezia*-specific IgE tests cannot differentiate between patient groups, so such tests should not be used to diagnose otitis externa due to *Malassezia*. In contrast, multiple studies have reported a positive correlation between *Malassezia*-specific IgE levels and clinical severity of "head and neck dermatitis" for *M. furfur* in humans, with as many as 100% of affected patients

having increased *Malassezia*- specific IgE (Bayrou et al., 2005). It is possible that measuring *Malassezia*-specific IgE during active otitis externa would increase the number of positive samples. Another limitation of the retrospective nature of the study of Layne et al. (2016), was the investigators' inability to assess clinical and cytologic disease severity. Affected dogs positive for *Malassezia*-specific IgE might have had more clinically severe disease than the dogs without *Malassezia*-specific IgE. Because *Malassezia pachydermatis* is a commensal organism of canine ears it could have been useful to compare quantitative cytologic results for all affected dogs in order to determine if increased yeast number was related to presence of *Malassezia*-specific IgE (Campbell et al., 2010). Otitis disrupts the local environment of the ear canal. Epithelial changes include hyperplasia and hyperkeratosis. Moreover, glandular changes lead to changes in secretions and cerumen (Figure 19).
In otitis, there is failure of epithelial migration, increased cerumen with decreased lipid content, increased humidity and a higher pH in the ear canal. More severe and deeper changes occur over time and include stenosis, fibrosis and sometimes ossification of the ear canal (Clark, 2005).
A complete medical history, general physical exam and thorough otoscopic exam are necessary to discover and diagnose these problems. It is important to select initial cleaners that are safe for use with a ruptured tympanic membrane until one can see that it is intact. Cytology is the primary method of identifying these infections. In chronic and recurring cases involving bacteria, culture and sensitivity testing is necessary to determine treatment.

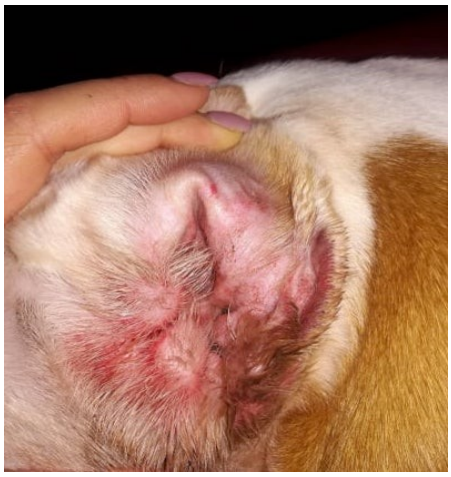


Figure 19. *Otitis externa with signs of scratching of ears. Picture taken by me*

Cytology using swabs of lesions rolled onto glass slides is normally best restricted to use in the ear canal, as the yield of squames and yeast from the skin is inferior to that obtained by tape

strips and dry scrapes (Bond and Sant, 1993; White et al., 1998; Bensignor and Carlotti, 1999). In an effort to improve upon the sensitivity of cytological sampling for *M. pachydermatis* in the canine ear, Puig et al. (2019) have developed a quantitative PCR method based on amplification of the single copy ß-tubulin gene. The authors judged that the results were accurate and showed improved sensitivity over cytology; this method may have useful applications in diagnosis and therapeutic monitoring, and in studies of pathogenesis and therapeutic product development (Guillot et al., 2020). There can be episodes of *Malassezia* in which otitis externa is defined by the clinical signs of otitis externa combined with demonstration of yeast presence in ear swab cytology. Diagnosis of *Malassezia* otitis externa in dogs, however, is generally straightforward based on clinical and cytologic criteria (Miller et al., 2013).

Overall, the presence of *Malassezia*-specific IgE in dogs affected with a clinical problem commonly associated with atopic disease might be an indication that immediate-type hypersensitivity to *Malassezia* is present and might contribute to recurrence of otitis externa in IgE-positive affected dogs (Layne et al., 2016). In addition, there are breeds of dogs that have a well-documented predisposition to atopic dermatitis and has been reported in some studies as more frequently affected with *Malassezia* otitis externa than with other pathogens (Favrot et al., 2010).

It is well-recognized that serum IgE against environmental allergens does not always correlate with clinical signs and cannot be used to diagnose atopic dermatitis (Pucheu-Haston et al., 2015). The presence of allergen-specific IgE in healthy dogs does not however negate the potential importance of those tests for dogs diagnosed with atopic dermatitis, particularly for the formulation of allergen-specific immunotherapy (Olivry et al., 2015).

Similarly, it appears that *Malassezia*-specific IgE also does not necessarily correlate with clinical signs of *Malassezia*-related skin or ear disease. *Malassezia*-specific IgE has previously been detected in the sera of healthy dogs at a level not significantly different from that detected in the sera of atopic dogs affected with *Malassezia* dermatitis (Farver et al., 2005).

4.2.1 Routine physical examination of ears in dogs

Routine physical examination of the dog's ears in search for disease symptoms are as follows:

1. Examination of the pinna

Evaluation of the dorsal and ventral surfaces of the pinna visually and by lightly touching them with the fingers. The presence of small swellings or thickened areas should not be overlooked.

Attention should be paid to unexpected high temperature, sensitivity and changes in tissue as indications of inflammation. The margin of the pinna should also be examined in search for possible hair loss and the presence of lesions with scales or crusting.

2. Check the external ear canal

Next, lift the pinna toward the dorsal midline to evaluate the external ear canal. Beginning with a visual exam, determine the presence of masses. Check for exudate and note the color, amount and whether an odor is present. Palpate the outer wall of the vertical canal for flexibility and to determine whether the canal has narrowed. Suspected otits media may also be an indication for radiographs.

3. Break out the otoscope

The otoscopic examination provides a detailed view of the ear canal. Pets may need to be sedated or anesthetized. Otoscopic exams are necessary for all pets, even healthy ones. However, they are particularly important for pets who have characteristic clinical signs of otitis externa. To conduct an otoscopic exam, first select the appropriate ear cone size. For pets with healthy ears, choose the largest cone that fits well in the ear and does not cause discomfort. For an inflamed ear, choose a cone that fits without rubbing against the canal wall to minimize pain. It is important to remember that this is one of the most painful examinations for pets.

Elevate the pinna dorsally and introduce the cone's tip into the external portion of the vertical canal. If proper traction and direction are not applied, the canal cannot be clearly observed. Continue guiding the cone deeper into the canal to view the horizontal portion. Note the colour and health of the entire canal, as well as the colour and consistency of any discharge and tissue pathology. Note any ear canal abnormalities, including masses, ulcerations and foreign bodies, in the medical records.

4. View the tympanic membrane

The tympanic membrane, or eardrum, should be visible during the otoscopic examination. If it is not, consider the following possibilities: Proper tension is not being applied to the pinna (i), exudate or a foreign body is obstructing the view (ii), the ear cone is too short (iii), stenosis or fibrosis is prohibiting the ear cone (iv), the eardrum is ruptured and only a black area is visible (v). Sedation may be necessary with fibrosis or if the dog is too painful. Healthy eardrums are flat or slightly concave, semitranslucent, light gray and somewhat glistening. A diseased eardrum may have a convex bulge from fluid collection in the middle ear. It also may be opaque

and have an erythematous rim at the margins. In cases of trauma or advanced disease, the tympanic membrane may be only partially formed or missing altogether.

4.2.2 Collection of cytology samples

Diagnostic tests are essential to identify the cause of infection and treat and manage ear disease. Do not prescribe medication or clean the ear until have collected a cytology sample to help identify the presence of mites, yeast, neoplastic cells or bacteria.

First, lift the pinna and insert the tip of a cotton swab into the vertical portion of the canal. Gently roll the swab tip against the canal wall to obtain the material needed.
Once collected a sample, roll the swab against a glass slide. Do not make the material too thick or smear evaluation will be difficult. Perform the cytologic staining procedures. Examine the dried slide under oil immersion to determine whether yeast, bacteria or fungi are present. To see mites or eggs, examine the slide on low-power magnification.
Organisms found in the ear include the following:

a) *Yeast* (Figure 20) (*Malassezia pachydermatis*);

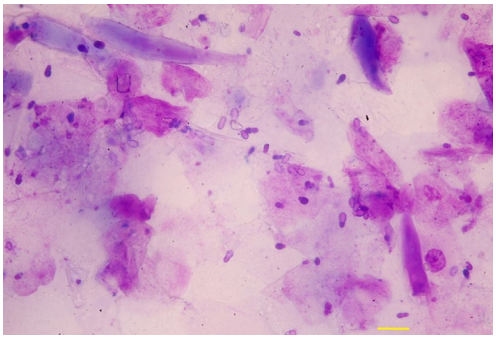


***Figure 20**. Numerous Malassezia yeasts visible on skin cytological sample (adhesive tape preparation, 40×). Angileri et al., 2019.*

b) *Bacteria*: When found on an ear swab, bacteria can be normal or secondary flora. *Staphylococcus intermedius* is the most common bacteria in otitis externa and media, but *Pseudomonas*, *Proteus* and *Corynebacterium* species and *Escherichia coli* are also found. If the percentage of bacteria is beyond normal flora numbers or if there is a prevalence of Gram-negative rods, the sample should be submitted for bacterial culturing. Bacterial culture should

also be considered if the ear infection does not resolve with standard therapy (Figure 21).

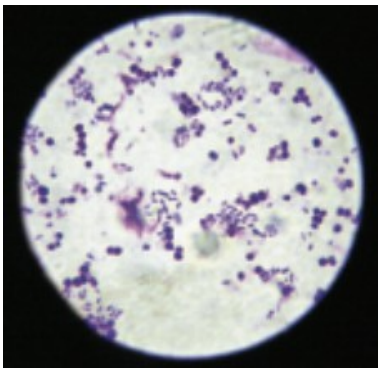


Figure 21. *Mixed rod-shaped and cocci bacteria (1,000x): Severe infection identified using Wright's stain. McQuillan, 2005*

c) Ear mites: Applying a few drops of mineral oil to the cotton-tipped swab before placing it in the ear canal allows for better sample collection. Roll that swab into a small well of mineral oil on a glass slide and place a cover slip over the area. When collecting a culture sample, watch for signs of a bacterial infection, such as odor or a yellowish-green debris.
Perform a culture if bacteria are present, if bacterial numbers are above the normal flora range, if rods and cocci are present or if polymorphic bacteria are in the ear (Figure 22);

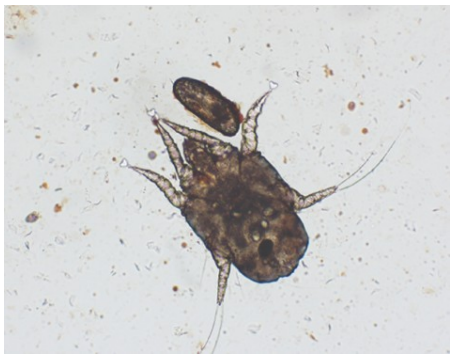


Figure 22*. An image of an ear mite (Otodectes cynotis). McQuillan, 2005.*

4.2.3 Evalutation of otoscope cone disinfection techniques

Malassezia yeasts can be very easily carried by different fomites. Human hands bring also the question of a risk of transmission of resistant/tolerant strains from infected individuals (Bourdeau et al., 2008).
However, very sparse information is available on the susceptibility of *M. pachydermatis* to common disinfection methods (Madrid et al., 2013). There is a concern that otoscope cones could serve as a carrier for the spread of infection (Korkmaz et al., 2013).

Otoscope cones are used in veterinary medical practices to examine healthy or infected ears in dogs and cats. At this respect, Rethore et al. (2019) evaluated the efficacy of different disinfection procedures applied on otoscope cones contaminated with *Malassezia pachydermatis*. Otoscope cones were soaked for 10 min either in ethanol, chlorhexidine, hydrogen peroxide, peracetic acid disinfectant, enilconazole or water (positive control). They were then paper wiped or left to air dry. Finally, each otoscope cone was sampled with two swabs that were inoculated on modified Dixon's agar and incubated at 32◦C. On day 3, the number of Colony Forming Unit (CFU) was counted.

At the end of the experimentation, enilconazole 0.4%and 0.8% were perfectly efficient and gave negative results also. These results demonstrate that disinfection by soaking for 10 minutes the otoscope cones in common disinfectants (with or without paper wiping) can be efficient against *M. pachydermatis* for all products tested, except enilconazole 0.2%.

Overall, the usual disinfectants are efficient against *M.pachydermatis*, these results demonstrate that disinfection by soaking the otoscope cones for 10 minutes in the disinfectant bath should be enough. Moreover, as in every disinfection procedure, an optimal method for application in the clinical setting requires thorough physical cleaning to remove any organic debris and lipid substrates prior disinfection, since these elements have a huge impact on the efficacy of most disinfectants (Sauders et al., 2012).

5. Managing of ear and skin diseases of caused by *Malassezia* in dogs

As yeast (and bacterial) infections of the skin and ear canals are thought to be secondary problems in most cases, it is universally agreed that it is of utmost importance to identify and resolve the primary underlying condition (Scott et al., 2013). On the other hand, it is essential to remember that in some cases, especially in predisposed breeds, there is no identifiable underlying cause and the dog's skin disease resolves completely with antifungal therapy (Chen et al., 2005). The antifungals most commonly used the treatment of *Malassezia* infections in dogs belong to various chemical classes with different mechanisms of action (Table 6) (Peano et al., 2020).

***Table 6**. Antifungal agents used to treat Malassezia in the dog. Peano et al., 2020*

Mechanism of Action	Class	Agent	Use *	Notes
Interaction with sterol-14α-demethylase, involved in the biosynthesis of ergosterol	Azole derivatives (imidazoles)	KTZ	O/T	Recognised clinical efficacy for canine *Malassezia* dermatitis (oral administration).
		MCZ	T	Principally available in aural formulations. Available in formulations for dermatological use in many countries.
		CTZ	T	Principally available in aural formulations.
		ECZ	T	-
	Azole derivatives (triazoles)	ITZ	O	Recognised clinical efficacy for canine *Malassezia* dermatitis (oral administration).
		FCZ	O	Traditionally employed in the case of systemic mycoses. One study has demonstrated some efficacy in treating *Malassezia* dermatitis.
		PSZ	T	Used mainly in human medicine for the treatment of invasive fungal infections. An aural PSZ-based formulation for dogs has also been recently marketed.
Action against membrane sterols	Polyene macrolides	NYS	T	Principally available in aural formulations.
Perturbation of fungal sterol synthesis by inhibiting squalene epoxidase	Allylamines	TER	O/T	Found effective for *Malassezia* dermatitis with oral treatment in some studies. Available for topical use in some countries.
Inhibition of microtubule assembly	Benzimidazoles	TBZ	T	Principally available in aural formulations.

*KTZ = ketoconazole; MCZ = miconazole; CTZ = clotrimazole; ECZ = econazole; ITZ = itraconazole; FCZ = fluconazole; PSZ = posaconazole; NYS = nystatin; TER = terbinafine; TBZ = thiabendazole. * The availability of formulations containing the different agents varies according to country. (O = oral; T = Topical)*

Several antifungal agents belong to the azole class, which exert their inhibitory function by interacting with the sterol-14α-demethylase, a fungal enzyme involved in the biosynthesis of ergosterol (Vanden Bossche et al., 2003).

These agents represent the most common choice for treating *Malassezia* otitis and *Malassezia* dermatitis in dogs. Many of the agents mentioned above are marketed for veterinary use, particularly in products for topical application, such as shampoos, dermatological solutions, and aural formulations. Generally, aural formulations are products which also contain glucocorticoids and antibiotics. Veterinary medicines containing these agents are less widely available for systemic use. Therefore, formulations for human medicine are often used off-label (Vanden Bossche et al., 2003; Bond, 2006). Polyene macrolides such as amphotericin B (AMB), though very active against *M. pachydermatis* (Theelen et al., 2018), are not employed to treat *Malassezia* dermatitis and *Malassezia* otitis in dogs due to their high toxicity, the difficulty of usage (an intravenous administration is necessary), and elevated cost.

5.1 Treatment of *Malassezia* otitis in dogs

The key steps in successful therapy of otitis are to diagnose and manage primary skin diseases affecting the ear, identify and treat secondary infections and then recognise any perpetuating or predisposing factors and manage them to prevent recurrence of disease. Options for the treatment of *Malassezia* dermatitis and otitis include, in addition to various antiseptics such as selenium sulphide and chlorhexidine, systemic and topical therapy with several antifungal agents.

5.1.1 Topical ear treatment

Topical otic products form an integral part of the overall management of otitis externa.
Topical agents include antibiotics, antifungals and anti-inflammatories; many commercial products are combinations of these.
Product choice should be based on cytology results, the condition of the tympanic membrane and the amount of inflammation. In recurrent or resistant cases, bacterial culture and sensitivity testing helps identify the correct treatment (Clark, 2005).
Allergy is the most common of the primary triggers and can account for up to 75% of all cases (Paterson, 2002). If otitis is part of a more generalised allergic skin disease, then management of the ear disease can often be achieved using systemic drugs or with allergen-specific immunotherapy. In some dogs, allergy only affects the ears, or affects the ears more severely than other areas. In other cases, systemic medication is inadequate in controlling allergic otitis and additional therapy is needed. In such situations, topical drugs can be used as the sole source of therapy or can be used to supplement other modalities such as allergen-specific immunotherapy (Colombo et al., 2007). The allergic reaction itself is best treated with anti-inflammatory therapy. In fact, topical products containing either glucocorticoids (Rougier et al., 2005) or tacrolimus (Kelley et al., 2010) have been described as useful forms of anti-inflammatory therapy in dogs, for both allergic and immune-mediated disease.
Licensed veterinary ear products containing glucocorticoids range from potent anti-inflammatory agents such as mometasone, hydrocortisone aceponate, fluocinolone, dexamethasone and betamethasone to moderately potent drugs such as triamcinolone acetonide and prednisolone (Koch et al., 2012). Almost without exception these glucocorticoids are found as components of compound products that also contain an antibiotic and an antiyeast drug.
Glucocorticoid-only drops tend to be human products used off-license for dogs and cats. An exception is fluocinolone acetonide combined with dimethyl sulfoxide (DMSO). DMSO has many potential effects, but in this preparation specifically acts as a drug delivery system to carry

the corticosteroid into the skin and is particularly suited to treating hyperplastic ear conditions (Parterson, 2016).Two studies looking at the anti-inflammatory effects of glucocorticoids in otitis showed that topical prednisolone produced a significant reduction in ear thickness and erythema (Bolinder et al., 2006) but, when compared to dexamethasone, prednisolone was less effective in reducing pain, production of exudate, and odour (Rougier et al., 2005).
Almost without exception these glucocorticoids are found as components of compound products that also contain an antibiotic and antifungal compound (Paterson, 2016).

As for tacrolimus, it is a macrolide lactone drug labelled for use in human patients with moderate to severe atopic dermatitis. Recently tacrolimus ointment has been found to be effective in treating refractory non-infectious otitis in humans (Lennon & Fenton, 2010). In contrast, there are limited reports of its use in dogs and cats. One report has shown that when a sterile olive oil-based 0·1% tacrolimus suspension was used in the ears of atopic beagles without otitis externa it produced no signs of adverse local reactions, development of otitis externa, change in otic cytology, vestibular dysfunction or hearing loss (Kelley et al., 2010).

The choice of an appropriate corticosteroid preparation should be based on a variety of factors, including the potency of the glucocorticoid that is required for therapy, the potency and concentration of the glucocorticoid in the topical preparation, the base that the glucocorticoid is in and the potential for systemic absorption of the drug relative to the animal's general health and the length of course that is needed.
As parasites, and in particular *Otodects cynotis*, can be primary agents of otitis in dogs, the use of topical acaricidal drug is often required. Demodectic otitis externa is an uncommon primary trigger for ear disease. Most products contain a recognised acaricide but a number of non-acaricidal products can be effective as well (Scherk-Nixon et al., 1997).

Nystatin is the principal polyene antifungal found in veterinary ear drops. It works by binding to sterols in the fungal cell membrane, leading to changes in permeability and fungal death due to osmotic destruction. Nystatin is available as a combination ear product in both Europe and America. In Europe it is combined with framycetin, prednisolone and fusidic acid, in America it is combined with triamcinolone acetonide, neomycin and thiostepton.

Azole antifungal drugs disrupt the biosynthesis of the ergosterol in the fungal cell wall. Topical azoles are available in ear products as imidazoles (clotrimazole, miconazole, ketoconazole) or triazoles (itraconazole, posaconazole). All of the azoles have excellent *in vitro* activity against

Malassezia species (Chiavassa et al., 2014). Several studies which have used different methodologies have tried to establish the relative potency of the different azoles. One study suggested that itraconazole was the most potent followed by ketoconazole, miconazole and clotrimazole (Lorenzini et al., 1985). However, another *in vitro* study suggested ketoconazole, itraconazole and terbinafine were equipotent (Gupta et al., 2000). More recently a comparison of miconazole and clotrimazole suggested miconazole was the more potent (Peano et al., 2012). The triazole posaconazole was found to be more effective than three other antifungal drugs (miconazole, clotrimazole and nystatin) when used to treat *Malassezia* infection in dogs (Bordeau et al., 2004). A study looking at a product combining marbofloxacin, clotrimazole and dexamethasone showed that although drops consisting of miconazole only treated the Malassezia as well as the combined drops, the dexamethasone-based product reduced signs of erythema, pruritus and wax production.

Allylamines disrupt ergosterol biosynthesis and prevent fungal cell wall formation. Terbinafine is the most widely used product in this class and has recently become available as a long acting veterinary ear drop combined with betamethasone, licensed for once weekly aural application for two consecutive weeks per month (Paterson, 2016).

5.1.2 Topical therapy to manage predisposing factors

Although predisposing factors do not cause otitis, they need to be managed to promote resolution of disease and prevent its recurrence.

Many of the veterinary ear cleaners have excellent antibacterial and antifungal activity, but their ability to effective clean and dry the ear is often overlooked. Hairy ear canals, stenotic ear canals and dogs with pendulous ears need to have their ears cleaned effectively. Where animals with predisposing conformational problems such as these have primary triggers, e.g. allergy causing inflammation within the ear canal, the prudent use of antibacterial ear flushes can help to reduce the risk of secondary infection.

Effective ear cleaning is also important in to remove wax, which allows the penetration of other drugs and helps the assessment of the integrity of the tympanic membrane (Paterson, 2016). First, cleaning may be necessary to visualize the canal and eardrum adequately. It also removes exudates that cause inflammation, interfere with topical medications and act as a barrier between the tissues and medications. The intensity of the cleaning process should be adjusted to the

severity of the case and amount of exudate present.
The simplest method is to fill the ear canal with cleaning solution and gently massage the base of the ear, then gently wipe away the solution and dislodged exudates with a soft cloth tissue or cotton ball. When necessary, the pet should be sedated or anesthetized to clean the ear thoroughly. An otoscope with an operating head, a red rubber or tomcat catheter and a syringe for this procedure are needed.
Attach the catheter to the syringe and clean the ear canal with gentle flushing and suctioning. In addition, when the status of the tympanum is not known, it is important to use warmed saline or water. Use the otoscope for visualization while flushing the deeper ear canal to avoid damage to the tympanum. Nevertheless, in most cases it is difficult to compare the exact reason for the in vitro activity of the ear cleaners tested, because these vary widely in their ingredients, and often include several components with proven antifungal activity (Figure 23).

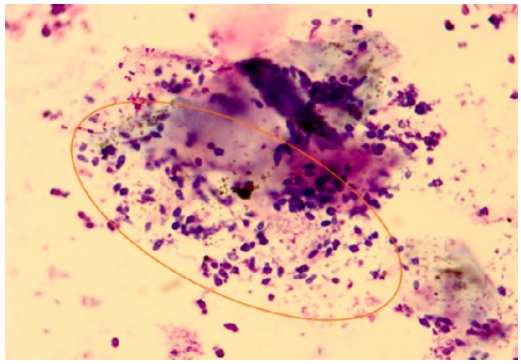


***Figure 23**. Dense population of Malassezia in a cytology smear of ear cerumen, Kamaljyoti 2017.*

The azole compound ketoconazole has good activity against *Malassezia*, as demonstrated by different studies testing ear cleaners (Mason et al., 2013). Isopropyl alcohol and propylene glycol (Larson & Morton, 1991) may also provide antimicrobial benefits.
Chlorhexidine at a concentration of 2 to 4% is an antiseptic with good activity against *Malassezia* as demonstrated by several shampoo studies (Bond et al., 1995; Lloyd & Lamport 1999). In addition, chlorhexidine at a concentration of 0.15% is found in several European and American ear cleaning products and has good activity against both Gram-positive and Gram-negative organisms (Steen & Paterson, 2012).

The ability to effective clean ears to remove wax is an important part of the treatment and then ongoing maintenance therapy for otitis. Cleaning agents can be classified as ceruminolytic or drying agents. Ceruminolytic agents break down cerumen and cells, and may be oil-based

(squalene, propylene glycol, glycerin and mineral oil) or water-based (dioctyl sodium sulfosuccinate, calcium sulfosuccinate, and carbamate and urea peroxide). Truly ceruminolytic ear cleaners disrupt the integrity of cerumen by inducing lysis of the squames (Robinson et al., 1989).

Squalene is the most potent of these wax-removing agents and it is found in several veterinary ear cleaners. Squalene deserves special mention because it is unlikely to cause ototoxicity, although oil-based preparations can accumulate in the ear canal and may require additional cleaning. Some cleaners have it present at low concentrations of 2% whilst other more potent cerumino-solvent cleaners have squalene at concentrations of 22 to 25% (Koch et al., 2012).
In all cases, antiseptic ear cleaning solutions that contain components such as chlorhexidine, without a recognised ceruminolytic, show poor ability to remove wax.

Astringents dry the ear canal surface to prevent maceration (Nuttall & Cole, 2004). Drying agents are typically used after the ear has been cleaned with a ceruminolytic/ceruminosolvent product or are used prophylactically after water is introduced into the ear canal either by aqueous-based treatments, bathing or swimming (Koch et al., 2012). Astringents are usually isopropyl alcohol or an acid.
Organic acids such as lactic acid, salicylic acid, acetic acid, boric acid, oleic acid and citric acid are ingredients in many of the cleaners showing good anti-yeast activity because of all of which tend to decrease the pH and humidity. In Europe, drying agents are often incorporated into more general ear cleaning products and many preparations combine ceruminolytics and drying agents. Regular cleaning at home by the client is important for controlling and preventing chronic otitis.

5.1.3 Topical therapy to manage perpetuating factors

Management of perpetuating factors is important to facilitate resolution of otitis. Chronic change is often inadequately addressed in cases of otitis and is a common reason for disease relapse. Where there is marked hyperplastic change of the walls of the canal and/or the glandular tissue within the wall, potent steroid treatment is needed to reverse these changes.
Some of the liquid bandages, ear packs and other “stay in place” otics, together with topical corticosteroids in ear drops, in aqueous solution in ear wicks or systemic corticosteroids are useful (Harvey & Paterson, 2014).

Otitis media is a common sequel to chronic otitis externa and it is another perpetuating factor that needs to be treated. Propylene glycol, a solvent and penetration enhancer, is a common component of many different ear drops and cleaners. This compound has been shown to be ototoxic when instilled into the middle ear (Little et al., 1991). Ear drops and cleaners containing propylene glycol should therefore be used with care if the ear drum cannot be seen.
Anecdotal reports suggest that a 2 to 2.5% solution of acetic acid solution is safe in the face of a ruptured tympanic membrane (Rosychuk, 1994). Other topical aminoglycosides such as tobramycin, as well as the semisynthetic penicillin ticarcillin, have been associated with severe hearing loss when used to treat otitis media (Paterson & Payne, 2008). Aqueous solutions of fluoroquinolones including enrofloxacin and marbofloxacin appear to be safe when used in the canine middle ear (Paterson & Payne, 2008; Paterson, 2012). Conflicting information appears to be available regarding the use of topical azoles in cases of otitis media.

Many products commonly used in the external ear can be toxic to the inner ear if the tympanum is ruptured (Table 7). Therefore, when choosing the optimal treatment for a particular case, the potential ototoxicity of all the product's ingredients should be considered. It is important to know which products can be used safely with a ruptured tympanum and in the middle ear. The safest cleaning solutions are saline and water. Two percent acetic acid and 2 percent boric acid, along with tris EDTA (Gotthelf, 2004) are also considered safe in the presence of a ruptured tympanum. Conflicting information appears to be available regarding the use of topical azoles in cases of otitis media, most of the commonly used antifungals appear to be safe in the middle ear, including nystatin, thiabendazole, clotrimazole, miconazole, ketoconazole, itraconazole and terbinafine (Morris, 2004). Chlorhexidine at concentrations up to 0.2% appears to be safe as an irrigating solution in dogs (Merchant, 1994).

Table 7. *Ototoxic Products. Ettinger et al. (2004)*

Antibiotics	Antiseptics	Ceruminolytics and Cleaners
Aminoglycosides	Chlorhexidine	Propylene glycol
Polymyxins	Iodines and iodophors	Detergents, surfactants
Minocycline	Alcohols	Salicylates
Erythromycin	Benzalkonium chloride	
Chloramphenicol		
Vancomycin		

5.1.4 Systemic ear therapy

Systemic medications are sometimes used to treat otitis, especially systemic corticosteroids that decrease inflammation, so they are especially useful in cases with considerable swelling, hyperplasia and proliferation of the ear canal (Clark, 2005). However, corticosteroids are sometimes necessary as part of the management regime for atopy.

Systemic formulations of some antifungals, such as ketoconazole and itraconazole, are sometimes indicated in otitis treatment. Though there is some controversy regarding their efficacy (Ettinger et al., 2004) they are indicated when yeasts are involved in otitis media, when there is associated dermal yeast infection or when there are marked proliferative changes in the ear canal.

5.2 Treatment of *Malassezia* dermatitis in dogs

Multiple treatment options for *Malassezia* dermatitis are available. It is preferable to use a combination of topical and systemic therapy in order to achieve rapid and complete remission of clinical signs. Prescribing topical or systemic therapy alone may be adequate for some patients. Often, treatment of *Malassezia* dermatitis is accompanied by other recommendations such as a dietary elimination trial, antibiotic therapy, and antipruritic therapy. Follow-up examination is usually recommended 3 to 4 weeks after initiation of treatment in order to evaluate clinical response and re-evaluate cytological *Malassezia* numbers.

For mild cases or localized lesions, frequent topical therapy with antifungal products containing ingredients such as 2% ketoconazole, 1% ketoconazole- 2% chlorhexidine, 2% miconazole, 2% climbazole, 2% chlorhexidine, 3% chlorhexidine, 2% miconazole-2% chlorhexidine, 2% lime sulfur, 0.2% enilconazole, or 1% selenium sulfide is usually effective (Miller et al., 2013). Shampoos containing two active ingredients may provide better efficacy (Hnilca, 2011). Medicated antifungal wipes or pads such as those containing 0.3% chlorhexidine, 0.5% climbazole, and Tris- EDTA solution are effective against *M. pachydermatis* (Cavana et al., 2015a).

For patients with generalized or multifocal lesions, oral antifungal therapy in combination with topical therapy is most effective. Oral antifungal drugs effective against *Malassezia* include ketoconazole, fluconazole, terbinafine, and itraconazole (Patterson et al., 2002).

Patient factors such as age, clinical history, underlying or concomitant disease, and breed predisposition should be considered before use of systemic drugs.

Among the various treatments used to treat *Malassezia* dermatitis in dogs, strong evidence is

available only for the use of 2% miconazole and 2% chlorhexidine shampoos, used twice weekly (Bond et al., 2020). Moderate evidence is available for a 3% chlorhexidine shampoo and ketoconazole at 5-10 mg/kg orally once or twice daily and itraconazole at 5 mg/kg orally once daily or two consecutive days per week (Bond et al., 2020). In addition, topical treatments are preferred to systemic treatments for long-term therapy because of a lower risk of toxicity.
Topical prevention of *Malassezia* dermatitis in dogs might be achieved using 2% chlorhexidine/2% miconazole or 3% chlorhexidine shampoo twice weekly, as has been previously recommended for treatment. Pulsed therapy with itraconazole (5 mg/kg orally once daily, two days on/five days off for three weeks) has been found to be efficacious in the treatment of *Malassezia* dermatitis in dogs and thus should be effective as a preventative.
In dogs, persistent or recurrent *Malassezia* dermatitis, are usually associated with failure to identify and correct predisposing and perpetuating factors.

5.2.1 Efficacy of a 2% climbazole shampoo for reducing *Malassezia* skin infection

Cavana et al. (2005b), has undertaken a study in which the objective was to find out if shampoo therapy, often recommended for the control of *Malassezia* overgrowth in dogs, is useful for controlling yeast infection. They wanted to evaluate the *in vivo* activity of a 2% climbazole shampoo against *Malassezia pachydermatis* yeasts in naturally infected dogs.
Shampoo therapy is especially suitable for generalized *Malassezia* infection but may also be beneficial as a preventive to decrease the recurrence rate (Miller et al., 2013).

However, only few studies have evaluated the effectiveness of shampoos against *Malassezia* yeasts in dogs. The efficacy of shampoos containing from 2% to 4% chlorhexidine was demonstrated *in vitro* (Lloyd et al., 1999) as well as *in vivo* in association with miconazole (Maynard et al., 2011) or alone.
Climbazole is a member of the azole chemical group and is incorporated in some veterinary products (shampoos or wipes). It has been found effective *in vitro* against *Malassezia* yeasts (Schmidt et al., 1996). In dogs, a 3% chlorhexidine and 0.5% climbazole shampoo was effective to reduce *Malassezia* yeasts in naturally infected dogs (Bourdeau et al., 2011).
The present study demonstrated clearly that a 2% climbazole shampoo application is effective to reduce *M. pachydermatis* population sizes on canine skin. The climbazole shampoo was quick and effective in decreasing *Malassezia* population sizes on the skin of all dogs with a significant reduction percentage already one and five hours after one shampoo application. Significant

reduction (> 60%) was maintained in the four days following the first application. In clinical practice, the use of antifungal shampoos is commonly recommended twice or thrice weekly initially tapering to once weekly depending on clinical response. However, there is no accepted regimen for use of antimicrobial shampoos in superficial skin infections (Maynard et al., 2011). In the present study, a weekly climbazole shampoo application for two weeks achieved a *Malassezia* reduction of 94% after the second application and 15 days after the last shampoo a *Malassezia* CFU reduction of 69% was found. A shampoo that allows to control *Malassezia* populations with a limited number of applications could decrease therapy costs and favour owner compliance, a key factor in course of local therapy.

In conclusion, the present study showed that once a week application of 2% climbazole shampoo is effective to reduce *M. pachydermatis* population sizes on canine skin. This shampoo may be useful, alone or in association with systemic antifungal therapy, for treatment of *Malassezia* dermatitis. It may be also proposed as prevention to decrease yeasts CFU if recurrences of *Malassezia* dermatitis are frequent (Cavana et al., 2015b).

6. Mechanisms of possible drug resistance in *M. pachydermatis*

Antimicrobial resistance has emerged as a serious threat to human and animal health. Recent publications (Brilhante et al., 2018; Schlemmer et al., 2019) support previous observations that most wild-type *Malassezia* yeasts remain susceptible to commonly used azole drugs such as itraconazole, ketoconazole and miconazole, although efficacy of fluconazole is more variable (Velegraki et al., 2004; Weiler et al., 2013). In particular, itraconazole and ketoconazole are the most active agents against all *Malassezia* species, while fluconazole and voriconazole are the least active (Theelen et al., 2018). In *M. pachydermatis* isolates from canine otitis externa, synergistic interactions have been reported between caspofungin and itraconazole or fluconazole (Schlemmer et al., 2019), whereas amphoterecin B antagonized the activity of itraconazole and voriconazole in some of the stains tested (50% and 6.7%, respectively; n=30), but not fluconazole or posaconazole (Álvarez-Pérez et al., 2019).

The development of newer improved techniques for antifungal susceptibility testing of *Malassezia* species, such as the colorimetric-based assay recently proposed by Leong et al. (2017), may reduce incubation times and increase the sensibility of the checkerboard approach for detecting the effect(s) of antifungal combinations on this group of slow-growing yeasts and thus lead to a more accurate determination of MIC results. Furthermore, the Etest, which is a rapid and user-friendly way of determining antimicrobial sensitivity by placing a strip

impregnanted with antimicrobials onto an agar plate, has shown a great potential for routine antifungal susceptibility testing of *M. pachydermatis*.

Of greater concern are recent sporadic reports of therapeutic failure with azoles in cases of canine *M. pachydermatis* dermatitis that were associated with increased azole tolerance *in vitro*. For example, Angileri et al. (2019) isolated *M. pachydermatis* from an azole- unresponsive toy poodle that had MICs that were several folds higher when compared with strains from untreated dogs. Previous reports have highlighted varied *in vitro* drug susceptibility results among the different *Malassezia* species and within genotypes of selected species. In particular, in the case that Italian clinical isolated of *M. pachydermatis* from canine skin lesions had low susceptibility to azoles compared with those from normal canine skin (Cafarchia et al., 2012c).

This might reflect the chronic and relapsing course of *Malassezia* dermatitis and otitis that often necessitate frequent and lengthy treatment courses. MICs were higher in *M. pachydermatis* isolates from chronic otitis dogs that had been previously treated with various topical ear products containing miconazole and clotrimazole (Chiavassa et al., 2014; Watanabe et al., 2014). Further data are urgently required to establish whether topical therapies are preferable to systemic treatments in this context, and to guide antimicrobial stewardship policies for antifungal therapy in small animal practice.

Regarding the mechanisms of antifungal resistance, Jesus et al. (2011), using the technique described by Fekete-Forgács et al. (2000) (which consists of exposing a microorganism to increasing concentrations of a drug in a broth medium) reported a dramatic increase in the MICs of FCZ, KTZ, and ITZ against 30 isolates of *M. pachydermatis*. Interestingly, they exposed the isolates only to FCZ, which exhibits the possibility of cross-resistance phenomena among various azoles. Similar results were obtained in another study (Cafarchia et al., 2012b) using the same approach (Fekete-Forgács et al., 2000) to induce “resistance” in one isolate that was used to investigate the suitability of different growth media in a broth-dilution procedure.

Nakano et al. (2005) obtained increased MICs using a different approach. They performed 30 subcultures in the presence of drug concentrations (KTZ, NYS, TER) around the MIC. Other studies generated mutants resistant/tolerant to NYS (Uchida et al., 1994), KTZ (Kim et al., 2018), and MCZ to investigate possible mechanisms of resistance using the following methods: exposure to N-methyl-N’-nitrosoguanidine and UV radiation (Uchida et al., 1994) and serial subcultures of a yeast colony on a solid medium containing increasing concentrations of KTZ and MCZ (Peano et al., 2020).

Uchida et al. (1994) showed that the quantity of membrane sterols in NYS-resistant mutants of the reference strain CBS 1879 (resistance induced *in vitro*) was significantly decreased compared to the original strain. As sterols are the main target of polyene antifungal agents which include NYS (Vanden Bossche et al., 2003). Interestingly, the proportion of fecosterol in mutants was significantly increased, which may suggest a mechanism of resistance similar to the one described for *Candida* against azoles: the development of bypass pathways, through which ergosterol is replaced by its precursor 14a-methylfecosterol, with the latter product leading to still-functional membranes (Kanafani et al., 2008).

Another study (Iatta et al., 2016) reported that the defence mechanisms against azoles by *M. pachydermatis* might depend on efflux pumps - a common mechanism of azole resistance in *Candida* species (Sanguinetti et al., 2005) particularly those belonging to the "major facilitator superfamily". This was shown by the combination of FCZ and a substance able to inhibit these pumps (haloperidol (HAL)), resulting in an increased *in vitro* drug activity (MIC for 14 isolates: without HAL 8–512 µg/mL; with HAL 2–64 µg/mL).

Kano et al. (2019) showed that an isolate with proved clinical resistance had missense mutations in the ERG11 gene that encodes lanosterol 14 α demethylase, the target site for antifungal azoles (Kano et al., 2019).

Another recently proposed possibility is that of chromosomal rearrangement and gene over-expression, which are quite common mechanisms of resistance in other fungal species (Feng et al., 2017).

A final consideration regards the possibility that a lower azole susceptibility occurs due to the uptake of exogenous sterols provided by sebum or cerumen. The ability to import and use host sterol when ergosterol biosynthesis is blocked is considered a likely cause of the azole resistance typical of certain yeast species, such as *Candida glabrata* (Bard et al., 2005). This mechanism of resistance may be present in the *Malassezia* species, too, as the members of this species cannot produce fatty acids themselves but need lipids from the environment for growth (Theelen et al., 2018). Overall, these findings indicate that *M. pachydermatis* is capable of developing resistance mechanisms that is present in some strains of *Malassezia spp.*

Concern over azole resistance has prompted heightened interest in alternative antifungal agents. There are reports of *in vitro* efficacy against *M. pachydermatis* of a honey-based gel (Oliveira et al., 2018), and narasin, a polyether ionophor originally marketed as anti- coccidials and growth-promoting modifiers of the bovine rumen flora (Chan et al., 2019). Multiple recent publications have explored the potential antifungal utility of essential oils (Manion and Widder, 2017).

There are recent reports of synergistic interactions between essential oil components and azoles or nystatin against *M. pachydermatis*, including carvacrol and miconazole or nystatin, thymol and nystatin (Schlemmer et al., 2019), and between clotrimazole and essential oils of *Melaleuca alternifolia*, *Mentha piperita,* and *Origanum vulgare* (Bohmova et al., 2019).

6.1 Resistance to Azoles

The primary target of azoles is the haeme protein, which cocatalyses cytochrome P-450-dependent 14-α demethylation of lanosterol. Inhibition of 14α- demethylase leads to depletion of ergosterol and accumulation of sterol precursors resulting in the formation of a plasma membrane leading to increased cellular permeability.
It seems that azole resistance develops gradually as a result of sequential alterations due to the continuous pressure exerted by the drug.

6.2 Resistance to Polyenes

The target is the fungal membrane. They bind to ergosterol leading to the formation of aqueous pores leading to enhanced permeability to protons, leakage of vital cytoplasmic components and ultimately, death of the organism (Canuto et al., 2002).
Potential molecular mechanisms of polyene resistance include the decrease in the total ergosterol content of the cell, replacement of some or all of the polyene-binding sterols, and reorientation or masking of existing ergosterol. The emergence of polyene resistant fungi due to both qualitative and quantitative changes in the sterols of the cell membrane has been reported. Mutant strains of *Malassezia pachydermatis* resistant to nystatin produced with UV radiation have been documented (Uchida et al., 1994).

6.3 Resistance to Allylamines

The antifungal action is mediated by inhibition of squalene epoxidase in the early steps of ergosterol biosynthesis leading to accumulation of the sterol precursor squalene, even if resistance to allylamines has been reported only rarely (Robson, 2007).

6.4. Biofilm development

Biofilms are differentiated microorganism communities formed by a single microbial agent or by a mixture of fungal and bacterial species. Biofilms adhere to a biotic or abiotic surfaces, and its structure contributes to the innate physical and chemical resistance of the microorganisms (Costerton et al., 1999).

Some studies (Brilhante et al., 2018; Figueredo et al., 2013) have demonstrated that most isolates of *M. pachydermatis* can produce biofilms and, in this form, the yeast has significantly reduced antifungal susceptibility. Some authors gave these results great clinical importance. For example, Figueredo et al. (2013) stated, "It is known that canine *Malassezia* infections are usually chronic, with conventional therapy largely ineffective. This may be due to the ability of *Malassezia* to form biofilms, consequently requiring higher drug concentrations than are currently used to cure infection".

Most importantly, the biofilm was obtained only *in vitro*, in microplate wells (Figueredo et al., 2013) or on the surface of segments of catheters (Jerzsele et al., 2014). In fact, a biofilm can adhere to surfaces (catheter, implant or dead tissue) and colonize them. Moreover, the biofilm resists removal by fluid movement and have a decreased susceptibility to antimicrobials (Robson, 2007). Catheters are frequently used in modern medicine, particularly in intensive care units. However, such use may contribute to local and systemic infections. In fact, catheter-related fungemia due to *M. pachydermatis* has been described in premature neonates (Mickelsen et al., 1988) and immunodeficient children and adults most of them receiving parenteral nutrition (Weiss et al., 1991). Cannizzo et al. (2007), have reported a study in which they evaluated the ability of *M. pachydermatis* to form biofilms *in vitro* on a polystyrene microplate model and on polyurethane catheters commonly used in medical practice. Biofilm formation showed variability among the different strains studied but was unrelated to the clinical origin isolates or the genotype of the strains. Mature *M. pachydermatis* biofilms, evident on the intraluminal surfaces of both venous and gastro-duodenal catheters, showed a heterogeneous architecture.

It is necessary to clarify whether and how often *M. pachydermatis* can produce a biofilm on the skin and in the ears of dogs. The authors believe that this possibility should be explored with regard to, in particular, the ear localisation (Peano et al., 2020).

A quite common finding in the course of otitis, especially in chronic forms, is the presence in the ear canal of abundant material composed of exudates, cerumen, and debris in which bacteria and yeasts can be harboured. It is considered of fundamental importance to apply medicaments able to penetrate (or remove) this material to allow antimicrobials to reach effective local

concentrations (Morris et al., 2004). According to Nuttall et al. (2016), this material would represent an actual biofilm. In particular, the biofilm would be easy to recognise clinically as an adherent, thick, and slimy discharge that is frequently dark brown or black, and on cytology as a variably thick veil-like material including bacteria and cells. In the case of *Malassezia* otitis, it is common to detect, on cytology from ear discharge, clumps including numerous yeast cells and amorphous material (Figure 24). These findings may be due to the active overgrowth of the yeasts which remain "entrapped" in the cerumen and debris. However, it would be interesting to investigate the possibility that, at least in some cases, these clumps correspond to the sites of the formation of actual biofilm (Peano et al., 2020).

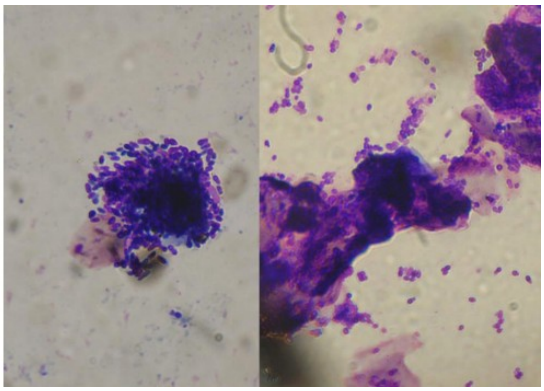


Figure 24. *Cytology from ear discharge in two dogs with Malassezia otitis. Clumps including numerous yeast cells (400-fold magnification, Hemacolor® rapid staining kit). Peano et al., 2020*

The ability to form biofilms has been associated with infections caused by other pathogens and it is considered an important virulence factor (Donlan et al., 2002).
Biofilm formation is different in timing and structure among different bacterial, protozoan and fungal species studied (Ramage et al., 2001).
In addition, *M. pachydermatis* biofilms required 5 days to develop in contrast to the 48h needed by *C. albicans* biofilms (Douglas et al., 2002) and they are structurally simpler than those of C. *albicans* (Chandra et al., 2001).
Multifactorial mechanisms of resistance in fungal biofilms constitute a broad-spectrum defence that is effective against many types of antifungal agents.

7. Antifungal susceptibility testing in *Malassezia* species

In vitro susceptibility testing is critical for the selection of optimal treatment strategies and the control of drug-resistant pathogens, especially when the microorganism's susceptibility cannot

be reliably predicted by its species identity (Bond et al., 2020).
The Clinical and Laboratory Standards Institute (CLSI) regularly publishes reference standards for antigungal susceptibility testing of yeasts (CLSI, 2008), but *Malassezia pachydermatis* does not exhibit sufficient growth in the standard defined (and lipid-free) RPMI 1640 medium to permit use of the reference method for this species.
Modified Etest protocols and conventional disc diffusion have also been used to determine the in vitro susceptibility of *M. pachydermatis*. These tests are more suited to routine diagnostic use than broth dilution methods (Nijima et al., 2011), but they can be less reproducible. The CLSI recommended medium (Mueller-Hinton agar supplemented with glucose and methylene blue) optimally supports the growth of *M. pachydermatis* provided the inoculum is incubated with a lipid source (Tween 40 or 80).
Breakpoints are usually defined as "specific values of parameters such as minimum inhibitory concentrations (MICs) or inhibition zone diameters on the basis of which microbes can be assigned to the clinical categories "susceptible (sensitive)", "intermediate" and "resistant" (EUCAST, 2019). MIC values obtained *in vitro* are indicators of the relative susceptibility of fungi to the agent under consideration. Still, these values alone are insufficient to predict the clinical outcome *in vivo*. Instead, these measurements are more practically linked with the therapeutic outcome when integrated with clinical information and pharmacokinetic data to provide interpretive clinical breakpoints (CBPs) thus they are specific MIC values (Johnson, 2008).

It is almost universally accepted that the conditions employed for antifungal susceptibility testing of *Candida spp.* are not suitable for testing *M. pachydermatis* due to the different physiologic features. For example, the medium for *M. pachydermatis* needs a lipid supplementation to reach an adequately vigorous growth (Beccati et al., 2012). Moreover, *M. pachydermatis* has a slower growth rate compared to that of *Candida* species and tends to form clusters.
The lipid sources more frequently employed are tween, glycerol, olive oil, oleic acid, ox bile and cow's milk fat (Pasquetti et al., 2017). It is now clear that, in addition to the intrinsic drug susceptibility, the final therapeutic outcome in the course of infections for any infectious disease, depends on several other factors. These include factors regarding drugs (e.g., impaired drug absorption, accelerated drug metabolism, and poor penetration into the site of infection), general host factors (e.g., inflammatory response, hypersensitivity reactions, phagocyte function, and underlying diseases), and factors pertaining to pathogen activity (e.g., toxin production and other virulence factors). For these reasons CBPs have been established, taking into account the MIC

distributions, PK data (such as the maximum concentration of drug in the serum) and pharmacodynamic (PD) parameters, resistance mechanisms, and clinical outcomes, as these all relate to MIC values (Pfaller et al., 2012).

It is possible to draw a ranking with regard to the absolute potency against *M. pachydermatis* by the different agents tested. ITZ and PSZ were the most active agents (MIC_{90} for most studies < 0,5 µg/mL), followed by KTZ (MIC_{90} <1 µg/mL), whereas FCZ had higher absolute values (>4 µg/mL in most cases). With regard to the other agents, the MIC_{90} went from 0.25 to 20 µg/mL (MCZ), 0.125 to 3.2 µg/mL (TER), 0.5 to 40 µg/mL (NYS), 0.5 to 16 µg/mL (CTZ), and 7 to 32 µg/mL (TBZ) (Table 8).

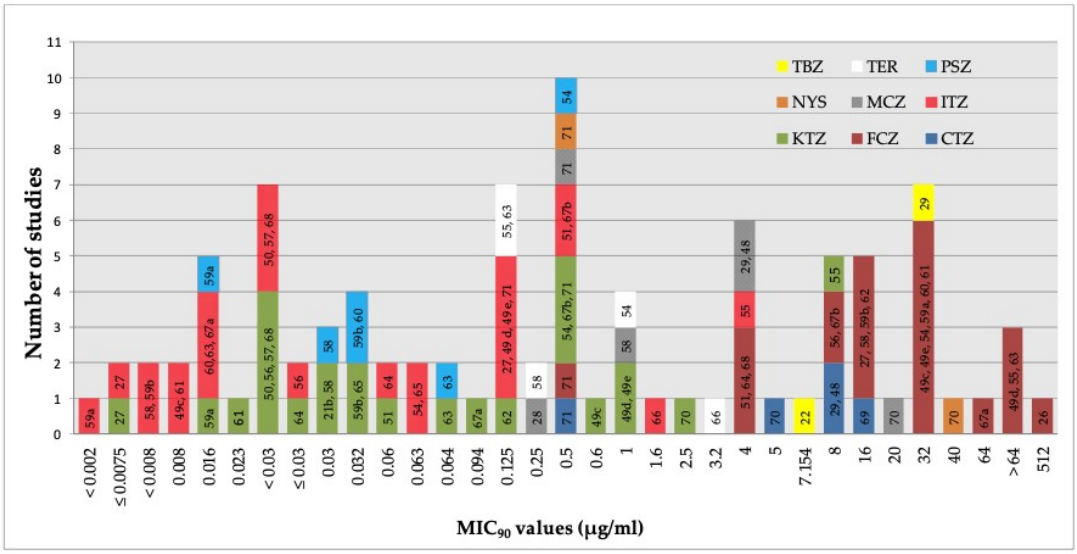


Table 8. *MIC90 values obtained for different antifungal agents against M. pachydermatis (MIC90 is the MIC required to inhibit the growth of at least 90% of the isolates tested). References are inside the bars. a = MICs obtained with an E-test; b = MICs obtained with the BMD method; c,d, e= MICs obtained in Sabouraud Broth+ Tween, Urea Christensen Broth+ Tween, and Dixon broth, respectively. KTZ= ketoconazole; MCZ= miconazole; CTZ= clotrimazole; ECZ= econazole; ITZ= itraconazole; FCZ= fluconazole; PSZ= Posaconazole; NYS= nystatin; TER= terbinafine; TBZ= thiabendazole. Peano et al., 2020*

In general MICs are higher for isolates from dogs with lesions than isolates from healthy dogs. Three independent studies (Cafarchia et al., 2012b; Weiler et al., 2013; Watanabe et al., 2014) reported that the MICs of various antifungal agents were significantly higher for isolates from animals with otitis/dermatitis than isolates from healthy animals. In two cases (Cafarchia et al., 2012b; Watanabe et al., 2014), it was hypothesised that increased MICs were due to the exposure of isolates from symptomatic animals to antifungal drugs, which may support the idea that resistance can develop during treatment. Although information on the clinical history and eventual treatments (agents employed, dosages, length of therapy, etc.) of the sampled animals was not available, Watanabe et al. (2014) explained that "due to the fact that *Malassezia* is

considered to be an exacerbating factor of canine atopic dermatitis (AD), we expect that many of the cases of canine AD were treated with either oral or topical azoles".
Moreover, Chiavassa et al. (2012) tested the antifungal susceptibility of isolates of *M. pachydermatis* obtained from cases of chronic otitis that had been previously treated with various topical ear products containing MCZ and CTZ. These isolates were associated with significantly higher MIC values for both the agents compared to isolates from dogs with acute forms of the disorder that had never received antifungal treatments. Although the authors themselves observed that increased MICs are unlikely to have any clinical relevance due to the topical use of the agents being tested, these findings may indicate the possibility of resistance developing during treatment.
In addition, in the article of Álvarez-Pérez et al. (2016), a total of 216 colonies of *M. pachydermatis* from 28 cases of fungal otitis or dermatitis in pets were genotyped by M13 fingerprinting and tested for antifungal susceptibility. A huge genetic diversity was found (157 M13 types in total), with all animals having a polyclonal pattern of infection. In particular, all animals had polyclonal otitis or dermatitis with 3 to 10 different genotypes. Moreover, most isolates displayed high *in vitro* susceptibility to amphotericin B, terbinafine and all azoles tested except fluconazole, for which MIC values were always ≥4 μg/ml. However, the experience gained from other fungal species demonstrates that polyclonal colonization or infection is possible (Álvarez-Pérez et al., 2009). Different body locations of the same individual can harbor different yeast genotypes (Cafarchia et al., 2008).

7.1 Azole antifungal drugs

Azole compounds, which inhibit the fungal biosynthesis of ergosterol by interacting with sterol-14α-demethylase (Vanden Bossche et al., 2003), are widely employed in the treatment of *Malassezia* infections (Chen et al., 1996). The possibility of developing azole resistance by *M. pachydermatis* has been frequently claimed in the literature on the basis of *in vitro* tests but there are also reports of *in vivo* resistance (i.e treatment failure) in dogs. For example, a multi-azole-resistant strain of M. pachydermatis was isolated from a dog with *Malassezia* dermatitis in Japan (Kano et al., 2018) (Figure 25), where resistance was suspected because of the lack of response despite 23 days of once-daily oral therapy with ITZ (8 mg/kg) and weekly shampooing with 2% miconazole/ 2% chlorhexidine shampoo.

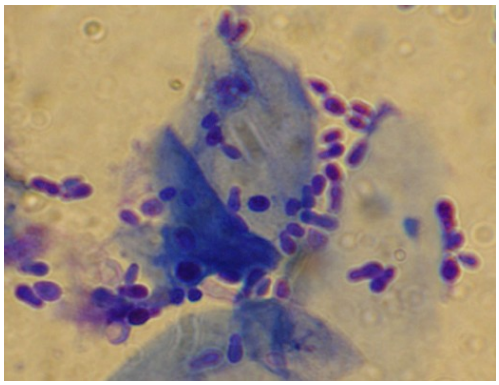


Figure 25. *Skin citology from a dog with Malassezia dermatitis. Peano et al., 2020*

Similarly, another case of *M. pachydermatis* that displayed *in vivo* resistance to azole compounds (Angileri et al., 2019) has been reported. In this last case, for comparison, a reference strain (strain CBS 1879, which is *M. pachydermatis* "type strain") was included in the tests. For ITZ, KTZ, MCZ and CTZ, MICs were also compared with MICs- available in the database of the laboratory - previously obtained for 10 isolates of the yeast coming from dogs never subjected to antifungal therapies. MICs of different azoles – in particular of ITZ, KTZ and MCZ- were increased by several-fold compared with MICs obtained for the control isolates (Table 9).

Table 9*. MICs (μg/ml) for six isolates of M. pachydermatis obtained from the toy Poddle dog, a reference strain and 10 isolates of the yeast coming from dogs never subjected to antifungal therapies. Angileri et al., 2019*

		ITZ		KTZ		MCZ	TER	CTZ	PSZ	FCZ
Number	**Provenance**	**MiB**	**E-test**	**MiB**	**E-test**	**MiB**	**MiB**	**MiB**	**E-test**	**E-test**
CBS 15214[a]	skin (abdomen)	0.5	0.75	2	0.75	16	1	32	1.5	> 256
CBS 15215[a]	skin (dorsum)	1	0.75	2	1	16	1	16	1.5	128
CBS 15216[a]	right ear	8	> 32	16	1.5	> 32	4	16	4	> 256
CBS 15208[b]	right ear	1	0.75	4	1	16	1	32	2	128
CBS 15212[b]	skin (neck)	1	0.5	4	0.75	16	2	32	1.5	> 256
CBS 15211[b]	skin (left thigh)	1	0.75	4	0.5	16	2	32	1.5	> 256
CBS 1879	reference strain	0.03	0.125	0.25	0.125	2	0.5	4	0.25	12
/	dogs never subjected to antifungal therapies[c]	0.016–0.06	0.023–0.094	0.03–0.125	0.19–0.125	1–4	/	1–16	/	/

Imidazoles such as clotrimazole, climbazole and miconazole are used topically (most commonly in otic products, creams or shampoos) although ketoconazole and its triazole derivative itraconazole are widely available for oral use in dogs. Posoconazole is used orally or intravenously for the treatment and topically in dogs with *Malassezia* otitis.

7.2 Terbinafine

Terbinafine is a synthetic allylamine derivative that inhibits fungal ergosterol biosynthesis at the point of squalene epoxidation. In general, *M. pachydermatis* shows greater susceptibility to this compound than other *Malassezia* species (Gupta et al., 2000).
Further studies in collections of field isolates of *M. pachydermatis* have routinely indicated susceptibility at concentrations similar to those obtained with azole antifungal drugs.

7.3 Nystatin

The polyene cyclic macrolides, amphotericin B and nystatin, were among the earliest broad-spectrum antifungals introduced for clinical use (Odds, 1996). The potential toxicity of amphotericin B generally limits its use in veterinary medicine to serious progressive or disseminated systemic mycoses, whereas nystatin is active when applied topically (Rausch et al., 1978). Its mode of action is via altered cell membrane permeability mediated by preferential binding to ergosterol.

Combination therapy has become popular in clinical practice, but limited data on the effects of combinations of antifungal agents is still available. The susceptibility of *Malassezia spp.* to combinations of antifungals, in fact, has only been assessed for miconazole-polymyxn B mixtures and in a limited number of studies (Pietschmann et al., 2009).
Álvarez-Pérez et al. (2019) studied the *in vitro* response of 30 genetically diverse clinical strains of *M. pachydermatis* obtained from cases of canine otitis attended between 2004 and 2016 at the Clinical Veterinary Hospital of Complutense University of Madrid (Spain) to several amphotericin B (AMB)-azole combinations. Broth microdilution checkerboard tests revealed that AMB antagonized the effects of itraconazole (ITC) and voriconazole (VRC) in 50% and 6.7% of the strains, respectively, but did not interact with fluconazole or posaconazole. Overall, the results of this study suggest that antagonistic combination effects between AMB and azoles might occur when tested against *M. pachydermatis* and it is strain-dependant. In particular, AMB antagonized the effect of ITC in a high proportion of tested strains.
In another study, Álvarez-Pérez et al. (2014), determined the *in vitro* amphotericin B susceptibility of 60 *Malassezia pachydermatis* isolates by the CLSI broth microdiluition method and the E-test using lipid-enriched media. All isolates were susceptible at MICs of <1 µg/ml, the results confirm that amphotericin B is very active against *M. pachydermatis.* Notably, this

contrasts with the decreased *in vitro* susceptibility to amphotericin B detected by Velegraki et al. (2004) for a significant proportion of isolates belonging to other *Malassezia* species, such as *M. furfur, M. restricta, M. globosa, and M. slooffiae.*

7.4 Chlorhexidine

Various antiseptics, such as chlorhexidine, are also effective treatments (Chen et al., 2005), in fact *M. pachydermatis* is typically susceptible to dilutions of chlorhexidine products, in accordance with reports of clinical efficacy. It only allowed a partial control of clinical signs. This is surprising as an antiseptic should also maintain its activity against an azole-resistant strain of *M. pachydermatis.*

7.5 Gentamicin and other aminoglycosides

Gentamicin is an aminoglycoside antibiotic with activity against many aerobic Gram-positive and negative bacteria, commonly formulated in polypharmacy otic products for dogs (Bond et al., 2020). Netilmicin, tobramycin and framycetin also have variable but often high activity against *M. pachydermatis* (Silva et al., 2017).

7.6 Reports of treatment failure due to resistance

Three publications- two recent case reports (Angileri et al., 2019; Kano et al., 2019) and a prospective study (Robson et al., 2010)- provide strong evidence in support of the presence of clinically relevant azole resistance in dog isolates coming of *M. pachydermatis.* The results of these three studies are briefly described below.

The prospective study of Robson et al. (2010) was conducted in a veterinary clinic in Australia. The criteria for trial admission were the presence of *Malassezia* otitis, *Malassezia* dermatitis, or both. *Malassezia* yeasts identified on cytology and the failure to respond to typically clinically effective empirically selected antifungal therapies. The correlation of a clinical lack of response with *in vitro* results was noted for five out of eight cases under study. To justify the three cases with the clinical suspicion of resistance not confirmed by *in vitro* tests, authors recalled the consideration mentioned above that a failure of response might occur because of several factors. For two dogs, they suspected that there had been problems with the topical drug administration.

Host factors were claimed for the remaining case (the dog was affected by a severe keratinisation defect) (Robson et al., 2010).
As regards the two other publications, the first concerned a dog in Japan (Kano et al., 2019). The clinical case was not described in detail, in fact, information about previous antifungal treatments was not clear.

The other publication is the case of Angileri (2019), as reported above, that included an exhaustive description of the case, which was followed for an extended period. This case, found in Italy, was "idiopathic" as possible underlying systemic or dermatological problems were carefully and repeatedly ruled out by extensive clinical and laboratory investigations. The same finding supports the fact that resistance to the isolates of the yeast was an acquired characteristic rather than an intrinsic feature. Moreover, different degree of *in vitro* susceptibility was noted for one of the isolates (no MIC of MCZ and ITZ detected). This suggests that the skin of a given dog may be colonised by strains of *M. pachydermatis* with different antifungal susceptibility profiles (a similar result was also obtained for a dog in the Australian study). Even azole agents that had not been used in the dog (PSZ, FCZ, KTZ) showed a reduced activity *in vitro*.
This may confirm that the phenomenon of the cross-resistance of M. pachydermatis to different azoles demonstrated during *in vitro* experiments (Jesus et al., 2011) also occurs *in vivo*. In fact, Jesus et al. (2011), valued the induction of high fluconazole MICs in 30 strains of *M. pachydermatis* by prolonged exposure. It was associated with elevated MICs to other azoles, suggesting that the molecular basis for these effects may in some cases confer cross-resistance to this drug class. This is could be a real important problem for long-term drug use in clinical practice (chronic cases). The Australian study too may indicate that resistance to *M. pachydermatis* is an acquired slow-developing phenomenon.
An *in vitro* result common to these publications is that, for many isolates coming from the "resistant" cases, an MIC of different antifungal agents was not obtained. This result may indicate a complete lack of- or at least a highly reduced- efficacy by the antifungal agents under testing (Peano et al., 2020). This finding supports the correlation between *in vitro* results and treatment failure as regards topically employed principles. For topical antifungals, resistance based on increased MIC values (measured in μg/mL) would be indeed poorly significant from a clinical perspective, since topical medications may have a 1000-fold higher concentration (mg/mL).

8. New therapeutic candidates for the treatment of *Malassezia* pachydermatis infections

The opportunistic pathogen *M. pachydermatis* causes bloodstream infections in preterm infants or individuals with immunodeficiency disorders and has been associated with a broad spectrum of diseases in animals such as seborrheic dermatitis, external otitis and fungemia (Sastoque et al., 2020). Many times, *Malassezia* dermatitis or otitis have been classified as chronic, which may require prolonged treatment and thereby causing adverse effects (Brilhante et al., 2018). Moreover, the current approaches to treat these infections are failing as a consequence of changes in susceptibility and antifungal resistance. Thus, the identification of novel therapeutic targets against *M. pachydermatis* infections are highly relevant.

8.1 Identification of novel therapeutic targets

In the Sastoque et al., (2020)'s study, was applied to a previously reported *M. pachydermatis* metabolic network to identify enzymes that, when absent, negatively affect biomass production. Searching therapeutic targets through metabolic network reconstructions has been proposed as a strategy to control the virulence of pathogens (Bazzani et al., 2012).

A frequently used approach is Gene Essentiality Analysis (GEA) that uses in silico deletions to identify potentially essential genes for growth of an organism (Rawls et al., 2019). This approach provides useful information about the metabolism of target organisms, which can be used to nominate new therapeutic candidates (Uddin et al., 2019).

Three nomination criteria were defined in order to identify potential therapeutic targets: (i) Target enzymes must not have counterpart versions within the human proteome, which was expected to reduce the effect of inhibitors on host's metabolism. This was verified by comparing the sequences of potential enzymes with those reported in the human genome; (ii) Target enzymes were selected to be easily quantified (i.e. there are commercially available quantification kits), and (iii) Inhibitors must be reported for selected enzymes in specialized databases (Sastoque et al., 2020). Three novel therapeutic targets: homoserine dehydrogenase (MpHSD), homocitrate synthase (MpHCS) and saccharopine dehydrogenase (MpSDH) were identified that are absent in humans.

HSD is involved in the aspartate route resulting in the L-amino acids lysine, threonine, cysteine and methionine. This enzyme catalyzes the third reversible reaction in this pathway producing L-

homoserine (Dong et al., 2016). Notably, this "sulfur assimilation pathway" is present in the fungal kingdom, but not in humans (Schroeder et al., 2010).
HCS and SDH catalyze the first and last step of the α-aminoadipate pathway that results in L-lysine biosynthesis. HCS and SDH are regulated by feedback inhibition by an excessive amount of L-lysine, while HSD is similarly regulated by the end products of the aspartate route including L-threonine (Tomonaga et al., 2015).
Notably, L-lysine was shown to be an inhibitor of the enzymatic activity of MpHCS and MpSDH at concentrations of 1 mM and 75 mM, respectively, while L-threonine (1 mM) inhibited MpHSD. Interestingly, L- lysine was also shown to inhibit growth of *Malassezia spp.*, even if the amino acid L-lysine has a mild cytotoxic effect on HEKa cells (Primary Epidermal Keratinocytes) but present no cytotoxic activity in keratinocytes at a concentration < 150 mg/mL. Microdilution and agar well diffusion assays were used to determine the effect of L-lysine and L-threonine on growth of reference strains of *Malassezia spp.* and canine isolates of *M. pachydermatis.*
Overall, results suggest that L-lysine could be used as a potential treatment against *M. pachydermatis* infections. It is important to mention that the concentrations required for growth inhibition could limit their clinical utility. Thus, L-lysine could be used to treat dermatological infections instead of sepsis where higher concentrations will be required. Additional cytotoxicity, pharmacokinetic and toxicodynamic studies will be needed to confirm its potential as a novel pharmaceutical product and possible treatment against *M. pachydermatis* infections.

8.2 *In vitro* and *in vivo* efficacy of tea tree essential oil for ear infections in dogs

People have become increasingly demanding and more discerning about the quality of life of their pets and the quality of the product they consume. There is a growing concern in making use of less aggressive and of natural origin products (Packer & Luz, 2007), such as essential oils formulations. Essential oils are volatile compounds produced by plants for their survival, both for defense or to attract pollinators. These oils are complex mixtures of liquids, of intense and pleasant odor, which main characteristic is volatility, that differentiates them from fixed oils, extracted from lipid seed (Jesus et al., 2007).

Melaleuca alternifolia, also known as tea tree plant, is native from Australia, (Silva et al. 2009), and, such as *Myrtaceae* thin skin trees, it can reach 5-7 meters tall, and have long thin pointed

leaves that, when broken, loose a strong aroma. There is evidence that the Australian aborigines crushed *M. alternifolia* leaves to make antibacterial poultices, centuries before the scientific knowledge about microorganisms (Simões et al. 2002).
Scientific data shows that essential oil extracted from the leaves by hydrodistillation has proven antibacterial, antifungal and antiviral properties (Silva et al. 2009). Its main constituents are α-terpinene (10%), γ-terpinene (23%) and terpinen-4-ol (40%), with antimicrobial effect (Oliva et al. 2003).
Reichling et al. (2004) showed the efficacy of 10% tea tree oil cream in dogs presenting yeast and bacterial isolates from pruriginous cutaneous lesions, caused by localized acute and chronic pyoderma. Another study demonstrated that some strains of *Pseudomonas aeruginosa* were resistant to tea tree oil, while 51% of the pathogenic microorganisms, including yeasts and bacteria, found in humans with otitis were susceptible to this oil (Farnan et al., 2005).

"Oil of *Melaleuca* - Terpinen-4-ol type (tea tree oil)" (Carson & Riley, 2001) is obtained from the leaves may contain varying amounts of terpenes (pinene, terpinene and cymene), terpineol (terpinen-4-ol), sesquiterpenes and cineole, which are the most important constituents with antimicrobial activity.
The Australian committee of standardization (Australian Standard AS 2782-85) states that the oil should contain cineol, a skin irritant (Simões et al., 2002) below 15% and terpinen-4-ol between 30-40%, in order to have any antiseptic efficacy (Simões et al., 2002).
Terpinen-4-ol is touted as the largest contributor of antimicrobial activity among the components (Simões et al., 2002). In addition to its antibacterial properties, even against bacteria resistant to antibiotics, tea tree essential oil is also effective against pathogenic molds, some viruses, and has been used as a strong repellent against mosquitoes, fleas, lice (Silva et al., 2009) and mites, such as *Otodectes cynotis* (Neves et al., 2013). Moreover, tea tree essential oil 100% has got a low risk of allergic reactions.

Neves et al. (2018) wanted to evaluate *in vitro* and *in vivo* efficacy of tea tree essential oil for bacterial and yeast ear infections in dogs. During their study, they took twenty-eight dogs from a particular shelter in Cuiabá (Mato Grosso, Brazil) presenting clinical signs of otitis externa were eligible for enrollment in this clinical trial, regardless of size, sex, breed or age. Patients who have received antibiotics, antifungal or anti-inflammatory as topical or systemic treatment in the previous 15 days were excluded from the study, as well as infants and the ones presenting signs of pregnancy, parasitic ear infection, tumors or foreign bodies in the ears.

Treatment of otitis externa began with flushing the ears twice a day in ear canals with waxy material. After cleansing phase, the 28 enrolled dogs were divided into three groups: fungal otitis, bacterial ear infections and mixed ear infections (bacterial and fungal). All right ears received 5% tea tree essential oil lotion, while left ears received treatment according to group classification: 0,15% nystatin lotion for fungal otitis (Lorenzini et al., 1985); 0,3% gentamicin lotion (Leite, 2008) for bacterial ear infection and both of them when there was mixed otitis. Efficacy of treatment was assessed through clinical findings and cytology and compared to susceptibility testing results. In particular, tea tree essential oil treatment efficacy for ear infections was evaluated through a paired T-test, applied separately to each lotion, considering the score before and after its administration. Regarding the efficacy as an anti-infection agent (Fig.26), tea tree oil showed a significant reduction for *Malassezia* because of *Malassezia* species presented similar sensitivities as to ketoconazole, econazole and miconazole.

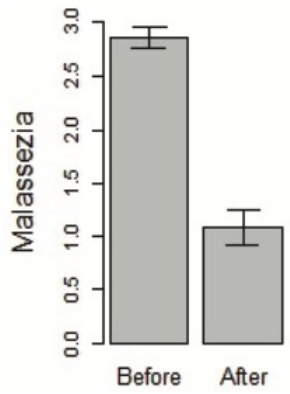


***Figure 26**. Results of cytological examination before and after treatment with tea tree oil (M) and Nystatin (N) of the yeast otitis group. Neves et al., 2018*

Despite not all dogs treated with tea tree essential oil have healed completely in this 14 days trial, a significant improvement of clinical and cytological were evident in all patients. All 28 dogs treated with tea tree essential oil showed significant improvement in clinical parameters (inflammation, pruritus, ear discharge) and in cytological evaluation (quantification of microorganisms and inflammatory cells), when comparing findings before and after treatment. Good antimicrobial spectrum and the absence of allergic reactions confirm the importance of developing a tea tree formulation as a possible alternative therapy for ear infections in dogs.

In addition to this, antifungal activity of *Cinnamomum cassia, Mentha piperita, Origanum vulgare* and *Syzygium aromaticum* essential oils were tested against 19 strains of *M. pachydermatis* isolated from healthy dogs and reference strain *M. pachydermatis* CBS 1879

(Bohmova et al., 2019). The checkerboard assay was used to search for interactions. Synergism was observed for the combination of clotrimazole with *Melaleuca alternifolia* essential oil, *Mentha piperita* and *Origanum vulgare.*
The combinations of *Cinnamomum cassia* and *Syzygium aromaticum* essential oils with clotrimazole showed indifferent effect.

Additive antimicrobial activity was observed for the combination of clotrimazole with *Syzygium aromaticum* and *Melaleuca alternifolia* essential oils against reference strain. The obtained results showed synergistic interactions between essential oils and clotrimazole which could improve effectiveness of this antifungal drug (Bohmova et al., 2019) (Table 10).

***Table 10**. Oils and their source. Table created by me*

Name of the oil	Source	Plant family
Tea tree oil	Melaleuca alternifolia	Myrtaceae
Peppermint	Mentha piperita	Lamiaceae
Oregano	Origanum vulgare	Labiate
Chinese cinnamon	Cinnamomum cassia	Lamiaceae
Eugenia caryophyllata	Syzygium aromaticum	Myrtaceae

8.3 *In vitro* efficacy of a honey-based gel (HBO) against canine clinical isolates of *M. pachydermatis*

Oliveira et al. (2018), during her study, wanted to determine the *in vitro* efficacy of a honey-based gel (HBO) against *M. pachydermatis*, by minimum fungicidal concentration (MFC) and time-kill assay (TKA). Efficacy of the product's honey component (HO) also was evaluated. During the experiment, 10 *M. pachydermati*s canine isolates were selected. All isolates were tested against serial dilutions of an HBO. Microbroth assay followed by subculture was used to determine MFC. All isolates were killed after 4 h of exposure. Overall, *M. pachydermatis* are susceptible to the HBO and these results can be used for future clinical trials.

8.4 *In vitro* antimicrobial activity of narasin against pathogens associated with canine otitis externa

Antimicrobial resistance and antimicrobial stewardship are of ever-increasing importance in veterinary medicine.
Chan et al. (2019), during his study wanted to determine the antimicrobial activity of narasin, a

polyether ionophore, against common clinical isolates of canine otitis externa.
Polyether ionophores are highly lipophilic molecules which transport monovalent or divalent cations across the cell membrane of susceptible bacteria. Narasin, a monovalent polyether ionophore discovered in the late 1970s by the fermentation process of *Streptomyces aureofaciens*, has been shown to be effective against Gram-positive bacteria, anaerobic bacteria and fungi (Berg etl al., 1978). There is a little evidence of baceterial resistance or co-selection for resistance to other clases of antimicrobials by the ionophores (Subbiah et al., 2016).
These drugs are only licensed for animal use in which they are commonly used as rumen modulators and anticoccidial agents in production animals (Kevin li DA et al., 2009). These drugs are not suitable for development as systemic antibiotics due to a low margin of safety and potential toxicity in nontarget species. It has a very low therapeutic index and has resulted in neurotoxicity, cardiac toxicity and toxic myopathy in dogs following consumption of narasin-contaminated commercial dog food (Karsai et al., 1990). Despite this, nasarin may have value as a topical agent for the treatment of surface infections such as those found on the skin or in the ear (Chan et al., 2018). Narasin MIC for *M. pachydermatis* ranged from 32 to >128 lg/mL, with MIC_{50} and MIC_{90} values of 128 lg/mL and >128 lg/mL. Narasin demonstrated antifungal activity against *M. pachydermatis* but only at higher concentrations, as a topical product, such as in an ear preparation, it is likely that the concentration in the formulation would exceed these higher MICs. Narasin may therefore also have potential as a treatment for canine otitis externa caused by *M. pachydermatis*.
However, further studies would be required to assess suitable formulations and topical safety in dogs. To date, there is only limited information about the potential of narasin to cause cutaneous irritation in laboratory animals.

Narasin did not demonstrate any antibacterial activity against the Gram-negative otic pathogens (Butaye et al., 2003). In general, it is not suitable as a sole antimicrobial agent in cases of otitis externa with mixed infections or those associated with Gram-negative organisms.This problem could be solved by the addition of a second antimicrobial agent, but there is also a possibility that addition of an adjuvant that weakens the external cell wall of Gram-negative bacteria could remove this resistance mechanism. This could lead to the organisms becoming susceptible to narasin. Further studies are underway to test this hypothesis (Chan et al., 2018).
The mechanism of action of narasin relates to its highly lipophilic properties, allowing it to insert into bacterial cell membranes. It then chelates reversibly with Na^+ and K^+ conveying them rapidly across the cell membrane where they exchange for other monovalent cations such as

hydrogen ions, disrupting the normal ionic and pH gradients (Russel et al., 1989).
In Gram-positive bacteria, it is likely that narasin can penetrate the porous peptidoglycan cell wall layer and cause an imbalance of intracelular concentration gradients, disrupting cellular division and potentially leading to cell death (Callaway et al., 2003).
The inherent resistance of Gram-negative bacteria is most likely due to the two-layered cell wall structure of Gram-negative bacteria preventing narasin from permeating into the inner cytoplasmic membrane.

9. Conclusions

Drug resistance is a well-known problem regarding many infective microorganisms, not only bacteria, but also some fungal species of medical importance.
The mechanisms of antifungal resistance are related to the intrinsic or acquired characteristics of the fungal pathogen that interfere with the antifungal mechanism of the respective drug, or that lower target drug levels (Pfaller, 2012).
As reported above, azole-resistant strains of *M. pahydermatis* have been found in some studies (e.g. Kano et al. 2019; Angileri et al., 2019) and evidence suggests that the susceptibility of *M. pachydermatis* to amphotericin B, azoles and terbinafine varies from strain to strain (Figure 27).

The skin and ear of a given dog may be colonised by strains of *M. pachydermatis* with different antifungal susceptibility profiles (Álvarez-Pérez et al. 2016).
Moreover, in the few well-documented cases available, treatment failure occurred after months or years of therapy, which may indicate that resistance in *M. pachydermatis* is an acquired slow-developing phenomenon. This information is extremely important for the treatment of *Malassezia* otitis and dermatitis, as these often necessitate frequent and lengthy treatment courses (especially in the cases of atopic dermatitis, seborrhoeic dermatitis, and chronic otitis externa). The chronicity of treatment provides ideal opportunities for the selection of antifungal resistance (Peano et al., 2020).

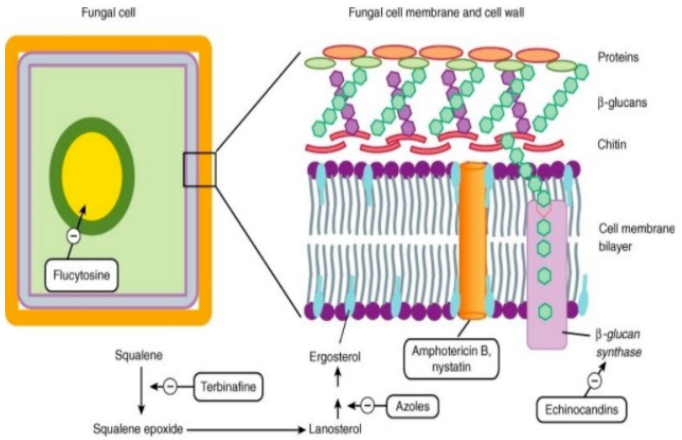


Figure 27*. Different sites of action of antifungal agents. Strange (2017)*

Further evidence in support of the capacity of *M. pachydermatis* to develop antifungal resistance comes from: (i) the higher MICs found for isolates from animals with probable/confirmed exposure to antifungal drugs and isolates exposed to antifungal agents *in vitro*; (ii) the description of possible resistance mechanisms in field isolates and in mutant isolates obtained *in vitro*; (iii) the reports of isolates with MICs significantly higher within a certain population of isolates. Notably, most strains of *M. pachydermatis* can produce biofilm *in vitro*, and in this form of the yeast has a significantly reduced antifungal susceptibility.

On the other hand, the growing importance of *M. pachydermatis* as an emergent pathogen of humans and animals has been recently reviewed (Guillot et al., 1999). Detailed documentation of future cases of *Malassezia* infection in human an animal patients and concomitant susceptibility testing of yeast isolates would help to correlate *in vitro* results with clinical outcomes and clarify the prospects of antifungal therapy for the treatment of these conditions (Álvarez-Pérez et al., 2018). In this context, *in vitro* antifungal susceptibility testing of *M. pachydermatis* has great value, as the presence of strains with decreased susceptibility to antifungals on the skin of per animals and their possible zoonotic spread pose important threats for the treatment of eventual systemic infections in both humans and animals (Álvarez-Pérez et al., 2016).

New possible treatments for *Malassezia* infections are essential oils. In particular, tea tree essential oil ear solution significantly induced remission of clinical signs both in bacterial and yeast ear infections. It also reduced *M. pachydermatis* growth as much as a nystatin solution, while gentamycin showed better antibacterial effect (Neves et al., 2018). In fact, another alternative antimicrobial is narasin, which is effective against Gram-positive bacteria and, at higher concentrations, has antifungal activity against *M. pachydermatis*.

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