



Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

**Faculty of Veterinary Medicine
and Animal Science**

Department of Clinical Sciences

Is topical treatment with Crystavet® an effective treatment of *Malassezia* overgrowth in dogs? – a pilot study.

Jenny Lithner

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Is topical treatment with Crystavet® an effective treatment of *Malassezia* overgrowth in dogs?

– a pilot study.

Är topikal behandling med Crystavet® effektiv vid behandling av jästsvampsöverväxt hos hund?

– en pilotstudie.

Jenny Lithner

Supervisor: Kerstin Bergvall, Department of Clinical Sciences

Assistant Supervisor: Elisabeth Bagge, National Veterinary Institute, Sweden

Examiner: Ann Pettersson, Department of Clinical Sciences

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SUMMARY

Malassezia spp. are commensals of the canine skin flora. Opportunistic skin infections involving overgrowth of *Malassezia* are often found in intertriginous areas or associated with one or several underlying medical conditions affecting the host defence of the skin barrier. Symptoms include pruritus, erythema, alopecia, lichenification, hyperpigmentation, scaling and moist skin surface with a greasy, malodorous exudate. A diagnosis of *Malassezia* dermatitis is indicated when skin with cutaneous lesions and *Malassezia* overgrowth responds to antifungal therapy.

Treatments available with the indication of *Malassezia* overgrowth include both systemic treatment with azole derivatives or allylamine and a wide variety of agents for topical treatment. Available topical treatment options include azole derivatives and antimicrobial agents, many of which lack sufficient evidence base. Therefore, the need for randomized, controlled trials evaluating topical antimicrobial agents available and marketed for *Malassezia* overgrowth is urgent.

The purpose of this study was to evaluate the efficacy of a new topical product, Crystavet® (1% hydrogen peroxide, Bioglan, Malmö, Sweden), for the treatment of *Malassezia* overgrowth in a prospective, open, randomized, controlled study design using a split-body protocol. In total, 22 anatomically distinct skin areas from ten dogs were included. The efficacy of Crystavet was compared to a reference product of 4% chlorhexidine and evaluated based primarily on the reduction of *Malassezia* yeast obtained by cytology. Secondly, the efficacy was evaluated by quantitative culture of *Malassezia* spp., clinical skin lesion score and degree of pruritus pre and post treatment.

Treatment with both Crystavet and the reference product reduced *Malassezia* population sizes and clinical parameters significantly, without any difference in efficacy noted between the products. No adverse reactions were recorded and owners rated the treatment as easy to perform. These results suggest that topical treatment with Crystavet (1% hydrogen peroxide) is effective and safe for the treatment of localized *Malassezia* overgrowth in dogs. The results also suggest that the antifungal efficacy of 1% hydrogen peroxide is similar to that of 4% chlorhexidine for *Malassezia* species. Further studies including a larger sample size are needed to confirm these results.

SAMMANFATTNING

Malassezia spp., även kallad jästsvamp, är en del av hundens normala mikroflora. Opportunistiska hudinfektioner med jästsvampsöverväxt uppstår ofta i intertriginösa områden eller sekundärt till medicinska tillstånd som påverkar hudens barriär och försvarsmekanism. Kliniska tecken som uppstår är klåda, erytem, alopeci, lichenifikation, hyperpigmentering, mjäll samt en fuktig hudyta med illaluktande sekret. Diagnosen jästsvampsdermatit är verifierad när en inflammerad och skadad hudyta med jästsvampsöverväxt läker av efter svampdödande behandling.

Både systemiska och topikala behandlingsalternativ finns att tillgå för behandling av jästsvampsöverväxt. För systemisk behandling används azolderivat eller allylamin medan topikala alternativ innefattar både azolderivat samt en mängd antimikrobiella substanser. För flertalet av dessa saknas tillräcklig evidens. Det finns därför ett stort behov av randomiserade, kontrollerade studier som utvärderar effekten av topikala behandlingsalternativ för jästsvampsöverväxt.

Syftet med den här studien var att utvärdera effektiviteten av en 1% väteperoxid baserad produkt, Crystavet® (Bioglan, Malmö, Sweden), vid behandling av jästsvampsöverväxt hos hund i en prospektiv, öppen, randomiserad, kontrollerad studie. Ett split-body protokoll användes och Crystavets effektivitet jämfördes med en 4% klorhexidinprodukt som referens. Totalt inkluderades 22 anatomiskt skilda hudområden från tio hundar. Behandlingens effektivitet utvärderades i förstahand baserat på minskning av antalet jästsvampar påvisade via cytologi. I andra hand utvärderades behandlingens effekt genom kvantitativ odling av jästsvamp, kliniska lesionscore och grad av klåda innan och efter behandling.

Behandling med både Crystavet och referensprodukt resulterade i en signifikant minskning av jästsvampar och kliniska mätvärden, behandlingarna skiljde sig inte signifikant åt i sin effektivitet. Inga biverkningar rapporterades och djurägarna ansåg att behandlingen var enkel att utföra. Resultat från den här studien indikerar att topikal behandling med Crystavet (1% väteperoxid) är effektivt och säkert att använda vid behandling av lokaliserad jästsvampsöverväxt hos hund. Resultaten tyder även på att den jästsvampsdödande effekten av 1% väteperoxid är jämförbar med den hos 4% klorhexidin för jästsvampar av genuset *Malassezia*. Vidare studier med en större studiepopulation krävs för att bekräfta dessa resultat.

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INTRODUCTION

Malassezia is the scientific name of one genus of microorganism known as yeast. *Malassezia* spp. are recognized as commensals of the canine skin flora but are also known to cause opportunistic skin and ear infections (Cafarchia et al., 2005; Machado et al., 2011; Nardoni et al., 2004; Nuttall, 2012; Prado et al., 2008).

Treatments available for the indication of *Malassezia* dermatitis include both systemic with azole derivatives and a wide variety of antimicrobial topical treatments. Out of the several available alternatives for topical treatment only a few have been reported effective in treating *Malassezia* dermatitis as reviewed by Mueller et al (2012) as well as Negre et al (2009).

The purpose of this study was to evaluate the efficacy of a new topical product, Crystavet® (1% hydrogen peroxide, Bioglan, Malmö, Sweden), for the treatment of *Malassezia* overgrowth in canines.

LITERATURE REVIEW

***Malassezia* species**

Malassezia species are eukaryotic, unicellular organisms of the Fungi kingdom, also known as yeasts, which reproduce by budding (Miller et al., 2013a; Nuttall, 2012). The genus of *Malassezia* has been revised several times and new species are continuously discovered. Currently 14 species are accepted which have been isolated from both healthy and diseased skin of mammals (Gaitanis et al., 2012). The genus is divided into one lipophilic, non-lipid dependent species, *M. pachydermatis*, and 13 lipid dependent species. Although, recently published data of whole genome sequencing suggests lipid-dependence of all *Malassezia* spp. (Gaitanis et al., 2012; Wu et al., 2015)

***Malassezia* species in canines**

Malassezia is part of the normal micro flora of skin in healthy dogs and several other mammals (Kennis et al., 1996; Miller et al., 2013a; Nuttall, 2012). Based on their study of early colonization of commensal *Malassezia* in healthy puppies, Wagner and Schadler (2000) concluded that colonisation occurs soon after birth, since they isolated *Malassezia* from puppies as young as 3 days old.

Out of the 14 known species, *Malassezia pachydermatis* is especially prevalent in canine isolates from both healthy and dermatologically diseased dogs (Cafarchia et al., 2005; Machado et al., 2011; Nardoni et al., 2004; Nuttall, 2012). Some of the lipid-dependent *Malassezia* spp. have also been isolated from both skin of dogs with cutaneous lesions and from healthy dogs. These include: *M. furfur* and *M. sympodialis* (Cafarchia et al., 2011; Machado et al., 2011; Nardoni et al., 2004; Raabe et al., 1998) as well as *M. globosa*, *M. restricta*, *M. sloofiae*, *M. nana* and *M. obtusa* (Khosravi et al., 2016). Within each species of the *Malassezia* genus there are different genotypes that may cohabitate or infect the same animal or anatomical site (Cafarchia et al., 2011, 2008; Castellá et al., 2005; Puig et al., 2016).

In healthy dogs of various breeds, the *Malassezia* population size varies widely in different anatomical areas within and between individuals, but will in most areas be < 10 yeasts/1.25 cm² (Bond et al., 1995c; Cafarchia et al., 2005; Kennis et al., 1996, 1996; Nardoni et al., 2007). However, Bond and Lloyd (1997, 1998) found that population sizes of *M. pachydermatis* was significantly higher in healthy Basset hounds compared to other dogs of various breeds.

Many anatomical areas, such as periorbital, perianal, inguinal, the dorsal neck and the conjunctival sacs, have been found to be populated with *Malassezia* spp./*M. pachydermatis* in healthy dogs. The most frequent areas of isolation in healthy dogs varies between studies but includes the perioral, interdigital and perianal skin and the external ear canal (Bond et al., 1995c; Bond and Anthony, 1995; Cafarchia et al., 2005; Kennis et al., 1996; Prado et al., 2008, 2004).

Age does not seem to influence the frequency of isolation in canines (Mauldin et al., 1997; Miller et al., 2013a; Plant et al., 1992) although Nardoni et al (2004) did isolate *Malassezia* spp. more frequently in dogs aged 1-5 years compared to dogs of other ages. In another study by Machado et al (2011), age-related differences found in *Malassezia* spp. isolation were by the authors themselves believed to be due to a, false positive, statistical error.

***Malassezia* associated dermatitis in canines**

Malassezia dermatitis is an inflammation of the skin associated with *Malassezia* yeast overgrowth (Miller et al., 2013a). The first report of *Malassezia* as a cause of canine dermatitis was published in 1970s by Dufait (1975) and almost twenty years later Plant et al (1992, pp. 881–882) suggested that “the prevalence of cutaneous *Malassezia* infection in dogs may not be rare, as previously reported.” This is further supported by several more recent studies where the frequency of isolation and population size of *Malassezia* yeast in dogs with cutaneous lesions is reported to be significantly higher compared to healthy dogs (Cafarchia et al., 2005; Machado et al., 2011; Nardoni et al., 2004; Prado et al., 2008). For example Machado et al (2011) isolated *Malassezia* spp. in culture from 52.9% of the

dogs with cutaneous lesions compared to 15.6% of the healthy dogs. In the same study a linear trend was found between increasing lesional scores and *Malassezia* population counts obtained by fungal culture. Several studies have also shown that treatment targeted on eliminating *Malassezia* yeasts in dogs with *Malassezia* overgrowth and concurrent dermatitis results in a decrease of cutaneous lesions (Bensignor, 2008; Bond et al., 1995b; Maynard et al., 2011; Pinchbeck et al., 2002).

Clinical lesions and signs

Canine *Malassezia* dermatitis presents as a pruritic skin disease with one or several of the following manifestations: erythema, alopecia, lichenification, hyperpigmentation, scaling and moist skin surface with a greasy, malodorous exudate. Chronic cases often present with more pronounced alopecia, lichenification and hyperpigmentation. The manifestations are either generalized or localized (Bond et al., 1995b; Cafarchia et al., 2005; Mason and Evans, 1991; Mobley and Meyer, 1994; Nuttall, 2012). Intertriginous areas such as skin folds and interdigital skin as well as areas perioral, ventral abdomen, inguinal, the ventral neck, axillae and the medial aspects of the extremities, are often affected (Bond et al., 1995b; Cafarchia et al., 2005; Mason and Evans, 1991; Mauldin et al., 1997; Mobley and Meyer, 1994).

Malassezia spp. can also cause inflammation of the external ear canal, which can be concurrent with *Malassezia* dermatitis, but is defined as an ear inflammation (otitis externa) and not as dermatitis (Bond et al., 1995b; Miller et al., 2013b). The findings of Prado et al (2004) also suggest that *Malassezia* spp. could be involved in the pathogenesis of corneal ulcers, or at least be an aggravating factor.

Pathogenicity and risk factors

For *Malassezia* to become pathogenic, overpower of the host defence (physical, chemical and immunological defences) is necessary, which is achieved by changes in both the host and the yeast organism (Nuttall, 2012). For *Malassezia pachydermatis* this includes virulence factors such as the ability to produce enzymes like lipase, phosphatase, protease and urease. These enzymes may alter the local pH and break down dermal components, causing activation of inflammatory mediators (Coutinho and Paula, 2000; Lautert et al., 2011; Mathieson et al., 1998; Nuttall, 2012). Phospholipase activity seems to play a particularly important role as a virulence factor (Cafarchia et al., 2008; Machado et al., 2010).

Mason and Evans (1991) stated two categories of host related factors that contributes to allowing commensals to become pathogenic: the first being changes in microclimate of the skin that benefits proliferation of the organisms and the second being failure of the host's immune defence to respond to and control the overgrowth. An example of a factor that can change the microclimate is occlusion, something that occurs in intertriginous areas such as skin folds or interdigital skin areas. Occlusion leads to increased humidity and accumulation of debris, desquamated epidermal cells, sweat and sebum on the skin, which induces dermal inflammation single-handedly and creates a microenvironment favourable for yeast organisms (Bond et al., 2004). The *Malassezia* overgrowth further drives the inflammation and both clinical and histopathological lesions increase in severity. Bond et al (2004) also showed that a larger *Malassezia* population size can by itself lead to dermal inflammation. In contrast Bensignor et al (2002) found large population sizes on healthy, non lesional, pinnae of Basset Hounds. Supporting the findings of Bond et al (2004), regarding the fact that large *Malassezia* populations and an occluded, moist skin climate can cause disease, are the findings of Uchida et al (1992). Uchida et al (1992) induced otitis externa following a single application of a *M pachydermatis* suspension into the external ear canal of dogs. Noteworthy is that in the study by Bond et al (2004) in which *Malassezia* dermatitis was induced, the majority of the dogs recovered spontaneously after ceasing to apply the *Malassezia* solution and occluding the skin surface. This illustrates the importance of host defence in the pathogenesis of *Malassezia* dermatitis, as some of the dogs were not able to clear the infection when the underlying trigger of disease was no longer present (Bond et al., 2004).

Factors that also affect both microclimate of the skin and the host's capability of defence are underlying medical conditions leading to dermatoses that precedes the *Malassezia* dermatitis, only to mention a few: ectoparasitosis, cutaneous adverse food reactions, endocrinopathies and seborrhoeic dermatitis/keratinization defects (Bond and Lloyd, 1997, 1998; Mauldin et al., 1997; Nuttall, 2012; Plant et al., 1992). Another frequently reported association is that between atopic dermatitis and *Malassezia* dermatitis (Bond et al., 1996, 1994; Mauldin et al., 1997; Nardoni et al., 2004). The association is complex, since the highest population sizes of *Malassezia* yeasts are sometimes, but not always, found on lesional skin (Bond et al., 1994; Nardoni et al., 2007) and even normal population sizes of *Malassezia* might cause inflammation in dogs with atopic dermatitis if an underlying hypersensitivity for *Malassezia* yeasts is present (Nuttall and Halliwell, 2001). In fact, independent of *Malassezia* counts, atopic dogs show higher levels of *Malassezia*-specific IgG and IgE antibodies compared to non-atopic dogs. In non-atopic dogs these levels remain low also during concurrent *Malassezia* dermatitis (Nuttall and Halliwell, 2001).

Since *Malassezia* is a commensal, there is no transmission of disease in the true sense of the word. There is, however, some evidence that leads to speculations that scratching and licking in dogs with *Malassezia* dermatitis could spread *Malassezia* organisms to other areas of the body (Cafarchia et al., 2005). One example is in the situation when dogs have perioral lesions and larger populations of *Malassezia* yeasts on interdigital skin, which might reflect consequences of scratching and licking (Cafarchia et al., 2005).

Several breeds have been reported to be at an increased risk for *Malassezia* dermatitis. These are: Basset hounds, American and English Cocker Spaniels, West Highland Terriers, English Setters, Shih Tzus and Dachshund (Bond et al., 1996; Mauldin et al., 1997; Plant et al., 1992). Tendencies of increased risk have also been reported for Boxers, Springer Spaniels and German Shepherds (Bond et al., 1996; Plant et al., 1992). Miller et al (2013a) also report Toy and Miniature Poodles, Cavalier King Charles Spaniel, Australian and Silky Terriers as breeds at an increased risk.

No significant difference could be proven between males and females in several studies (Machado et al., 2011; Nardoni et al., 2004; Plant et al., 1992). In contrast, Mauldin et al (1997) have reported an increased risk for *Malassezia* dermatitis in spayed females and castrated males.

Diagnostic testing in *Malassezia* dermatitis

When presented with a clinical case with symptoms of *Malassezia* dermatitis, it is especially important to remember that there are several differential diagnoses associated with *Malassezia* dermatitis and that more than one might be relevant in one patient (Miller et al., 2013a). For detection of *Malassezia* overgrowth there are several available techniques; cytology, culture or histopathology/skin biopsies (Miller et al., 2013a; Nuttall, 2012). Although detection of *Malassezia* is important for the diagnosis, it has been suggested that a diagnosis should only be considered confirmed when a dog with increased *Malassezia* populations on skin with cutaneous lesions, responds to treatment with antifungal therapy (Bond and Lloyd, 1997; Nuttall, 2012).

Cytology

On microscopic examination the *Malassezia* yeast, with its diameter of 3-5µm and typical "peanut" or "Russian doll" shape, is detectable on dry lens (x400) but preferably oil immersion lens (x1000) should be used (Hensel et al., 2015; Nuttall, 2012). The colour will vary from light to dark blue and some *Malassezia* yeasts will not stain, but are still detectable by their visible cell wall.

Sampling for microscopic examination of the skin surface can be done by superficial scraping, the rubbing of a cotton swab, direct impression of a microscopic glass slide onto the skin surface or by the adhesive tape strip technique, in which a clear acetate adhesive tape is pressed on the skin surface, then stained and placed adhesive down on glass slide (Bond et al., 1995a, 1994; Cafarchia et al., 2005; Machado et al., 2011; Plant et al., 1992; White et al., 1998). None of these techniques are regarded as gold standard (Miller et al., 2013a). When comparing the techniques of direct impression, swabbing

and superficial skin scrapings in healthy dogs Kennis et al (1996) found no significant difference in their ability to recover yeast organisms. In contrast, White et al (1998) found that superficial scraping yielded higher quantities of yeasts compared to cotton swabs.

No exact number of *Malassezia* organism have been established as a criteria to diagnose *Malassezia* dermatitis and therefore different cut off values are used in different studies with varying study protocols (Bond et al., 1996, 1994; Bond and Lloyd, 1997; Cafarchia et al., 2005; Nuttall, 2012; Nuttall and Halliwell, 2001; Plant et al., 1992). In the study by Cafarchia et al (2005) a sterile NaCl moistened cotton swab was used to sample the skin surface and then rolled onto glass slides for microscopic evaluation. A skin sample was considered positive for *Malassezia* overgrowth if it had >5 yeasts in five random fields at 40X magnification. Bond et al (1996, 1994) suggested that populations should be considered elevated when > 10 *Malassezia* yeasts are detected in 15 random fields at X100/oil immersion lens after sampling with tape strip technique.

The sensitivity and specificity for cytology when considering fungal culture as a gold standard for detection of *Malassezia* spp in dogs was studied by Machado et al (2011). The specificity was found to be good, 100% in dogs with no cutaneous lesions and 92.7% in dogs with cutaneous lesions; while the sensitivity was much lower, 25% and 53.2% respectively. Similar results have been obtained by Cafarchia et al (2005) as well as Prado et al (2008). Especially in healthy dogs sensitivity is reported to be low for several cytological sampling techniques (Bensignor et al., 2002; Kennis et al., 1996)

According to Mason and Evans (1991) cytology is the most useful diagnostic technique for the everyday clinician since it easily available.

Culture

For culture of all *Malassezia* species a lipid supplemented media is essential, for example modified Dixons' medium, Ushijima's medium A and Leeming's media (Bond and Lloyd, 1996; Miller et al., 2013a). For isolation of *Malassezia pachydermatis* exclusively, Sabouraud's medium is used (Bond et al., 1995a; Bond and Lloyd, 1996; Gordon, 1979; Miller et al., 2013a). *Malassezia pachydermatis* colony appearance is reported most distinct on modified Dixons medium compared to several other medias, where they form buff-coloured dome shaped colonies of 1-3 mm in diameter (Bond and Lloyd, 1996; Nuttall, 2012). To detect and quantify *Malassezia* in culture an incubation time of three up to ten days and a temperature of 28-37°C should be used (Bond et al., 1996, 1995a, 1995c, 1994; Bond and Lloyd, 1996; Cafarchia et al., 2011, 2005; Kennis et al., 1996; Machado et al., 2011; Nardoni et al., 2007, 2004; White et al., 1998). Noteworthy is that population sizes of *Malassezia pachydermatis* do not vary significantly between an incubation time of three and seven days at 32°C but will be significantly lower on day three compared to day seven if cultivated at 26°C (Bond and Lloyd, 1996).

An aerobic atmosphere is sufficient and most commonly used (Bond et al., 1996, 1995a, 1995c, 1994, Cafarchia et al., 2011, 2005; Kennis et al., 1996; Machado et al., 2011; Nardoni et al., 2007, 2004; White et al., 1998) but carbon dioxide enriched (5-10%) air might be used and is proven to give an increased frequency of isolation and population size of *Malassezia pachydermatis* on Sabouraud's medium, but not on modified Dixons medium (Bond and Lloyd, 1996; Pinchbeck et al., 2002).

Fungal culture is used for both detection of *Malassezia* organisms and estimating their population sizes by quantitative or semi-quantitative methods. No exact number have been established for the diagnosis of *Malassezia* dermatitis although it has been proposed that populations higher than 70 colony forming units (CFU) when sampling a ≤ 25 cm² skin area with a cotton swab could be indicative of *Malassezia* overgrowth (Cafarchia et al., 2005). This suggestion was based on the mean of 70 CFU obtained from the healthy dogs negative on cytology but positive on culture in the study.

Sampling techniques include sampling by direct impression of culture medium onto the skin with, for example, contact plates of various sizes; or by using a variety of tools such as an adhesive tape strip, a

sterile carpet or cotton swab for rubbing the skin, or by a detergent scrub technique (Bensignor et al., 2002; Bond et al., 1995a, 1995c; Bond and Lloyd, 1997; Cafarchia et al., 2011, 2011, 2005; Kennis et al., 1996; White et al., 1998). For mucosal sites or sampling of the external ear canal cotton swabs are used. Few comparative studies of the techniques have been done, although one published study by Bond et al (1995c) found no significant difference between results obtained using contact plates and the detergent scrub technique in healthy dogs. A correlation between the contact plates and the detergent scrub technique was reported in samples from dogs with dermatitis and otitis by Bond et al (1995a) but populations sizes tended to be about 100 times greater when using the detergent scrub technique. In addition, Bond et al (1994) found fungal culture with contact plates in healthy dogs to be lacking in sensitivity compared to cytology as gold standard.

Correlation of population size based on cytology and fungal culture

Reports of correlations for population sizes between cytology and fungal culture are diverse and inconclusive. Correlations for calculation of population size between cytology using a tape strip technique and culture using contact plates was reported significant by Nuttall and Halliwell (2001) for dogs with both elevated/high and normal/low populations sizes. Using similar sampling techniques Bond et al (1995a, 1994) came to the opposite conclusion when sampling healthy dogs as well as dogs with dermatitis or otitis. No correlation between cytology and culture results were found in the study by Machado et al (2011) although high populations on cytology correlated with a positive fungal culture. A weak correlation between cytology and culture was reported in healthy canines by Bensignor et al (2002), although ultimate population sizes obtained by cytology was reported to be >100 times higher than counts obtained by culture. In contrast Kennis et al (1996) found the population sizes obtained by culture to be significantly higher compared to cytology when sampling healthy dogs.

Species differentiation

Culture can be used for species differentiation between lipid dependent species and *Malassezia pachydermatis* due to the non-lipid dependent property of *M. pachydermatis*. For this purpose *M. pachydermatis* is cultured on Sabouraud's medium which is lipid deficient (Bond et al., 1996; Bond and Lloyd, 1996; Gordon, 1979; Nuttall, 2012). Further species identification cannot be done by culture alone and might include evaluating morphology, immunological properties and molecular biology or use of methods like polymerase chain reaction (PCR), rRNA sequencing analysis or Matrix Assisted Laser Desorption Ionization Time of Flight mass spectrometry (MALDI-TOF MS) (Gaitanis et al., 2012; Kolecka et al., 2014). Kolecka et al (2014) reported MALDI-TOF MS to correctly identify clinical isolates of *Malassezia* yeast by 100% on genus level and that 99.2-100% of the isolates were identified by species.

Histopathology

From samples taken for skin biopsies, *Malassezia* yeasts can be detected on histopathology mainly in surface and infundibular keratin and sometimes in hair follicles (Mauldin et al., 1997; Miller et al., 2013a). Surface yeasts are not necessarily indicative of *Malassezia* dermatitis as it may occur on histopathology for other canine dermatological diagnoses as well, but when detected in hair follicles it should be considered pathognomonic for *Malassezia* dermatitis (Miller et al., 2013a; Scott, 1992). Although *Malassezia* yeasts are readily detectable on cytology prior to skin biopsies, they are not always detectable on histopathology (Mauldin et al., 1997; Mobley and Meyer, 1994). In the study by Mauldin et al (1997) *Malassezia* organisms were detectable in 73.3% of the histological preparations whereas Mason and Evans (1991) reported detectable yeast in 100% of their samples from biopsies.

Malassezia dermatitis is consistent with a histopathological reaction pattern of epidermal reactions and lymphocytic superficial perivascular to interstitial dermatitis (Mauldin et al., 1997). This reaction pattern is based on findings of parakeratotic hyperkeratosis, lymphocytic exocytosis, diffuse intercellular oedema and irregular epidermal hyperplasia. Other findings might be acanthosis, orthokeratotic hyperkeratosis, focal parakeratosis and neutrophil infiltration into epidermis and under

stratum corneum (Mason and Evans, 1991; Mobley and Meyer, 1994). When yeasts are detectable, a higher number of yeasts correlates with a more severe and pronounced parakeratosis (Mauldin et al., 1997). The superficial dermis, is according to findings of Mason and Evans (1991), characterized with mild to moderate oedema and a mixed pattern of inflammatory cells including neutrophils, lymphocytes, plasma cells, mast cells and occasionally eosinophils.

After challenging the skin of healthy dogs with *M. pachydermatis* solutions for 7 days, histopathological findings were similar to those of natural occurring cases of *Malassezia* dermatitis but changes were milder in degree of severity (Bond et al., 2004).

Treatments of *Malassezia* dermatitis

A wide variety of agents for both systemic and topical use have been proposed and evaluated for the treatment of *Malassezia* dermatitis (Miller et al., 2013a).

Treatment of choice might include either or both systemic and topical treatments and should depend on distribution pattern, degree of severity, owner's resources, possible adverse effects and legislation in the present country (Nuttall, 2012). If any underlying diseases or symptoms (e.g. dryness of skin) are related to the *Malassezia* dermatitis, they also need to be addressed with appropriate measures.

As an example, early case series reported by Mason and Evans (1991) included the use of systemic ketoconazole at a dosage of 10 mg/kg twice daily for one month, combined with one or more of the following topical treatments: selenium sulphide shampoo twice weekly for one month, miconazole cream twice daily for 2 weeks, or focal treatment daily with povidone-iodone. The protocols were curative for all the patients (Mason and Evans, 1991).

Systemic therapy

Therapies available and evaluated *in vivo* for the treatment of *Malassezia* dermatitis includes azole derivatives (ketoconazole, itraconazole, fluconazole) and the allylamine terbinafine (Bensignor et al., 2002; Kumar et al., 2002; Rosales et al., 2005; Sickafoose et al., 2010). Both azole derivatives and allylamine inhibit synthesis of ergosterol, an essential component of cell membrane in *Malassezia* yeasts, ultimately resulting in the death of the yeast cell (Miller et al., 2013a).

There are several *in vitro* studies reporting a high antifungal activity against *Malassezia pachydermatis* for itraconazole, ketoconazole and terbinafine (Álvarez-Pérez et al., 2016; Cafarchia et al., 2012; Carrillo-Muñoz et al., 2013; Gupta et al., 2000; Yurayart et al., 2013). In contrast, the *in vitro* susceptibility of *Malassezia pachydermatis* to fluconazole has been reported to be much lower compared to other azole derivatives (Álvarez-Pérez et al., 2016; Brito et al., 2009; Cafarchia et al., 2012; Carrillo-Muñoz et al., 2013). There is some contradictory reports regarding these *in vitro* susceptibilities, Lyskova et al (2007) reported a high antifungal activity for fluconazole and Cafarchia et al (2012) found the antifungal activity for terbinafine to be low.

However, an *in vitro* isolate from a clinical case of canine seborrhoeic dermatitis was reported resistant to ketoconazole and itraconazole (Nijima et al., 2011) and Cafarchia et al (2012) found several strains of *Malassezia pachydermatis* to be cross-resistant to numerous azole compounds from dogs with and without skin lesions. As an example, one strain found was resistant to fluconazole, ketoconazole, itraconazole, voriconazole and posaconazole (Cafarchia et al., 2012).

Fluconazole

In a double blinded non inferiority trial by Sickafoose et al (2010) the efficacy of fluconazole was compared to that of ketoconazole. Twenty-five dogs were divided into two groups, either receiving fluconazole 5-10mg/kg q24h or ketoconazole 5-10mg/kg q24h during a period of three weeks. In addition, all dogs received cephalexin 22-30 mg/kg q12h since pyoderma was also an inclusion criteria of the study. Statistically significant reduction of cytological yeast count, clinical signs and pruritus was achieved by both treatments, without any statistical difference between the treatments. The

majority of the dogs in the study by Sickafoose et al (2010) obtained clinical resolution but a small majority required further treatment.

Adverse side effects reported by Sickafoose (2010) for fluconazole include vomiting, soft stool and diarrhoea. These findings are also reported in the textbooks by Koch et al (2012) and Miller et al (2013a) with the addition of hepatotoxicity as a possible side effect.

Itraconazole

In a randomized controlled trial by Pinchbeck et al (2002) with 20 dogs divided into two groups, two dose regimes of itraconazole were compared. These were a pulse administration of itraconazole 5mg/kg q24h for two days per week and itraconazole 5mg/kg q24h continuously. The treatment period for both regimes was three weeks. A significant decrease of yeast organisms as well as clinical severity and pruritus was reported for both regimes without any significant difference in efficacy between the two regimes (Pinchbeck et al., 2002). The efficacy of a pulse administration regime of itraconazole as described by Pinchbeck et al (2002) was also reported efficient by Bensignor (2008) who compared it to the efficacy of ketoconazole 10mg/kg q24h for a treatment period of three weeks. Both treatment regimens resulted in a reduction of *Malassezia* yeast population, clinical lesions and pruritus and no statistical significant difference in efficacy could be reported between the regimes (Bensignor, 2008).

Kumar et al (2002) also reported that the use of itraconazole, 5mg/kg q24h, in combination with selenium sulphide shampoo twice weekly, was efficient and led to clinical resolution in 35-40 days. Another study by Sai Prasanna et al (2006) with the same dose regime, reported a shorter treatment period, the resolution of the infection was then also confirmed by cytological examination.

No adverse side effects were reported for itraconazole in these study populations (Bensignor, 2008; Pinchbeck et al., 2002), although side effects reported elsewhere are hepatotoxicity, gastrointestinal toxicity and cutaneous vasculitis (Grooters and Taboada, 2003; Koch et al., 2012; Miller et al., 2013a). In addition Koch et al (2012) report anorexia as a common side effect and ulcerative dermatitis as an uncommon side effect.

Ketoconazole

The efficacy of ketoconazole in reducing yeast count, clinical lesions and pruritus have been reported by Bensignor (2008) in a randomized, blinded controlled study, in which ketoconazole was administered at 10mg/kg q24h for three weeks. Similar results were reported in the study by Sickafoose et al (2010) where dogs with *Malassezia* dermatitis and concurrent pyoderma were treated with ketoconazole 5-10mg/kg q24h and cephalexin 22-30 mg/kg q12h daily for three weeks.

Rosales et al (2005) studied the efficacy of ketoconazole as a therapy for *Malassezia* infection concurrent with pyoderma. The study was randomized, single blinded and included 20 dogs divided into three groups. All groups received cephalexin 22-30 mg/kg q12h, one group only received cephalexin and was used as a control group. The other two groups received either terbinafine at 30 mg/kg q24h or ketoconazole 5-10 mg/kg q12h. Reduction of yeast, clinical lesions and pruritus was noted for all groups but only reduction of yeast was found statistically significant for ketoconazole and reduction of clinical lesions was only significant for the group treated exclusively with cephalexin (Rosales et al., 2005). In the study by Rosales et al (2005) only two of the dogs treated with ketoconazole resolved completely both on cytology and clinically.

Sai Prasanna et al (2006) reported ketoconazole to be efficient at 5mg/kg once daily when combined with topical treatment using ketoconazole 2% shampoo twice weekly and ketoconazole cream 2% applied twice daily. The treatment period ranged from 15-25 days and results were evaluated by clinical observations and cytology.

Rosales et al (2005) did not observe any adverse side effects in dogs receiving ketoconazole, 5-10 mg/kg twice daily during 3 weeks, in their study. However, several studies have reported of adverse

side effects for the systemic treatment with ketoconazole, which include: vomiting, anorexia/inappetence, exhaustion and soft stool/diarrhoea (Bensignor, 2008; Mason and Evans, 1991; Sickafoose et al., 2010). Furthermore, ketoconazole is reported to be hepatotoxic and teratogenic, as well as to cause fever, thrombocytopenia, non-regenerative anemia, pruritus and alopecia (Koch et al., 2012; Miller et al., 2013a). Listed possible side effects for ketoconazole tablets approved for dogs in Sweden include apathy, ataxia, tremor, hepatotoxicity, vomiting, anorexia and diarrhoea (Läkemedelsindustriföreningen, 2014). In addition, ketoconazole is reported to have antiandrogenic and antiglucocorticoid effects (Läkemedelsindustriföreningen, 2014; Miller et al., 2013a).

Terbinafine

In a randomized, single blinded study by Berger et al (2012) two dose regimes of terbinafine were compared. Terbinafine at a dosage of 30mg/kg was either administered q24h or as a pulse administration q24h for two consecutive days per week. The treatment periods lasted three weeks. Clinical lesions, mean *Malassezia* population and pruritus was statistically significantly reduced for both treatment regimens with a significantly larger reduction of pruritus achieved by the pulse administration of terbinafine (Berger et al., 2012). Noteworthy is that in the study by Berger et al (2012) 17 of the 20 dogs treated with terbinafine required further treatment after the end of the trial.

The *in vivo* efficacy of terbinafine was compared to that of ketoconazole by Rosales et al (2005) and found to be as efficient in reducing *Malassezia* populations and more efficient in reducing pruritus in dogs with *Malassezia* dermatitis and concurrent pyoderma. Another study, by Kumar et al (2002), reported successful treatments within a duration of 44 days using terbinafine at a dosage of 2.5 mg/kg q24h in combination with topical terbinafine 1% cream and selenium sulphide 2.5% shampoo. The duration of treatment for ketoconazole in the same study was less than half than that of terbinafine (Kumar et al., 2002).

Berger et al (2012) reported adverse effects for terbinafine to be vomiting, anorexia, diarrhoea, excessive panting, and in one dog elevated hepatic enzymes. Rosales et al (2005) did not observe any adverse effects of terbinafine in their study.

Topical therapy

Topical therapy can be used as a sole therapy or combined with systemic treatment options (Mueller et al., 2012). Topical therapy solely is often curative in the treatment of superficial *Malassezia* infections and might be especially effective for breeds with short coats or areas of focal alopecia (Miller et al., 2013a). It may also be the most cost effective and safest treatment option in regards of possible adverse side effects (Nuttall, 2012). Agents are available in several topical formulations including shampoos, rinses, lotions, solutions, gels, creams and ointments (Miller et al., 2013c). Type of formulation might also influence the efficacy of a product. This was illustrated in the study by Stroh et al (2010) in which the shampoo vehicle was as effective in reducing superficial bacterial counts as the shampoo containing the active agents chlorhexidine and phytosphingosine.

Therapies available and evaluated *in vivo* includes chlorhexidine, miconazole, a combination of chlorhexidine and miconazole, piroctone olamine, selenium sulphide, a herbal formulation and climbazole (Bond et al., 1995b; Cavana et al., 2015a, 2015b; Jasmin et al., 2003; Lloyd and Lamport, 1999; Marsella et al., 2000; Maynard et al., 2011; Nardoni et al., 2014; Rème et al., 2003)

A number of *in vivo* studies report the topical use of enilconazole (Carlotti and Laffort-Dassot, 1996; Mueller et al., 2012; Negre et al., 2009), ketoconazole (Kumar et al., 2002; Sai Prasanna et al., 2006) and terbinafine (Kumar et al., 2002) although always in combination with systemic antimycotic therapy.

Chlorhexidine

Several *in vitro* studies reports chlorhexidine products of 2-4% concentration to have low minimum fungicidal concentration for isolates of *Malassezia pachydermatis* (Lloyd and Lamport, 1999; Uri et

al., 2016; Young et al., 2012). Loyd and Lamport (1999) reported that products with lower concentrations needed more time to eliminate the *Malassezia* organisms, although the product of 3% chlorhexidine tested was more efficient than the 4% chlorhexidine product in this *in vitro* study, in which chlorhexidine gluconate was used. The authors suggested that the formulation other than the chlorhexidine concentration was important for the efficacy of the product. In another *in vitro* study, Uri et al (2016) reports a lower minimum fungicidal concentration (MFC) for a 4% chlorhexidine gluconate product versus 3% chlorhexidine digluconate. The authors suggested that this difference might not only be due to the concentration of chlorhexidine, as additional ingredients varied in the products tested. In the 4% chlorhexidine product an additive effect of isopropyl alcohol was possible, whereas the 3% product reported as less effective also contained 0.5% climbazole and phytosphingosine.

The *in vivo* effect of chlorhexidine was studied by Maynard et al (2011) in a prospective, controlled, randomized, single blinded study. The trial compared the efficacy of a 3% chlorhexidine shampoo with a control shampoo containing 2% chlorhexidine and 2% miconazole. The trial included 54 dogs that were evaluated until cytological recovery or a maximum of six weeks. Treatment regime for the chlorhexidine shampoo was three times weekly for two weeks, then twice weekly for two weeks and lastly once weekly for two weeks. The control product was applied twice weekly. Maynard et al (2011) based the evaluation of treatment efficacy primarily on reduction of *Malassezia* counts and secondary on reduction of twelve clinical parameters. Treatment with both products resulted in a large reduction of *Malassezia* count and reduction of all clinical parameters measured. Most cases recovered within four weeks of treatment and a good clinical response was reported in 86.4-90.6% of the cases (Maynard et al., 2011). None of the efficacy parameters measured differed significantly between the two products tested. Maynard et al (2011) reported treatment failure for one dog in each product group.

Jasmin et al (2003) found a 3% chlorhexidine shampoo to be efficient in the treatment of *Malassezia* dermatitis. The study included 28 dogs and shampoo was applied twice weekly for three weeks. The treatment resulted in a statistically significant reduction of clinical lesions, pruritus and *Malassezia* population scores. In fact, *Malassezia* population scores were reduced by more than 97% and 93% of the dogs had a yeast score regarded as normal flora at the end of the trial (Jasmin et al., 2003).

Jasmin et al (2003) did not observe any adverse side effects in their trial using a 3% chlorhexidine shampoo. However, transient adverse effects in 4 of 31 cases for a shampoo containing 3% chlorhexidine was reported by Maynard et al (2011). The adverse effects reported were acute pododermatitis in one forelimb, exfoliation and scaling of the skin and increased pruritus. In addition, contact allergy to chlorhexidine have been reported in humans (Opstrup et al., 2016; Toholka and Nixon, 2013).

Chlorhexidine/Miconazole

A randomized, double blinded study set up by Bond et al (1995b) evaluated the antifungal efficacy of a shampoo containing 2% chlorhexidine and 2% miconazole for treatment of seborrhoeic dermatitis associated with *Malassezia pachydermatis*. The study included 33 dogs and the efficacy of 2% chlorhexidine and 2% miconazole shampoo was compared to a 0.25% selenium sulphide shampoo. Shampoos were applied at a three day interval during three weeks. Bond et al (1995b) reported a statistically significant reduction of both clinical lesions and *Malassezia* population counts for dogs treated with the 2% chlorhexidine and 2% miconazole shampoo. Compared to the selenium sulphide shampoo the chlorhexidine and miconazole shampoo showed a significantly greater reduction of pruritus, erythema, exudation, overall severity and *Malassezia* population counts. Bond et al (1995b) did not observe any adverse reactions.

Maynard et al (2011) confirmed the good efficacy of a shampoo containing 2% chlorhexidine and 2% miconazole in the treatment of *Malassezia* dermatitis. In their study the shampoo significantly reduced *Malassezia* populations and clinical lesions. Cytological recovery was obtained within 28 days for a

majority of the cases and no adverse effect were reported for the treatment with combined chlorhexidine and miconazole shampoo (Maynard et al., 2011).

Malassezia pachydermatis is highly susceptible to both chlorhexidine and miconazole as reported by several *in vitro* studies (Carrillo-Muñoz et al., 2013; Lyskova et al., 2007; Uri et al., 2016). Although Cafarchia et al (2012) reported the antifungal activity to be low for miconazole.

Climbazole

Based on *in vitro* data published by Schmidt (1997) climbazole has a good antifungal activity against *Malassezia pachydermatis*.

The antifungal *in vivo* efficacy of a 2% climbazole shampoo was reported by Cavana et al (2015b) in a randomized study of healthy dogs. In the study the efficacy of the climbazole shampoo was compared to that of a physiological shampoo base without antifungal properties. Shampoos were applied once weekly for two weeks. The climbazole shampoo showed significant reduction of *Malassezia* population sizes already after one application and remained significantly decreased fifteen days after the second application of the climbazole shampoo. Cavana et al (2015b) did not observe any significant reductions of *Malassezia* populations in dogs treated with the physiological shampoo. No adverse effects were observed for the 2% climbazole shampoo during the study period (Cavana et al., 2015b).

In another study Cavana et al (2015a) evaluated the *in vivo* activity of wet wipes, containing chlorhexidine 0.3%, climbazole 0.5% and Tris-EDTA, against *Malassezia pachydermatis*. The study included five healthy dogs with cutaneous elevated *Malassezia* populations. Using a split body protocol, wipes were applied once or twice daily for three consecutive days. Significant reduction of *Malassezia* counts was obtained 30 min after the first application and remained significantly reduced, compared to the initial count, seven days after the last application. There was no difference in results between the application once or twice daily (Cavana et al., 2015a). Transient erythema and pruritus at the application site was noted in one of the dogs.

Essential oils

The efficacy of a herbal formulation consistent of essential oils as sole treatment of *Malassezia* dermatitis was studied by Nardoni et al (2014). The formulation contained: Citrus aurantium 1%, Lavandula officinalis 1%, Origanum vulgare 0.5%, Origanum majorana, 0.5%, Mentha piperita 0.5% and Helichrysum italicum var. italicum 0.5%. Nardoni et al (2014) set up a prospective, randomized, single blinded study in which 35 dogs were divided into three groups. Five dogs were left untreated and the remaining received one of the following treatments: topical herbal formulation applied twice daily for one month or treatment for three weeks with a combination of systemic ketoconazole 10mg/kg q24h and topical 2% chlorhexidine twice a week. Based on evaluation performed thirty days after initiated treatment Nardoni et al (2014) reported significant reduction of clinical lesions and *Malassezia* population size for both treated groups but not for the untreated group. The efficacy did not differ between the two groups receiving treatment although there were two dogs that did not show a good clinical response, both belonging to the group treated with the herbal formulation. Recurrence of clinical signs at 180 days post initiation of treatment was not observed for the group treated with the herbal formulation but reported in the group treated with ketoconazole and chlorhexidine (Nardoni et al., 2014).

In the same study, *in vitro* data revealed the active major components of the herbal formulation to be thymol, carvacrol, p-cymene, 1,8-cineol, limonene and menthol; and the minimum inhibitory concentration for the product as a whole was reported to be low (Nardoni et al., 2014). Khosravi et al (2016) studied the effect of several essential oils *in vitro* and reported low minimum inhibitory concentrations for *Zataria multiflora* and *Thymus kotschyanus*. The main constituent of these oils were thymol and carvacrol which are both phenolic compounds.

Miconazole

A randomized double blinded study evaluated the use of a 1% and 2% miconazole conditioner and controlled their efficacy against a placebo vehicle conditioner (Marsella et al., 2000). The study included 18 dogs divided into three groups, each group was shampooed once weekly with a nonmedicated shampoo and conditioner was then applied three times weekly for two weeks and then twice weekly for an additional two weeks. Marsella et al (2000) reported that *Malassezia* population size had decreased significantly for both two groups treated with miconazole after two weeks treatment, while the dogs treated with the vehicle had significantly higher counts at that point. However, by the end of the treatment period all treatments regimes had significantly reduced the *Malassezia* population sizes and there was no significant difference between any of the groups. All three treatment regimens also resulted in a significant reduction of erythema and pruritus at the end of the study period without any significant difference between the treatment groups at any point during the study (Marsella et al., 2000). In the study by Marsella et al (2000) eight dogs required further systemic treatment after the study period, out of which half had been treated with the vehicle product and the other half with one of the miconazole products.

In vitro studies are inconclusive regarding the antifungal activity of miconazole, both high antifungal activity (Carrillo-Muñoz et al., 2013; Lyskova et al., 2007) and low antifungal activity is reported (Cafarchia et al., 2012).

Piroctone olamine

In a study by Rème et al (2003) a combination of piroctone olamine containing shampoo and lotion was evaluated. The study included 14 dogs with keratoseborrhoeic disorder and high *Malassezia* populations. Treatment regime consisted out of shampoo once weekly and lotion twice weekly for the duration of three weeks and resulted in a significant reduction of excoriations, keratoseborrhoeic disorder, erythema, pruritus and *M. pachydermatis* populations (Rème et al., 2003). Piroctone olamine have also been reported to reduce *Malassezia pachydermatis* populations on the skin of healthy dogs (Bourdeau et al., 2006).

Selenium sulphide

In a randomized, double blinded study 0.25% selenium sulphide shampoo was one of the treatments evaluated on dogs with seborrhoeic dermatitis associated with *Malassezia pachydermatis* (Bond et al., 1995b). The dogs in the study were shampooed with selenium sulphide at three day intervals for three weeks which resulted in a statistically significant reduction of clinical manifestations and *Malassezia pachydermatis* populations for the dogs treated. Although six of the seventeen dogs treated with the selenium sulphide shampoo did not respond clinically and had persistently high *Malassezia pachydermatis* population counts (Bond et al., 1995b).

Selenium sulphide 2,5% shampoo have been used successfully in combination with systemic treatment of itraconazole (Kumar et al., 2002; Sai Prasanna et al., 2006). It has also been reported in successful treatments combined with systemic and topical terbinafine (Kumar et al., 2002). Mason and Evans (1991) reported using selenium sulphide shampoo in combination with systemic ketoconazole and other topical agents. They argued that the keratolytic effect of selenium sulphide removes excess keratin and thereby eliminates the yeast, although they did not recommend it to be used as the only acting agent in treatment of *Malassezia* dermatitis (Mason and Evans, 1991).

In vitro

Based on *in vitro* data reported by Young et al (2012) it was concluded by Mueller et al (2012) that treatment with acetic acid and boric acid or benzoyl peroxide may be useful in treatment of *Malassezia* dermatitis.

Other agents reported to have good antifungal activity with low minimum inhibitory concentrations for *Malassezia pachydermatis* are numerous and include: amphotericin B, bifonazole,

ciclopiroxolamin, clotrimazole, econazole, ethyl lactate, nystatin, pimaricin, posaconazole and voriconazole (Álvarez-Pérez et al., 2016; Brito et al., 2009; Lyskova et al., 2007; Schmidt, 1997; Young et al., 2012; Yurayart et al., 2013).

Evidence based therapies

In a systematic review based on *in vivo* studies of interventions for *Malassezia* dermatitis in canines Negre et al (2009) concluded that the only treatment options that fulfilled the criteria of being evidence based were:

- Systemic treatment with ketoconazole 5-10 mg/kg q24h for 3 weeks OR itraconazole: 5 mg/kg q24h for 3 week
- Topical treatment with 2% chlorhexidine in combination with 2% miconazole nitrate shampoo twice weekly for 3 weeks

In a review by Mueller et al (2012), focusing on topical therapies for yeast and bacteria, the authors concluded that there is good evidence for the use of 2% chlorhexidine in combination with 2% miconazole for the treatment of cutaneous infections by *Malassezia* spp..

There is also good evidence for the use of 3% chlorhexidine in shampoo formulation as a treatment of cutaneous *Malassezia* infections. This evidence is mainly based on the prospective, controlled, randomised, single-blinded study by Maynard et al (2011). Although the efficacy of chlorhexidine has also been reported in several other studies (Jasmin et al., 2003; Lloyd and Lamport, 1999; Uri et al., 2016; Young et al., 2012).

BACKGROUND

Hydrogen peroxide

Hydrogen peroxide (H₂O₂) is an effective germicide widely used for antiseptics, disinfection and sterilization. In contact with organic tissue it decomposes into water and oxygen, making it safe for medical use and environmentally friendly (Block, 2001). The decomposition is fast in water solution but when stabilized in a cream formula of 1% hydrogen peroxide a slow decomposition is obtained thereby prolonging the effect (Christensen and Anehus, 1994; Mueller et al., 2012). Hydrogen peroxide has both antibacterial and antifungal activity, although the activity is greater against bacteria compared to yeast (Block, 2001). In contrast, Millers et al (2013c) graded hydrogen peroxide as a weak germicide and concluded that it was of limited use in treatment of skin disease in animals.

Acting as an oxidant, hydrogen peroxide produces hydroxyl free radicals which damages lipids, proteins, DNA and other essential cell components (Block, 2001; McDonnell and Russell, 1999).

Hydrogen peroxide occurs naturally in bodily tissues as a result of cellular metabolism (Block, 2001). Furthermore it plays an important part of early inflammatory defence response against bacteria in which the response includes release of hydrogen peroxide by phagocytising neutrophils (Block, 2001; Mueller et al., 2012). The enzymatic production of hydrogen peroxide is also reported to play an important role for the antimicrobial effects of honey (Feás et al., 2013; French et al., 2005; Miller et al., 2013c).

The use of a 1% hydrogen peroxide cream for topical treatment has been reported safe and effective for both a human bacterial skin infection called impetigo contagiosa (Christensen and Anehus, 1994) and human acne vulgaris (Capizzi et al., 2004; Milani et al., 2003). In veterinary medicine a 1% hydrogen peroxide cream was reported to efficiently prevent bacterial colonization during wound healing in a randomized, blinded, controlled study in horses (Tóth et al., 2011).

To the best of my knowledge neither *in vitro* nor *in vivo* data regarding the antifungal activity of hydrogen peroxide for *Malassezia* spp. has yet been reported.

Crystavet®

Crystavet® is an antimicrobial cream product with 1% hydrogen peroxide produced by Bioglan. The product was at the time of this study not yet commercially available.

AIM OF THE STUDY

The purpose of this study was to, in a prospective, open, randomized, controlled study, evaluate the efficacy of a new topical product, Crystavet® (1% hydrogen peroxide, Bioglan, Malmö, Sweden), for the treatment of *Malassezia* overgrowth in dogs. Evaluation of treatment efficacy was primarily based on changes in mean *Malassezia* count obtained by cytology. Secondary evaluation criteria were changes in clinical skin lesion score, pruritus score and *Malassezia* count based on quantitative culture.

MATERIAL AND METHODS

Literature review

With the use of the databases PubMed, Google Scholar, Science Direct, Web of Science, Wiley Online Library and Primo, literature was searched regarding *Malassezia* spp. and *Malassezia* dermatitis in canines. Included search words were: *Malassezia*, *Malassezia* dermatitis, yeast dermatitis, canine*, dog* skin, *Malassezia* isolates*, treatment*, therapy, diagnose*, hydrogen peroxide etc. Reference list in articles and books found relevant were also used in the search.

Information was gathered April-December 2016. Articles and texts were restricted to publications in English and Swedish.

Clinical study

Study design

The study was set up as a prospective, open, randomized, controlled study using a split-body protocol. The study took place during 2016, divided into two-study periods: May and September/October.

Privately owned dogs with *Malassezia* overgrowth was recruited through the University Animal Hospital, Uppsala, Sweden and the dermatology department at DjurAkuten AB, Stockholm, Sweden. The recruitment also included advertising in social media and e-mailing veterinary- and veterinary nurse students at the Swedish University of Agricultural Sciences.

Inclusion criteria were:

- Dogs presented with two or more anatomically distinct sites with an mean of ≥ 4 *Malassezia* yeast organisms per 10 microscopic fields at 1000 magnification
- Signed informed owner consent form

If the patient presented with more than three sites available for inclusion, the lesions with the highest average numbers of organisms were chosen for the study.

Exclusion criteria were:

- Treatment with systemic antimycotic substances or topical antimycotic substances on the skin site included in the study ≤ 10 days prior to enrolment and throughout the study period.
- Adverse reactions incompatible with continued treatment, after recording data.
- Non-compliance to study protocol

After signing an informed owner consent form, a clinical history was taken and a general physical exam was conducted. The study group was divided into two subgroups, based on prior treatments. Inclusion in subgroup A required that the patient had not initiated or changed any treatment, systemic or topical of, or adjacent to, area of inclusion, of any substances ≤ 28 days prior to entry or throughout the study period. Patients that did not fulfil this criteria were included in subgroup B.

The general pruritus pre-treatment was estimated by the patient's owner using a validated pruritus visual analogue scale (PVAS). Cytology was performed on clinical skin lesions and if compatible with inclusion criteria, contact plates were used to take samples for fungal culture from the chosen lesions. The skin at each site was assessed for clinical lesions using clinical lesion scoring.

Patients were treated at home by the owner according to written and oral instructions given at start of the study period and the treatment was thus not blinded for the owner or the investigator. Each patient was used as their own control by treating the anatomically distinct lesion sites with either Crystavet® (1% hydrogen peroxide, Bioglan, Malmö, Sweden) 0.05 ml/cm² twice daily or Trikem spray (4% chlorhexidine, Trikem AB, Klågerup, Sweden) 0.025 ml/cm² twice daily for seven days. The choice of treatment for the lesions was randomized using a dice for the site with the highest average of *Malassezia* yeast organisms on cytology. The site with the second highest average of *Malassezia* yeast

organisms was then treated with the other treatment protocol. If possible, when more than two lesional sites were present and the owner complied, one randomized lesion was left untreated.

Evaluation of the treatment was performed after seven days and included a short owner interview, a general physical examination and owner estimation of pruritus post-treatment using PVAS. The owners had been instructed to not treat the sites on the same day as the evaluation. Sampling for cytology and culture was performed and skin lesions were scored according to the same procedure as on the inclusion day. Cytological recovery post treatment was defined as a result of ≤ 0.2 mean *Malassezia* yeast organism on cytology. Any kind of side effect was recorded. The owners were given an evaluation form in which they were asked to evaluate treatment outcome, possible adverse effect, user friendliness of products and quality of instructions. Any kind of adverse effect was treated *lege artis*, after recording.

Pruritus visual analogue scale, PVAS

To assess the effect on pruritus, a pruritus visual analogue scale (PVAS) with descriptors was used, one pre- and one post-treatment. From the visual analogue scale a number between 0 and 10, representing the severity of pruritus, was calculated where 0 represented normal/no itching and 10 extremely severe itching. The descriptors were translated into Swedish by K. Bergvall and based on the validated canine pruritus visual analogue scale (Hill et al., 2007; Rybniček et al., 2009).

Cytology

Cytology was performed with tape strip technique: using a clear acetate tape pressed to the skin once. The sample was stained with a drop of basophilic thiazine dye Hemacolor rapid staining kit (Merck KGaA, Darmstadt, Germany) and the mean of *Malassezia* yeast organisms per 10 oil immersion fields (HPF), under 1000 magnification was calculated.

Fungal culturing

Custom made contact plates were manufactured with modified Dixon's medium (National veterinary institute, Uppsala, Sweden) in test tubes with a 13 mm diameter, see Figure 1. These were made in two separate batches for the different study periods: 2016-05-04 for May and 2016-09-02 for September/October.



Figure 1. Contact plate

Sampling of lesions for fungal culture was performed by lightly pressing the contact plates against the skin surface for 10 seconds. Within 12 hours after sampling the contact plates were cultured at $30^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for 4-5 days. Quantification of colonies was performed by manually counting colony forming units (CFU). For cultures of confluent growth on the contact plates the CFU was determined to be >100 CFU.

Colonies from the contact plates were then cultured for species differentiation using agar plates of modified Dixon's medium (National veterinary institute, Uppsala, Sweden) and Sabouraud's medium (National veterinary institute, Uppsala, Sweden) at $30^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for 4-5 days. This process was overseen by a third party, to whom the cultures were blinded.

- If pure culture and colony appearance corresponded with that of *Malassezia pachydermatis* on the contact plate, a colony was transferred to both modified Dixon's medium and Sabouraud's medium to confirm or exclude *Malassezia pachydermatis*.
- If pure culture and colony appearance did not correspond with that of *Malassezia pachydermatis* on the contact plate but other fungal species was suspected, a colony was transferred to modified Dixon's medium.
- If cultures were of mixed species on the contact plates, pure culture was obtained by transferring a colony for each different fungal macroscopic appearance to modified Dixon's

medium separately. When pure culture was obtained, a colony was transferred to Sabouraud's medium to confirm or exclude *Malassezia pachydermatis*.

Suspected fungal colonies that could not be confirmed as *Malassezia pachydermatis* were analysed using Matrix Assisted Laser Desorption/Ionization Time of Flight mass spectrometry (MALDI-TOF MS, Bruker Daltonics Microflex, MALDI Biotyper, Bremen, Germany) performed at the National Veterinary Institute laboratory according to standard laboratory procedure. Growth of colonies with bacterial macroscopic appearance on the contact plates was noted but not cultured for species differentiation.

Clinical lesion score

Each lesional skin area included in the study was rated according to 11 clinical skin lesions including erythema, lichenification, hyperpigmentation, alopecia, papules, pustules, collarettes, crusts, excoriations, greasy seborrhoea and scaling. Lesion parameters were rated according to severity with 0 representing no lesions, 1: mild, 2: moderate and 3 representing severe lesions. From this, a total clinical lesion score was calculated with a minimum value of 0 to a maximum value of 33.

Statistical analysis

Calculations were made using Microsoft Excel for PC and analyses performed using the statistical software Minitab version 17 for PC.

Statistical analysis was performed on data obtained from cytology, clinical lesions score, PVAS and the owner evaluation form. A probability plot was used to determine whether data was para-metric or not. Further, Wilcoxon Signed Rank Test was used to analyse if there was a significant reduction of parameters post treatment compared to pre-treatment. Kruskal-Wallis test was used to evaluate if there was a difference between subgroup A and B for the cytology results obtained for Crystavet as well as between subgroup A and B for the reference product. The test was also used to evaluate whether there was any difference on inclusion day in mean yeast for cytology and clinical lesion scores between the sites included for the different therapies. Furthermore the test was applied on data from the owner evaluation form. For fungal culture data, the signtest was applied. For all tests, results with a P-value of < 0.05 were regarded as statistically significant.

RESULTS

In total, ten dogs contributed with 22 anatomically distinct skin areas. In eight of the dogs, two skin sites were included and in the remaining two dogs, three skin sites were included. In the dogs with three skin sites, one site acted as an untreated control. All lesions were localized and anatomical areas included in the study were interdigital skin, lip folds and nose folds.

The dogs included in the study were of the breeds: Nova Scotia duck tolling retriever, German shepherd, Labrador retriever, Pug, Beagle, Great Pyrenees and crossbreed. The age ranged from 1 year to 11 years with a mean of 4.5 and median of 3.6 years. Six of the dogs were female and four male, two of the males were neutered. Seven of the dogs fulfilled the criteria for subgroup A and the remaining three dogs were included in subgroup B. The treatments which placed the test subjects/patients in subgroup B were as follows: in one patient a single dose of carprofen seven days prior to inclusion, another patient had a 16 day treatment duration of cetirizine which had ceased eight days prior to inclusion day and had also received a single dose of imidacloprid and moxidectin 14 days prior to inclusion day. The third test subject in subgroup B underwent surgical removal of a mastocytoma on the day of inclusion, which included both anaesthesia and analgesia: cepromazin, metadon, haloperidol, atropine, prednisolone, propofol, isoflurane, and meloxicam followed by post-operative carprofen.

Six of the dogs had a history of diagnosed dermatitis and six dogs had a history of otitis externa. Two dogs were previously diagnosed with atopic dermatitis and four dogs were under ongoing investigations for suspected allergies. See Table 1 for details.

Table 1. *Dermatological clinical history*

Subject	Previous diagnoses of dermatitis, otitis externa or allergy	Ongoing investigation of allergy
1	<i>Malassezia</i> overgrowth, otitis externa	✓
2	<i>Malassezia</i> overgrowth mixed with pyoderma, otitis externa	-
3	-	-
4	Dermatitis (no cytology), multiple otitis externa	✓
5	Dermatitis (no cytology), otitis externa, atopic dermatitis	-
6	-	-
7	Otitis externa	-
8	Dermatitis (no cytology), multiple otitis externa	✓
9	<i>Malassezia</i> overgrowth, otitis externa, atopic dermatitis	✓
10	Otitis externa	-

All dogs followed the study protocol and completed the study, none were lost to follow up or excluded during the study period.

Pre-treatment observations

Results include areas treated either with Crystavet or the reference product. Results from untreated areas are reported separately.

Cytology

The mean of *Malassezia* yeast organisms per 10 oil immersion/high power field (HPF), under 1000 magnification at the time of inclusion is presented in Table 2.

Mean cytology results on the inclusion day did not differ significantly between the sites included for the different therapies (P=0.850). For subgroup A and B there was a statistical significant difference (P=0.017) in pre-treatment cytology data for Crystavet with a higher average of mean yeast for subgroup B. There was no statistical significant difference between subgroup A and B regarding pre-treatment cytology data for the reference product (P=0.305).

Table 2. Mean (median) *Malassezia* yeasts obtained by cytology at the time of inclusion for sites of each treatment group.

	Product	
	Crystavet	Reference
Total	46.16 (28.35)	40.08 (25.20)
Subgroup A	21.66 (20.70)	31.84 (23.9)
Subgroup B	103.33 (60.40)	59.3 (59.5)

Fungal culture

Microorganisms were cultured on all contact plates and in 11 cultures *Malassezia pachydermatis* was confirmed. No other *Malassezia* spp. was found. Of the sites selected for treatment with Crystavet or reference product, five and six sites respectively were positive for *Malassezia*. The quantity of CFU for all *M. pachydermatis* positive cultures was >100 CFU. In addition, *Candida albicans* was confirmed in one of the samples with growth of *Malassezia pachydermatis*. This finding was recorded in a culture from interdigital skin.

Clinical lesions

On inclusion day the mean total lesion score was 3.9 and median 3, minimum total clinical lesion score noted was 1 and maximum 10. There was no statistical significance difference (P=0.789) for total lesion scores between sites included for the different therapies nor between subgroups within each treatment group of Crystavet (P=0.083) or the reference product (P=0.107). Erythema was noted in all sites included on inclusion day and the erythema score did not differ significantly between the sites included for the different therapies (P=0.490) or between subgroups within each therapy, Crystavet (P=0.602) and reference product (P=1.000).

The patients' mean and median pruritus visual analogue scale (PVAS) score was 3.28 and 3.6 respectively. PVAS was noted at a minimum of 0.4 and maximum of 6.4.

Post treatment observations

Treatment duration varied from 5.5 days to 10 days. One dog was included for a prolonged treatment of 17 days total. Results obtained from that dog at the first revisit are included in the results reported here. Results of the prolonged treatment and from untreated areas are reported separately. No adverse side effects from either treatment was noted by the investigator.

Cytology

Malassezia populations were significantly reduced by both Crystavet (P=0.003) and the reference product (P=0.003). There was no statistical significance found between treatment efficacies of reducing the mean number of yeast organisms in absolute numbers (P=1.00) or expressed as reduction in percent (P=1.00). Mycological recovery based on cytology was obtained in eight out of ten areas treated with Crystavet and in seven out of ten areas treated with the reference product. Four of the sites that did not recover came from the same two test subjects and the one additional site for the reference product, came from a third test subject. The mean and median reduction of *Malassezia* yeast organisms per 10 oil immersion/ HPF, with 1000 magnification at post treatment evaluation is

presented in Table 3. Pre- and post treatment cytology data for each site and treatment is illustrated in Figure 2.

For treatment with Crystavet there was a significant difference in reduction of absolute numbers of yeast organisms when comparing subgroup A and B (P=0.017) but not when mean yeast reduction was expressed as percent (P=0.521). For the reference product there was no significant difference between subgroup A and B for either reduction in absolute numbers (P= 0.305) or reduction expressed as percent (P = 0.888).

Table 3. Mean (median) reduction of *Malassezia* yeasts obtained by cytology at time of evaluation, presented as number of yeast organisms and percentage (%) for sites of each

	Product	
	Crystavet	Reference
Total	-43,66 (-28.3)	-36.9 (-23)
Total (%)	-0.89 (-1)	-0.90 (-1)
Subgroup A	-18.1 (-19.4)	-28.03 (-22.1)
Subgroup A (%)	-0.84 (-1)	-0.89 (-1)
Subgroup B	-103.3 (-60.4)	-57.6 (-59.5)
Subgroup B (%)	-1 (-1)	-0.92 (-1)

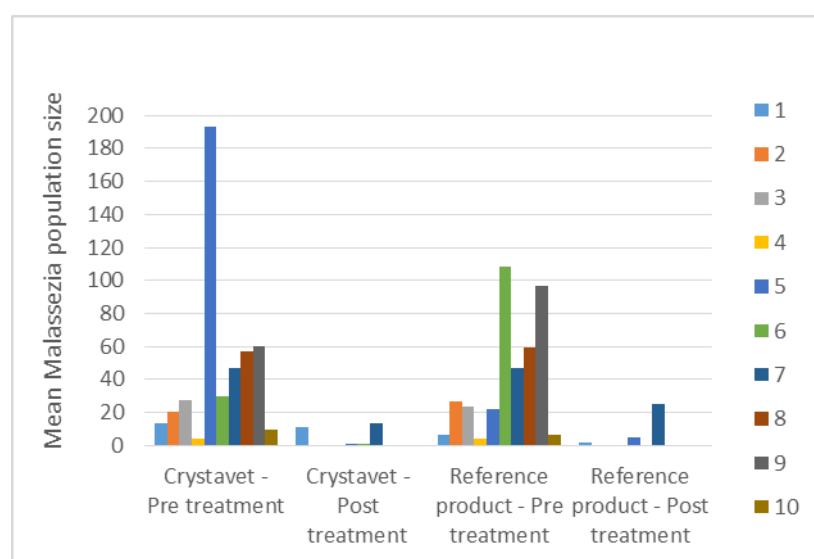


Figure 2. Mean *Malassezia* population size showed for each site and treatment group pre- and post treatment with columns of same colour originating from the same test subject.

Fungal culture

Reduction of *Malassezia* populations based on fungal culture was significant with both Crystavet (P=0.03125) and the reference product (P=0.03125). Complete mycological recovery was obtained in

Table 4. Quantitative results of *Malassezia* populations from samples positive for *M.pachydermatis* post treatment. Fungal culture expressed as CFU and cytology as mean yeast organisms.

three of five previously positive cultures from the Crystavet group and four out of six for the reference product group. One area treated with Crystavet found negative on fungal culture pre-treatment was positive post treatment with growth of 3 CFU, the species was confirmed as *M. pachydermatis*. Of the samples that were negative for *Malassezia* on culture post treatment, two sites, belonging to the same test subject, had detectable *Malassezia* yeasts on cytology. For quantitative results of positive fungal cultures and correlation with cytology results see table 4.

Sample	Therapy	<i>Malassezia</i> population	
		Fungal culture	Cytology
1	Crystavet	5	0
2	Crystavet	3	0.1
3	Crystavet	32	13.5
4	Reference	5	5.1
5	Reference	23	25.1

Clinical lesions

Reduction of total clinical lesion score was significant both for treatment with Crystavet (P=0.011) and the reference product (P=0.007). There was no significant difference in efficacy between products (P=0.789). Based on total clinical lesion score, two skin sites did not improve clinically using the reference product, nor did three sites for Crystavet. Out of those that had not improved clinically, two out of three treated with Crystavet and one out of two from the reference product group obtained complete mycological recovery based on cytology. The remaining two sites were from the same test subject.

Reduction of erythema as a clinical lesion subscore was found to be significant for Crystavet (P=0.018) and the reference product (P=0.018). Improvement was seen in six out of ten sites for both treatments respectively. In the sites where erythema had not been reduced, the cytological recovery was complete in three sites treated with Crystavet and two sites treated with the reference product. There was no significant difference in the products' efficacy of reducing erythema (P=0.789).

The PVAS score was significantly reduced (P=0.008) post treatment, the reduction did not vary significantly between subgroup A and B (P=0.286). The PVAS was reduced by a mean of -0.96 and median of -0.8.

Owner evaluation form

Ten owners participated in the study, all of them filled out the owner evaluation form at the follow up appointment. In general owners commented that they had a good to moderately good experience using both therapies, although seven reported that the dog found the spray application of the reference product unpleasant. When asked to rate how easy the product was to use both products were rated as easy to use and there was no statistical significant difference between the products (P=0.291). Instructions on how to use the products was reported as easy to understand and there was no statistical difference between Crystavet and the RP (P=0.503).

Owners were asked if they experienced a difference in the efficacy of the products. Six owners deemed the efficacy equal, one believed that the reference product was more efficient and remaining three owners thought that Crystavet had been more efficient. Owners did not report any adverse side effects.

Other observations

Untreated control areas

In two of the subjects included in the study, one area was left untreated. Both these dogs fulfilled the criteria for inclusion in subgroup A and areas included were both interdigital skin. In one of the test subjects cytology of the untreated area showed a decrease in mean *Malassezia* organisms from 31.1 to 2.2 yeasts while culture was positive with a growth of >100 CFU on both pre and post treatment. For the same subject areas treated according to study protocol had responded with an elimination of *Malassezia* based on cytology for both therapies tested. Culture was also negative for *M. pachydermatis* in the area treated with the reference product but positive with 5 CFU for the area treated with Crystavet. The second test subject who had an untreated area, also showed a decrease of mean *Malassezia* yeasts post treatment in the area left untreated, from 13.5 to 1.4. Culture for the untreated area was positive for *M. pachydermatis* (>100 CFU) pre-treatment but negative post treatment. The areas that were treated in this test subject, were both negative on cytology and culture post treatment. Culture pre-treatment for these sites was negative for *Malassezia*, although growth of unknown yeast was noted with a quantity of >100 CFU. Total clinical lesion score, including the erythema score, did not change for either of the two untreated control areas during the study.

Prolonged treatment of “non-responder”

Out of the five different skin sites in which treatment according to the study protocol failed to reduce the cytology population size to ≤ 0.2 as a mean, two sites belonged to the same test subject. This dog was further included in the study to evaluate whether a prolonged treatment was efficient. In this patient both skin sites, which were interdigital skin, were still positive on cytology and culture for *Malassezia* spp. after an initial study period of 10 days, although all samples had reduced in population size. The treatment period was prolonged for another seven days, resulting in a treatment period of seventeen days total. After seventeen days of treatment, cytology and culture was still positive for *Malassezia* spp. at the site treated with Crystavet. The cytology showed a decrease in population size of *Malassezia* spp. from a mean of 46.9 to 3 yeasts, and culture from >100 CFU to 48 CFU. The site treated with the reference product was negative on both cytology and culture after the prolonged treatment. Total clinical lesion score had not changed during the initial treatment period for either of the areas. After the prolonged treatment both areas had reduced the total clinical lesion score with one point respectively, this was represented by a reduction of erythema in both areas.

DISCUSSION

As stated by both Mueller et al (2012) and Negre et al (2009) *in vivo* trials assessing the efficacy of various therapies available for the treatment of *Malassezia* overgrowth are sparse. *In vivo* trials of chlorhexidine, either combined with miconazole or solely, at a concentration of $\geq 2\%$, have reported the efficacy of chlorhexidine in reducing mycological as well as clinical parameters (Bond et al., 1995b; Jasmin et al., 2003; Maynard et al., 2011). *In vitro* studies reporting chlorhexidine to have a low minimum fungicidal concentration for isolates of *Malassezia pachydermatis* further supports the fact that chlorhexidine has fungicidal properties (Lloyd and Lamport, 1999; Uri et al., 2016; Young et al., 2012). Based on this, a 4% chlorhexidine formulation was chosen as a reference product.

The *in vivo* studies of chlorhexidine accounted for in the present study evaluated the efficacy of shampoo formulations at a concentration of $\geq 2\%$. In contrast, the reference product used in this study was a spray lotion containing 4% chlorhexidine. This spray lotion was chosen with the purpose of avoiding possible vehicle (detergent) effects of shampoo since the product to be evaluated, Crystavet, had a cream formulation. Shampoo vehicle has been suggested to reduce antimicrobial and clinical parameters (Marsella et al., 2000; Stroh et al., 2010). Furthermore, it was desirable that the reference product, much like the cream formulation of Crystavet, remained on the skin for a longer time to produce a “leave on” effect. Since a shampoo is rinsed off after application it would not have achieved the same effect.

At the time of inclusion there was no significant difference between sites that were to be treated with the two topical agents in regards to both cytological and clinical parameters. The distribution of cytological and clinical findings in sites included for the different therapies might thereby be considered equally distributed. However, there was a significant difference for cytological data observed between subgroups within the sites to be treated with Crystavet, with a higher mean of yeast for subgroup B. A significant difference between these subgroups was also seen on post treatment cytology data regarding reduction of numbers of organisms, in which the mean was higher for subgroup B. This significance was not found when the cytological reduction was expressed in percent. These findings suggest that inclusion in subgroup A or B did not affect the cytological or clinical outcome of this study.

Cytological recovery was not defined as zero detectable *Malassezia* yeasts since the normal micro flora of healthy canine skin includes *Malassezia* spp (Kennis et al., 1996; Miller et al., 2013a; Nuttall, 2012). The criteria for cytological recovery defined as ≤ 0.2 mean *Malassezia* yeast per 10 oil immersion fields/HPF, under 1000 magnification, was previously used by Maynard et al (2011). This criterion meant that a maximum of two yeast organisms were allowed in samples that were reported as cytologically recovered. The criterion for negative fungal culture was determined as zero CFU since one CFU on a contact plate might be the result of several closely adjacent micro colonies or *Malassezia* organisms, as stated by Bond et al (1994). Since several studies report of positive culture samples being negative on cytology (Bensignor et al., 2002, 2002; Cafarchia et al., 2005; Machado et al., 2011) fungal culture might be a more sensitive diagnostic tool for detection of *Malassezia* organisms. However, mycological recovery in the present study was primarily based on data from cytology due to its practical and common use in research and clinical practice (Bond et al., 1995a; Cafarchia et al., 2005; Machado et al., 2011; Mason and Evans, 1991; Miller et al., 2013a; Nuttall, 2012). In the present study, only one sample, taken post treatment, was positive on culture but negative on cytology.

Both therapies evaluated in this study achieved a significant reduction of mycological data without a statistically significant difference in the results. Mycological recovery based on cytology was reported in 8 out of 10 areas treated with Crystavet and in 7 out of 10 areas treated with the reference product (RP). Two sites for each product that did not achieve cytological recovery, came from the same two test subjects. This suggests that the efficacy of reducing *Malassezia* yeasts is equal for the two therapies tested and that the failure of therapy in these sites might be dependent on environmental or

host related factors. In another test subject, treatment with Crystavet, but not treatment with the RP, led to cytological recovery. This may be coincidental or indicative of a higher efficacy of Crystavet. In contrast, the results of the test subject enrolled for prolonged treatment showed the opposite trend, since the site treated with the RP had recovered mycologically but not the site treated with Crystavet. No conclusions can be drawn from these findings, but it is possible that a larger study population would reveal a significant difference in antifungal efficacy between Crystavet and the RP.

Treatment with both products resulted in a significant reduction of total clinical lesion scores and there was no significant difference in efficacy between the treatment options. The total clinical lesion score included both acute manifestations such as erythema and alopecia as well as the more chronic manifestations of hyperpigmentation and lichenification (Miller et al., 2013a; Nuttall, 2012). Since the maximum treatment period of ten days in this study was relatively short compared to other studies (Bond et al., 1995b; Jasmin et al., 2003; Marsella et al., 2000; Maynard et al., 2011; Nardoni et al., 2014), all measured parameters may not have been able to present with a notable change within the study period. Reduction of lesions such as alopecia, lichenification and hyperpigmentation likely take longer than 10 days due to the complex nature of repair and regeneration of skin (epidermis, dermis and hair follicles), while the inflammatory response manifested by erythema changes faster (Zachary and McGavin, 2011). Therefore erythema was also analysed separately as a clinical lesion subscore. In addition, erythema was also the only clinical manifestation present in all the sites included for the study, which is similar to findings by Maynard et al (2011), who reported erythema to be the most frequent clinical observation in their study. Both products in the present study resulted in a significant reduction of erythema and there was no significant difference in efficacy between Crystavet and the RP. For both therapies the P-value for reduction of total clinical lesion score was lower than the P-value for reduction of the erythema score exclusively, which suggests that the significant reduction of the total clinical lesion score was not only dependent on reduction of erythema. Reduction of erythema as well as pruritus are reported as important outcome parameters in several studies of agents found efficient for topical treatment of *Malassezia* overgrowth with concurrent cutaneous lesions (Bond et al., 1995b; Jasmin et al., 2003; Marsella et al., 2000; Maynard et al., 2011; Rème et al., 2003). When evaluating clinical parameters, Marsella et al (2000) evaluated reduction in erythema and pruritus exclusively, while other studies have included additional manifestations such as excoriations, greasy exudate, lichenification and hyperpigmentation as well as a score of lesion extent. It is highly relevant to include clinical signs in the outcome analysis since reduction of clinical parameters is important to verify the diagnose *Malassezia* dermatitis (Bond and Lloyd, 1997; Nuttall, 2012). Reduction of clinical manifestations, especially erythema, may also act as a control for the mycological data obtained. It can be assumed that if changes in mycological and clinical parameters correlate, the risk of a false positive reduction value for mycological data is lower than if solely mycological data is reduced. This is relevant since untreated controls in this study did not show any reduction of clinical signs, including erythema, even though the cytology data was reported as significantly reduced. Even though the untreated controls do provide some interesting results, it is important to remember that no statistics could be applied due to low sample size. Nonetheless, the reduction of *Malassezia* organisms in the untreated sites resulted in a mean *Malassezia* count below the limit of the inclusion criteria, although neither of the sites fulfilled the criteria for cytological recovery. This reduction of *Malassezia* organisms noted in the untreated controls indicates that reduction of *Malassezia* attributed to the effect of products in the treated sites might not be solely dependent on the treatment. Since both untreated control sites were interdigital skin areas, it is possible that this noted reduction might be due to owners unconsciously drying the paws more vigorously after receiving the diagnosis of *Malassezia* overgrowth. Another possible cause of reduction in the untreated control sites could be that the relief of host response in the treated sites resulted in a redirection of host defense mechanisms to the untreated sites. Any systemic antifungal effects of the topical products used is unlikely but cannot be excluded. There are several possible factors, other than the therapies tested, that might have affected the *Malassezia* population size and clinical lesions for both untreated and treated sites. These include both changes in microclimate such as humidity (Bond et al., 2004) and capabilities of the host defence,

possibly affected by underlying medical conditions and dermatoses (Bond and Lloyd, 1997, 1998; Mauldin et al., 1997; Nuttall, 2012; Plant et al., 1992). An underlying hypersensitivity for *Malassezia* spp., as seen in some dogs with atopic dermatitis may be especially relevant (Nuttall and Halliwell, 2001). In this study two dogs included had previously been diagnosed with atopic dermatitis and four dogs were under clinical evaluation for allergies.

Out of the samples taken pre-treatment, of which all were positive on cytology, 50-60% were positive for fungal culture of *Malassezia* spp. Negative cultures for *Malassezia* spp. might be due to overgrowth of other microbes (Cafarchia et al., 2005), low detection sensitivity of contact plates (Bensignor et al., 2002; Bond et al., 1994), residual active product post treatment or failure to correctly identify *Malassezia* spp. The technique used to identify *Malassezia pachydermatis* by culture on Sabouraud's medium is widely used and reported in studies as well as textbooks (Bond et al., 1996; Bond and Lloyd, 1996; Gordon, 1979; Miller et al., 2013a; Nuttall, 2012) and should therefore not affect the results unless the isolated strains were lipid dependent (Gaitanis et al., 2012; Wu et al., 2015). Detection of other *Malassezia* spp. was dependent on MALDI-TOF MS, which is reported to identify *Malassezia* yeast by 100% on genus level (Kolecka et al., 2014). However, it is possible that samples analysed by MALDI-TOF MS were not sufficiently pure cultured or that the isolate was not included in the database used as reference (Bagge. E., National Veterinary Institute, Sweden, personal communication, 2016).

Other possible factors affecting the outcome of this study might be sources of error such as faulty handling of samples, poor sampling technique and contaminated dye solution. To prevent this, hands were disinfected, gloves were used before handling the samples and samples were transported in a special designed container to prevent contamination. Furthermore, sample technique was standardized and performed by the same investigator for all samples collected. To minimize risk of dye contamination, solutions used in the clinics are changed regularly and controlled once weekly. Noncompliance to study protocol could have affected the outcome of the study and it is possible that some noncompliance was not reported by owners but this is less likely as great attention was paid to this in interviews performed at post treatment follow-ups. To prevent noncompliance due to instructions being unclear or difficult to understand, the owners received both written and pictorial information as well as a demonstration by the investigator at point of inclusion. At follow-ups, owners reported the treatment instructions as easy to understand. The lack of blinding in this study might have led to bias influencing the results. The risk for bias would be higher for the clinical lesion scoring since this was based on the investigators subjective assessment of lesions changing gradually along a scale. This is in contrast to the mycological measures obtained, since they were quantified according to standardized techniques. Lack of blinding in the study also affects the results obtained by reducing power. Low intra-rater reliability may also be a source of error but any possible inter-test variability was avoided since the same investigator performed all sampling.

A significant reduction of pruritus score was observed for the test subjects. However, this reduction was not specified for each therapy option since the collected data regarded the estimation of general pruritus and not separate for each site included. Due to the split-body protocol used in the study, estimations of pruritus for each site would have been required to apply data of reduced pruritus to outcome results. Owners were not asked to rate the pruritus for each separate site as they are only able to estimate general pruritus and cannot distinguish differences between focal areas (Bergvall. K., Swedish University of Agricultural Sciences, personal communication, 2016).

Owners reported both products to be easy to use, although Crystavet cream formulation might be preferable since seven of the ten dogs found the spray application of the RP lotion unpleasant. No adverse side effects of either treatments were noted for the products tested, not by owners nor the investigator. In comparison, transient adverse reactions of chlorhexidine have been reported in dogs (Maynard et al., 2011) and chlorhexidine contact allergy in humans (Opstrup et al., 2016; Toholka and Nixon, 2013).

CONCLUSIONS

The results of this prospective, open, randomized, controlled pilot study suggest that topical treatment with Crystavet (1% hydrogen peroxide) is an effective and safe alternative in the treatment of localized *Malassezia* overgrowth in dogs. The results also suggest that the antifungal efficacy of 1% hydrogen peroxide is similar to that of 4% chlorhexidine for *Malassezia* species. Further studies are needed to confirm these results and should include a larger sample size for both treated and untreated sites. Blinding of further studies is recommended to increase power of presented statistics.

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