Universidade de Trás-os-Montes e Alto Douro

Medical honey: activity towards antibiotic resistant *Staphylococcus pseudintermedius* and *Malassezia pachydermatis* in canine folliculitis and otitis

Doctoral Thesis in Veterinary Sciences - Clinic

ANA MARGARIDA PEDROSO DE OLIVEIRA

Special Regimen Thesis

Vila Real, 2019

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Original thesis presented by Ana Margarida Pedroso de Oliveira at the Universidade de Trás-os-Montes and Alto Douro, to obtain the doctor's degree on Veterinary Sciences. The author is fully responsible for the work presented in the thesis.

To my family, Eric and friends.

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RESUMO

O mel de grau médico é considerado uma opção eficaz e económica no tratamento de feridas infetadas. O mel manuka (MH) e um gel à base de mel (HBO) encontram-se disponíveis no mercado permitindo a prescrição de produtos seguros, sem antibióticos, eficazes e padronizados.

Neste trabalho, o primeiro objetivo consistiu em determinar a metodologia de diagnóstico e tratamento das doenças dermatológicas causadas por *Staphylococcus pseudintermedius* e *Malassezia pachydermatis* em clínica de pequenos animais em Portugal. Inicialmente, demonstrámos que ambos os microrganismos causam frequentemente várias doenças, nomeadamente, a foliculite bacteriana superficial (SBF), a dermatite das pregas de pele (FD), a dermatite generalizada por *Malassezia* spp. (MD) e otite externa (OE). Os antibióticos orais foram largamente prescritos para o tratamento de SBF, particularmente amoxicilina-ácido clavulânico (100%), cefalexina (94%), enrofloxacina (67%) e marbofloxacina (60%). Para o tratamento de FD e OE, os antibióticos orais foram administrados em 88% e 82% dos casos, respetivamente. Os antifúngicos orais foram prescritos para o tratamento da MD (85%), FD (70%) e OE (59%). Todas as doenças foram tratadas topicamente com antibióticos, antifúngicos e glucocorticóides. Alternativas, como produtos à base de mel, não foram frequentemente prescritas pelos clínicos portugueses.

Casos clíncos causados por *S. pseudintermedius* multirresistentes (MDR) são frequentemente observados em clínica o que reduz dramaticamente as opções de antibioterapia. O segundo trabalho avaliou a incidência de multirresistência em *S. pseudintermedius* sensíveis (MSSP) e resistentes à meticilina (MRSP), previamente recolhidos de cães com SBF. Observou-se que todos os MRSP eram resistentes à amoxicilina-ácido clavulânico, clindamicina e eritromicina. Foram observados elevados níveis de resistência ao trimetoprim-sulfametoxazol (97%), tetraciclina e gentamicina (87%), cefalotina (83%), enrofloxacina (83%), pradofloxacina (80%) e minociclina (50%). Observou-se um baixo nível de resistência ao cloranfenicol (17%), amicacina (7%) e rifampicina (7%). A maioria dos isolados eram MDR (38/60). Todos os isolados não-MDR eram MSSP. A resistência à meticilina foi associada à multirresistência a outras classes de antibióticos.

O terceiro trabalho apresentava como objetivos determinar a eficácia *in vitro* do MH contra *S. pseudintermedius* e *M. pachydermatis* e o tempo necessário para obter o efeito bactericida ou fungicida. Sessenta isolados de *S. pseudintermedius* recolhidos de cães com SBF, foram divididos em grupos MRSP/MSSP e MDR/não-MDR. Testaram-se também vinte isolados de *M. pachydermatis* recolhidos de cães com OE. O MH foi testado não diluído e diluído a 80%, 40%, 30%, 20%, 15%, 10%, 7,5%, 5%, 3,7% e 2,5% p/v. A concentração mínima bactericida (MBC) de *S. pseudintermedius* foi de 20% p/v, sem diferença entre MSSP/MRSP ou MDR/não-MDR. Para *M. pachydermatis* a concentração mínima fungicida (MFC) foi de 40% p/v. Realizou-se um ensaio de "time-kill" em períodos distintos. O MH diluído eliminou ambos os microrganismos após 4 horas. O MH diluído a 40% p/v manteve atividade contra *S. pseudintermedius* mas demorou mais tempo a eliminar os isolados de *M. pachydermatis*.

Finalmente, determinou-se a eficácia *in vitro* do HBO contra os isolados de *S. pseudintermedius* e *M. pachydermatis*. Avaliou-se a eficácia da componente de mel (HO) do produto. Os isolados de *S. pseudintermedius* e *M. pachydermatis* foram testados contra diluições seriadas de HBO (40%, 20%, 10%, 5% e 2,5% p/v). O HBO contém HO na concentração de 40%. O HO foi testado puro e em diluições seriadas (40%, 20%, 10%, 5% e 2,5% p/v). Aplicou-se o mesmo protocolo após exposição à catalase para avaliar a presença de peróxido de hidrogénio. Realizou-se um ensaio de "time-kill" para determinar tempo de exposição para obter efeito bactericida/fungicida. O MBC para S. *pseudintermedius* foi de 20% p/v (5-20% p/v) para HBO e HO. O HBO demonstrou valores de MBC inferiores em comparação com o HO (P=0,003). Não houve diferença entre MSSP/MRSP (HBO P=0,757; HO P=0,743). Apenas o HO foi afetado pela catalase (P=0,015). O MFC para o HBO foi de 10% p/v (5-10% p/v) e 40% p/v para o HO (20-≥40% p/v). Todos os isolados foram eliminados após 4 horas de exposição ao HBO.

Resumindo, este trabalho contribui para melhorar o nosso conhecimento sobre o efeito do mel sobre o *S. pseudintermedius* e a *M. pachydermatis.* Demonstra especificamente que ambos microrganismos são sensíveis ao MH, HBO e ao mel que compõem este produto. Tratamentos à base de mel não são, no entanto, prescritos frequentemente pelos clínicos portugueses e, os antibióticos orais, são frequentemente utilizados na prática clínica, apesar da propagação do *S. pseudintermedius* resistente à meticilina. Finalmente, este trabalho acrescenta evidências de que *S. pseudintermedius* resistente à meticilina pode ser

multirresistente, reforçando a necessidade de opções não-antibióticas. Os resultados poderão ser usados futuramente em ensaios clínicos que tenham como objetivo tratar infecções cutâneas e de canal auditivo causados por *S. pseudintermedius* e/ou *M. pachydermatis.*

Palavras-chave: antibiótico, antifúngico, foliculite, manuka, mel, meticilina, *Malassezia, pachydermatis,* otite, *Staphylococcus pseudintermedius.*

SUMMARY

Medical honey is considered an effective economic option for the treatment for infected wounds. Medical grade Manuka honey (MH) and a honey-based gel (HBO) are now available, allowing for the prescrition of safe, non-antibiotic, effective and standardised products.

In the present doctoral thesis, we aimed to determine how clinicians diagnosed and treated dermatological conditions caused by *Staphylococcus pseudintermedius* and *Malassezia pachydermatis* in Portugal. We demonstrated to both pathogens often cause superficial bacterial folliculitis (SBF), fold dermatitis (FD), *Malassezia* dermatitis (MD) and otitis externa (OE). Oral antibiotics were widely prescribed for treatment of SBF, particularly amoxicillin-clavulanic acid (100%), cephalexin (94%), enrofloxacin (67%), or marbofloxacin (60%). Fold dermatitis and OE, were also treated with oral antibiotics in 88% and 82% of cases, respectively. Oral antifungals were often prescribed for MD (85%), FD (70%), and OE (59%). All the diseases were frequently treated topically with antibacterials, antifungals, and glucocorticoids. Alternative options such as honey-based products were not frequently used by clinicians.

Multidrug resistant (MDR) *S. pseudintermedius* isolates are frequently observed, a fact that dramatically decreases antimicrobial treatment options. The second part of this work evaluated the incidence of multidrug resistance in methicillin-susceptible and resistant *S. pseudintermedius* isolates (MSSP/MRSP) previously collected from dogs with SBF. All MRSP exhibited resistance to amoxicillin-clavulanic acid, clindamycin and erythromycin. High resistance levels were observed to trimethoprimsulfamethoxazole (97%), tetracycline and gentamicin (87%), cefalothin (83%), enrofloxacin (83%), pradofloxacin (80%) and minocycline (50%). Low resistance level was observed for chloramphenicol (17%), amikacin (7%) and rifampicin (7%). Most isolates were multidrug resistant (MDR, 38/60). All non-MDR isolates were MSSP. Methicillin resistance was associated with MDR to other classes of antibiotics.

The third part of this work, aimed to determine the *in vitro* efficacy of MH against *S. pseudintermedius* and *M. pachydermatis* and necessary exposure time for a bactericidal or fungicidal effect. Sixty *S. pseudintermedius*, previously isolated from dogs with canine SBF,

were divided into MRSP/MSSP and MDR/non-MDR groups. Twenty *M. pachydermatis* isolates, also previously isolated from dogs with OE were tested. Manuka honey was tested undiluted and diluted at 80%, 40%, 30%, 20%, 15%, 10%, 7.5%, 5%, 3.7% and 2.5% w/v. For *S. pseudintermedius* the minimal bactericidal concentration (MBC) was 20% w/v with no difference between MSSP/ MRSP or MDR/non-MDR. For *M. pachydermatis* the minimal fungicidal concentration (MFC) was 40% w/v. A time-kill assay was performed at different times. Undiluted MH killed both microorganisms after 4 hours of exposure. Diluted MH at 40% w/v maintained activity against *S. pseudintermedius* but took longer time to kill *M. pachydermatis* isolates.

Finally, we determined the *in vitro* efficacy of HBO, against isolates of *S. pseudintermedius* and *M. pachydermatis.* The efficacy of the product's honey component (HO) was also evaluated. All isolates were tested against serial dilutions of HBO (40%, 20%, 10%, 5% and 2.5% w/v). HBO contains HO at 40%. HO was tested pure and after serial dilutions (40%, 20%, 10%, 5% and 2.5% w/v). The same protocol was applied after exposure to catalase to determine the influence of hydrogen peroxide. A time-kill assay was performed determine the period of time necessary for a bactericidal/fungicidal effect. MBC for *S. pseudintermedius* was 20% w/v (5-20% w/v) for HBO and HO. HBO had lower MBC values when compared to HO (P=0.003). No difference was observed between MSSP/MRSP isolates (HBO P=0.757, HO P=0.743). Only HO was affected by catalase (P=0.015). MFC for HBO was 10% w/v (5-10% w/v) and 40% w/v for HO (20-≥40% w/v). All isolates were killed after 4 h of exposure to HBO.

In summary, our work indicates that honey was effective against *S. pseudintermedius* and *M. pachydermatis.* We could demonstrate that both microorganisms are susceptible to MH, HBO and the honey that composes this product. Nevertheless, honey treatments are not frequently prescribed by Portuguese clinicians and oral antibiotics are overused in small practice, despite the spread of antibiotic resistant *S. pseudintermedius.* Finally, the work adds further evidence that methicillin resistant *S. pseudintermedius* may be multidrug resistant which reinforces the need for non-antibiotic options. The results obtained can be used in future clinical trials aiming to treat skin and ears infections caused by *S. pseudintermedius* and *M. pachydermatis.*

Palavras-chave: antibiotic, antifungal, folliculitis, honey, manuka, methicillin, *Malassezia, pachydermatis,* otitis, *Staphylococcus pseudintermedius.*

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LIST OF ABBREVIATIONS AND SYMBOLS

®	Registered trademark
™	Trademark
%	Percentage
µL	Microliter
CLSI	Clinical and Laboratory Standards Institute
FD	Fold dermatitis
g	Grams
H_2O_2	Hydrogen peroxide
HBO	L-Mesitran Soft®, Triticum, The Nederlands
HO	Honey that composes L-Mesitran Soft®
IP	Initial population
ISCAID	International Society for Companion Animal Infectious Diseases
MBC	Minimum bactericidal concentration
MD	*Malassezia* dermatitis
MDR	Multidrug-resistant
MFC	Minimum fungicidal concentration
mL	Milliliter
mm	Millimeter
MH	Manuka honey
MSSA	Methicillin-susceptible *Staphylococcus aureus*
MSSP	Methicillin-susceptible *Staphylococcus pseudintermedius*
MRSA	Methicillin-resistant *Staphylococcus aureus*
MRSP	Methicillin-resistant *Staphylococcus pseudintermedius*
nm	Nanometer
OE	Otitis externa
PCR	Polymerase chain reaction
rpm	Rotations *per* minute
SBF	Superficial bacterial folliculitis
SD	Standard deviation
w/v	Weight/volume percentage

INTRODUCTION

1. *Staphylococcoccus pseudintermedius*: A MULTIDRUG RESISTANT CANINE PATHOGEN

1.1. Characteristics of S. pseudintermedius

Staphylococcus pseudintermedius is a bacterium with specific features, like virulence factors and ability to produce biofilms. The knowledge of these specifications allows a better understanding of the pathogenicity of this microorganism (Fazakerley *et al.*, 2009; Futagawa-Saito *et al.*, 2006; McEwan *et al.*, 2006; Simou *et al.*, 2005; Singh *et al.*, 2013).

The *Staphylococcus* genus (from the Greek σταφυλή, *staphylē*, "grape" and κόκκος, *kókkos*, "granule") is composed of Gram-positive bacteria. Under the microscope they appear similar to grape clusters (LSPN, 2015). This genus includes 52 species and 28 subspecies, which are classified by their genotypic differences, habitat and pathogeny. They are differentiated into coagulase-positive and coagulase-negative *Staphylococci* according to their ability to coagulate plasma by converting fibrinogen into fibrin (LSPN, 2015).

S. intermedius was described for the first time in 1976 by V. Hajeck after being isolated from pigeons, dogs, minks and horses, allowing for an important distinction between *S. aureus* and this new species (Hajek, 1976; Devriese *et al.*, 2009; Bannoehr and Guardabassi, 2012; Bond and Loeffler, 2012). Although Hajeck (1976) observed some heterogeneity between the strains described as *S. intermedius*, the species name remained unaltered until 2005, when Devriese proposed a new species named *S. pseudintermedius* (Devriese *et al.*, 2005). In 2007, a genetic population study, using a multilocus sequence phylogenetic analysis, confirmed three distinct species, namely *S. intermedius*, *S. pseudintermedius* and *S. delphini* in isolates previously assumed to be *S. intermedius* (Bannoehr *et al.*, 2007). These three species represent the *S. intermedius* group, and *S. pseudintermedius* was found to be the main pathogen involved in canine pyoderma (Bannoehr *et al.*, 2007). The routine identification of *S. pseudintermedius* in diagnostic laboratories is based on the fact that other species of the *S. intermedius* group are practically non-existent in dogs (Fitzgerald, 2009; Bannoehr and Guardabassi, 2012). More recently, polymerase chain reaction-restriction fragment length polymorphism was developed after sequence analysis of one of the loci, *pta*, which encodes the enzyme phosphoacetyltransferase and is a restriction site unique to *S. pseudintermedius*, making it an accurate and simple test that allows differential identification (Bannoehr *et al.*, 2009).

S. pseudintermedius is part of the normal cutaneous microbiotic, constituting about 90% of a healthy dogs staphylococci population, colonizing the skin, hair follicles and particularly mucosae like the nose, mouth and anus (Allaker *et al*., 1992; Griffeth *et al.*, 2008; Fazakerley *et al.*, 2009; Bannoehr and Guardabassi, 2012). The skin of puppies is colonized by *S. pseudintermedius* shortly after birth, probably as a result of vertical transmission (Saijonmaa-Koulumies and Lloyd, 2002; Paul *et al*., 2014). Clones can persist in the skin of the puppies up to 48 months after separation from the dam (Paul *et al.*, 2014).

A study performed with 50 healthy dogs and 59 dogs suffering from inflammatory skin disease detected that 68% and 92% of the healthy and affected dogs respectively, were colonized with *S. pseudintermedius* (Griffeth *et al.*, 2008). Another study reported that more than 94% of the *S. pseudintermedius* isolates recovered from cutaneous lesions of dogs with superficial pyoderma, were genetically similar to the ones recovered from carriage sites (Pinchbeck *et al.*, 2006).

Although *S. pseudintermedius* is the most common pathogen in canine pyoderma, other pathogens like *S. aureus, S. schleiferi* and *S. hyicus* are occasionally encountered (Medleau *et al.*, 1986; Frank *et al.*, 2003; Cain *et al.*, 2011). Rarely, coagulase-negative Staphylococci (*S. epidermidis, S. xylosus, S. simulans* and *S hominis*), *Streptococcus canis* and *Pseudomonas aeruginosa* can be found solely or in association with *S. pseudintermedius* (Medleau *et al.*, 1986; Fortin and Higgins, 2001; Hillier *et al.*, 2006).

S. pseudintermedius pathogenesis is not yet fully understood. Virulence factors include: enzymes such as coagulase, thermonuclease and proteases; surface proteins such as clumping factor and protein A; and toxins such as cytotoxins, exfoliative toxin, enterotoxins, leukocidin and pore-forming toxins (Bannoehr and Guardabassi, 2012; Abouelkhair *et al,* 2018; Maali *et al,* 2018). *S. pseudintermedius* adheres to the epidermal cells of healthy dogs and appears to show greater adherence in atopic dogs (Simou *et al.*, 2005; McEwan *et al*., 2006; Fazakerley *et al.*, 2009).

S. pseudintermedius also produces biofilms (Futagawa-Saito *et al.*, 2006; Singh *et al.*, 2013; Stefanetti *et al,* 2017; Arima *et al,* 2018), which are known to be resistant to antibiotics, environmental stress and macrophage phagocytosis (Shiau and Wu, 1998; Olson *et al.*, 2002; Stefanetti *et al,* 2017). No association has been found between biofilm production and the presence of methicillin resistance genes, isolate source (infection site or colonization)

or clonal complex (Singh *et al.*, 2013). Methicillin-resistant *S. pseudintermedius* (MRSP) isolates are not more virulent when compared with methicillin-susceptible *S. pseudintermedius* (MSSP) isolates (Morris *et al.*, 2006; Loeffler *et al.*, 2007). However, infections with MRSP biofilm producers constitute an additional risk factor, since antibiotics cannot easily penetrate biofilm layers reducing the therapeutic options (Venkatesan *et al*., 2015; Stefanetti *et al*, 2017).

1.2. S. pseudintermedius in canine dermatology

Canine pyoderma is a common cause of canine skin disease, with superficial bacterial folliculitis the most common presentation (Hillier *et al.*, 2014). Pyoderma can be classified, in accordance with the depth of the lesions, into: superficial, when the infection involves the epidermis and/or hair follicles; and deep pyoderma when deeper tissues and possible furunculosis are involved (Beco *et al.*, 2013; Bloom, 2014). Surface pyoderma involves the trauma of the surface of the epidermis only with colonization of the area by *S. pseudintermedius* (Beco *et al.*, 2013; Bloom, 2014).

Moreover, *S. pseudintermedius* can cause wound infections. In Sweden, a study analysed the pathogens involved in canine surgical wound infections and their susceptibility patterns. Out of 194 samples, the most prevalent was *S. pseudintermedius* (46%) and three isolates were MRSP. No relation was found between the pathogen and classification of the surgical procedure, duration of hospitalization or depth of the surgical site infection (Windahl *et al.*, 2015).

Furthermore, *S. pseudintermedius* was identified as a complicating factor of immuno-modulatory-responsive lymphocytic-plasmacytic pododermatitis in a study with 20 dogs (Breathnach *et al.*, 2005). A fatal case of necrotizing fasciitis, with unknown source of infection and caused by MSSP was also described in a dog (Weese *et al.*, 2009). *S. pseudintermedius* has also been isolated from abscesses in dogs (Hoekstra and Paulton, 2002).

S. pseudintermedius is a very important pathogen in canine otitis externa (OE). This microorganism can cause otitis solely or concomitantly with *Malassezia pachydermatis* and is responsible for the largest number of canine OE cases (Lyskova *et al*., 2007). The most frequently isolated bacteria from the ears of affected dogs is *Staphylococcus*

pseudintermedius (Kiss *et al.,* 1997a; Rougier *et al.,* 2005; Lyskova *et al.,* 2007). A study performed in 515 dogs affected with OE reported *S. pseudintermedius* as the most commonly isolated bacteria, with 202 isolates recovered from pure culture or associated with other microorganisms (Lyskova *et al.,* 2007). Another study, also reported *S. pseudintermedius* as the most frequently isolated bacteria, from 97 dogs with OE (Rougier *et al.,* 2005).

In short, a large number of dermatological cases encompass either skin or otitis infections that are caused by *S. pseudintermedius.*

1.3. Zoonotic aspects of S. pseudintermedius

Humans are not natural hosts for *S. pseudintermedius* which explains its low impact in public health. However, it is unknown if *S. pseudintermedius* strains containing mobile genetic elements could represent a reservoir for the spread of resistant genes to the human commensal skin microbiotic (van Duijkeren *et al.,* 2011a).

Recently, *S. pseudintermedius* has been implicated in occasional human infections (Van Hoovels *et al.,* 2006; Stegmann *et al.,* 2010; Kuan *et al,* 2016). Although *S. pseudintermedius* rarely colonizes the human skin, in those cases when individuals have regular contact with dogs, the colonization rate rises, particularly in the nasal cavities (Harvey *et al.,* 1994; Goodacre *et al.,* 1997; Guardabassi *et al.,* 2004; Stegmann *et al.,* 2010; van Duijkeren *et al.,* 2011b). It has been demonstrated that dog owners with dogs affected by deep pyoderma can carry the same genetic MRSP strain through nasal colonization as their pets, which supports an interspecies transmission (Guardabassi *et al.,* 2004; Somayaii *et al,* 2016; Lozano *et al,* 2017). Veterinarians in contact with infected animals also appear to have a higher risk of being MRSP nasal culture positive (Morris *et al.,* 2010; Espadale *et al,* 2018; Worthing *et al,* 2018a).

1.4. Antibiotic resistance of S. pseudintermedius

MRSP isolates were first reported in 1999 in North America and throughout Europe between 2005 and 2006, and they are now recognized as having a worldwide distribution (Gortel *et al.,* 1999; Loeffler *et al.,* 2007; Schwarz *et al.,* 2008; Perreten *et al.,* 2010; Onuma

et al., 2012; Wegerner *et al,* 2018; Worthing *et al,* 2018b). The North America strain (ST68-C-t06-V) is still susceptible to chloramphenicol, rifampicin and amikacin, while the predominant MRSP clone in Europe, sequence type (ST71-J-t02-II–III) is normally resistant to beta-lactams, aminoglycosides, macrolides, lincosamides, tetracyclines, chloramphenicol, trimethoprim and fluoroquinolones, but remains susceptible to amikacin, fusidic acid, minocycline, rifampicin, vancomycin, teicoplanin and linezolid (Perreten *et al.*, 2010; Frank and Loeffler, 2012; Somayaii *et al,* 2016; Wegener *et al,* 2018). This demonstrates the importance of recognizing MRSP susceptibility patterns, according to the clone distribution, in order to apply effective antibiotherapy (Han *et al,* 2018).

Generally, it is not advised to empirically switch antibiotic classes if treatment fails with first-line antimicrobials. In this case, culture and susceptibility testing should be performed before a second antibiotic is prescribed (Hillier *et al.*, 2014). Differentiation between susceptible and resistant *S. pseudintermedius* strains, based on the clinical presentation, is not possible, since MRSP is not more virulent than MSSP (Morris *et al.*, 2006; Loeffler *et al.*, 2007). Although resistance in staphylococci is not always associated with multidrug resistance, most of the MRSP isolates described in the literature display resistance to the majority of clinically relevant antibiotics (Bond and Loeffler, 2012; Somayaii *et al,* 2016). Reports from referral practices, with chronic or recurrent cases of pyoderma, in which previous antibiotherapy has been attempted, frequently report high levels of MRSP (Morris *et al.*, 2006; Loeffler *et al.*, 2007; Ben Zakour *et al.*, 2012). Multidrug resistance is present in cases of pyoderma but also in cases of OE. One hundred and fifty one samples were obtained from dogs with unmedicated otitis, from which 35 isolates were *S. pseudintermedius*, with the majority displayed multidrug resistance (Penna *et al.*, 2010).

Currently, general practitioners are strongly recommended to follow the guidelines for diagnosis and antimicrobial therapy of canine superficial bacterial folliculitis developed by the Antimicrobial Guidelines Working Group of the International Society for Companion Animal Infectious Diseases (ISCAID). These guidelines provide updated information for adequate treatment of canine folliculitis and rational use of antibiotics (Hillier *et al.*, 2014).

2. *Malassezia pachydermatis*: A RELEVANT PATHOGEN IN CANINE DERMATOLOGY

2.1. Characteristics of M. pachydermatis

Malassezia yeasts are unicellular eukaryotic symbionts that contribute to the microbiotic of the skin of several warm blooded species, including humans, dogs and cats (Gaitanis *et al.*, 2012). Currently, 14 species of *Malassezia* are recognized, namely *M. pachydermatis*, *M. furfur*, *M. globosa*, *M. obtusa*, *M. restricta*, *M. slooffiae*, *M. sympodialis*, *M. dermatis*, *M. nana*, *M. japonica*, *M. yamatoensis*, *M. equina*, *M. caprae* and, the last species identified, *M. cuniculi* (Cabañes *et al*., 2011; Gaitanis *et al.*, 2012).

Malassezia spp. is classified in the Phylum Basidiomycota, subphylum Ustilaginomycotina, class Exobasidiomycetes, order Malasseziales, and family Malasseziaceae (Baillon, 1889). The genus *Malassezia* can be differentiated based on culture, biochemical and molecular testing (Makimura *et al.*, 2000; Mirhendi *et al.*, 2005; Kaneko *et al.*, 2007). In humans, *Malassezia* causes pityriasis versicolor (Borgers *et al.*, 1987; Hay and Midgley, 2010) and can be implicated in the pathogenesis of atopic dermatitis (Hay and Midgley, 2010). *M. pachydermatis* is the only non-lipid-dependent *Malassezia* species, while other species depend on lipids for their growth (Kaneko *et al.*, 2007). This species was first described by Fred Weidman as *Pityrosporum pachydermatis*, after isolation from an Indian rhinoceros (*Rhinoceros unicornis*) with a severe exfoliative dermatitis (Weidman, 1925).

M. pachydermatis is able to produce several virulence factors including esterases, lipases, lipoxygenases, proteases, hyaluronidases and chondroitinsulfatases. However, the phospholipase activity has been the most studied (Coutinho and Paula, 2000; Cafarchia and Otranto, 2004; Juntachai *et al.*, 2009). *M. pachydermatis* has the highest secreted phospholipase activity among other species (Juntachai *et al.*, 2009). It has been postulated that hydrolyzation of glycerophospholipids of host cell membranes by phospholipase is involved in the pathogenesis of these species in canine dermatitis (Cafarchia and Otranto, 2004) and OE (Teramoto *et al.*, 2015). Virulence factors including production of biofilms can vary according to the strain of *M. pachydermatis* (Buommino *et al*, 2016).

2.2. M. pachydermatis in canine dermatology

M. pachydermatis is one of the major pathogens of the skin and ear canals of the dog. In 1983, Dufait reported for the first time *M. pachydermatis* as the etiologic agent of generalized dermatitis in dogs (Dufait, 1983). Particularly, *M. pachydermatis* is associated with atopic dermatitis, OE and seborrheic dermatitis and contributes to the worsening of clinical signs (Machado *et al.*, 2011). Other species, including *M. furfur, M. obtuse, M. globosa* and *M. sympodialis*, have been described in healthy and diseased skin and ears (Raabe *et al*., 1998; Crespo *et al*., 2000; Cafarchia *et al.*, 2005).

Predisposing factors, for the cutaneous proliferation of this commensal, include allergic diseases, cornification disorders, bacterial skin infections, recent antibiotic therapy and long-term glucocorticoid administration (Plant *et al*., 1992). Genetic predisposition for *Malassezia* dermatitis appears to be important in certain breeds, including West Highland White Terriers, Basset Hounds, English Setters, Shih Tzus and American Cocker Spaniels (Bond *et al.*, 1996; Mauldin *et al.*, 1997). In the Basset Hound breed, the disease can be severe and yeast proliferation has been associated with a primary keratinization disorder (Power *et al.*, 1992).

There are several factors that favour *Malassezia* growth, such as humidity, high temperature and an environment rich in fat (Weiler *et al.*, 2013). Additionally, the skin microbiotic, pH, salts, immune response and other physiological characteristics are also considered important in colonization by *Malassezia* (Cafarchia *et al.*, 2008).

Malassezia dermatitis lesions in dogs are normally intensely pruritic and the main primary lesion is erythema (Morris, 1999). Secondary lesions normally consist of excoriations, hyperpigmented areas, erythematous lesions, varying degrees of traumatic alopecia and scaling that normally affect the ventral neck, face, axillae, interdigital areas, perineal regions and skin folds (Morris, 1999; Bond, 2010). The lesions may be confined to one area or affect multiple regions (Morris, 1999; Bond, 2010; Bond *et al*., 2010). Lesions in the interdigital skin may progress to involve the claw folds, producing exudation and red-brown discoloration of the hairs or claws (Morris, 1999; Bond *et al*., 2010).

Malassezia otitis in the dog is a common occurrence and a source of distress to the dog owner (Bernardo *et al*., 1998). It is a saprophytic yeast that is normally present in the external ear canal and, in favorable conditions, it can become pathogenic and cause an otitis (Crespo *et al*., 2002; Korbelik *et al*, 2018). *Malassezia* otitis, is presented initially with erythema and

pruritus. Chronic changes like stenosis and hyperpigmentation of the ear canal can develop, contributing to perpetuate the condition (Uchida *et al.*, 1992; Bensignor and Grandemange, 2006).

2.3 Resistance of M. pachydermatis to antimycotics

Several types of antifungal drugs have been used to treat *Malassezia* dermatitis and otitis, including azoles, allylamines and polyene macrolides (Gupta *et al*., 2000; Yurayart *et al.*, 2013). Other antifungals include chlorhexidine (Young *et al.*, 2012), piroctone olamine (Rème *et al*., 2005), salicylic acid (Ghibaudo and Graziano, 2002), and selenium sulphide (Van Cutsem *et al.*, 1990). Most of those drugs can be used topically for the treatment of dermatitis and otitis in the dog avoiding side-effects associated with oral medication (Van Cutsem *et al.*, 1990; Morris, 1999; Bensignor and Grandemange, 2006).

Although topical and systemic therapy is usually effective in controlling *M. pachydermatis* dermatitis and OE, treatment failure can, potentially be attributed to resistance to antimycotics but clinical data is lacking (Chiavassa *et al*., 2014). There are several *in vitro* studies reporting the presence of resistance of *M. pachydermatis* to antimycotics (Nascente *et al.*, 2009; Jesus *et al.*, 2011; Nijima *et al.*, 2011; Cafarchia *et al.*, 2012a; Chiavassa *et al.*, 2014).

In a study with *M. pachydermatis* isolates recovered from dogs with acute and dogs with chronic otitis, Chiavassa and colleagues (2014) reported an increase of minimal inhibitory concentration values of miconazole and clotrimazole in the isolates from dogs with chronic otitis. The reduced *in vitro* susceptibility may be associated with repeated exposure to these agents, as they are common in otic preparations. Association of therapeutic failure to the presence of resistance is arguable, as these products have concentrations that exceed the minimal inhibitory concentration value by at least 1000 times, and, as such, it should be enough to guarantee an effective treatment (Chiavassa *et al*., 2014).

M. pachydermatis resistance to azoles can be induced *in vitro.* Exposure to subtherapeutic concentrations of azole agents can result in a decrease susceptibility of these agents in isolates collected from healthy and diseased canine ears. This is highly suggestive that *M. pachydermatis* is able to develop resistance mechanisms (Nakano *et al.*, 2005). Jesus

et al. (2011) evaluated the *in vitro* antifungal activity of fluconazole, ketoconazole, itraconazole, and voriconazole against clinical isolates of *M. pachydermatis* susceptible to fluconazole. This isolates were exposed *in vitro* to fluconazole. It was observed that *M. pachydermatis* can acquire resistance through prolonged exposure to fluconazole under laboratory conditions.

Cross-resistance between fluconazole-resistant isolates to other azoles has also been reported. Cafarchia and colleagues (2012b) observed low susceptibility to fluconazole and miconazole, mainly in isolates recovered from animals with skin lesions. It is worth noticing that the strains resistant to fluconazole, in that study, were also resistant, or showed intermediate susceptibility, to other azoles (Cafarchia *et al.*, 2012b). This demonstrates that cross-resistance may occur in *M. pachydermatis*, as previously described in *Candida glabrata* (Sanguinetti *et al*., 2015).

Candida species can display variable susceptibility to different antifungal agents. *C. glabrata*, for example, has a reduced susceptibility to fluconazole, in comparison with other species. *C. albicans*, on the other hand, rarely displays primary resistance to fluconazole (Pfaller *et al.*, 2010). In addition to the intrinsic resistance to antifungals, the development of acquired resistance is a fundamental issue in *Candida* species (Sanguinetti *et al.*, 2015).

Resistance may be also associated with biofilm formation (Bumroongthai *et al*, 2016). An *in vitro* study with 60 *M. pachydermatis* isolates, collected from dogs with and without skin lesions, evaluated the antifungal resistance of sessile (attached to an underlying base) and planktonic cells (free cells). In sessile cells, a high percentage of resistance to ketoconazole (98.3%), terbinafine (96.7%), itraconazole (95%), posaconazole (93.3%), fluconazole (90%) and voriconazole (90%) was observed. Planktonic cell resistance was low, meaning that free cells are more susceptible to antifungals. This data suggests that biofilm formation, in *M. pachydermatis* may be associated with antifungal resistance (Figueredo *et al*., 2013). Previous studies with other yeast species, namely *Candida albicans* are in accordance with these findings (Al-Fattani and Douglas, 2004). The mechanisms of resistance in *M. pachydermatis* have not been described (Peano *et al.*, 2012). In *Candida* spp. the mechanisms for azole resistance include reduced affinity for lanosterol demethylase, the target of other azoles, and a second mechanism involving an energy-dependent efflux pump which results in a decrease of intracellular accumulation of azoles (Chen *et al.*, 2009).

There is a clear lack of standardization in methodology and interpretation of the susceptibility testing of *M. pachydermatis* to antifungals (Chiavassa *et al*., 2014). The interpretation of how presence of *in vitro* resistance translates into clinical outcome is currently unknown but might be useful for better understanding of the pathogenesis of this yeast (Alvarez-Perez *et al*, 2016; Buomino *et al*, 2016).

3. THE NEED FOR ALTERNATIVE ANTIBACTERIALS AND ANTIMYCOTICS

The quick emergence of methicillin resistance, as well as the rise of multidrug resistance strains in *S. pseudintermedius* is becoming a therapeutic challenge, particularly in veterinary medicine, severely restricting the available antimicrobial options (Hillier *et al.*, 2014).

This problem requires a careful and focused use of antimicrobials, with susceptibility testing being a fundamental tool, as empiric use of these drugs is becoming limited (Gold *et al*., 2014). In referral clinics, where most pyoderma infections have been treated several times with various antimicrobials, or even in dogs that never received antibiotics, the percentage of methicillin resistant or multidrug resistant *S. pseudintermedius* isolates is alarming (Hensel *et al*., 2016). This leads to the frequent use of second-tier antibiotics that are associated with serious adverse effects or development of further antimicrobial resistance (Hillier *et al.*, 2014).

In cases where no antimicrobials are available to treat these serious infections, or even as a first approach when there are only present few lesions, topical treatment becomes indispensable. Exploring this option is of extreme importance in order to reduce the use of antimicrobials in small animal clinical practice.

It is for these reasons that the search for other alternatives, particularly natural ones, with virtually no probability of development of resistance is essential. Walking towards a post-antimicrobial era, it is extremely important to understand the reality of ever increasing bacterial resistance and start considering the value of natural products, like honey.

Due to the reasons stated above, namely high prevalence, antibiotic resistance and treatment failures, the search for alternatives becomes a pressing issue. A recent study addressed the use a honey-based gel in the treatment of canine OE. The study suggested that the product is effective when the causal agent is bacterial and/or *Malassezia* spp. (Maruhashi *et al.*, 2016). All enrolled patients had intact tympanic membranes, and the treatment was well tolerated with no noticeable side-effects. This raises the question of the use of honey in the treatment of otitis, although care should be taken if the tympanic membrane is ruptured. In an experimental study with five chinchillas, manuka honey (MH) diluted at 50% caused ototoxicity after myringotomy with inflammatory changes of the inner ear which resulted in facial paralysis, head tilt and loss of hearing capacity (Aron *et al.*, 2012). In a later study, MH appeared safe when diluted at 4% and applied transtympanic in 11 chinchillas during a month (Aron *et al.*, 2015).

In the following chapter we will discuss the origins and benefits of honey in medicine as well as why MH is an exceptional alternative to conventional topical products.

4. MEDICAL HONEY

4.1. Bees: the honey producers

Honey has been a natural food source for human civilizations for centuries, with early Neolithic farmers being the first documented consumers (Roffet-Salque *et al.*, 2015). According to the International Foods Standards OMS/FAO, honey is a naturally sweet substance produced by honey bees from the nectar of plants which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in the honey comb to ripen and mature (Codex Alimentarius Commission FAO/OMS, 2001).

Bees produce honey because it provides a good food source for the colony during the Winter. In order to produce honey, worker bees collect nectar from flower blossoms with their tongue and store it in the honey stomach. When the stomach is full, the bee returns to the hive and regurgitates the nectar directly into a processor bees' mouth or into a honeycomb cell. A processor bee stores the nectar in the honey stomach. In the stomach, the nectar sucrose and complex sugars are converted into fructose and glucose by enzymes like invertase and amylase. Honey is then regurgitated into a honeycomb cell and sealed with wax. The wax

allows the water to evaporate which, along with the fan effect of the bees wings and the warm temperature inside the hive, gives honey its characteristic thick texture (Langstrom, 1853).

Honey can be contaminated due to the treatment of hives, for example, with antibiotics or toxic substances. Chemical residues and pollution fallout, observed in certain regions of the world, can be detected in honey (Rial-Otero *et al.*, 2007). Organic honey must be produced under the European directive for organic products, avoiding toxic contamination (European Union, 2007). It is recommend that honey used for medical purposes should be harvested without contamination (Feás and Estevinho, 2011). Most of the research into honey use for medical purposes has focused on the honey produced by the European honeybee *Apis mellifera*, although honey can also be produced by stingless bees. These species of bees (*Apidae* spp., *Meliponini* spp.) are found in certain areas of the world like South America and Australia, and include species such as *Trigona carbonaria* (Souza *et al.*, 2006; Boorn *et al.*, 2010).

4.2. Honey composition

Honey is an inhospitable environment for microorganisms. The antibacterial effect of honey is multifactorial and includes osmotic effects due to a high sugar content in a low volume of water, acidity (Karabagias *et al.*, 2014) and hydrogen peroxide activity (Molan, 1992).

Honey is naturally rich in simple sugars and has a lower content of proteins, amino acids, vitamins, antioxidants, aromatic substances, minerals and organic acids (Alqarni *et al*., 2016; da Silva *et al.*, 2016). The floral source, components of the honey and water content determine the physical characteristics of each type of honey, such as color, smell, taste, viscosity, solubility and conservation (Escuredo *et al.*, 2013).

Sweetness is due to monosaccharides like fructose, glucose, sucrose and maltose which are the main components of the honey. Honey is composed of roughly 68% sugars and 17% of water, with slight variations depending on the botanical source (Escuredo *et al.*, 2013). In Europe, the honey marketed for human consumption should meet the following criteria: no less than 60g/100g of fructose and glucose content in blossom honey, no more than 5g/100g of sucrose, no more than 20% moisture content, no more than 0.1g/100g of water-insoluble

content (European Union, 2002). Additional criteria are applied, for example, for conservation purposes. Minor constituents include enzymes, protein, amino acids, organic acids, minerals, phenolic and volatile compounds (da Silva *et al.*, 2016).

One of the characteristics that differentiate honey from other sweeteners is the presence of enzymes. These enzymes are produced by the bee during the conversion of nectar into honey in the nectar stomach. The most important enzymes are amylase, invertase and glucose oxidase. Honey contains amylase, which hydrolyses starch into short-chain sugars like maltose. This is called the diastase activity of the honey (Boukraa *et al*., 2008). Invertase converts sucrose from nectar into glucose and fructose. Glucose oxidase is the enzyme that converts glucose into gluconolactone, which in turn yields gluconic acid and hydrogen peroxide. Catalase converts hydrogen peroxide into water and oxygen (Bogdanov *et al.*, 2008).

Another main feature of honey is the presence of natural antioxidants, like phenolic compounds and flavonoids. These are biologically active substances with antioxidant and anti-inflammatory effects by scavenging free radicals in aerobic metabolisms (Pietta, 2000; Erejuwa *et al*., 2012; Alvarez-Suarez *et al*., 2013; Alqarni *et al.*, 2016). Antioxidants, along with nutritional components, vary between honeys depending on the floral source. Darker honeys are associated with a higher content of flavonoids (Bogdanov *et al.*, 2008; Escuredo *et al.*, 2013).

4.3. Medical use of honey

The first documented use of honey for medical purposes was found in a clay tablet from the Sumerian civilization in Mesopotamia 2600 B.C.: "Grind to a powder river dust ... and then knead it in water and honey, and let oil and hot cedar oil be spread over it" (*Sumerian clay tablet,* c. 2000 B.C.). Honey was used in ancient Egypt to manage trauma wounds in battles and was mentioned in the first surgical papyrus: "Thou shouldst bind fresh meat upon [the wound] the first day, thou shouldst apply two strips of linen; and treat afterward with grease, honey, (and) lint every day until he recovers" (Smith surgical papyrus, 1700 BC).

Until the first part of the 20th century, honey dressings were commonly used for everyday wound care of patients with traumatic wounds, surgical incision sites, burns,

sloughy wounds, pressure ulcers and skin grafts preservation (Postmes *et al*., 1993; Seckam and Copper, 2013). With the introduction of antibiotics, honey became less used in clinical practice. Lately, the emergence of antibiotic resistant bacteria renewed the interest in honey as a safe and wide-spectrum antibacterial product for human use (Shenoy *et al.*, 2012; Gobin *et al,* 2018). Honey was accepted in wound healing, ulcers and superficial partial thickness burns treatment (Al-Waili *et al*., 2011; Samarghandian *et al,* 2017). Later a Cochrane systematic review stated that honey appears to heal partial thickness burns faster than conventional treatments and infected post-operative wounds faster than antiseptics and gauze although recommendations for other clinical applications could not be made due to the low quality of the studies (Jull *et al.*, 2013). In veterinary medicine, the application of honey in a wound created under laboratory conditions in White New Zealand rabbits resulted in faster wound healing with increased tissue strength (Oryan *et al.*, 2013).

Medical grade honey means that the honey has been filtered, gamma-irradiated and handled under strict hygiene conditions to ensure a standardized product. Medical honey is sterilized by gamma-radiation to eliminate contaminating microorganisms, including spores of *Clostridium botulinum* and *Bacillus subtilis* (Postmes *et al*., 1995; Carnwath *et al,* 2013). Gamma-radiation does not affect the antibacterial effect of the honey but heat sterilization does (Molan and Allen, 1996).

4.4. Manuka honey: its uniqueness

Manuka honey (MH) is a monofloral dark honey derived from the manuka tree, *Leptospermum scoparium*, which grows in New Zealand and eastern Australia (Molan, 1999; Adams *et al*., 2009; Blair *et al.*, 2009). It is important to note that not all honeys are the same. The antibacterial quality of the honey depends on the source of the nectar as well as when and how it was harvested and stored (Al-Waili, 2004; Mandal and Mandal, 2011; Gobin *et al,* 2018). Some honeys, like MH, have a very potent and well recognized antibacterial activity. The activity of MH is due to the same characteristics that are common to other honeys and some particular features that make it an unique product (Mavric *et al.*, 2008). The antibacterial effect of MH is associated with methylglyoxal (Mavric *et al.*, 2008; Jenkins *et al*., 2011; Cokcetin *et al,* 2016) and this component is found in much higher quantities in MH when compared to conventional honeys (Mavric *et al.*, 2008). This flavonoid is the most important

antibacterial component in MH (Rabie *et al.*, 2016) and is produced from the dihydroxyacetone found in the nectar of manuka flowers (Adams *et al.*, 2009). Another important flavonoid found in MH is leptosin, also known as bee defense, a glycoside peptide found in the insects innate immune system (Kato *et al.*, 2012). Other important antimicrobial compounds found in MH include different 1,2-dicarbonyl compounds, such as glyoxal and 3-deoxyglucosulose (Weigel *et al*., 2004) and phenolic acids although their concentration is too low for antibacterial activity (Alvarez-Suarez *et al.*, 2014).

Manuka honey has been proven to be effective against bacteria (French *et al*., 2005; Boorn *et al.*, 2010; Shenoy *et al.*, 2012; Hillitt *et al*, 2017), *C. albicans* (Patton *et al.*, 2006) and influenza virus (Watanabe *et al.*, 2014). The antibacterial effect of honey has been extensively studied and mainly focused in the pathogens implicated in wounds, burns and ulcers in human medicine (French *et al*., 2005; Boorn *et al.*, 2010; Shenoy *et al.*, 2012).

Staphylococcus aureus is typically the most susceptible organism to honey and has proven to be susceptible to several types of honey, including MH, pasture honey and honey produced from stingless bees (Cooper *et al*., 1999; Boorn *et al.*, 2010). MH also has bactericidal activity against coagulase negative staphylococci and vancomycin-resistant Enterococci (French *et al*., 2005; George and Cutting, 2007). *Pseudomonas aeruginosa,* which is another common pathogen present in wounds and burns proved to be susceptible to MH (Roberts *et al*., 2012; Shenoy *et al.*, 2012). Other Gram-negative bacteria to which MH has antibacterial activity include *Escherichia coli* (Blair *et al.*, 2009) and *Actinobacter baumanni* (George and Cutting, 2007). Cell wall-free bacteria like *Ureaplasma parvum* are also susceptible to MH (Hillitt *et al*, 2017).

In veterinary medicine, a pilot study reported MH to lack complete bactericidal effect against one isolate of MSSP, multi-resistant *P. aeruginosa, E. coli* and extended-spectrum beta lactamase *E. coli* (Uri *et al*, 2016). Another study, reported strong antibacterial effect of a membrane composed of MH and pectin against one strain of MRSP, *Proteus* mirabilis, *P. aeruginosa* and extended-spectrum beta lactamase *E. coli* all obtained from a canine wound infections (Tramuta *et al*, 2016). A study with equine isolates reported *in vitro* efficacy of MH and other types of honey against MRSA, *S. aureus*, *E. coli*, *Streptococcus equi* subs. zooepidermicus, *Enterococcus faecalis*, *Acinetobacter baumanni*, methicillin-resistant *Staphylococcus epidermidis* and *Staphylococcus sciuri* (Carnwath *et al*, 2013). A later study

in an equine model of second intention healing suggested that MH has a beneficial effect in wound healing by reducing healing time (Tsang *et al,* 2018).

MH is approved as a medical grade honey. Medical grade honey allows for the treatment of topical infections with a safe and standardized product. It can be used to treat antibiotic-susceptible infections in order to preserve antibiotics. It can also be used to treat antibiotic-resistant infections when antibiotic options are not available. It is a significant asset now that the battle against resistant microorganisms is increasingly pressing (Hillitt *et al,* 2017; Hussain *et al,* 2017).

In fact, if honey could be used to treat not only bacterial infections but also fungal infections, such as in cases of canine OE, it would be a great asset. The possibility of treating these conditions with a product that has virtually no possibility of development of bacterial/fungal resistance would be excellent news. The use of MH seems, therefore, to present a major opportunity for in canine dermatology.

4.5. Should we be concerned about bacterial resistance to manuka honey?

Antibiotic resistant bacteria have provided the leverage for research into natural alternatives like honey, aloe vera and tea tree oil (Boorn *et al.*, 2010; Cataldi *et al.*, 2015; Falci *et al.*, 2015). Currently, MH is recognized as an option against infections caused by antibiotic resistant bacteria (Jenkins *et al.,* 2011).

Several studies failed to induce MH resistance in either antibiotic-susceptible or antibiotic-resistant bacteria. Cooper *et al.* (2010) demonstrated a lack of resistance after continuous exposure to MH up to 28 days in two reference strains (*S. aureus* and *P. aeruginosa*) and four clinical isolates of *Escherichia coli*, methicillin-resistant *Staphylococcus aureus* (MRSA), *P. aeruginosa* and *Staphylococcus epidermitis*. Another study revealed that resistance to honey is not acquired when *S. aureus* and *P. aeruginosa* are exposed to continuous sublethal concentrations (Blair *et al.*, 2009). *E. coli* shows a unique transcriptional response when exposed to sublethal concentrations of honey, suggesting a different mode of action from antibiotics (Blair *et al.*, 2009).

In summary, lack of resistance to MH may be due to the multifactorial antibacterial nature of the honey. Current findings do not rule out eventual future development of resistance, although it seems unlikely (Seckam and Copper, 2013; Hillitt *et al,* 2017).

4.6. Honey-based products: a clinical option

In human medicine, a honey-based gel (HBO, L-Mesitran® Soft, Triticum, The Nederlands) is licensed for the treatment of wounds, either acute or chronic like pressure, venous, arterial and diabetic ulcers. It is also licensed for first and second degree burns and colonized and postoperative surgical wounds. Oncological wounds and donor sites for skin grafts can also be managed with the product. Its composition includes 40% medical-grade honey, medical-grade hypoallergenic lanolin, propylene glycol, polyethylene glycol 4000, and vitamins C and E (Triticum, 2016a).

The uses of this product in veterinary medicine has not been extensively investigated (Overgaauw and Kirpensteijn, 2005) but a pilot study reported favorable results for treatment of canine otitis externa either caused by bacteria or *M. pachydermatis* (Maruhashi *et al.*, 2016). Another similar product produced by the same company (L-Mesitran® Ointment Triticum, The Nederlands) was used in preliminary veterinary clinical trials for the treatment of intertrigo and wounds in dogs and "proud flesh" in horses (Jakobsson, 2011; Wijnmaalen and Brander, 2012). Similar indications apply for this ointment. It contains 48% medical grade honey, medical grade hypoallergenic lanolin, sunflower oil, cod liver oil, *Calendula officinalis*, *Aloe barbadensis*, vitamin C and E, and zinc oxide (Triticum, 2016b).

The antibiotic resistance developed by *S. pseudintermedius* confirms the urgent need for non-antibiotic alternatives in the treatment of skin infections in the dog, cat, equines and animals used for food consumption. Honey-based products could be an asset in a clinical setting by providing extra benefits when compared to pure honey.

AIMS

Honey has been used since ancient times in the treatment of infected wounds and its antimicrobial effect has been proven against human bacterial and fungal pathogens. The work in veterinary medicine is scarce and the emergence of methicillin-resistant *S. pseudintermedius* has led to interest in topical non-antibiotic based treatment options.

The present doctoral thesis has as main goals:

1- To investigate how clinicians in Portugal currently diagnose and treat skin and ear infections due to *S. pseudintermedius* and *M. pachydermatitis.* We aimed to evaluate systemic and topical treatment options including antibiotics, antifungals and medical honey.

2- Characterize the antibiotic resistant profile of *S. pseudintermedius* isolates obtained from dogs with superficial bacterial folliculitis in referral practice.

3- To determine if manuka honey (MH) and a honey-based gel (HBO) are effective against methicillin-resistant and methicillin-susceptible *S. pseudintermedius* and to assess for how long is necessary for *S. pseudintermedius* to be exposed to MH and HBO in order to obtain a killing effect.

4- To determine if MH and HBO have fungicidal activity against *Malassezia pachydermatis*, and to assess for how long is necessary for *M. pachydermatis* to be exposed to MH and HBO in order to obtain a killing effect.

CHAPTER I

TREATMENT OF SELECTED CANINE DERMATOLOGICAL CONDITIONS IN PORTUGAL - A RESEARCH SURVEY

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1. TREATMENT OF SELECTED CANINE DERMATOLOGICAL CONDITIONS IN PORTUGAL - A RESEARCH SURVEY

1.1. Introduction

Canine pyoderma, particularly superficial bacterial folliculitis (SBF), and otitis externa (OE) are common reasons for veterinary consultation.

Staphylococcus pseudintermedius, although normally isolated from healthy skin, mucosa, and ear canals, can also act as an opportunist pathogen. It is invariably associated with SBF, as well as commonly found in cases of OE (Allaker *et al*., 1992; Kiss *et al*., 1997a; Fazakerley *et al*., 2009; Hillier *et al*., 2014).

Malassezia pachydermatis is a normal inhabitant of canine skin and ears, although, when an adequate environment is created, it can also act as an opportunistic pathogen. This yeast commonly causes *Malassezia* dermatitis (MD) in dogs and is frequently associated with canine OE (Kiss *et al*., 1997; Morris, 1999; Lyskova *et al*., 2007). Skin infections can be treated with antibiotics and topical antiseptics like chlorhexidine (Beco *et al,* 2013; Hillier *et al*., 2014). Bacterial culture and antibiotic susceptibility testing increases the likelihood of prescribing the correct antibiotic (Bryan *et al*., 2012; Beco *et al,* 2013). An increase in the proliferation of antibiotic resistance has led to the demand for alternative treatments, for example with natural products, preferably to which microorganisms cannot acquire resistance. The widespread appearance of methicillin-resistant *S. pseudintermedius* (MRSP) are well documented, and resistance to azoles in *M. pachydermatis* isolates has also been reported (Loeffler *et al*., 2007; Jesus *et al*., 2011; Nijima *et al*., 2011; Bryan *et al*., 2012; Detwiler *et al*., 2013; Cafarchia *et al*., 2015).

This study had three objectives: first, to evaluate the current practice in Portugal regarding diagnosis and treatment of SBF, fold dermatitis (FD) and bacterial OE caused by *S. pseudintermedius* and compare it with International Society for Companion Animals Infectious Diseases (ISCAID) recommendations. Secondly, to investigate the diagnostic methodology and treatment for dermatitis, FD and OE caused by *M. pachydermatis*. Finally, to determine if alternative topical products, namely medical honey, are used in the management of these conditions.

1.2. Material and methods

1.2.1. Survey

A 18-question survey (Annex I) was developed in Google Forms to interrogate practitioner approach to the diagnosis and management of skin and ear infections associated with *S. pseudintermedius* or *M. pachydermatis* in dogs. Two questions were designed to assess diagnostic approaches, 13 questions addressed treatment choices and, three questions covered participant demographics. The questions were designed to avoid bias by multiple-choice and permitting only option would be selected (with the exception of one question).

An e-mail was sent nationwide in October 2017 through Mailchimp® software (The Rocket Science Group, Atlanta, USA) with the link to the survey. It was directed to 740 veterinary hospitals and clinics located in Portugal and was intentionally limited to only one survey *per* practice.

1.3. Results

1.3.1. Total replies

From the total of 740 e-mails, we obtained 103 replies (a 14% response rate). Three surveys were incomplete and thus excluded. A total of 100 replies were considered valid.

1.3.2. Demographics

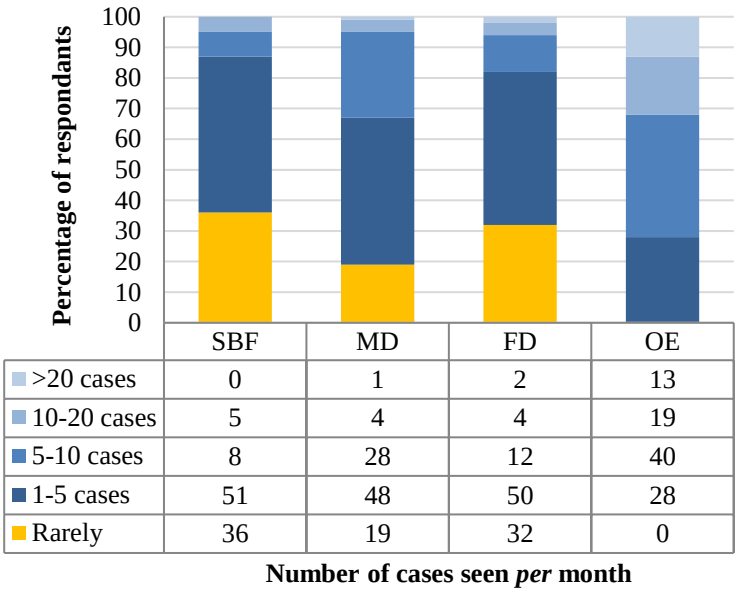
Fifty-two respondents were located in the centre of the country, 31 were from the south and 15 from the northern regions. One response came from the Azores and one from the Madeira archipelago. Thirteen percent of respondents had been in practice for less than 5 years, 26% between 5 and 10 years, 42% between 10 and 20 years, and 19% had more than 20 years of clinical experience.

1.3.3. Use of the ISCAID guidelines

About a third (32%) of the respondents applied the ISCAID guidelines for the diagnosis and treatment of SBF in practice. Most participants were not aware of the guidelines (53%) or did not apply them in practice (15%).

1.3.4. Diagnosis

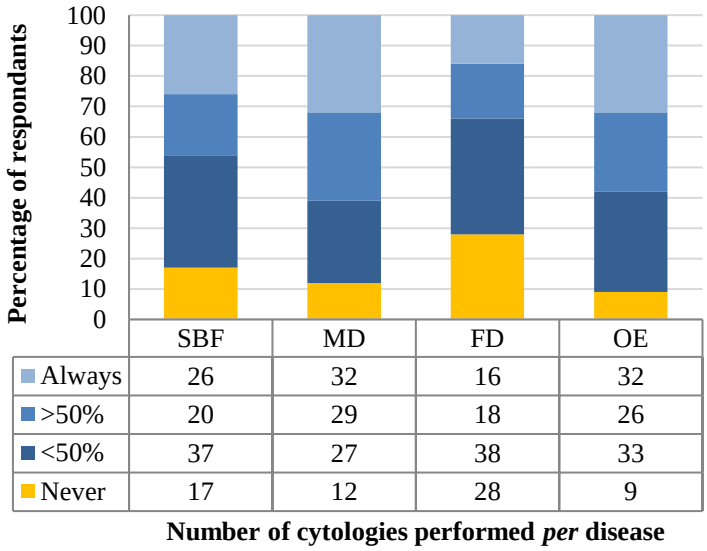
Of the four conditions surveyed, the prevalence was highest for OE. On a monthly basis, all clinicians diagnosed at least one case of OE (100%). *Malassezia* dermatitis was the next disease most commonly seen (81%), followed by FD (68%) and SBF (64%) (Figure 1.1).



	SBF	MD	FD	OE
>20 cases	0	1	2	13
10-20 cases	5	4	4	19
5-10 cases	8	28	12	40
1-5 cases	51	48	50	28
Rarely	36	19	32	0

Figure 1.1. Number of superficial bacterial folliculitis (SBF), *Malassezia* dermatitis (MD), fold dermatitis (FD) and otitis externa (OE) cases observed *per* month.

Cytology evaluation was more commonly used in cases of OE (91%), followed by MD (88%), SBF (83%) and FD (72%) (Figure 1.2).



	SBF	MD	FD	OE
Always	26	32	16	32
>50%	20	29	18	26
<50%	37	27	38	33
Never	17	12	28	9

Figure 1.2. Cytological evaluation for diagnosis confirmation of superficial bacterial folliculitis (SBF), *Malassezia* dermatitis (MD), fold dermatitis (FD) and otitis externa (OE).

1.3.5. Presence of antibiotic resistance in SBF

All clinicians observed cases of SBF caused by antibiotic-resistant *S. pseudintermedius.* In fact, most clinicians (57%) declared an increase in the number of antibiotic-resistant cases seen in the last 5 years, whereas 33% did not think this was the case. Ten percent did not have an opinion on the prevalence of antibiotic resistance in *S. pseudintermedius*.

Most clinicians treated SBF with empirical antibiotic therapy and considered only bacterial culture and antibiotic susceptibility testing after unsuccessful empirical treatment. Cases suspected at the initial stage of being aggravated by bacterial resistance were another reason for culture and antibiotic susceptibility, and in such cases this step was taken prior to treatment (Figure 1.3).

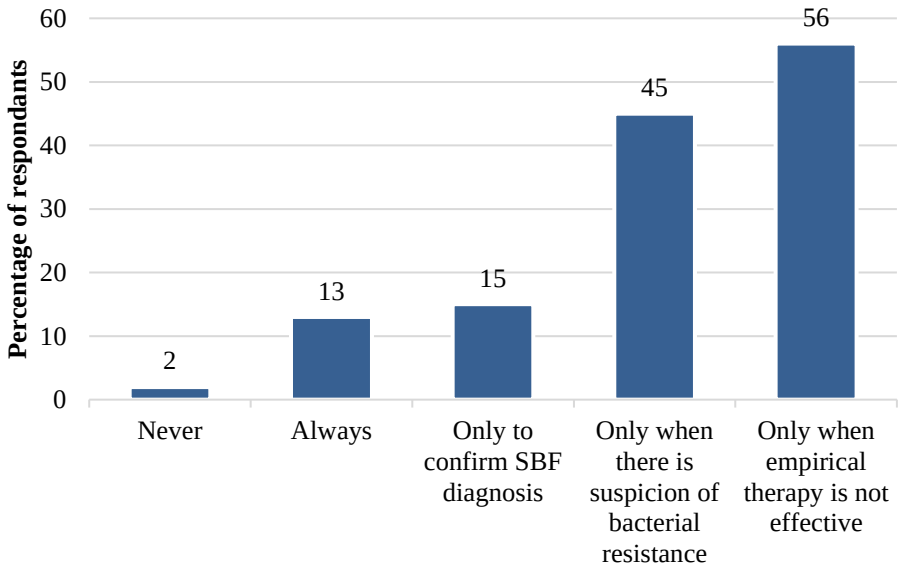
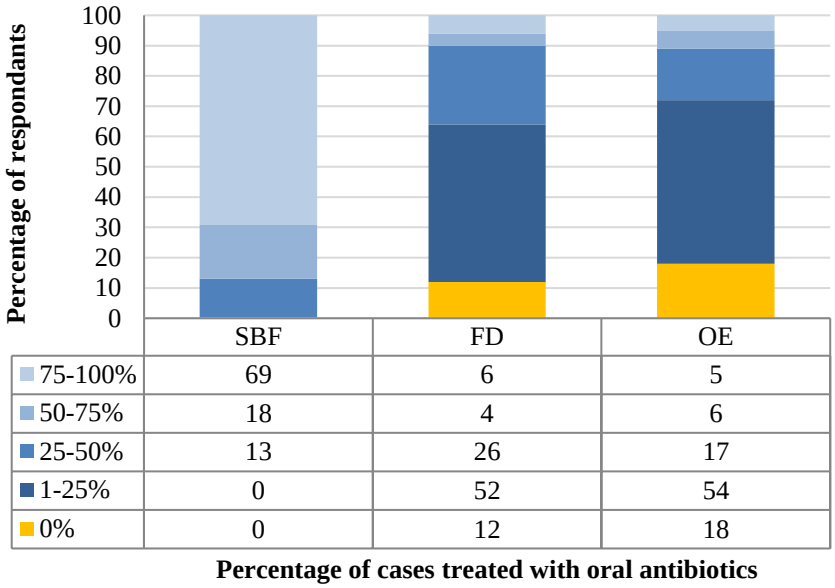


Figure 1.3. Reasons for the use of bacterial culture and antibiotic susceptibility testing in cases of superficial bacterial folliculitis (SBF).

1.3.6. Treatment of bacterial infections

1.3.6.1. Oral antibiotherapy

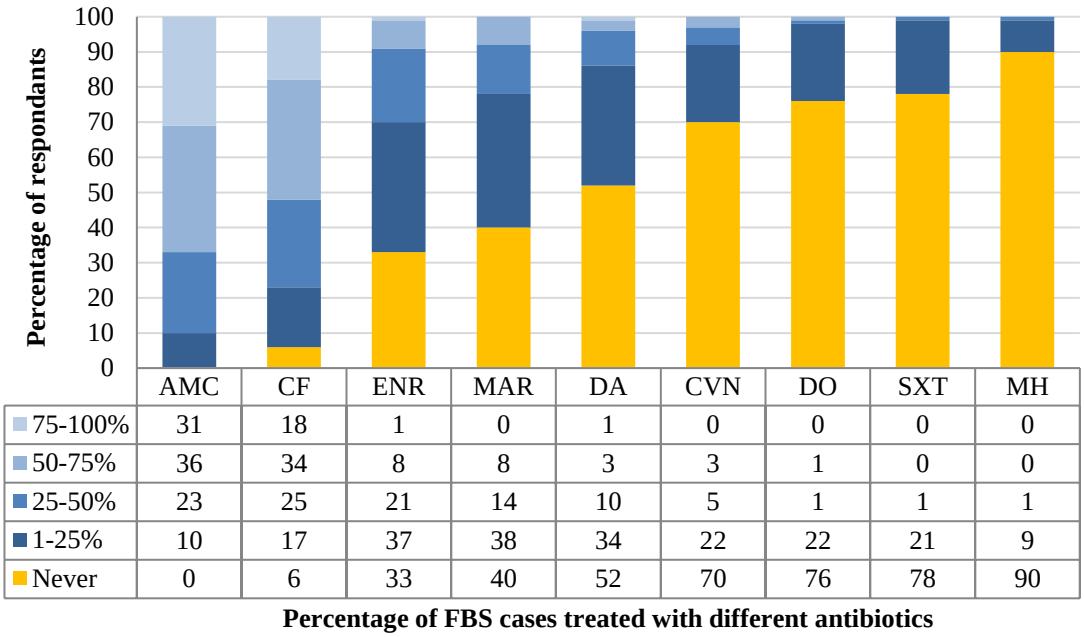
Oral antibiotics were frequently prescribed to manage infections due to *S. pseudintermedius* (Figure 1.4). The results showed that SBF cases are very likely to be treated with oral antibiotics as 100% of the participants considered prescribed them in this circumstance. In FD and OE, the clinician still considered prescribing oral antibiotics in 88% and 82% of the cases, respectively.



	SBF	FD	OE
75-100%	69	6	5
50-75%	18	4	6
25-50%	13	26	17
1-25%	0	52	54
0%	0	12	18

Figure 1.4. Oral antibiotherapy use in superficial bacterial folliculitis (SBF), fold dermatitis (FD) and otitis externa (OE) due to *S. pseudintermedius.*

For the treatment of SBF, amoxicillin with clavulanic acid was considered by all the clinicians. Cephalexin was also very commonly used (94%), followed by enrofloxacin (67%) and marbofloxacin (60%). Antibiotics less commonly used were clindamycin (48%), cefovecin (30%), doxycycline (24%), trimethoprim-sulfamethoxazole (22%) and minocycline (10%) (Figure 1.5).



	AMC	CF	ENR	MAR	DA	CVN	DO	SXT	MH
75-100%	31	18	1	0	1	0	0	0	0
50-75%	36	34	8	8	3	3	1	0	0
25-50%	23	25	21	14	10	5	1	1	1
1-25%	10	17	37	38	34	22	22	21	9
Never	0	6	33	40	52	70	76	78	90

Figure 1.5. Use of oral antibiotherapy for superficial bacterial folliculitis (SBF): amoxicillin-clavulanic acid (AMC); cephalexin (CF); enrofloxacin (ENR); marbofloxacin (MAR); clindamycin (DA); cefovecin (CVN); doxycycline (DO); trimethoprim-sulfamethoxazole (SXT) and minocycline (MH).

1.3.6.2. Topical therapy in bacterial infections

Participants prescribed therapeutic baths for SBF treatment followed by skin disinfection. The treatment was performed with topical antibiotics (either associated with antifungals or glucocorticoids). Fold dermatitis was managed by disinfection of the skin followed by topical antibiotics. Ear cleaning was frequently prescribed, and topical treatments contain a combination of antibiotic, antifungal and glucocorticoids were the main choice. If honey-based products are considered, they would mainly be used for SBF and hardly used at all in the treatment of FD and OE. Other products (not specified) were also used by the participants (Table 1.1).

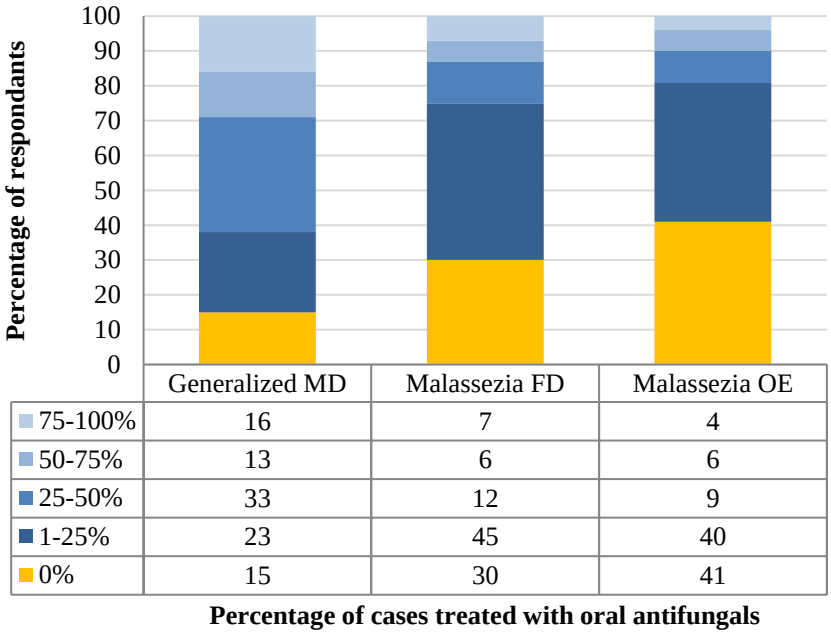
Table 1.1. Topical therapy in superficial bacterial folliculitis (SBF), bacterial fold dermatitis (FD) and bacterial otitis externa (OE).

Type of topical therapy	**Frequency of prescription**				
	Never	**<25%**	**25-50%**	**50-75%**	**75-100%**
Superficial bacterial folliculitis					
Therapeutic baths	0	4	18	14	64
Skin disinfection	9	8	15	12	56
Product with antibiotic, antifungal and glucocorticoid	43	31	15	8	3
Product with only antibiotic	43	32	11	7	7
Honey-based products	67	23	6	3	1
Other products	69	17	6	4	4
Bacterial fold dermatitis					
Skin disinfection	0	3	2	15	80
Product with antibiotic, antifungal and glucocorticoid	29	26	8	21	16
Product with only antibiotic	34	25	7	20	14
Honey-based product	80	12	2	3	3
Other products	72	12	1	8	7
Bacterial otitis externa					
Ear cleaning	3	0	6	4	87
Product with antibiotic, antifungal and glucocorticoid	2	8	6	23	61
Product with only antibiotic	54	24	10	9	3
Honey-based product	92	6	2	0	0
Other products	74	14	7	2	3

1.3.7. Treatment of Malassezia infections

1.3.7.1. Oral antifungals

For the treatment of generalized MD, oral antifungals were used by 85% of the clinicians. Concerning *Malassezia* FD and OE, oral antifungals were prescribed by 70% and 59% of the clinicians, respectively (Figure 1.6).



	Generalized MD	Malassezia FD	Malassezia OE
75-100%	16	7	4
50-75%	13	6	6
25-50%	33	12	9
1-25%	23	45	40
0%	15	30	41

Figure 1.6. Oral antifungal use in generalized *Malassezia* dermatitis (MD), *Malassezia* fold dermatitis (FD) and *Malassezia* otitis externa (OE) cases.

1.3.7.2. Topical therapy in Malassezia infections

Topical treatment of generalized MD was performed with bathing and skin disinfection, and with products containing only antifungals. *Malassezia* FD was managed with skin disinfection followed by application of products containing antibiotic and antifungal agents and glucocorticoids. Ear cleaning followed by use of products with antibiotic and antifungal effect and glucocorticoid content was the treatment adopted for OE. Honey-based products are hardly used for any of the diseases caused by *Malassezia.* Other products that were not specified were also used by the clinicians (Table 2).

Table 1.2. Use of topical therapy in *Malassezia* dermatitis (MD), fold dermatitis (FD) and otitis externa (OE).

Type of topical therapy	**Frequency of prescription**				
	Never	**<25%**	**25-50%**	**50-75%**	**75-100%**
Generalized *Malassezia* dermatitis					
Therapeutic baths	0	1	7	5	87
Skin disinfection	20	12	7	12	49
Product with antibiotic, antifungal and glucocorticoid	53	21	13	10	3
Product with only antifungal	36	17	19	12	16
Honey-based product	96	3	0	1	0
Other products	78	6	3	7	6
***Malassezia* fold dermatitis**					
Skin disinfection	1	2	6	7	84
Product with antibiotic, antifungal and glucocorticoid	29	20	17	16	18
Product with only antifungal	34	18	25	10	13
Honey-based product	90	6	2	1	1
Other products	74	11	5	5	5
***Malassezia* otitis externa**					
Ear cleaning	4	1	3	8	84
Product with antibiotic, antifungal and glucocorticoid	7	6	12	15	59
Product with only antifungal	61	12	9	10	8
Honey-based product	95	2	2	0	1
Other products	80	9	3	0	8

1.4. Discussion

This study showed that largely oral antibiotics were used for the treatment of SBF, FD and OE. Diagnostic approach is another issue, in veterinarians´ failure to use of appropriate diagnostic tests for the conditions considered in the survey.

The majority of clinicians who collaborated in this survey were experienced in small animal practice and had been working for over 10 years. The conditions considered were observed routinely by the practitioners. This is in accordance with previous literature which states that SBF is a common disease and also one of the main reasons for antimicrobial prescription in small animal practice (Rantala *et al*., 2004; Baker *et al*., 2012). Otitis externa is also a common cause for consultation (August, 1988; O'Neill *et al*., 2014; Perry *et al*., 2017). *Malassezia* dermatitis is another frequent disease and is normally associated with an underlying cause, such as atopic dermatitis (Bond *et al*., 1996; Machado *et al*., 2011). Skin fold dermatitis is also very common, particularly in brachycephalic dogs and breeds with excessive skin folds (Beco *et al*., 2013). Lately, brachycephalic breeds have become very common in Portugal, exemplified most clearly by the French Bulldog, and this might be the reason for the high prevalence of FD observed by the clinicians in this study.

In general, the survey demonstrated that cytology could be more thoroughly used by clinicians for diagnostic purposes. In fact, only approximately a quarter of the clinicians performed it in every case of SBF. Cytology is a simple, inexpensive, and reliable diagnostic test that can easily be performed in a consultation by the clinician (Beco *et al*., 2013). Unfortunately, 12% of the clinicians never used this diagnostic tool for generalized MD, which is surprising, bearing in mind that adhesive cellophane testing is the most suitable test for diagnosis of MD (Bensignor *et al*., 2002). Otic cytology allows discrimination between bacteria and *Malassezia* yeasts, as the appearance and odour of ear exudate cannot be used to reach a reliable diagnosis (Kiss *et al*., 1997a; Angus, 2004).

Clinicians always considered the use of oral antibiotherapy in SBF cases. Overall, clinicians preferred to begin therapy with oral antibiotics, empirically, and if clinical improvement was not observed, they resorted to bacterial culture and susceptibility testing. When bacterial resistance is suspected, the clinicians will also perform culture and susceptibility testing. There are situations when bacterial culture is particularly important, mainly in cases of apparent antimicrobial resistance (Hillier *et al*., 2014).

However, the number of clinicians who never used bacterial culture, never tested for antibiotic susceptibility, or only use culture for diagnostic purposes is surprising. In fact, most of the participants had diagnosed cases SBF with antibiotic resistance to *S. pseudintermedius* and had recognized an increase of antibiotic resistance in the last five years. The problem of their diminishing effectiveness is therefore escalating. In Portugal, methicillin and multidrug resistant *S. pseudintermedius* were reported for the first time in 2010 and in other papers thereafter (Pomba *et al*., 2010; Couto *et al*., 2011; Couto *et al*., 2014; Beça *et al*., 2015).

Bacterial FD and bacterial OE were also largely treated with oral antibiotics, which adds further concern. Exposure to antibiotics has been associated with the development of resistance by *S. pseudintermedius* isolates, either from skin lesions or from OE (Weese *et al*., 2012; Hillier *et al*., 2014; Ludwig *et al*., 2016; Zur *et al*., 2016).

This survey showed that amoxicillin-clavulanic acid and cephalexin were the most frequently prescribed antibiotics for SBF treatment, followed by enrofloxacin and marbofloxacin. Other antibiotics such as clindamycin, trimethoprim-sulfamethoxazole, cefovecin, doxycycline, and minocycline were less frequently used. This is in accordance with official data: penicillins, first and second generation cephalosporins, and fluoroquinolones are the most prescribed antibiotics in small animal practice. Macrolides and tetracyclines are less often used, as well as sulphonamides and lincosamides (DGAV, 2015). A 16-year study in Portugal documented an increase in *S. pseudintermedius* resistance against oxacillin, ampicillin, amoxicillin, penicillin, cefovecin, cefalexin, enrofloxacin, clindamycin and trimethoprim-sulfamethoxazole. Cephalosporins have been putatively implicated in the development of MRSP (Couto *et al*., 2016). Another study reports misuse of antimicrobials such as fluoroquinolones, macrolides, and third generation cephalosporins, demonstrating correlation with MRSP colonization (Rota *et al*., 2013). Based on the results of this survey, we recommend that fluoroquinolones should be used with more caution. Clinicians principally use antibiotics in the treatment of SBF, FD, and OE, in spite of the conservative recommendations of the ISCAID guidelines. The guidelines developed by ISCAID are a great asset to help the clinician recognise the signs of canine SBF, choose the correct diagnostic tools, and determine the most appropriate topical or systemic antimicrobial therapy (Hillier *et al*, 2014). In reality, most of the practitioners do not follow or are not aware of the ISCAID guidelines, although the reasons are not explained in this survey.

In general, clinicians recommended the application of therapeutic baths and skin disinfection in cases of bacterial or *Malassezia* infections. The use of antibiotic and antimycotic based-products was also frequent, in contrast to honey-based products which were rarely applied.

According to our findings, topical treatment with honey-based products is seldom prescribed. The efficacy of a honey-based gel was also confirmed for the treatment of bacterial and/or *Malassezia* OE and canine intertrigo (Jakobsson, 2011; Maruhashi *et al*., 2016). The same product has been proven to be effective against MSSP and MRSP originated from SBF cases. The product also eradicated *M. pachydermatis* originated from OE (Oliveira *et al*., 2018). Medical honey or honey-based products are potential treatments for the diseases considered in this study and could be used more often in Portugal.

4.5. Conclusion

This survey contributed to the understanding on how Portuguese clinicians are diagnosing and treating superficial bacterial folliculitis, *Malassezia* dermatitis, fold dermatitis and, otitis. It uncovered a lack of awareness of the ISCAID guidelines, an increasing perception of antibiotic-resistant *S. pseudintermedius,* potential overuse of antibiotics and, lack of antibiotic-free products. Educational actions should be undertaken to increase awareness about the correct use of antibiotics to avoid promulgating bacterial resistance in our country.

CHAPTER II

MULTIDRUG RESISTANT *Staphylococcus pseudintermedius* ISOLATED FROM SUPERFICIAL BACTERIAL FOLLICULITIS IN DOGS FROM PORTUGAL AND SPAIN

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2. MULTIDRUG RESISTANT *Staphylococcus pseudintermedius* ISOLATED FROM SUPERFICIAL BACTERIAL FOLLICULITIS IN DOGS FROM PORTUGAL AND SPAIN

2.1. Introduction

In the nineties, most infections caused by *Staphylococcus pseudintermedius* in dogs were effectively treated with empiric antibiotherapy with beta-lactams, macrolides or potentiated sulphonamides antibiotics (White, 1996; Ganiere *et al*., 2005). At least in Europe, multidrug resistance was extremely rare (Lloyd *et al*., 1996; Guardabassi *et al*., 2004; Rantala *et al*., 2004). Between 1987 and 1995, resistance to cephalexin, amoxicillin-clavulanic acid, oxacillin/methicillin and enrofloxacin had never been reported in the UK (Lloyd *et al*., 1996).

The first multidrug resistant (MDR) methicillin resistant *Staphylococcus pseudintermedius* (MRSP) isolates were reported at a dermatology referral centre in Germany in 2005 (Loeffler *et al*., 2007). Multidrug resistance is considered as resistance to three or more classes of antibiotics (Coombs *et al*., 2004; Schwarz *et al*., 2010).

Two MRSP strains developed simultaneously in Europe and USA with different resistant patterns. MRSP isolates were first reported in 1999 in North America and throughout Europe between 2005 and 2006, and are actually recognized as having a worldwide distribution (Gortel *et al*., 1999; Loeffler *et al*., 2007; Schwarz *et al*., 2008; Perreten *et al*., 2010; Onuma *et al*., 2012). The North America strain is still susceptible to chloramphenicol, rifampicin and amikacin. Regarding the predominant MRSP strain in Europe, resistance to beta-lactams, aminoglycosides, macrolides, lincosamides, tetracyclines, chloramphenicol, trimethoprim and fluoroquinolones is normally observed, although susceptibility to amikacin, fusidic acid, minocycline, rifampicin, vancomycin, teicoplanin and linezolid is still maintained (Perreten *et al*., 2010; Frank *et al*., 2012). This demonstrates the importance of recognizing MRSP isolates susceptibility in order to apply effective antibiotherapy.

The aim of the present work was to evaluate the MDR profile of 60 *S. pseudintermedius* isolates from two referral veterinary hospitals in Portugal and Spain.

2.2. Material and methods

2.2.1. Characterization of the isolates

Sixty *S. pseudintermedius* isolates, previously collected from dogs with superficial bacterial folliculitis (SBF) presented to the Dermatology Service of the Faculty of Veterinary Medicine at Universidade Lusófona de Humanidades e Tecnologias and Universitat Autònoma de Barcelona, were used in this study. These isolates were collected between January and December of 2014. Isolates were stored in a mixture of glycerol 30% (Scharlab S.L., Barcelona, Spain) and nutrient broth at -80˚C. All media used were supplied by Oxoid (Oxoid, Hampshire, UK) unless stated otherwise.

The isolates were previously characterized as Gram-positive *cocci*, catalase and coagulase positive. They were also purposely chosen as MRSP (30/60) and methicillin resistant *Staphylococcus pseudintermedius* (MSSP, 30/60) after disk diffusion susceptibility test to oxacillin by the Kirby-Bauer technique following the Clinical Laboratory Standards Institute (CLSI) guidelines (CLSI, 2013).

A multiplex PCR (polymerase chain reaction) of 16S rRNA (*Staphylococcus* genus specific), *nuc* (*Staphylococcus aureus* species specific) and *mecA* (a determinant of methicillin-resistance) genes was used for identification of the isolates (National Food Institute, 2009). The isolates were then identified as *S. pseudintermedius* by polymerase chain reaction-restriction fragment length polymorphism assay, as described by Bannoehr and collaborators (Bannoehr *et al*., 2009).

2.2.2. Antibiotic-Susceptibility Testing

Antibiotic susceptibility testing was performed in accordance with the Kirby-Bauer methodology described in the CLSI guidelines (CLSI, 2013). Antibiotics were chosen based on the recommendation of the Working Group of the International Society for Companion Animal Infectious Diseases (ISCAID) guidelines for the treatment of SBF (Hillier *et al*., 2014). The inhibition halos were interpreted as susceptible or resistant using the recommended diameters by the CLSI guidelines and, if not available, by the European Committee on Antimicrobial Susceptibility Testing (CLSI, 2013; EUCAST, 2017). The following antibiotic disks were tested: oxacillin (1µg), amoxicillin-clavulanic acid (20/10µg),

cefalothin (30µg), clindamycin (2µg), erythromycin (15µg), trimethoprim-sulfamethoxazole (1.25/23.75 µg), tetracycline (30µg), minocycline (30µg), enrofloxacin (5µg), pradofloxacin (5µg; Mast Diagnostics, UK), chloramphenicol (30µg), rifampicin (5µg), gentamicin (10UI; Bio-Rad, France) and amikacin (30µg). *Staphylococcus aureus* (Culti-Loops™, ATCC® 29213™, Thermo-Fisher™, USA) strain was used as the quality control recommended by the CLSI. Isolates were classified as MDR or non-MDR according to the number of classes to which they were resistant (Coombs *et al*., 2004; Schwarz *et al*., 2010). The following antibiotics were used to determine the MDR pattern: oxacillin (beta-lactam class), clindamycin (lincosamide class), erythromycin (macrolide class), trimethoprim-sulfamethoxazole (sulphonamide class) tetracycline (tetracycline class), enrofloxacin (fluoroquinolone class), chloramphenicol (phenicol class), rifampicin (ansamycin class) and gentamicin (aminoglycoside class) (Table 2.1).

Table 2.1. Antibiotic classification according to the Working Group of the International Society for Companion Animal Infectious Diseases (ISCAID) guidelines.

FIRST-TIER ANTIBIOTICS	SECOND-TIER ANTIBIOTICS
Amoxicillin-clavulanic acid	Tetracycline
Cefalothin	Minocycline
Clindamycin	Enrofloxacin
Erythromycin	Pradofloxacin
Trimethoprim-sulfamethoxazole	Chloramphenicol
	Rifampicin
	Amikacin
	Gentamicin

2.2.3. Statistical analysis

Statistical analysis was performed with *Statistical Package for the Social Sciences* 16.0 (IBM SPSS Chicago, IL).

2.3. Results

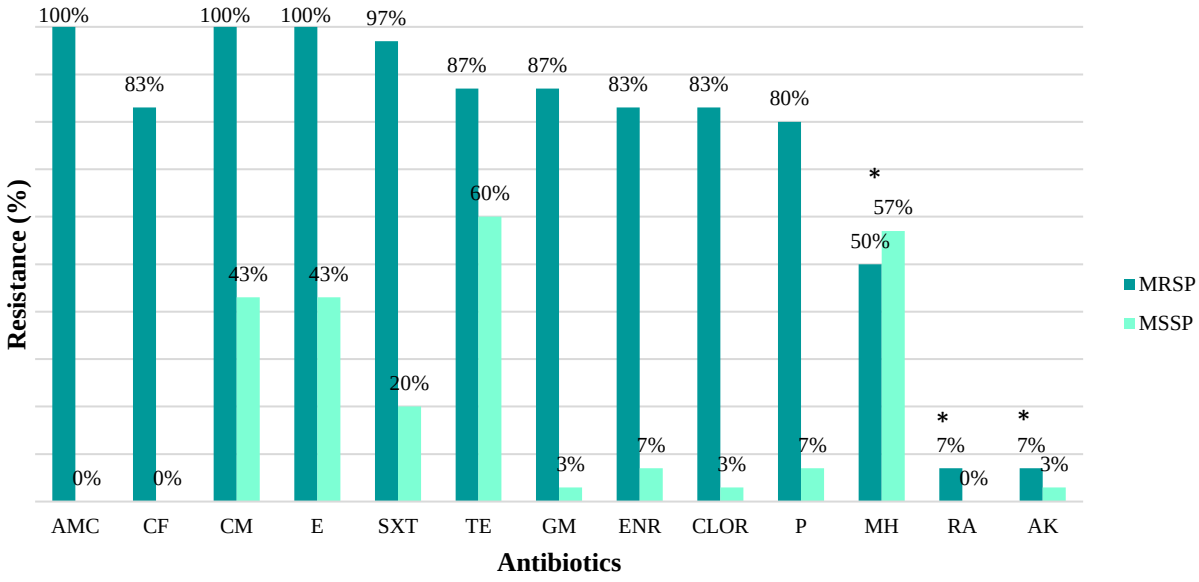
2.3.1. Identification of MRSP and MSSP isolates

All isolates were identified as *S. pseudintermedius.* The *mecA* gene was present in all MRSP isolates and absent in MSSP isolates, confirming the oxacillin-resistance phenotype.

From the total of 60 isolates, 38 were of Portuguese origin (19 MSSP/ 19 MSRP) and 22 originated from Spain (11 MSSP/ 11 MRSP).

2.3.2. Antibiotic susceptibility testing

All MRSP isolates displayed resistance to amoxicillin-clavulanic acid. Isolates also exhibited resistance to clindamycin and erythromycin. High level of resistance was observed against trimethoprim-sulfamethoxazole, tetracycline, gentamicin, cefalothin, enrofloxacin, chloramphenicol, pradofloxacin and minocycline. Low level of resistance was observed for, amikacin and rifampicin. Within the MSSP group, all isolates were susceptible to amoxicillin-clavulanic acid, cefalothin and rifampicin. A high number of isolates exhibited resistance to tetracycline, minocycline, clindamycin and erythromycin (Figure 2.1).



* Statistically significant differences between MSSP and MRSP groups (P<0.05).

Figure 2.1. Relative frequency (%) of antibiotic resistance in the MRSP and MSSP group. First-tier antibiotics: AMC, Amoxicillin-clavulanic acid; CF, Cefalothin; CM, Clindamycin; E, Erythromycin; SXT, Trimethoprim-sulfamethoxazole. Second-tier antibiotics: TE, Tetracycline; GM, Gentamicin; ENR, Enrofloxacin; CLOR, Chloramphenicol; P, Pradofloxacin; MH, Minocycline; RA, Rifampicin; AK, Amikacin.

Overall, resistance to oxacillin was associated with resistance to other antibiotics, except for minocycline (P=0.796), rifampicin (P=0.492) and amikacin (P=1.000). Out of 60

isolates, 11 were only susceptible to rifampicin and/or amikacin, and none of them to chloramphenicol.

Within the tetracycline class, 12 isolates (11 MRSP and 1 MSSP) were susceptible to minocycline but resistant to tetracycline (12/44; *P*=0.000); amongst the fluoroquinolone class, only 2 (1 MSSP and 1 MRSP) enrofloxacin-resistant isolates were pradofloxacin susceptible (2/27; *P*=0.000). Within the aminoglycoside group, 24 gentamicin-resistant isolates (all MRSP) were amikacin susceptible (24/27; *P*=0.085).

Most isolates were MDR (63%, 38/60). All non-MDR isolates belonged to the MSSP group. Methicillin resistance was associated with multidrug resistance to other classes of antibiotics (*P*=0.0001) (Table 2.2).

Table 2.2. Association between multidrug resistance and methicillin resistance.

	MDR	**Non-MDR**
MRSP	79% (30/38)	0% (0/22)
MSSP	21% (8/38)	100% (22/22)

MSSP (methicillin susceptible *Staphylococcus pseudintermedius*), MRSP (methicillin resistant *Staphylococcus pseudintermedius*) and MDR (multidrug resistance).

2.4. Discussion

This study demonstrates the presence of MDR *S. pseudintermedius* isolates in dermatology referral patients from the Iberian Peninsula. Multidrug resistance was associated with the presence of methicillin resistance. The percentage of MDR isolates was extremely high within the MRSP isolates obtained in this study, however, multidrug resistance was also observed in MSSP isolates. The presence of these MDR patterns has become a clinical challenge for veterinarians, since it reduces the number of antibiotic alternatives for the successful treatment of canine SBF. This is in accordance with other studies reported in Germany, UK and other European countries (Loeffler *et al*., 2007; Holm *et al*., 2002; Nienhoff *et al*., 2011). In Portugal, multidrug resistance has also been detected in colonization and infection cases in dogs (Couto *et al*., 2014).

Since the first report in 2005, there has been increased incidence of MRSP across Europe (Loeffler *et al*., 2007; Perreten *et al*., 2010). In Spain, MRSP has been reported in healthy dogs with isolates being resistant to beta-lactams, tetracycline, macrolides,

lincosamides, aminoglycosides, trimethoprim-sulfamethoxazole and, in some cases, to fluoroquinolones (Gómez-Sanz *et al*, 2011). More recently, a human infection caused by *S. pseudintermedius* originating in dogs has been described (Lozano *et al*, 2017). Cases of MRSP have been described since 2010 and a trend towards multidrug resistance has been suggested (Pomba *et al*, 2010; Couto *et al*, 2011; Couto *et al*, 2014; Beça *et al*, 2015; Couto *et al*, 2016).

Nowadays, it is recommended to follow the guidelines for the diagnosis and antimicrobial therapy of SBF developed by the ISCAID. These guidelines provide updated information for adequate treatment of canine SBF and rational use of antibiotics (Hillier *et al*., 2014).

Staphylococcus pseudintermedius is the most common bacterial pathogen associated with canine SBF (Bannoehr *et al*., 2007). Treatment of these infections typically involves administration of broad-spectrum antibiotics, such as beta-lactams, clindamycin, erythromycin or potentiated sulphonamides (Hillier *et al*., 2014). This study demonstrates the presence of high levels of resistance to first line antibiotics, often used empirically, which significantly limits treatment options by the veterinarian, particularly in MRSP isolates. In fact, only one MRSP isolate was susceptible to trimethoprim-sulfamethoxazole. However, MSSP isolates were also resistant to first line antibiotics, such as clindamycin, erythromycin and trimethoprim-sulfamethoxazole. This is one of the reasons why bacterial culture and susceptibility testing should always be performed in case of lack of clinical response after two weeks of appropriate empirical antibiotherapy (Hillier *et al*., 2014).

Based on the resistance profile to oxacillin, the representative antibiotic for the beta-lactam class, the isolates were divided into two groups: MRSP and MSSP. It is important to evaluate the susceptibility to the oxacillin disk in *S. pseudintermedius* isolates, since it is an indicator of resistance mediated by the *mecA* gene, when its detection by PCR, the gold standard method, is not possible. The link between MDR and MRSP has been reported (Holm *et al*., 2002; Ganiere *et al*., 2005; Nienhoff *et al*., 2011). In this study a clear association was observed between MRSP and multidrug resistance. Resistance to methicillin causes a major impact on treatment using beta-lactam class antibiotics, which are administrated empirically for the treatment of staphylococcal infections. Although some MRSP displayed susceptibility to cefalothin, according to the CLSI recommendations and the ISCAID guidelines, they should be considered and reported as resistant, as methicillin resistance confers resistance to

virtually every beta-lactam antibiotic, with the exception of anti-MRSA cephalosporins (Jones *et al*., 2007; Bemis *et al*., 2009; Magiorakos *et al*., 2012; Papich, 2013).

Lincosamides and macrolides are considered good antibiotic choices for the treatment of canine SBF caused by staphylococci due to its good oral absorption, distribution in tissues and high intracellular concentration (Ganiere *et al*., 2005; Hillier *et al*., 2014). However, especially in recurrent infections, their use is limited by a high level of resistance, particularly when there is cross-resistance between the two antibiotics (Ganiere *et al*., 2005). The interpretation of susceptibility to lincosamides should be considered carefully since cross-resistance with macrolides can occur. The presence of cross-resistance should be considered when the isolate demonstrates *in vitro* resistance to erythromycin and susceptibility to clindamycin (Steward *et al*., 2005; Perreten *et al*., 2010).

Potentiated sulphonamides are first line antibiotics frequently used in the treatment of canine SBF (Dowling, 1996; Hillier *et al*., 2014). The use of potentiated sulphonamides as first line antibiotics is limited by the relatively high incidence of resistance and potential side effects (Trepanier, 2004; Jones *et al*., 2007).

Since tetracycline is only the marker for resistance to the tetracycline class, doxycycline is usually the administered antibiotic. Even though minocycline is not licensed for use in dogs, results of the current study suggest that minocycline could have been used to treat 12 patients which were tetracycline-resistant (Maaland *et al*., 2014). Therefore, in addition to its use being recommended in the ISCAID guidelines, minocycline represents a useful alternative.

Fluoroquinolones, particularly in MSSP isolates in which a high susceptibility rate is observed, can be administered for staphylococcal infections. The association between the use of quinolones and carbapenems and an increased risk for MRSA is reported in a large hospital study among hospitalized people (Ascioglu *et al*., 2014).

Chloramphenicol is rarely used in the treatment of *S. pseudintermedius* infections as it may cause severe adverse effects, particularly in humans (Short *et al*., 2014). Susceptibility in MSSP isolates was still observed, although resistance to this antibiotic is common in Europe, due to the expression of the chloramphenicol acetyltransferase (Schwarz *et al*., 1995; Ganiere *et al*., 2005; Kadlec *et al*., 2012). Isolates resistant to chloramphenicol are still susceptible to

florfenicol, a derivative of chloramphenicol, so it may be a safer option (Schwarz *et al*., 2000; Kehrenberg *et al*., 2004; Ganiere *et al*., 2005).

Resistance to rifampicin is normally rare and the presence of resistant isolates can be associated with previous administration of this antibiotic, since mutations for resistance can persist for months (Perreten *et al*., 2010, Kadlec *et al*., 2011). Even when the isolate displays susceptibility, the administration of rifampicin as monotherapy or in combination with another antibiotic to treat MDR-MRSP infections, can result in high levels of resistance (Kadlec *et al*., 2011).

Aminoglycosides can be used in the treatment of staphylococcal infection, if no other safer alternatives are available. Against resistant isolates, amikacin is more active than gentamicin and resistance is less likely to occur, which was verified in this study as amikacin could have potentially been used to treat most of the gentamicin-resistant patients (Papich, 2013). Inactivation by aminoglycoside modifying enzymes is the main resistance mechanism to aminoglycosides (Perreten *et al*., 2010; Gold *et al*., 2014). These antibiotics are not routinely used in the treatment of staphylococcal infections, particularly due to their nephrotoxic and ototoxic effects and the inconvenience of parenteral administration (Hillier *et al*., 2014). Their administration should be avoided in animals with renal insufficiency and in healthy animal monitoring of renal function, according to International Renal Interest Society guidelines for prevention of aminoglycoside-induced acute kidney injury is advised (International Renal Interest Society, 2015). However, with the increasing prevalence of MRSP, their use is becoming a necessary alternative and they are recommended in the ISCAID guidelines when no other safer alternatives are present (Hillier *et al*., 2014).

Ideally, second choice antibiotics like fluoroquinolones should only be used in MRSP infection cases (Hillier *et al*., 2014). In general, higher susceptibility rates were associated with minocycline, amikacin and rifampicin, but side-effects can deem their use unacceptable in certain patients. MRSP were less likely to be susceptible to doxycycline, enrofloxacin, pradofloxacin and gentamicin.

2.5. Conclusion

Although this study was performed in a limited number of animals, the isolates were recovered from dogs attending referral consultation and, therefore, these data further supports the fact that first line antibiotics are extremely limited to treat these patients.

The association between MRSP and the presence of MDR was evident and it was also observed in MSSP isolates.

This problem requires a prudent, targeted use of antibiotics and the development of novel topical treatments to control infections caused by MDR *S. pseudintermedius.* Additionally, bacterial culture to identify the bacteria and sensitivity testing are essential tools to determine the most appropriate treatment plan, which can include antibiotics with potential severe side-effects.

CHAPTER III

IN VITRO EFFICACY OF MANUKA HONEY AGAINST *Staphylococcus pseudintermedius* AND *Malassezia pachydermatis*

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3. *IN VITRO* EFFICACY OF MANUKA HONEY AGAINST *Staphylococcus pseudintermedius* AND *Malassezia pachydermatis*

3.1. Introduction

Honey is a natural product with an interesting source of nutrients and it is known for its beneficial effects in human health. It has long been used in human medicine but evidence for its benefits are really just starting to be shown (Carter *et al*., 2016). Manuka honey (MH) is produced by the European honey bee (*Apis mellifera*) foraging on pollen from the *Leptospermum scoparium* tree and has a proven antibacterial efficacy against many types of bacteria commonly involved in skin infections (Alvarez-Suarez *et al*., 2014). In general, honey has antibacterial properties, an autolytic debriding action, is anti-inflammatory and enhances healing (Jull *et al*., 2015). The use of honey has been increasing, especially in those cases where conventional antibiotic therapy is failing (French *et al*., 2005). Nowadays, honey intended for medical purposes is sterilized by gamma-radiation and produced under standard conditions to ensure safety and efficacy (Postmes *et al*. 1995). Another advantage associated with honey use is the apparent lack of bacterial resistance development (Cooper *et al*., 2010). In general, honey has hydrogen peroxide in its composition produced by the enzyme glucose oxidase. The hydrogen peroxide against pathogens but can be inactivated by enzyme catalases present in the wounds. Catalase converts hydrogen peroxide into water and oxygen (Bogadnov *et al*., 2008, Honnegowda *et al*., 2015). Interestingly, MH does not contain hydrogen peroxide, which is seen as a main advantage when compared with other types of honey. The antimicrobial activity of MH is maintained even in the presence of catalases (Mavric *et al*., 2008). Honey is also well known for its antifungal properties, with activity reported against several species of yeasts and dermatophytes, like *Candida albicans* and *Cryptococcus neoformans* (Boorn *et al*., 2010; Feás and Estevinho, 2011). It has also been documented to have effects against several *Aspergillus* species and *Rhodotorula* spp. (Moussa *et al*., 2012; Pradeep and Dhananjay, 2012).

Staphylococcus pseudintermedius is the most common bacteria implicated in canine folliculitis and otitis externa (OE) constituting a common cause for antibiotic prescription (Lyskova *et al*., 2007). Previous reports of chronic or recurrent cases of pyoderma, in which previous antibiotherapy has been attempted, frequently report high levels of methicillin-

resistant *Staphylococcus pseudintermedius* (MRSP) which can become a treatment challenge (Loeffler *et al*., 2007).

Malassezia dermatitis and otitis are commonly associated with allergic diseases, keratinization disorders, bacterial skin infections and long-term glucocorticoid administration (Plant *et al*., 1992). Clinical signs include severe pruritus, oily seborrhea, erythema and a malodorous brownish ear discharge (Bond *et al*., 2010). In practice, *Malassezia* dermatitis and otitis is managed with topical or systemic antimycotics like imidazoles, terbinafine and nystatin (Mueller *et al*., 2012).

The aim of this study was to evaluate the bactericidal and antifungal activity of MH against *S. pseudintermedius* and *M. pachydermatis.*

3.2. Materials and methods

3.2.1. Collection and identification of the isolates

A total of 60 *S. pseudintermedius* isolates, 30 methicillin-susceptible (MSSP) and 30 methicillin-resistant (MRSP) were included in this study. These isolates had been previously isolated from dogs with superficial pyoderma. Polymerase chain reaction was performed for species identification and detection of the *mecA* gene (Bannoehr *et al*., 2009; National Food Institute, 2015). Antibiotic susceptibility was tested according to the Clinical Laboratory Standards Institute (CLSI) for Kirby-Bauer disk methodology (CLSI, 2013). Isolates were tested for resistance to oxacillin, clindamycin, erythromycin, trimethoprim-sulfamethoxazole, tetracycline, enrofloxacin, chloramphenicol, rifampicin and gentamicin. The isolates were classified as multidrug resistant (MDR) if resistant to ≥ 3 classes of antibiotics and non-multidrug resistant (non-MDR) if resistant to 2 or less classes of antibiotics (Schwarz *et al*., 2008). Twenty *M. pachydermatis* isolates, also previously collected from dogs with *Malassezia* otitis externa were also used in this study. Yeast identification was based on macroscopic and microscopic characteristics and ability to grow in Sabouraud glucose agar with chloramphenicol (SGC) (Kaneko *et al*., 2007). All media were obtained from Oxoid (Oxoid, Hampshire, UK). *Staphylococcus* isolates were stored at -80˚C in a mixture of glycerol 30% (Scharlab S.L., Barcelona, Spain) and nutrient broth and *Malassezia* isolates at -20˚C in a mixture of 10% glycerol and milk broth until further analysis.

3.2.2. Determination of minimum bactericidal and fungicidal concentrations

3.2.2.1. Product preparation

The product used in this experiment was medical grade MH (Activon tube, Advancis Medical, UK) and we confirmed its sterility by culturing 10 µL in 5% Sheep Blood agar and MacConkey agar followed by incubation for 24 h at 37˚C under aerobic conditions.

Manuka honey was tested undiluted and diluted at 80%, 40%, 30%, 20%, 15%, 10%, 7.5%, 5%, 3.7% and 2.5% w/v in nutrient broth for *S. pseudintermedius* and in Sabouraud broth for *M. pachydermatis* isolates.

Synthesized honey was used to mimic the high osmolality and pH of honey and prepared by mixing 1.5 g sucrose, 7.5 g maltose, 40.5 g D-fructose and 33.5 g D-glucose (all products supplied by Sigma-Aldrich, St Louis, USA) in 17 mL of sterile deionized water. The solution was dissolved by heating at 56°C in a water bath, and sterilized by autoclaving at 120°C for 20 minutes (Feás *et al*., 2013). Concentrations of synthesized honey matched those of MH.

An initial stock solution of the disinfectant, chlorhexidine digluconate (Sigma-Aldrich, St Louis, USA) was used as positive control against both microorganisms. The chlorhexidine solution was prepared in distilled water in a concentration of 1% w/v and 2-fold serial dilutions (1.28-0.0025% w/v) were prepared for testing (Valentine *et al*., 2012).

3.2.2.2. Inoculum preparation

3.2.2.2.1. Staphylococcus pseudintermedius

The isolates were cultured overnight at 37ºC and diluted in sterile saline to an optical density of 0.05 at 600 nm (CLSI, 2013). Bacterial count was performed according to the spread plate protocol (Wise, 2006).

3.2.2.2.2. Malassezia pachydermatis

The yeasts were inoculated on SGC agar and incubated for 3 days at 37ºC. Colonies were suspended in sterile saline, homogenized and centrifuged at 448 rpm for 10 minutes. The

pellet was diluted in sterile saline and adjusted at 570 nm to an optical density of 0.8 as recommended (Young *et al*., 2012).

3.2.2.3. Microbroth dilution assay protocol

3.2.2.3.1. Staphylococcus pseudintermedius

Microbroth dilution assay was performed according to the CLSI guidelines (CLSI, 2013). First, bacterial suspensions were diluted 1:10 in nutrient broth. Plates with 96-well microtiter and round bottom wells (Deltalab, Barcelona, Spain) containing 90 µL of progressive dilutions of the products were inoculated with 10 µL of the bacterial suspension and slowly agitated for 10 minutes at 100 rpm. To prevent drying, the plates were covered with a lid and incubated at 37°C for 20 hours in aerobic atmosphere. Minimum bactericidal concentration (MBC) was determined by subculturing 10 µL of each well in Muller Hinton agar and subsequently incubated at 37ºC for 24 hours. Plates with visible colony growth were considered to correspond to bacteriostatic activity, while those with no growth were considered as representing bactericidal activity. The MBC was considered as the lowest concentration where growth was not detected with the unaided eye. *S. aureus* ATCC® 29213™ (Scharlab S.L., Barcelona, Spain) was used as growth control.

Positive plate controls (growth-wells with isolates) and negative controls (wells with only MH at all concentrations and nutrient broth) were included in all plates. All experiments were performed in duplicate.

3.2.2.3.2. Malassezia pachydermatis

For *M. pachydermatis* the experiment was performed as previously described for *S. pseudintermedius*, with the exception of minimum fungicidal concentration (MFC) determination, in which 10 µL of each well was subcultured in SGC agar and incubated at 37ºC for 3 days.

3.2.3. Catalase treatment of MH

In order to determine hydrogen peroxide activity, MH was treated with catalase. Bovine liver catalase (Sigma-Aldrich; St Louis, MO, USA) diluted to a ratio of 1000 units/ml was

added to the MH dilutions and incubated for 1 h at 37˚C. MBC was determined according to previous methodology.

3.2.4. Time-kill assay for S. pseudintermedius and M. pachydermatis

A time-kill assay was performed based on "Standard Guide for Assessment of Antimicrobial Activity Using a Time-Kill Procedure" by the American Society for Testing and Materials (ASTM, 2016). The aim was to evaluate the efficacy of undiluted and diluted MH at 40% in sterile saline against 10 *S. pseudintermedius* (5 MSSP and 5 MRSP) and 10 *M. pachydermatis* isolates after exposure to different time points.

3.2.4.1. Product and inoculum preparation

Manuka honey was tested undiluted and diluted at 40% w/v in sterile saline shortly before testing. Manuka was tested undiluted based on the clinical application of the product and MBC and MFC results (40% w/v). The inoculum was prepared for both microorganisms as previously stated.

3.2.4.2. Time-kill assay

A time-kill assay was performed using 15 mL conical tubes (Deltalab, Barcelona, Spain) with each tube representing a time point: 1 hour (T1), 4 hours (T4), 8 hours (T8), 12 hours (T12) and 24 hours (T24). Contact times were determined based on previous published studies for honey (Boorn *et al*., 2010; Shenoy *et al*., 2012).

For each microorganism, at each time point and for both MH concentrations, 5 tubes were used. To each tube filled with 1 mL of testing product was added 10 µL of bacterial suspension, followed by mixing and vortexing for 1 min at 1200 rpm. The tubes were incubated at 37ºC and, at each designated time, neutralization was performed by adding 9 mL of sterile saline, followed by homogenization through vortexing and tube inversion. The neutralization process stops the honey's action by dilution with saline. The surviving microorganisms were counted at each time point, by spreading 10 µL of the solution in Muller Hinton agar followed by incubation at 37ºC for 24 hours. For *M. pachydermatis* the inoculum obtained after the neutralization process was cultured in SGC agar and incubated at 37ºC for 3

days.

3.2.4.3. Controls

For each microorganism, the initial number of controls was established as recommend by the ASTM guidelines: 10 µL of bacterial suspension was added to 1 mL of saline, vortexed and immediately cultured in growth agar (ASTM, 2016). Positive control (chlorhexidine at 0.1% w/v) was also included in the experiment (Shenoy *et al*., 2012). Other controls included *S. aureus* ATCC® 29213™ (growth control) and tubes with testing substances and saline (sterility control).

3.2.4.4. Percent reduction calculation

The reduction of the viable microorganisms at each time was calculated based on the following formula: percent reduction (PR) = (IP - T/IP) x 100 (IP, number of viable microorganisms in the initial population; T, number of viable microorganisms in MH at each time point).

3.2.5. Statistical analysis

Data were analysed using Statistical Package for Social Sciences (IBM SPSS Chicago, IL) for Windows. Means were compared with nonparametric tests (Mann-Whitney and Wilcoxon tests).

3.3. Results

3.3.1. Bactericidal effect of MH against S. pseudintermedius

Manuka honey had a bactericidal effect against *S. pseudintermedius*, with an MBC of 20% and ranging between 15-20% w/v. Most isolates revealed an MBC of 15% w/v (49/60 isolates) (Table 3.1). The MBC mean was 15.9% w/v (SD±1.9). After treatment with catalase, the MBC values were maintained.

Table 3.1. Minimum bactericidal concentration (MBC) with the percentage of dead *S. pseudintermedius* isolates for each manuka honey (MH) dilution.

Product concentration	**% death**
2.5% w/v	0
3.7% w/v	0
5% w/v	0
7.5% w/v	0
10% w/v	0
15% w/v	82%
20% w/v	100%
40% w/v	100%
80% w/v	100%
Undiluted	100%

MH, manuka honey; w/v, weight/volume.

3.3.2. Susceptibility of MRSP, MSSP and MDR isolates to MH

When the susceptibility of MSSP and MRSP isolates to MH were compared, the MBCs were 16.2% w/v and 15.7% w/v, respectively (Table 3.2). Comparison between susceptibility of MSSP and MRSP to MH showed no difference between groups (*P*=0.321).

Table 3.2. Minimum bactericidal concentration (MBC) of manuka honey (MH) for methicillin-susceptible (MSSP) and methicillin-resistant *S. pseudintermedius* (MRSP). The last line shows MBC mean and standard deviation (SD) for each group.

	MSSP (n=30)	**MRSP (n=30)**
MBC 15% (n=49)	87% (26/30)	77% (23/30)
MBC 20% (n=11)	13% (4/30)	23% (7/30)
MBC mean ±SD	16.2±2.2	15.7±1.7

MBC, minimum bactericidal concentration; SD, standard deviation; MSSP, methicillin-susceptible *S. pseudintermedius*; MRSP, methicillin-resistant *S. pseudintermedius*.

When the isolates were divided into MDR and non-MDR categories, the MBCs were 15.8% w/v and 16.1% w/v, respectively (Table 3.3). Comparison between susceptibility of MDR and non-MDR isolates to MH showed no difference between groups (*P*=0.507).

Table 3.3. Minimum bactericidal concentration (MBC) of manuka honey (MH) for multidrug (MDR) and non-multidrug resistant (non-MDR) *S. pseudintermedius*. The last line shows MBC mean and standard deviation (SD) for each group.

	MDR (n=38)	**non-MDR (n=22)**
MBC 15% (n=49)	84% (32/38)	77% (17/22)
MBC 20% (n=11)	16% (6/38)	23% (5/22)
MBC mean ±SD	15.8% ± 1.8	16.1% ± 2.1

MBC, minimum bactericidal concentration; SD, standard deviation; MDR, multidrug resistant; non-MDR, non-multidrug resistant.

3.3.3. Fungicidal effect of MH against M. pachydermatis

Manuka honey also had a fungicidal effect against *M. pachydermatis.* The minimum concentration was established at 40% w/v and ranged between 20-40% w/v, with most isolates revealing an MFC of 40% w/v (15/20 isolates) (Table 3.4).

Table 3.4. Minimum fungicidal concentration (MFC) with the number of dead *M. pachydermatis* isolates for each manuka honey (MH) dilution.

Product concentration	**% death**
2.5% w/v	0
3.7% w/v	0
5% w/v	0
7.5% w/v	0
10% w/v	0
15% w/v	0
20% w/v	20%
40% w/v	100%
80% w/v	100%
Undiluted	100%

MH, manuka honey; w/v, weight/volume.

3.3.4. Comparison between MH activity against S. pseudintermedius and M. pachydermatis

The MBC mean for *S. pseudintermedius* was 15.9% w/v (SD±1.9). The MFC mean for *M. pachydermatis* was 35.5% w/v (SD±8.2). Statistical analysis revealed a difference between means (P=0.001) suggesting that MH needs to be more concentrated in order to kill *M. pachydermatis* when compared to the concentration needed to kill *S. pseudintermedius.*

3.3.5. Time-kill assay

In general, results of the time-kill assay show that viability of *S. pseudintermedius* and *M. pachydermatis* decreases with time. Both microorganisms were unable to survive after 4 hours of exposure to undiluted MH. Diluted MH still allowed *M. pachydermatis* survival after 12 hours of contact time (Tables 3.5 and 3.6).

Table 3.5. Percentage of reductions of *S. pseudintermedius* isolates at different times versus control.

Product	**Time points**				
S. pseudintermedius	**T1**	**T4**	**T8**	**T12**	**T24**
Undiluted MH	98.24%	99.31%	100%	100%	100%
MH 40% w/v	97.94%	99.86%	100%	100%	100%

MH, manuka honey; w/v, weight/volume; T1, 1 hour; T4, 4 hours; T8, 8 hours; T12, 12 hours; T24, 24 hours of contact time.

Table 3.6. Percentage of reductions of *M. pachydermatis* isolates at different times versus control.

Product	**Time points**				
M. pachydermatis	**T1**	**T4**	**T8**	**T12**	**T24**
Undiluted MH	99.57%	99.95%	100%	100%	100%
MH 40% w/v	99.55%	99.75%	99.88%	99.94%	100%

MH, manuka honey; w/v, weight/volume; T1, 1 hour; T4, 4 hours; T8, 8 hours; T12, 12 hours; T24, 24 hours of contact time.

3.4. Discussion

This work documents the bactericidal effect of MH against *S. pseudintermedius* and fungicidal effect against *M. pachydermatis*. Our work also demonstrates that MRSP and MSSP, as well as MDR and non-MDR isolates, are equally susceptible to the effects of MH. Finally, we document the time necessary to obtain a killing effect against both microorganisms.

Manuka honey is a medical honey originated from New Zealand used for wound treatment (Davis, 2005). Several articles have proved its efficacy against a number of staphylococci isolates including *S. aureus*, coagulase negative staphylococci, *Staphylococcus epidermitis* and *Staphylococcus xylosus* (French *et al*., 2005; Boorn *et al*., 2010; Boateng and Diunase, 2015). Our study documents the efficacy of MH against MSSP and MRSP, revealing an MBC of 20% w/v. Manuka honey impairs cellular division of *S. aureus* (Henriques *et al.*, 2010) and has been reported to be effective against *S. aureus* with an MBC of 25% w/v and 24% w/v (Tan *et al*., 2009; Stobberingh and Vandersanden, 2010). Depending on the bacteria species, different MBC values are obtained, with *Stenotrophomonas maltophilia* displaying the lower MBC (11.25% w/v) and *Proteus mirabilis, Shigella flexneri* and *Enterobacter cloacae*, the highest MBC value (≥25% w/v).

The lack of hydrogen peroxide in MH is confirmed in this work. The bactericidal effect is attributed to phytochemicals, in special flavonoids. Methylglyoxal and other compounds, such as, 3-deoxyglucosulose and glyoxal, have been identified in MH and may also be responsible for its antimicrobial activity since they are involved in the non-peroxide antibacterial activity (Oelschlaegel *et al*., 2012). These compounds present marked antibacterial effects plus anti-inflammatory action in wound healing (Mavric *et al*., 2008; Kwakman and Zaat, 2012; Alvarez-Suarez *et al*., 2013). Other studies that evaluated the MH antimicrobial activity have determined that the other major flavonoids present in MH are pinocembrin and pinobanksin and chrysin, while 8-methoxykaempferol, luteolin, quercetin, kaempferol, isorhamnetin and galangin are present in lower concentrations (Alvarez-Suarez *et al*., 2014). The antibacterial effect of MH is also associated with leptosin (aka bee defensin), which is a peptide found in the insects' innate immune system, and to which the myeloperoxidase activity inhibition is attributed (Kato *et al*., 2012). Considering that MH is active against MRSP and MDR *Staphylococcus pseudintermedius*, its application could be extremely useful in the treatment of antibiotic resistant skin infections. Honey is being further recognized as a valuable treatment against antibiotic resistant infections. French *et al* (2005) reported that MH is active against antibiotic resistant coagulase-negative staphylococci. Other studies have demonstrated similar susceptibility to honey in methicillin-susceptible *Staphylococcus aureus* (MSSA) and methicillin-resistant *Staphylococcus aureus* (MRSA) (Cooper *et al*., 2002; Boorn *et al*., 2010). In fact, an *in vitro* study documented the ability of MH to restore MRSA susceptibility to oxacillin by down regulating the *mecR1* gene (Jenkins

and Cooper, 2012). Our work suggests that MH should be in close contact with the infection site without being removed, for example, by the licking behavior of the dog. For instance, it could be beneficial for the treatment of infected wounds or deep pyoderma if the animal is restricted from licking the area or if the area is covered with a bandage. We recommend future clinical trials to assess the efficacy of undiluted MH against cases of pyoderma and infected wounds with antibiotic resistant infections.

Manuka honey tested by Dryden *et al*. (2014) revealed a longer contact time (> 24 h) for a complete bactericidal activity. In our study, a complete bactericidal effect against MSSP and MRSP was noticed after 4h, which might be justifiable by the source of the MH. The specific plant nectar source has been implicated in the variability in the bactericidal effect of MH (Dryden *et al*., 2014). Likely, the species tested in this study (*S. pseudintermedius*) doesn't justify the result obtained since we also used a *S. aureus* ATCC reference. This isolate was completely eliminated between 4 and 8 hours of contact time. In other words, the bactericidal effect of our MH was similar for MSSP, MRSP and the *S. aureus* reference.

We demonstrated that MH decreases the number of *S. pseudintermedius* by 98.24% after 1 hour of exposure. This is in agreement with a previous work with MSSA and MRSA (Postmes *et al*., 1995). This effect was also observed with stingless bee honey diluted at 20% w/v, against a reference *S. aureus* isolate. After a contact time of 1 hour, it was observed a reduction of viability between 90-99.9%, which is within the range of what was observed in the same period of time in our study. In the same study, table honey revealed a decrease in viability below 90%. Stingless bee honeys demonstrated a quicker bactericidal effect when in comparison with *Apis mellifera* honeys (Boorn *et al*., 2010). To the author's knowledge there are no time-kill studies reported using *M. pachydermatis* isolates. In our study, a complete antifungal effect was observed after 4 hours of exposure for undiluted MH and after 12h for MH diluted at 40%. A few studies with fungal microorganisms have been reported. For a *C. albicans* reference isolate, a reduction of viability of less than 90% was observed for 3 different stingless bee honeys (*Tetragonisca angustula*, *Melipona quadrifasciata* and *Trigona carbonaria*) after an hour of exposure. *T. carbonaria* honey revealed a limited antifungal activity (Boorn *et al*., 2010). Our results demonstrate a quicker decrease of number of colony forming units, however this may be due to the type of honey, dilution and the microorganism itself. A time-kill assay with MH against filamentous fungi isolates was performed with dilutions of 40%, 60% and 80% and evaluated at 0, 5 min, 15 min, 30 min, 1 h, 1.5 h, 3 h, 6 h,

12 h, and 24 h. The majority of isolates tested displayed a reduction in number of colonies after the 3-hour time point, which is in accordance with our results (Yabes *et al*., 2017). When comparing the 2 microorganisms, it is possible to conclude that *M. pachydermatis* needs a longer period of exposure for complete antifungal activity if used diluted at 40% w/v when compared to *S. pseudintermedius*.

3.5. Conclusion

This work provides *in vitro* data that can be used for future clinical trials involving cutaneous infections with *S. pseudintermedius.* The clinical trial can encompass conditions like pyoderma, traumatic and post-surgical (infected) wounds, burns and impetigo. *In vitro* studies cannot predict the final outcome of a clinical situation since other factors, such as the presence of exudate in the lesions, may interfere with the efficacy of the treatment. Our work also suggests that a prolonged time between MH and the lesion might be more effective. Finally, our data propose that manuka honey should also be considered for antibiotic resistant cases. A more efficient use of non-antibiotic topical therapy can potentially decrease the use of antibiotics and, therefore, reduce the probability of further development of resistance in both *S. pseudintermedius* and commensal microbiotic.

CHAPTER IV

IN VITRO EFFICACY OF A HONEY-BASED GEL AGAINST CANINE CLINICAL ISOLATES OF *Staphylococcus pseudintermedius* AND *Malassezia pachydermatis*

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4. *IN VITRO* EFFICACY OF A HONEY-BASED GEL AGAINST CANINE CLINICAL ISOLATES OF *Staphylococcus pseudintermedius* AND *Malassezia pachydermatis*

4.1. Introduction

Staphylococcus pseudintermedius (formerly *S. intermedius*) is the most common pathogen causing canine pyoderma (Bannoehr *et al.*, 2007; Morris *et al.*, 2006). Methicillin-resistant *S. pseudintermedius* (MRSP) was first reported in North America followed by emergence in Europe and Asia (Gortel *et al.*, 1999; Loeffler *et al.*, 2007; Onuma *et al.*, 2012). Treatment of pyoderma caused by MRSP can be challenging due to the limitations in antibiotic choices, because many of these isolates are also multidrug-resistant (Hillier *et al.*, 2014). Guidelines have been published for the diagnosis and treatment of folliculitis which support the use of antibiotics and/or topical antibacterial therapy depending on several factors, which include extent of the lesions, and bacterial culture and susceptibility testing results (Hillier *et al.*, 2014). Topical therapy can be used as a sole treatment or in combination with systemic antibiotics allowing a reduction in duration of antibiotherapy (de Jaham, 2003; Loeffler *et al.*, 2011). *Malassezia pachydermatis* is a nonlipid-dependent yeast that inhabits the skin and ears of the dog and an important etiological agent of canine otitis externa (OE) (Bond *et al.*, 1995; Kiss *et al.*, 1997a; Crespo *et al.*, 2002). Otitis caused by *Malassezia* is normally managed with topical therapy (Kiss *et al.*, 1997b; Bensignor *et al.*, 2006; Hensel *et al.*, 2009).

Honey has been used from ancient times to treat several types of infected wounds including traumatic, venous and diabetic ulcers (Al-Waili *et al.*, 2011; Jull *et al.*, 2013). Recently, medical research has identified bactericidal, bacteriostatic, antiviral, antioxidant and anti-inflammatory activities of honey (Cooper *et al.*, 1999; Lusby *et al.*, 2005; Bardy *et al.*, 2008; Kassim *et al.*, 2010; Erejuwa *et al.*, 2012). The antibacterial effect of honey is in part due to its hydrogen peroxide (H_2O_2) activity, which can be inhibited through conversion into water and oxygen by the presence of catalases (Molan, 1992; Chelikani *et al.*, 2004). Catalases are antioxidant enzymes normally present in tissues and chronic wounds and confer protection against oxidative damage (Honnegowda *et al.*, 2015). L-Mesitran® Soft, (HBO, Triticum; Maastricht, the Netherlands) is a honey-based gel composed of 40% medical-grade honey (HO) which is marketed for the treatment of superficial and acute wounds, superficial

and partial thickness burns, chronic wounds, acute and postoperative surgical wounds (Triticum, 2016a). Preliminary veterinary clinical data of cases treated with HBO suggests efficacy in the treatment of canine intertrigo and otitis; *in vitro* documentation of antimicrobial activity is limited (Maruhashi *et al*., 2016; Jakobsson, 2011).

The objectives of the present study were to evaluate the *in vitro* bactericidal efficacy of HBO against *S. pseudintermedius* isolates from canine pyoderma and to compare susceptibility of both methicillin-susceptible and methicillin-resistant isolates. Additionally, we assessed the *in vitro* antifungal properties of HBO against *M. pachydermatis* using canine clinical isolates from cases of *Malassezia* otitis.

4.2. Materials and methods

4.2.1. Microbial isolation and identification

Sixty *S. pseudintermedius* isolates were collected from dogs with pyoderma. PCR (polymerase chain reaction) was used for speciation and detection of the *mecA* gene using a published method (Bannoehr *et al.*, 2009). Oxacillin susceptibility was determined by the Kirby–Bauer technique following the Clinical Laboratory Standards Institute (CLSI) guidelines (CLSI, 2013). The isolates were divided into MRSP (30 of 60) and methicillin-sensitive *S. pseudintermedius* (MSSP) (30 of 60). Isolates were stored at -80°C in a mixture of glycerol 30% (Scharlab S.L.; Barcelona, Spain) and nutrient broth until further analysis. Ten *M. pachydermatis* isolates were collected from canine ears with a diagnosis of *Malassezia* otitis. The isolates were identified based on colony macroscopic characteristics and microscopic cell characteristics after Gram staining. The isolates were stored at -20°C containing 10% glycerol in milk broth until further analysis. All media used were supplied by Oxoid (Oxoid; Hampshire, UK) unless stated otherwise.

4.2.2. Product preparation

The tested products were HBO and the honey that composes the product (HO) and provided by the manufacturer. HBO is composed of 40% HO after gamma-sterilization. Other components of HBO include medical-grade hypoallergenic lanolin, propylene glycol, polyethylene glycol 4000, and vitamins C and E. HBO was tested in undiluted form, followed by serial dilutions in nutrient broth, which resulted in final concentrations of 20%, 10%, 5%

and 2.5% w/v of HO content respectively (Patton *et al.*, 2006; Sherlock *et al.*, 2010; Feás *et al.*, 2013). HO was tested undiluted and diluted to 40% w/v in nutrient broth in order to match the concentration present in HBO. Further serial dilutions at 20%, 10%, 5% and 2.5% w/v were prepared. All solutions were prepared shortly before testing to ensure H_2O_2 activity. HO was handled aseptically in dark containers in order to prevent degradation of peroxide activity due to light exposure. Products were initially tested for sterility by culturing 10 µL of undiluted product on 5% Sheep Blood agar and MacConkey agar during 24 h at 37°C under aerobic conditions.

Synthesized honey was used as a control to mimic the high osmolality and acidity of the honey (Özbalci *et al.*, 2013). Laboratory honey was prepared by mixing 1.5 g sucrose, 7.5 g maltose, 40.5 g D-fructose and 33.5 g D-glucose (Sigma-Aldrich; St Louis, MO, USA) in 17 mL sterile deionized water. The solution was dissolved by briefly heating at 56°C in a water bath and autoclaved at 120°C for 20 min (Feás *et al.*, 2013). The concentrations of laboratory honey used in the experiment were undiluted, 40%, 20%, 10%, 5% and 2.5% w/v.

Triclosan was used as a positive control against *S. pseudintermedius*. An initial stock solution of triclosan (Irgasan, Sigma-Aldrich) was prepared in 40% dimethyl sulfoxide/water (DMSO 90%, Neogen; Lexington, KY, USA) with a concentration of 1 g/L. The solution was further diluted in nutrient broth (0.32–0.0003% w/v). Clotrimazole (Canesten 10 g/L solution, Bayer Portugal SA; Barcelona, Spain) was used as a positive control against *M. pachydermatis* after dilution in Sabouraud's broth (0.5–0.0078% w/v).

4.2.3. Well-diffusion assay for S. pseudintermedius

Well-diffusion assay was carried out as described previously with minor modifications (al Somal *et al.*, 1994; Patton *et al.*, 2006; Sherlock *et al.*, 2010). The isolates were diluted in saline to an optical density of 0.15 at 600 nm (previously determined to be approximately 1 x 10^7 colony forming units *per* mL). Muller-Hinton agar plates were inoculated with sterile cotton swabs, after being immersed in the bacterial suspensions and left to stand for 10 min. After inoculation, four wells were cut into the agar with an 8 mm biopsy punch and filled with 80 µL of each of the honey products. Plates were incubated overnight at 37°C. The diameter of the inhibition halos, including the diameter of the well, was measured using a ruler.

4.2.4. Microbroth dilution assay

4.2.4.1. Staphylococcus pseudintermedius

Microbroth dilution assay was performed following CLSI guidelines (CLSI, 2013). Briefly, 96-well microtitre plates with round bottom wells (Deltalab S.L., Spain) containing 90 µL of progressive dilutions of the products, were inoculated with 10 µL of bacterial suspension (final inoculum 1-5 x 10^4 colony forming units per well) and incubated at 37°C for 20 h in aerobic atmosphere. Positive and negative controls were included on all plates/lines including: one well with broth and the micro-organism being tested; one well with honey product and broth; and one well with only nutrient broth. For minimum bactericidal concentration (MBC) determination, 10 µL of each well was subcultured in Muller-Hinton agar and incubated at 37°C for 24 h. Due to the colour and density of HBO, it was not possible to read the minimum inhibitory concentration results in microtitre plates; therefore, subculture agar plates were deemed necessary to allow the determination of MBC. Plates with no growth were recorded as representing bactericidal activity. The MBC was recorded as the lowest concentration where growth was not detected with the unaided eye. All experiments were performed in duplicate. *S. aureus* ATCC® 29213™ (Scharlab S.L., Barcelona, Spain) was used as growth control.

4.2.4.2. Malassezia pachydermatis

In order to determine the minimum fungicidal concentration (MFC), the isolates were tested using a similar protocol to that used for *S. pseudintermedius*. A 96-well microtitre plate, containing 90 µL progressive dilutions of the products, was inoculated with 10 µL of the cell suspension (final inoculum 1-5 x 10^5 colony forming units per well). The plates were incubated at 37°C for 24 h. MFC was determined by subculturing 10 µL of each well in Sabouraud's chloramphenicol agar followed by incubation at 37°C for three days. Positive and negative controls were included on all plates. The experiment was performed in duplicate.

4.2.5. Catalase-treatment of products

Activity of H_2O_2 against the *S. pseudintermedius isolates* was determined by treating both products with catalase. Diluted catalase (Bovine liver catalase, Sigma-Aldrich) was

added (1,000 units/mL) to the dilutions of both products described previously and incubated for 1 h at 37°C. Microbroth assay for MBC determination was then repeated as described previously.

4.2.6. Time-kill assay

Time-kill assay was based on the Standard Guide for Assessment of Antimicrobial Activity Using a Time-Kill Procedure (American Society for Testing and Materials International, 2016). The efficacy of undiluted HBO was assessed against 10 *S. pseudintermedius* (five MSSP and five MRSP) and 10 *M. pachydermatis* isolates. The time-kill protocol was performed at 1 h (T1), 4 h (T4), 8 h (T8), 12 h (T12) and 24 h (T24), with contact times determined based on previously published studies for honey (Boorn *et al.*, 2010; Shenoy *et al.*, 2012). In short, 10 µL of *S. pseudintermedius* or *M. pachydermatis* suspension was added to 1 mL of product, followed by mixing and vortexing for 1 min at 161 g and incubation at 37°C. At each designated time, 9 mL of sterile saline was added to each testing tube suspension, in order to neutralize the action of HBO. Micro-organism counts at each time point were determined by spreading 10 µL of the solution onto Muller-Hinton agar, followed by incubation at 37°C for 24 h for *S. pseudintermedius*. For *M. pachydermatis* the inoculum was cultured in Sabouraud's chloramphenicol and incubated at 37°C for three days. Positive growth and negative controls were included. The Initial Population (IP) was determined by adding the same volume of inoculum suspension to a dilution blank containing the same volume as used for HBO testing followed by neutralization, culture and incubation as described before. The calculation of percentage of reduction was performed using the following formula: percentage reduction (PR) = (IP–T/IP) x 100 (IP number of viable micro-organisms in the initial population, *T* number of viable micro-organisms in HBO at each time point).

4.2.7. Statistical analysis

Data were analyzed using Statistical Package for Social Sciences, v25 (IBM SPSS; Chicago, IL, USA) for Windows.

4.3. Results

4.3.1. Results for well-diffusion assay

Partial growth inhibition of *S. pseudintermedius* was observed with HBO at concentrations of 40% and 20%, and HO in pure form and at 40% (Table 4.1). No inhibition was seen for HBO or HO at lower concentrations or with the synthesized honey.

Table 4.1. Comparison of zones of inhibition of growth of canine Staphylococci isolates in honey-based gel (HBO) and medical-grade honey (HO).

Composition of honey products	**Mean zone of inhibition (mm)**	**SD**	***P*-value**
HBO 40%	34.30	2.39	$P = 0.001$
HBO 20%	24.75	3.87	
HO undiluted	37.48	4.01	$P = 0.001$
HO 40%	30.85	2.20	
HBO 40%	34.30	2.39	$P = 0.5$
HO 40%	30.85	2.20	

SD, standard deviation.

4.3.2. Minimum bactericidal concentration results for S. pseudintermedius

For both HBO and HO, the MBC ranged between 5 and 20% w/v (Table 4.2). Sixteen isolates had a significantly lower MBC for HBO compared to HO ($P = 0.003$). No statistical difference was observed in MBC values between MSSP and MRSP isolates for any of the products tested (HBO, $P = 0.757$; HO, $P = 0.743$). Following incubation with catalase, there was no change in MBC values for HBO ($P = 0.072$). However, with HO, the MBC increased in 58 of 60 (97%) of the isolates ($P = 0.015$).

Table 4.2. Minimum bactericidal concentration (MBC) of HBO and HO with the percentage of dead isolates of *S. pseudintermedius* for each dilution.

Product concentration	**HBO**	**HO**
2.5% w/v	0	0
5% w/v	12	13
10% w/v	83	62
20% w/v	5	25
40% w/v	100	100
Undiluted	N/A	100

HBO, honey-based gel; HO, medical-grade honey; N/A, not applicable.

4.3.3. Minimum fungicidal concentration results for M. pachydermatis

The MFC for HBO ranged between 5 and 10% (Table 4.3). For HO, the MFC varied between 20 and 40%, apart from two isolates that had MFCs greater than 40%.

Table 4.3. Minimum fungicidal concentration (MFC) of HBO and HO with the percentage of dead isolates of *M. pachydermatis* for each dilution.

Product concentration	**HBO**	**HO**
2.5% w/v	0	0
5% w/v	20	0
10% w/v	80	0
20% w/v	100	40
40% w/v	100	40
Undiluted	N/A	20

HBO, honey-based gel; HO, medical-grade honey; N/A, not applicable.

4.3.4. Time-kill assay

Exposure of *S. pseudintermedius* and *M. pachydermatis* to pure HBO decreased viability and none of the microorganisms were able to survive after 4 h of exposure to the product (Table 4.4).

Table 4.4. Percentage of reductions of *S. pseudintermedius* and *M. pachydermatis* at different times.

Product	**Time points**				
Undiluted honey-based gel	**T1 (1 h)**	**T4 (4 h)**	**T8 (8 h)**	**T12(12 h)**	**T24(24 h)**
S. pseudintermedius	98.24%	99.31%	100%	100%	100%
M. pachydermatis	99.57%	99.95%	100%	100%	100%

4.4. Discussion

This study documents the antibacterial effect of a honey-based product against *S. pseudintermedius*, the most common agent causing canine pyoderma (Bannoehr *et al.*, 2007; Frank *et al.*, 2012). This product also was effective against *M. pachydermatis* which, along with *S. pseudintermedius*, frequently causes canine OE (Maruhashi *et al.*, 2016). The present work also shows that MSSP and MRSP isolates are equally susceptible to HBO. Bactericidal effect was observed at 20% w/v and no difference was seen between MSSP and MRSP isolates. The results obtained for HBO are in agreement with a previous study performed with a small number of methicillin-susceptible and -resistant *Staphylococcus aureus* isolates of human origin (Stobberingh *et al.*, 2010).

Our results suggest that HBO has a higher antibacterial activity when compared with HO. This is likely due to the presence of other components in the gel, such as medical-grade hypoallergenic lanolin, propylene glycol, polyethylene glycol 4000, and vitamins C and E. This work does not evaluate the antibacterial effect of each component, but it is likely that other components contribute to the enhanced antibacterial effect of the gel. Propylene glycol is widely used as an excipient, whereas it also has antibacterial activity against *S. aureus*, *Streptococcus mutans*, *Enterococcus faecalis* and *Escherichia coli* (Ballesteros *et al.*, 1993; Nalawade *et al.*, 2015). Vitamin C is an antioxidant and can improve healing in partial-thickness burns when mixed with honey, vitamin E and polyethylene glycol 4000 (Subrahmanyam, 1996).

The activity of H_2O_2 is one of the most important components in honey and can be inhibited by the presence of catalases (Molan, 1992). Catalase enzymes convert H_2O_2 into water and oxygen (Chelikani *et al.*, 2004). Catalases are antioxidant enzymes that are part of the natural defense against oxidative damage and are often found in tissue and chronic wounds, potentially rendering H_2O_2 inactive (Honnegowda *et al.*, 2015). Activity of H_2O_2 can be determined by testing the honey with catalase and observing a decrease in the bactericidal effect. This work shows that HBO's antibacterial effect is not dependent on H_2O_2. In contrast, HO alone was affected by catalase activity, resulting in an increase of the MBC. Our data suggest that during HBO preparation H_2O_2 is lost. It is known that H_2O_2 activity in honey decreases when honey is diluted, or with time due to degradation (Molan, 1992; Irish *et al.*, 2011). A bactericidal effect independent of H_2O_2 is an advantage, because antibacterial

activity will be less affected by the catalases present in wounds and fluids (Mavric *et al.*, 2008).

The discrepancy between MBC and inhibition halos might be due to the denser and stickier texture of HBO compared to HO, which probably affected the diffusion of the product in the agar plate. A similar study demonstrated that microbroth assay results had greater sensitivity when compared with well- and disk-diffusion assays for manuka honey (Patton *et al.*, 2006).

Time-kill tests are commonly used for the determination of bactericidal and antifungal effects. They can easily demonstrate the antimicrobial or antifungal effect of a product and evaluate it over time (Pfaller *et al.*, 2004). Our results demonstrate the effectiveness of HBO in killing *S. pseudintermedius* and *M. pachydermatis* isolates as quickly as after 1 h of exposure. A complete bactericidal and antifungal effect for both micro-organisms was observed after 4 h of exposure time. The biocidal activity of HBO was tested using a biocidal activity assay in five *S. pseudintermedius* isolates collected from canine ears (Maruhashi *et al.*, 2016). That study concluded that HBO had a biocidal activity but the time necessary for killing effect was not determined. Another study with a similar product demonstrated its effectiveness against *Candida albicans* (L-Mesitran® gel containing 48% of medical honey from Triticum). In the same study, yeast growth was reported within the first hour, followed by absent growth at 24 and 48 h. This study also reports its effectiveness in women with candidiasis vaginitis. A decrease or absence of the micro-organism and inflammatory cells was noticed microscopically after seven days of treatment (Boon, 2002). To the best of the authors' knowledge, there are no time-kill studies using HBO against *S. pseudintermedius* and *M. pachydermatis*.

Several studies have demonstrated the bactericidal effect of honey in the treatment of chronic ulcers, wounds, partial-thickness burns and post-surgical infection sites in human medicine (Bardy *et al.*, 2008; Al-Waili *et al.*, 2011; Vandamme *et al.*, 2013). Studies in animals are much more limited; a pilot study reported the efficacy of the application of HBO for the treatment of 13 surface pyoderma lesions in dogs in which 85% healing occurred after 14 days. The only adverse effect was pruritus after application in two dogs (Jakobsson, 2011). Considering our results, the application of HBO in superficial and deep pyoderma lesions seems to be a viable treatment option, particularly in focal lesions and in cases where no other antibacterial option is available.

HBO revealed fungicidal activity in all *M. pachydermatis* isolates. The MFC observed was 10% which suggests that the product can be diluted and still maintain antifungal activity. The gel had been previously reported to be effective in the management of canine otitis due to *Malassezia*. In a clinical trial, 15 dogs with bacterial and/or yeast otitis were treated daily with application of the product for 21 days. At the end of the study, 90% of the dogs were deemed to be clinically cured and there was a decrease in the number of micro-organisms on cytological samples (Maruhashi *et al.*, 2016). The proprietary honey content in the gel may not be the only ingredient active against *M. pachydermatis*. Propylene glycol is reported to be beneficial in the control of seborrhoeic dermatitis of the scalp due to *M. furfur*, formerly *Pityrosporum orbiculare* or *P. ovale* (Faergemann, 1988). Vitamin E is not an antimycotic agent, although one study reported low levels of vitamin E in individuals affected with seborrhoeic dermatitis due to *Pityrosporum* yeasts, when compared to normal controls (Ippolito *et al.*, 1989). To the best of the authors' knowledge, lanolin and polyethylene glycol have not been reported as antimycotics against *Malassezia* yeasts. Antifungal effects of lanolin, polyethylene glycol and vitamins, or the combination of all ingredients, cannot be ruled out in the present work.

4.5. Conclusion

The results of our study demonstrate that HBO is effective *in vitro* at killing *S. pseudintermedius* and *M. pachydermatis*; further studies are needed to describe the detailed mechanisms of action of the product against both pathogens with a larger number of isolates, as well as to further document the *in vivo* effectiveness of the product in the treatment of clinical cases of canine pyoderma and otitis externa.

GENERAL DISCUSSION

S. pseudintermedius and *M. pachydermatis* are important causes of skin and ear diseases in veterinary dermatology. *S. pseudintermedius* is the leading cause of canine superficial folliculitis but is also frequently encountered in otitis, fold dermatitis, deep pyoderma and post-surgical sites infections (Devriese *et al.*, 2005; Lyskova *et al.*, 2007; Beco *et al.*, 2013; Bloom, 2014; Hillier *et al.*, 2014; Diribe *et al.*, 2015; Couto *et al.*, 2016). *Malassezia pachydermatis* causes otitis and generalized and localized dermatitis in the dog (Plant *et al.*, 1992; Morris, 1999). Patients with atopic dermatitis frequently develop these infections and many times concurrently. For example, an atopic dog can present with otitis externa caused by *M. pachydermatis* and bacterial folliculitis caused by *S. pseudintermedius.* Despite the management of atopic dermatitis, infections need to be treated with appropriate antibacterial and/or antimycotic therapy (Olivry *et al.*, 2010). Generalized *Malassezia* dermatitis followed by fold dermatitis and superficial folliculitis are very commonly diagnosed in general practice. Our survey demonstrated that otitis was very commonly diagnosed with all clinicians diagnosing at least a case of otitis *per* month regardless the location of the clinic or the years in practice.

In the first study, we documented that MRSP is diagnosed by clinicians in first opinion practice in Portugal. In the second study, we demonstrated that MDR and MRSP represent a challenge regarding antimicrobial therapy. MRSP isolates were resistant to first choice antibiotics, which include beta-lactams, lincosamides and potentiated sulfonamides, antibiotics classified as first-tier by the ISCAID guidelines due to their efficacy and safety (Hillier *et al.*, 2014). If *S. pseudintermedius* is resistant to these antibiotics, the clinician is advised to use second-tier options, which include fluoroquinolones, rifampicin, tetracyclines, chloramphenicol or aminoglycosides which can potentially have serious side-effects (Hillier *et al.*, 2014). This study demonstrated that all MRSP isolates, except for one, needed a second-tier antibiotic for treatment of bacterial superficial folliculitis. Within second-tier antibiotics, eleven isolates exhibited susceptibility only to rifampicin and/or amikacin and none to chloramphenicol. This is a major concern, due to the risk of hepatotoxicity and nephrotoxicity, which can be life-threatening in some patients (Frank and Loeffler, 2012; Bajwa *et al*., 2013). The use of other drugs, like linezolid, teicoplanin, vancomycin, regardless of susceptibility, is discouraged, as these drugs should be 'reserved for the treatment of serious MRSA infections in humans' (Hillier *et al.*, 2014).

Observing the results of our survey, some first-tier antimicrobials, such as clindamycin and trimethoprim-sulfamethoxazole are clearly being disregarded in favor of fluoroquinolones. The excessive use of third generation cephalosporins and fluoroquinolones in small animal practice is documented (Watson and Madison, 2001; Murphy *et al*., 2012; Buckland *et al,* 2016 Hardefeldt *et al*., 2017). Fluoroquinolones should be used cautiously as they are considered a risk factor for MRSA in both humans and dogs (Taconelli *et al*., 2008). Fluoroquinolones and third generation cephalosporins can also select for extended-spectrum beta lactamase *E. coli* in humans and animals (Snow *et al*., 2012; Tinelli *et al*., 2012). Fluoroquinolones should be reserved for cases with documented resistance to first-tier antibiotics. Recently, exposure to multiple antibiotics (mainly beta-lactams) and concurrent immunomodulatory therapy, have been associated with pyoderma caused by MRSP (Hensel *et al.*, 2016). In another recent study, Couto *et al*. (2016) suggested that cephalosporins may select for MRSP isolates.

Another problem associated with MRSP is the zoonotic risk (Somayaii *et al*., 2016). The presence of MRSP and MDR is a pressing matter as its prevalence is rapidly increasing in the last few years in Europe (Jones *et al*., 2007; Ludwig *et al.*, 2016; Zur *et al*., 2016; Wegener *et al,* 2018). Clinicians are exposed daily to this microorganism and a survey found that MSSP and MRSP can be isolated amongst veterinary professionals (Beça *et al*., 2015; Espadale *et al,* 2018; Worthing *et al,* 2018a). In fact, it is recognized nowadays as an emerging zoonotic agent (Somayaji *et al*., 2016; Lozano *et al,* 2017). Reports of human infections with MRSP are being more frequently described in the literature and are most likely underestimated (van Hoovels *et al.*, 2006; Stegmann *et al.*, 2010; Kuan *et al.*, 2016). Clinical cases of infection and carriage of MRSP are described in owners of dogs with pyoderma and veterinary staff working in clinical practice, although the exact factors that lead to infection are unknown (Morris *et al.*, 2010; Kuan *et al.*, 2016; Espadale *et al,* 2018; Worthing *et al,* 2018a). An additional problem, is the fact that animals can act as reservoirs of MDR and methicillin-resistant staphylococci which can potentially disseminate human clones to other animals and humans (Couto *et al.*, 2016). For this reason, effective treatment of canine folliculitis and otitis is imperative for public health protection. Certainly, the implementation of strict hygiene strategies and education of owners and others in contact with dogs infected by MRSP is important to limit the transmission of nosocomial infections (British Small Animal Veterinary Association, 2014).

The general lack of awareness or non-application of the guidelines recommended by the ISCAID was raised in our survey. In fact, the omission of performing culture and susceptibility in cases of superficial bacterial folliculitis is illustrated as most of the clinicians consider to perform this diagnostic tool after unsuccessful empirical antibiotherapy. Certainly, it has been demonstrated that treatment failure after empirical therapy is possible (Jones *et al*., 2007). In our point of view, cytology could also be more frequently applied by the clinician for all the conditions covered in this study. The second study shows the importance of bacterial culture and susceptibility in order to choose the right antibiotic for the treatment of MRSP. In sum, antibiotic resistant folliculitis caused by *S. pseudintermedius* may represent a clinical challenge. The lack of antibiotic choices or, unacceptable side-effects along with the risk for public health, deemed other effective, safe and cost-effective treatments.

Lately, topical therapy for the treatment of infections caused by *S. pseudintermedius* has been rewarded with new insights (Uri *et al.*, 2016). Depending on the clinical case, topical treatment can be used as sole therapy (Loeffler *et al*., 2011) or to reduce the time of antibiotherapy (de Jaham, 2003). Topical treatment can be seen as a last resource in cases of *S. pseudintermedius* resistant to all available antibiotics or when antibiotherapy is not tolerated by the patient. However, new trends in antibiotic use recommend topical treatment with non-antibiotic based products as a first line treatment to treat superficial bacterial infections in companion animals (Uri *et al.*, 2016).

Honey has been used to treat infected wounds in people for centuries. However, not until recently and due to the emergence of antibiotic resistant pathogens, has the antibacterial activity of honey has been thoroughly investigated (Irish *et al.*, 2011; Seckam and Copper, 2013; Horn, 2013). In future studies, it will be interesting to evaluate if sub-inhibitory concentrations of MH can down-regulate the *mecA* gene. MH has been reported to down-regulate the *mec*R1 gene which resulted in restored oxacillin susceptibility to MRSA (Jenkins and Cooper, 2012). If *S. pseudintermedius* resistance to beta-lactams is reversed would be a major asset in the treatment of canine superficial bacterial folliculitis. Another major advantage would be interference with biofilm. *S. pseudintermedius* can be a biofilm producer (Futagawa-Saito *et al.*, 2006; Singh *et al.*, 2013; Stefanetti *et al*, 2017; Arima *et al*, 2018), but it is unknown if the disturbance of biofilm, in cases of infection, would count positively in the treatment outcome of the patient, either with folliculitis, otitis or infected wounds (Singh *et al.*, 2013). MH has been shown to inhibit MRSA biofilms at concentrations that can be

clinically achieved (Merckoll *et al.*, 2009; Cooper *et al*., 2011; Lu *et al*, 2014). This is a critical factor as biofilms are associated with antibiotic resistance and recurrent infections (Venkatesan *et al*., 2015; Hall and Mah, 2017). Therefore, determining the interference of MH in *S. pseudintermedius* biofilms could be an interesting option.

In the present work, we also evaluated the antibacterial effect of a gel composed of 40% medical honey (HBO) and the honey that is within it (HO). Our results shows that HBO has a stronger antibacterial effect when compared to HO. Biocidal activity of HBO has been previously reported for three MSSP and two MRSP isolates originating from canine otitis (Maruhashi *et al*, 2016). The present work, confirms that HBO is active against *S. pseudintermedius* both MRSP and MSSP with a higher number of isolates. For the first time, it is shown that the honey that is within HBO is active against MRSP and MSSP. Finally, we could observe that HBO has a lower MBC compared to HO suggesting that one or more components of the formulation also have antibacterial effects against *S. pseudintermedius.* After the publication of our work, a study reported biocidal efficacy of HBO against MRSA ST22 and MRSP ST71 (Maruhashi *et al*, 2018). Based on the author's clinical experience, HBO is easier to apply to the skin due to its thicker consistency. Potentially, it would be worth investigating owner compliance towards this product. Owners consider ease of application and cosmetic appearance important characteristics in a topical product (Bensignor and Fabries, 2018).

HBO and MH showed activity against MSSP, MRSP and *M. pachydermatis*. Since most skin lesions produce organic fluids, is important to know how many times a product can be diluted before it becomes inactive. *S. pseudintermedius* exhibited a MBC of 20% when tested against both products. Which suggests that the products can be diluted without losing the bactericidal activity. For *M. pachydermatis*, the MFC varied according to the product (MFC of 40% for MH and MFC of 10% for HBO). Of course, MBC and MFC values are not direct indicators of the clinical efficacy. Laboratory conditions, for example, cannot ascertain the influence of organic challenge of body fluids, variable bacterial load, pH and other factors like the patient's immune system (Al-Waili, 2004; Menke *et al.*, 2007; Mphande *et al.*, 2007; Guo and DiPietro, 2010; Boateng and Diunase, 2015). Nevertheless, there is a major advantage if a product is active against both microorganisms, avoiding prescrition of an antibiotic and an antifungal. Moreover, clinicians might find useful if the product can be

applied both to the skin and ear canal. Potentially this might enhance owner compliance by simplifying procedure and lowering the cost of the treatment.

Time-kill tests are frequently used for determining bactericidal and antifungal effects and are a tool for obtaining information about the dynamic interaction between the microorganism and the antimicrobial agent (Pfaller *et al.*, 2004). A complete bactericidal and antifungal effect was observed with HBO after 4h of exposure. Regarding MH, its complete antibacterial effect was noticed in *S. pseudintermedius* isolates after 4h of exposure, undiluted or diluted at 40% w/v. A partial killing effect of MH was observed after 1h of exposure. A previous pilot study tested the effect of MH against one isolate of MSSP, MRSP and *M. pachydermatis* and reported a decrease in colonies forming units after 3 and 10 minutes of contact time (Uri *et al,* 2016). Another study, tested a membrane composed of MH and pectin and reported a strong bactericidal effect after 1h against one clinical isolate of MRSP (Tramuta *et al*., 2016). In fact, honey has a slow killing effect and times are variable according to the type and concentration of honey (Shenoy *et al.*, 2012; Carnwath *et al,* 2013; Dryden *et al*., 2014). For example, the bactericidal effect for *S. aureus* was observed within 2 hours of contact time and 30 minutes of contact time for a medical grade honey mixture resulting from various types of honey and a novel engineered honey, respectively (Dryden *et al*., 2014). A polyfloral honey from India was tested against five *P. aeruginosa* isolates in concentrations of 20%, 25% and 50% displaying a bactericidal effect after 24 hours of exposure. For concentrations of 75% and 100% bactericidal effect was observed after 12 hours. Survival rates after 4, 8, 12 and 24 hours were 22.3%, 5.2%, 1.1%, and 0% respectively (Shenoy *et al.*, 2012). Killing time might also differ between different species of bacteria. Microorganisms such as *Enterococcus* spp., *E. coli* and *Pseudomonas aeruginosa*, might have different survival times and need additional time of exposure (Boorn *et al*., 2010; Henriques *et al*., 2011; Shenoy *et al*., 2012; Dryden *et al,* 2014).

For *M. pachydermatis*, a complete antifungal effect was also observed after 4h of exposure to HBO and pure MH. If MH is diluted at 40% it is necessary to have a longer exposure time. Although the reason is not depicted in this work, it is probably related to protection of the capsule and wall which are both present in this microorganism (Ashbee and Bond, 2010; Weiler *et al.*, 2013). *Candida* spp. isolates tested against a honey mixture and an engineered honey needed a higher concentration and exposure time for observation of antifungal activity, when in comparison to the time necessary to kill bacteria (Dryden *et al*.,

2014). This work documents for the first time that HBO is active against *S. pseudintermedius* and *M. pachydermatis* within 1 hour with a complete effectiveness after 4h of exposure. Certainly, it would be interesting to perform future studies in order to determine the exact killing time for HBO or MH that would kill different types of microorganisms implicated in skin and wound infections in the dog.

Laboratory assays are the starting point for clinical trials to prove efficacy and safety of medicinal products used in practice. Taking in account our results, the next step is a randomized, blinded clinical trial to test HBO and MH in the treatment of surface, superficial and deep canine pyoderma and otitis externa caused by *S. pseudintermedius.* Another clinical trial could aim to treat burns and wounds, either traumatic or post-surgical. Information about the concentration needed to kill each microorganism is crucial to decide, for example, the amount of product that should be applied in a wound. It is also important to decide for the time necessary for killing activity of the product. For example, the researcher might consider the use of a protective bandage in order to guarantee the contact time. We believe the information collected in this work allows for better clinical study designs.

However, sometimes the practitioner cannot wait for the publication of such clinical trials, which naturally take time to perform. The clinician might, therefore, consider prescribing treatments based on laboratory studies, small open trials, retrospective studies and/or peers experience. Based on our results, we suggest that HBO and MH be used in practice for the treatment of canine folliculitis and *Malassezia* dermatitis. However, clinical trials should be performed to assure it. Depending on the individual clinical case, products can be used either alone or associated with systemic antibiotics or antimycotics. The choice of solely using honey treatment or in association with systemic antibiotics would be dependent on the severity of lesions, practicability for the application of the product, owner compliance and cost. If antibiotics are less frequently used, it will potentially contribute to slower development of antibioresistance by *S. pseudintermedius.* Further laboratory work could be performed in order to evaluate the contribution of the products reversing MRSP to MSSP and inhibition of *S. pseudintermedius* biofilm formation. This can potentially add clinical value to these products, as natural products active against antibiotic resistant bacteria can be a major clinical asset against antibiotic resistant infections.

In summary, it was our aim to evaluate the *in vitro* efficacy of two honey products against MSSP, MRSP and *M. pachydermatis* which are commonly seen in small animal practice. The time-kill assays provided further insights about the activity against these pathogens. We hope to contribute for rational use of antibiotics and antimycotics in veterinary dermatology.

FINAL CONCLUSIONS

Honey is a natural product known to humankind for centuries. It can be a food source or a natural alternative in the treatment of many skin lesions. With the development of resistance to antibiotics by many bacteria honey became a more valuable option. This work led to the following conclusions:

- Portuguese clinicians seldom use honey as a topical treatment for skin and ears despite the recognition of antibiotic resistances by *S. pseudintermedius* in clinical practice.

- Multidrug-resistant *S. pseudintermedius* can be a challenge. Antibiotic choices left to treat these animals are few and, in some cases, limited to antibiotics with potential severe side-effects.

- Medical grade manuka honey is effective against *M. pachydermatis* and *S. pseudintermedius* regardless of methicillin resistance. The killing effect is still noticed after dilution of the honey.

- A honey-based gel is active *in vitro* against both methicillin susceptible and resistant *S. pseudintermedius* and *M. pachydermatis*. The activity of the gel is partly due to the honey component, although other ingredients contribute towards the overall antibacterial effect. Even after dilution the product maintains effectiveness.

- Both products are active in killing *S. pseudintermedius* and *M. pachydermatis* after one hour of exposure. A complete killing effect against both microorganisms is observed after four hours of contact time.

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ANNEX I

Questionário
"Tratamento de infeções de pele e ouvidos do cão"

Exmo(a). Colega,
O questionário destina-se ao estudo académico sobre o tratamento da piodermite superficial, dermatite por *Malassezia* e otites no cão em Portugal. As respostas são anónimas.
Os meus sinceros agradecimentos pela sua colaboração,

Ana Oliveira,
Diplomada pelo Colégio Europeu de Dermatologia Veterinária
Faculdade de Medicina Veterinária da Universidade Lusófona

1. Qual o número de casos que observa mensalmente de:

	Raramente	1-5 casos	5-10 casos	10-20 casos	Mais de 20 casos
Piodermite generalizada					
Dermatite por *Malassezia*					
Dermatite de pregas de pele					
Otite externa					

2. Recorre a citologia para confirmação de diagnóstico?

	Nunca	Menos 50% casos	Mais 50% casos	Sempre
Piodermite generalizada				
Dermatite por *Malassezia*				
Dermatite de pregas de pele				
Otite externa				

ANTIBIOTERAPIA ORAL

3. Que tipo de tratamento usa nas seguintes doenças?

	Antibiótico oral	Tratamento tópico	Combinação de antibiótico oral e tratamento tópico
Piodermite generalizada			
Piodermite das pregas			
Otite bacteriana externa			

4. Usa antibioterapia oral no tratamento das seguintes doenças?

	Nunca	< 25% casos	25-50% casos	50-75% casos	75-100% casos
Piodermite generalizada					
Piodermite das pregas					
Otite bacteriana externa					

5. Recorre à cultura e antibiograma antes de iniciar o tratamento de piodermite generalizada? (pode selecionar mais do que uma opção)

□ Nunca

□ Sempre

□ Somente nos casos que preciso de confirmar a minha suspeita de piodermite

□ Somente nos casos que suspeito de resistência aos antibióticos

□ Somente nos casos em que a antibioterapia empírica não se revelou eficaz

6. Com que frequência usa os seguintes antibióticos no tratamento de piodermite?

	Nunca	<25%	25-50%	50-75%	75-100%
Amoxicilina-ácido clavulânico					
Cefalexina					
Clindamicina					
Trimetoprim-sulfametoxazol					
Cefovecina					
Doxiciclina					
Minociclina					
Enrofloxacina					
Marbofloxacina					

7. Na sua opinião, qual a percentagem de casos de piodermite bacteriana que apresentam resistência a antibióticos?

□ 0%

□ <25%

□ 25-50%

□ 50-75%

□ 75-100%

8. Nos últimos 5 anos tem observado um aumento no número de casos de resistências a antibióticos?

□ Sim

□ Não

□ Não tenho opinião

TRATAMENTO TÓPICO

9. Que tratamento tópico aplica em piodermite bacteriana generalizada?

	Nunca	<25%	25-50%	50-75%	75-100%
Banho terapêutico					
Desinfecção das lesões					
Preparado com antibiótico, antifúngico e glucocorticóide					
Antibiótico tópico					
Produto à base de mel					
Outro tratamento não especificado					

10. Que tratamento aplica em piodermite das pregas?

	Nunca	<25%	25-50%	50-75%	75-100%
Desinfecção local					
Preparado com antibiótico, antifúngico e glucocorticóide					
Antibiótico tópico					
Produto à base de mel					
Outro tratamento não especificado					

11. Que tratamento aplica em otite externa bacteriana?

	Nunca	<25%	25-50%	50-75%	75-100%
Limpeza auricular					
Preparado com antibiótico, antifúngico e glucocorticóide					
Produto auricular somente com antibiótico					
Produto à base de mel					
Outro tratamento não especificado					

TRATAMENTO DE *MALASSEZIA*

12. Prescreve antifúngicos orais no tratamento das seguintes doenças?

	Nunca	<25%	25-50%	50-75%	75-100%
Dermatite generalizada por *Malassezia*					
Dermatite das pregas por *Malassezia*					
Otite externa por *Malassezia*					

13. Que tratamento aplica em dermatite generalizada por *Malassezia*?

	Nunca	<25%	25-50%	50-75%	75-100%
Banho terapêutico					
Desinfecção das lesões					
Preparado com antibiótico, antifúngico e glucocorticóide					
Antifúngico tópico					
Produto à base de mel					
Outro tratamento não especificado					

14. Que tratamento aplica em dermatite das pregas por *Malassezia*?

	Nunca	<25%	25-50%	50-75%	75-100%
Desinfecção local					
Preparado com antibiótico, antifúngico e glucocorticóide					
Pomada somente com antifúngico					
Produto à base de mel					
Outro tratamento não especificado					

15. Que tratamento aplica em otite externa por *Malassezia*?

	Nunca	<25%	25-50%	50-75%	75-100%
Limpeza auricular					
Preparado com antibiótico, antifúngico e glucocorticóide					
Produto auricular somente com antifúngico					
Produto à base de mel					
Outro tratamento não especificado					

PESSOAL

16. Em que área do país exerce?

□ Norte

□ Centro

□ Sul

□ Arquipélago dos Açores

□ Arquipélago da Madeira

17. Há quanto tempo exerce Clínica de Pequenos Animais

□ Há menos de 5 anos

□ Entre 5 e 10 anos

□ Entre 10 e 20 anos

□ Há mais de 20 anos

18. Aplica na prática clínica as *guidelines* para diagnóstico e tratamento de foliculite superficial bacteriana formuladas pela *International Society for Companion Animal Infectious Diseases*?

□ Sim

□ Não

□ Desconheço