UAB
Universitat Autònoma de Barcelona

STUDY OF MALASSEZIA SPECIES FROM THE SKIN OF DOGS AND RABBITS USING ROUTINE AND ADVANCED MOLECULAR METHODS

Leyna Díaz Álvarez

Universitat Autònoma de Barcelona

STUDY OF *MALASSEZIA* SPECIES FROM THE SKIN OF DOGS AND RABBITS USING ROUTINE AND ADVANCED MOLECULAR METHODS

Dissertation to obtain the PhD degree by:

Leyna Díaz Álvarez

Under the supervision of:

Dr. F. Javier Cabañes, Dra. Mª Rosa Bragulat and Dra. Gemma Castellá

PhD IN MEDICINE AND ANIMAL HEALTH
DEPARTMENT OF ANIMAL HEALTH AND ANATOMY
FACULTY OF VETERINARY MEDICINE

Bellaterra, May 2023

FRANCISCO JAVIER CABAÑES SAENZ y M. ROSA BRAGULAT ARARÁ, Catedráticos de Universidad y GEMMA CASTELLÁ GÓMEZ, Titular de Universidad, profesores del Departament de Sanitat i d'Anatomia Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona

CERTIFICAN:

que Doña **LEYNA DÍAZ ÁLVAREZ** ha realizado el presente trabajo sobre **"Study of *Malassezia* species from the skin of dogs and rabbits using routine and advanced molecular methods"**, bajo nuestra dirección en el Departament de Sanitat i d'Anatomia Animals de la Universitat Autònoma de Barcelona.

Y para que conste, a efectos de ser presentada como Memoria de Tesis para optar al título de Doctora por la Universitat Autònoma de Barcelona, firmamos el presente certificado en Bellaterra a 13 de abril de 2023.

F.J. Cabañes Saenz M.R. Bragulat Ararà G. Castellá Gómez

ACKNOWLEDGEMENTS:

La realización de esta tesis no habría sido posible sin la participación y el apoyo de muchas personas, a las cuales me gustaría agradecérselo.

En primer lugar, quisiera agradecer a mis directores de tesis por todo lo que me han enseñado, por todo el apoyo brindado y por su fe en mí. Concretamente, quisiera agradecer a Javier, por su exigencia y por sus consejos, a Maria Rosa, por su compresión y sus ánimos, y a Gemma, por su infinita paciencia y por guiarme por todo es mundo.

Quisiera agradecer también a mis compañeras, a Lourdes, por su simpatía y amabilidad y por nuestras charlas sobre música, a Carolina por abrirme todas las puertas, por comprensión, así como su ayuda en todo momento y por nuestras charlas sobre libros, y a Júlia por todas las charlas, cotilleos, comidas y tés compartidos en el despacho. No habría sido lo mismo sin vosotras.

También quisiera agradecer a mis mentores durante mi estancia en la Duke University, especialmente a Marco, Márcia y Joe, por todas sus enseñanzas y por toda su ayuda durante esos tres meses.

Finalmente, a mi familia, por su apoyo incondicional en todas mis aventuras. A mi madre por estar siempre ahí para mí y a mi abuela por apoyarme pese a no entender exactamente qué estoy haciendo. A mi abuelo, allá donde estés, lo he logrado. A mis amigos, Sergio, Marta, Karina, Grissel, Cherry, Iris, Noelia y Núria, por aguantarme durante estos años y por escucharme a diario. A Arnau, por estar siempre a mi lado, seguiremos asociados

muchos años. A mis perros, Kiva, Koira y Zeus por mostrarme su amor y lealtad incondicionalmente.

¡Mil gracias a todos!

This Doctoral Thesis dissertation is presented as a compendium of publications. The list of articles is as follows:

- Díaz L, Castellá G, Bragulat MR, et al. External ear canal mycobiome of some rabbit breeds. *Med Mycol*. 2021; 59(7): 683-693. doi:10.1093/mmy/myaa097.

- Díaz L, Castellá G, Bragulat MR, Paytuví-Gallart A, Sanseverino W, Cabañes FJ. Study of the variation of the *Malassezia* load in the interdigital fold of dogs with pododermatitis. *Vet Res Commun*. 2022; doi:10.1007/s11259-022-09951-2.

- Díaz L, Castellá G, Bragulat MR, Cabañes FJ. *ERG11* gene variability and azole susceptibility in *Malassezia pachydermatis. Mycopathologia*. 2022; doi:10.1007/s11046-022-00696-9.

Index

ABBREVIATIONS

AFLP	Amplified fragment length polymorphism
AMB	Amphotericin B
CFU	Colony forming units
CHS2	Chitin sintase 2
DA	Dixon agar
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
D1/D2	D1/D2 domain of the rRNA LSU
ERG11	Sterol 14-demethylase
FLZ	Fluconazole
ITS	Internal transcribed spacer
ITZ	Itraconazole
KTZ	Ketoconazole
LNA	Leeming and Notman agar
LSU	Large subunit of the rRNA
mDA	modified Dixon agar
MAT	Mating type
MCZ	Miconazole
MH-GM	Mueller Hinton glucose methylene blue agar
NGS	Next generation sequencing
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
qPCR	quantitative real-time PCR
RAPD	Random amplification of polymorphic DNA
RFLP	Restriction fragment length polymorphism
RNA	ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SGA	Sabouraud glucose agar

SSCP	Single strand conformation polymorphism
tFLP	Terminal fragment length polymorphism

SUMMARY

The yeasts of the genus *Malassezia* are part of the normal skin microbiota of a wide range of warm-blooded animals, including humans. The 18 species included in the genus have been isolated from different wild and domestic animals but also from humans. Some *Malassezia* yeasts appear to have a broad host range, whereas others are more host-specific with a close adaptation to the cutaneous ecosystem of a single animal species or a group of phylogenetically related animals like *Malassezia vespertilionis* that has only been isolated from bats.

A main characteristic of *Malassezia* spp. is their lipid-dependency, which is caused by the lack of the gene encoding the fatty acid synthase and genes involved in carbohydrate metabolism. Due to this, they are unable to synthetize long chain fatty acids *de novo* and they rely on the host as an exogenous source of fatty acids. Thus, *Malassezia* yeasts have a predilection for sebaceous skin sites. Also, specific media with a specific lipid composition are necessary to culture them. Despite being part of the normal skin microbiota, under certain circumstances the population of *Malassezia* can overgrow and the yeasts may act as an opportunistic pathogen causing *Malassezia* related diseases such as dermatitis, otitis, and other skin diseases. In dogs and cats, *Malassezia pachydermatis* is considered a frequent aetiological agent producing otitis and dermatitis. *Malassezia* related diseases are commonly treated with azole antifungals that inhibit the lanosterol-14α-demethylase involved in the ergosterol synthesis, a major component of the cell membrane. Although azole antifungals are usually effective resolving *Malassezia* diseases, some cases of treatment failure have been recently reported. Thus, it is necessary to test *in vitro* azole susceptibility of those isolates even though there is yet no

standardized method available for *Malassezia*. Some studies have associated azole resistance in *Malassezia* with mutations in the sequence of the *ERG11* gene.

In this thesis, the mycobiota of the external ear canal of healthy rabbits of different breeds was studied using cytology, culture, PCR and NGS. *Malassezia* species are rarely isolated in lagomorphs. In fact, only *Malassezia cuniculi* has been isolated in rabbits. The ear morphology has been suggested to be a predisposing factor for *Malassezia* overgrowth and thus, both lop-eared and erect-eared breeds were included. Also, farmed and pet rabbits were included. Although no growth was observed in any of the culture media used, the presence of *Malassezia* was detected by cytology. The NGS results demonstrated that *Basidiomycota* was the most abundant phyla in healthy rabbits followed by *Ascomycota*. *Malassezia* was the predominant genus in the external ear canal, however it was the *Malassezia* phylotype 131 the predominant taxa in all samples. *Malassezia cuniculi* was detected in all samples. No significant differences were observed in the results when comparing lop-eared and erect-eared rabbits.

Canine pododermatitis is a common veterinary disorder in which *M. pachydermatis* commonly acts as an opportunistic pathogen. Thus, the variation of the *Malassezia* load in samples from the interdigital fold of dogs with pododermatitis, dogs after antifungal treatment and healthy dogs was studied by cytology, culture, qPCR and NGS. The pododermatitis samples before treatment showed a higher population of *Malassezia* yeasts by cytology, CFU and qPCR. By NGS, *Ascomycota* was the main phylum in healthy and post-treatment samples while in the pododermatitis samples before treatment, *Basidiomycota*, and *M. pachydermatis* were predominant. In this study, a correlation between cytology and qPCR was observed.

Also, as *Malassezia* related diseases are commonly treated using azole antifungals, recent reports of treatment failure represent a major concern. Some studies reported cases of azole resistance associated with mutations in the *ERG11* gene, however there is no standardized method to test antifungal susceptibility in *Malassezia*. Thus, any modification of the test conditions could provide different susceptibility results. In this thesis, the *in vitro* antifungal susceptibility of strains of *M. pachydermatis* isolated from different animal species and health status was evaluated and the *ERG11* gene was sequenced. Also, multilocus sequence typing was performed using D1/D2, ITS, CHS2 and β-tubulin genes. The study revealed a high diversity in *ERG11* sequences, a correlation between certain genotypes and *ERG11* mutations, and a correlation between *ERG11* mutations and a reduced susceptibility to azoles.

RESUMEN

Las levaduras del género *Malassezia* forman parte de la microbiota normal de la piel de una amplia variedad de animales homeotermos, incluyendo los seres humanos. Actualmente, el género está constituido por 18 especies que se han aislado tanto en animales domésticos como salvajes, así como en humanos. Algunas especies de *Malassezia* parecen tener un amplio abanico de hospedadores, mientras que otras son especie-específicas, presentando adaptaciones al ecosistema cutáneo de una sola especie animal o de un reducido grupo de animales filogenéticamente relacionados. Así, por ejemplo, *Malassezia vespertilionis* ha sido aislada únicamente en murciélagos.

Una de las características principales del género *Malassezia* es su lipodependencia causada por la ausencia del gen codificante para una enzima sintasa de ácidos grasos y por la ausencia de genes relacionados con el metabolismo de los carbohidratos. Por este motivo, estas levaduras son incapaces de sintetizar *de novo* ácidos grasos de cadena larga y dependen del hospedador como fuente externa de ácidos grasos, presentando por ello predilección por las áreas sebáceas de la piel. Asimismo, para su cultivo es necesario emplear medios de cultivo específicos suplementados con ácidos grasos. Pese a ser parte de la microbiota normal de la piel, bajo determinadas circunstancias, la población de *Malassezia* puede incrementarse y estas levaduras pueden llegar a actuar como patógenos oportunistas, produciendo patologías como dermatitis, otitis y/u otras afecciones de la piel. En perros y gatos, *Malassezia pachydermatis* está considerada un agente etiológico frecuente en otitis y dermatitis. Las patologías causadas por *Malassezia* se tratan habitualmente con antifúngicos azólicos, los cuales inhiben la enzima lanosterol-14α-demetilasa relacionada con la síntesis del ergosterol, uno de los principales constituyentes de la membrana celular. Pese a que los antifúngicos azólicos son habitualmente efectivos para el tratamiento de las patologías producidas por *Malassezia*, recientemente se han

notificado algunos fallos terapéuticos. Por este motivo, es necesario evaluar la susceptibilidad *in vitro* de estas cepas de *Malassezia* frente a los diferentes antifúngicos azólicos aunque, actualmente, no exista un método de referencia estandarizado para estas levaduras. Además, algunos estudios han relacionado la resistencia a antifúngicos azólicos en *Malassezia* con mutaciones en la secuencia del gen *ERG11*.

En esta tesis, se ha estudiado la micobiota del conducto auditivo externo de conejos sanos de diferentes razas mediante citología, cultivo, PCR y NGS. Es poco frecuente el aislamiento de especies de *Malassezia* en lagomorfos, de hecho, *Malassezia cuniculi* es la única especie de este género aislada en conejos. La morfología de las orejas de estos animales ha sido propuesta como un factor de predisposición para el sobrecrecimiento de levaduras de *Malassezia*. Por ello, en este estudio se incluyeron razas de conejos de orejas erectas y de orejas caídas. Asimismo, se incluyeron conejos de granja y mascotas. A pesar de no obtener crecimiento en ninguno de los medios de cultivo utilizados, la presencia de *Malassezia* se detectó en las muestras mediante citología. Los resultados de la NGS demostraron que *Basidiomycota* fue el filo más abundante en conejos sanos, seguido de *Ascomycota*. *Malassezia* fue el género mayoritario y el filotipo 131 de *Malassezia* fue el taxón más abundante en el conducto auditivo externo. *Malassezia cuniculi* se detectó en todas las muestras. No se observaron diferencias significativas en los resultados obtenidos entre las dos tipologías de orejas.

La pododermatitis canina es un trastorno común en la clínica veterinaria en el cual *M. pachydermatis* actúa frecuentemente como patógeno oportunista. Por ello, en esta tesis se evaluó la variación de la carga de *Malassezia* mediante citología, cultivo, qPCR y NGS en muestras del espacio interdigital de perros con pododermatitis, perros sanos y perros tras un tratamiento antifúngico. Las muestras de pododermatitis presentaron unos

recuentos más elevados de *Malassezia* que el resto de las muestras tanto en el examen citológico, como en cultivo y qPCR. Los resultados de la NGS mostraron que *Ascomycota* fue el filo más abundante en las muestras de perros sanos y postratamiento. Sin embargo, el filo *Basidiomycota* y *M. pachydermatis* fueron los más abundantes en las muestras de pododermatitis. En este estudio, se demostró una correlación en la carga de estas levaduras entre la técnica de citología y el método qPCR.

Finalmente, dado que las enfermedades relacionadas con *Malassezia* se tratan comúnmente con antifúngicos azólicos, los fracasos terapéuticos recientemente notificados suponen un motivo de preocupación. Algunos estudios han relacionado la resistencia a antifúngicos azólicos en determinadas cepas con mutaciones en el gen *ERG11*. Cabe destacar que por el momento no existe ningún método estandarizado para evaluar la susceptibilidad antifúngica de *Malassezia*. Por ello, cualquier modificación de las condiciones de las pruebas puede dar lugar a resultados de susceptibilidad diferentes. En esta tesis, se evaluó la susceptibilidad antifúngica *in vitro* de una colección de cepas de *M. pachydermatis* aisladas de diferentes especies animales, procedentes de casos clínicos de otitis y/o dermatitis, así como de animales sanos. Además, se secuenció el gen *ERG11* y se tipificaron las cepas mediante un análisis multilocus de secuencias de los genes D1/D2, ITS, CHS2 y β-tubulina. Los resultados mostraron una gran variabilidad de secuencias en el gen *ERG11*, una correlación entre ciertos genotipos y mutaciones en dicho gen, y una correlación entre mutaciones del gen *ERG11* y la reducción de la susceptibilidad a los azoles.

1. JUSTIFICATION

The genus *Malassezia* includes, at present, 18 species of lipid-dependent yeasts. These species are part of the normal skin microbiota of a wide range of warm-blooded animals including humans. *Malassezia* species have been isolated from domestic animals, wild animals held in captivity, wild animals in their natural habitats and humans. Some species have been isolated from various animal hosts while other tend to be host specific. The *Malassezia* species present in humans and the main domestic animals such as cats and dogs have been well studied. However, *Malassezia* species are rarely isolated and studied in lagomorphs. Due to their lipid-dependent nature, isolating new *Malassezia* species could be fastidious and time-consuming as they have specific nutritional requirements to grow on culture media. Also, culturable microorganisms represent only a small fraction of the total microbiota. Thus, advanced molecular methods such as NGS have been used to study the skin microbiota of humans, dogs, and cats without the need of a culture. However, these studies have never been performed in rabbits.

Despite being part of the microbiota, under certain circumstances, the population of these yeasts can over proliferate, and the yeasts may act as opportunistic pathogens, causing dermatitis and otitis in dogs and cats. A common presentation of dermatitis diagnosed in the veterinary clinic is canine pododermatitis associated with *M. pachydermatis*. Different methods have been used to study the variation of the *Malassezia* load between healthy animals and animals with dermatitis. Molecular methods such as NGS or the qPCR that allows the study of the abundance of DNA have been applied in a few studies to demonstrate the increase of the yeast population in animals with otitis or dermatitis. However, these studies have never been focused on pododermatitis.

Malassezia related diseases are commonly treated using azole antifungals. Even though treatments are usually effective, some reports of antifungal resistance have recently emerged. Testing *in vitro* susceptibility of *Malassezia* isolates is necessary to fully understand their mechanisms of resistance. In *in vitro* susceptibility tests, a variation of the test condition could induce changes in susceptibility profiles. However, there is no standardized method to test *in vitro* antifungal susceptibility in *Malassezia* and due to their unique lipid-dependency, traditional methods available for yeasts cannot be used. Also, few studies on *M. pachydermatis* have associated *in vitro* azole resistance with mutations in the *ERG11* gene, which encodes for the lanosterol-14-α-demethylase, the primary target for azole drugs.

2. INTRODUCTION

2.1. The genus *Malassezia*

2.1.1. Historical perspective of the genus

The genus *Malassezia* was first described in 1846 by Eichstedt as a fungus associated with pityriasis versicolor lesions. However, it was not until 1853 that it was given the name *Microsporon furfur* [1]. The ancestors of the genus are plant- or soil-resident fungi that adapted to survive and develop in a cutaneous ecosystem [2].

The genus *Malassezia* (Baillon) was not created until 1889 and had a single species, *Malassezia furfur,* detected in human skin lesions. In 1925, Weidman isolated a yeast that could grow without lipid supplementation from the skin of a rhinoceros with exfoliative dermatitis and named them *Pityrosporum pachydermatis* [1]. In 1955, Gustafson isolated yeasts similar to *P. pachydermatis* from the external auditory canal of dogs with otitis and named them *Pityrosporum canis* [3]. *Pityrosporum pachydermatis* and *P. canis* shared morphological similarities such as the ability to grow on media without external lipid supplementation. As a result, *P. canis* became a synonym of *P. pachydermatis*. From 1970 the genus *Pityrosporum* included three species *P. ovale, P. orbiculare* and *P. pachydermatis* [4]. During this period, the morphological similarities between the genera *Pityrosporum* and *Malassezia* were evaluated. In 1986, the re-evaluation of previous studies led to the acceptance, by the International Commission on the Taxonomy of Fungi, of the name *Malassezia* for this genus of yeasts, displacing the term *Pityrosporum*. This decision was based on the morphology, ultrastructure, and immunological properties of these yeasts. The genus *Malassezia* included two species, *Malassezia furfur* associated with humans and *M. pachydermatis* in animals [3].

In 1990, Simmons and Guého described the new species *Malassezia sympodialis* that differed from *M. furfur* by morphological characteristics, the guanine-cytosine percentage of its nuclear DNA and by DNA/DNA hybridization [5]. In 1995, the sequencing of the D1/D2 region made it possible to study the diversity of the genus and to demonstrate that there were considerable differences that could represent new species [6]. Consequently, the following year four new species were described: *Malassezia globosa*, *Malassezia obtusa*, *Malassezia restricta* and *Malassezia slooffiae* [7].

In subsequent years, both new molecular and phenotypic techniques for the identification of new *Malassezia* species continued to be developed [8-12]. This allowed the description of new species in human skin such as *Malassezia dermatis* [13], *Malassezia japonica* [14], *Malassezia yamatoensis* [15] and *Malassezia arunalokei* [16]. New species associated with the skin of animals were also described, such as *Malassezia nana* in cats and cows [17], *Malassezia caprae* in goats, *Malassezia equina* in horses [18], *Malassezia cuniculi* in rabbits [19], *Malassezia psittaci* and *Malassezia brasiliensis* in birds [20] and *Malassezia vespertilionis* in bats [21].

2.1.2. Characteristics of the genus *Malassezia*

2.1.2.1. Current taxonomy

The genus *Malassezia* consists of 18 species which according to current taxonomy are located within phylum *Basidiomycota*, subphylum *Ustilaginomycotina*, class *Malasseziomycetes*, order *Malasseziales* and family *Malasseziaceae* [22]. Originally, its position within the subphylum *Ustilaginomycotina*, which includes mostly plant pathogenic fungi, was uncertain. The genus *Malassezia* constitutes a monophyletic lineage differentiated from the classes *Exobasidiomycetes* and *Ustilaginomycetes* that

form this subphylum. Also, the yeasts of the genus *Malassezia* have phenotypic characteristics at the morphological, ultrastructural, physiological, and biochemical level that differentiate them from the other classes of the subphylum. For all these reasons, the class *Malasseziomycetes* was proposed to include the genus *Malassezia* within the subphylum [22].

Phylogeny studies of the genus divide *Malassezia* species into three groups. A first approach using the genomes of 14 *Malassezia* species showed three main groups [23]. Group A includes *M. furfur,* and three other species rarely found on human skin (*M. yamatoensis, M. obtusa* and *M. japonica).* Group B includes a sub-group of the most common human skin residents *M. restricta* and *M. globosa* and the slightly less common *M. sympodialis* as well as related species in another sub-group (*M. pachydermatis, M. nana, M. caprae, M. equina* and *M. dermatis*). Group C includes two outliners, *M. cuniculi* and *M. slooffiae* [23]. Another approach using the D1/D2 sequences of the 18 *Malassezia* species, also showed three main groups (Figure 1). Group A included *M. furfur,* three other species rarely found on human skin (*M. yamatoensis, M. obtusa* and *M. japonica)* and two species isolated from parrots (*M. brasiliensis,* and *M. psitacci*). Group B includes a sub-group of human skin species (*M. restricta, M. anuralokei* and *M. globosa*) and a sub-group including species isolated from human skin (*M. sympodialis* and *M. dermatis*) and animal skin (*M. pachydermatis, M. caprae* and *M. nana*). Group C consists of more divergent species (*M. cuniculi, M. slooffiae* and *M. vespertilionis*) rare on human skin [24].

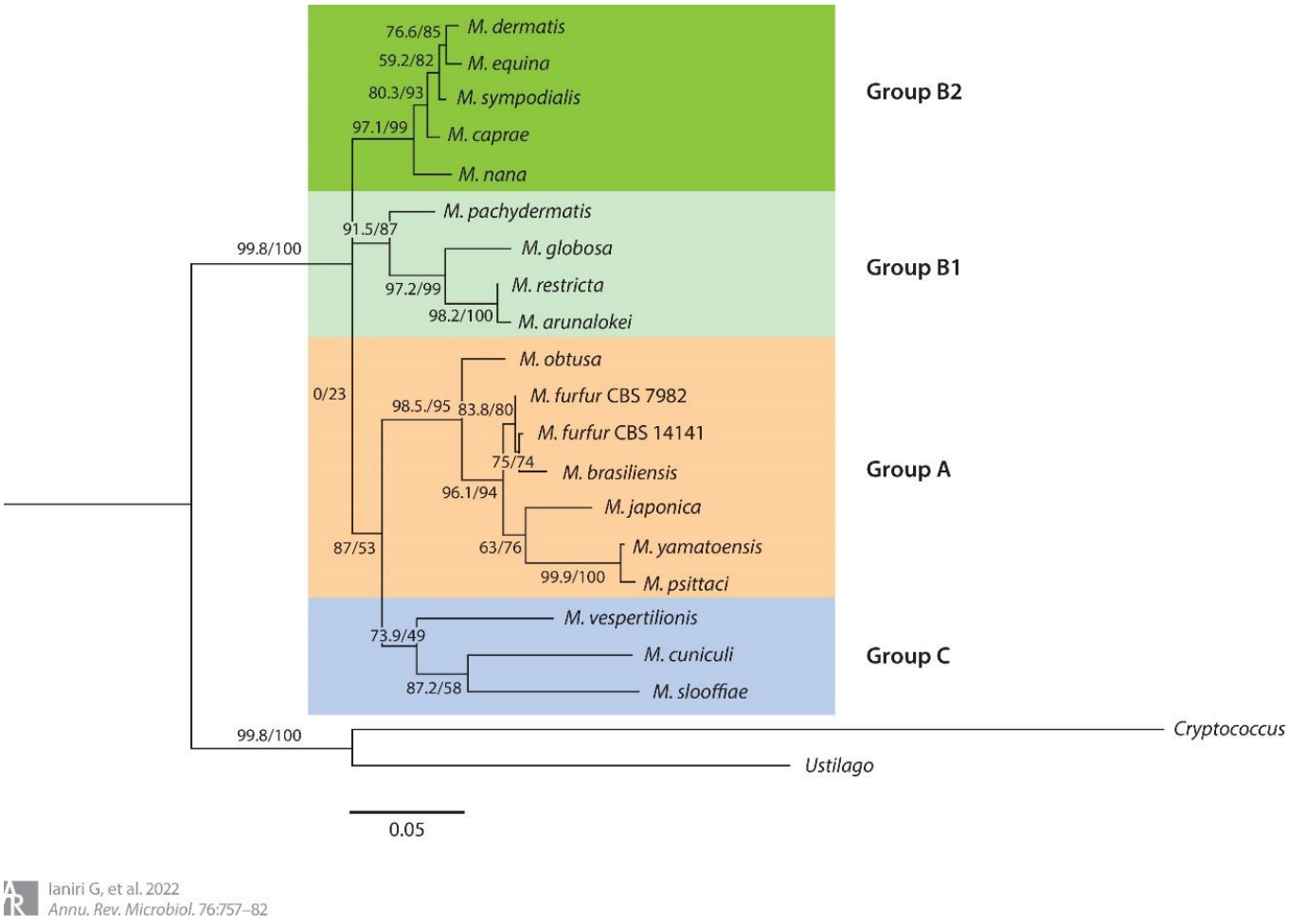

Figure 1. Phylogeny of 18 *Malassezia* species using D1/D2 sequences [24].

2.1.2.2. Morphology

At macroscopic level, after growth on mDA at 32ºC, single colonies of *Malassezia* spp. could be convex to flat, butyrous to brittle, smooth to slightly rough, pale yellow-cremish to brownish, shiny to matte, with smooth or folded margins depending on the species. The size of the colonies is also variable, between 1mm to 8mm [25].

At microscopic level, *Malassezia* yeast cells could be spherical, ovoid, ellipsoidal or cylindrical. Cells present budding in a monopolar pattern on a narrow to broad base. The

size of the cells is also variable depending on the species and some could form filaments or pseudo-hyphae [25].

2.1.2.3. Cell wall

The cell wall is essential for the viability of fungal cells. It is formed by high molecular weight polysaccharides that confer stability and rigidity and serves as an interface for recognition with the immune system. The *Malassezia* cell wall presents a series of characteristics that make it unique among the rest of fungal genera. Its wall thickness is 0.12µm and is formed by various layers including and outer lamella, a multilayer wall and a plasma membrane with a corrugate pattern and surrounded by an electro lucent band [25,26].

At the composition level, the wall of *Malassezia* spp. presents a high percentage of lipids (15%) compared to other genera such as *Saccharomyces* spp. (1-2%) [26]. This high lipid ratio may be responsible for the great resistance of *Malassezia* to external influences such as its resistance to osmotic changes or mechanical forces. It also contains a high proportion (~70%) of β-(1,6)-glucan compared to the rest of fungal walls that are generally rich in β-(1,3)-glucan. This may result in impaired recognition by the immune system since β-(1,3)-glucan plays an important role in this function. Other components of the *Malassezia* cell wall are chitin (5%), chitosan (20%) and β-(1,3)-glucan (5%) [27].

2.1.2.4. Reproduction

Asexual reproduction of yeasts of the genus *Malassezia* consists of monopolar, blastic, and percurrent budding. This budding can have a wider or narrower base depending on the species. The bud or daughter cell originates from the innermost layers of the cell wall,

leaving a visible collarette scar upon release. This scar becomes thicker with successive budding. These scars occur in all *Malassezia* species but are most clearly seen in those with broader based budding such as *M. pachydermatis, M. furfur, M. yamatoensis, M. slooffiae, M. japonica,* and *M. obtusa*. Sympodial budding also occurs in *M. sympodialis* and *M. dermatis*, with the buds occurring alternately on the right and left sides giving it a cloverleaf appearance [25].

It is hypothesized, that sexual reproduction in fungi contributes to gene content through recombination, which is associated with an increased virulence [28,29]. In *Malassezia*, the high genetic variability between species suggests the existence of genomic recombination even though its sexual cycle is still unknown [2,30]. However, genomic analysis studies have shown the presence of MAT genes in most *Malassezia* species [30,31]. Therefore, if *Malassezia* has a sexual cycle, it is probably bipolar or pseudo-bipolar with two types of alleles of the MAT gene instead of tetrapolar, which is more common in other basidiomycetes [23,31,32]. These MAT genes are related to sexual reproduction in other basidiomycetous species. It is still unknown whether these genes are associated with the yeast-hyphal transition, which has been rarely observed in *Malassezia* species [24].

2.1.2.5. Lipid dependency

Yeasts of the genus *Malassezia* are characterized by their inability to synthesize long-chain (C14 or C16) fatty acids *de novo* [33]. Due to this, they rely on the external contribution of lipids for their growth and to it is necessary to use specific media such as DA or LNA for their isolation [25]. There is some variability between the species of the genus, which has allowed the development of specific tests to identify them according to their lipid dependency [8].

This lipid dependency is caused by a defect in the synthesis of myristic acid, which is a precursor in the synthesis of long-chain fatty acids (C14 or C16) [26]. At the genomic level, it translates into an absence of the gene encoding the fatty acid synthase, compensated by an abundance of genes encoding hydrolases and enzymes related to carbohydrate metabolism [23]. Thus, yeasts of the genus *Malassezia* are capable of hydrolysing lipids in their environment to use them as a source of fatty acids [26]. This loss of genes is a consequence of the adaptation of these yeasts to the skin, which provides all the lipids necessary for their growth [34].

Historically, *M. pachydermatis* has been considered the only species, within the genus, not lipid-dependent but lipophilic, as it is the only one capable of growing in general media such as SGA [25,35]. Despite this, genome sequencing of *M. pachydermatis* showed that it also lacks the enzyme responsible for synthesizing long-chain fatty acids but is capable of using lipid fractions of the peptone on SGA [23]. Therefore, *M. pachydermatis* is unable to grow in lipid-free synthetic culture media and should also be considered as lipid-dependent [23]. However, there are strains of *M. pachydermatis* that grow poorly on SGA [36] or are unable to grow in this medium [37-39].

2.1.2.6. Genome

The genome of *Malassezia* is small and compact (~7-9Mb) and has a reduced number of genes (~4,400) [23,40]. Genes are arranged close together throughout the genome and are separated by few introns (present in ~27% of genes). Furthermore, they contain few repetitive elements (~0.78% of the genome) [2]. Comparative gene analysis revealed extensive turnover events characterized by gene gain and loss, probably a result of

evolutionary adaptation to colonize a specific niche such as animal skin. The loss of the fatty acid synthase and the δ-9 desaturase in all known *Malassezia* species may be due to the abundance of lipids in their natural habitat [23,41,42]. However, *Malassezia* genomes contain an abundance of genes encoding hydrolases to utilize the proteins, lipids, and glycerol of the media [43,44].

On the other hand, its genome also has a reduced number of transposable elements and lacks the key genes for the RNA interference mechanism. For this reason, it was hypothesized that *Malassezia* species could be associated with mycoviruses, as in the case of *S. cerevisiae* and *U. maydis*, to make up for this deficiency. In the case of *M. sympodialis, M. globosa, M. obtusa, M. pachydermatis, M. yamatoensis* and *M. restricta*, this hypothesis was confirmed by the presence of mycoviruses from the *Totiviridae* family. The presence of these mycoviruses is associated in *Malassezia* with a higher pathogenicity in *ex vivo* models [45,46].

Malassezia genomes are characterized by several genes of bacterial origin acquired through horizontal gene transfer. Those genes encode functions related to stress response, pathogenicity and probably contributed to *Malassezia* evolution and niche adaptation [47].

Haploid *Malassezia* species have six to nine chromosomes. The existence of an ancestral karyotype with nine chromosomes is hypothesized, which is conserved in *M. slooffiae, M. globosa* and *M. restricta*. Chromosome breakage phenomena and subsequent centromere inactivation resulted in eight chromosomes in *M. sympodialis, M. equina, M. caprae, M. nana, M. dermatis*, and *M. furfur*. In the case of *M. pachydermatis* and other *Malassezia* species, their chromosomal evolution is still unclear [24,40].

2.2. Isolation, detection, and identification methods for *Malassezia*

2.2.1. Isolation methods

Historically, culture of *Malassezia* yeasts has been the first step for its isolation and identification since Panja, in 1927, achieved the first culture of *Malassezia* in a medium containing egg and gentian violet [25]. From that moment on, different ways of supplementing the SGA medium with fatty acids were investigated, leading to inconstant and rapid loss of cultures [25]. With the development of the DA medium and its subsequent modification by Midgley [7], the culture of *Malassezia* experienced considerable progress. At the same time, LNA medium was developed which allows the growth of all *Malassezia* species [48].

Even *M. pachydermatis*, the least demanding species of the genus, needs culture media that contain peptone, such as SGA, which provides short-chain fatty acids [25]. Currently, the most widely used culture media to isolate *Malassezia* are SGA for *M. pachydermatis*, and DA, or mDA and LNA for the rest of species [25].

2.2.2. Detection methods

2.2.2.1. Cytology

Diagnosis of *Malassezia* otitis externa and dermatitis is commonly based on clinical signs, response to antifungal therapy and the presence of an elevated number of the yeasts by direct microscopy. In the veterinary practice, more than 3-5 cells per high power field in the skin could be indicative of infection by *Malassezia* spp. [49]

Different cytological techniques have been developed including scraping methods, tape stripping, direct impression with glass slides or smears on a slide from a swab previously rubbed onto the lesional skin. Tape stripping has been widely accepted in the veterinary clinical practice as a rapid and versatile method for recovering *Malassezia* yeast from the stratum corneum of the skin [50].

2.2.2.2. PCR and Real-time PCR

Molecular techniques, especially PCR approaches such as real-time PCR, namely also quantitative real-time PCR or qPCR, are useful and reliable to investigate the presence of *Malassezia* species without the need to culture the yeasts [51]. Different studies have developed PCR techniques to detect *Malassezia* species directly from samples. Those studies were based on the amplification of ribosomal genes [52-54]. Also, multiplex PCR techniques to detect several *Malassezia* species at once have been developed [55].

Real-time PCR has demonstrated to be fast, sensitive, and precise to reliably detect and quantify *Malassezia*. The use of the qPCR has allowed the detection and quantification of *Malassezia* in human skin [56]. Also, it has been used to study the increase of the population of these yeasts in cases of human pathologies [57,58]. Several qPCR assays have been designed to detect and quantify the most common *Malassezia* species in human skin, mainly *M. globosa* and *M. restricta*. These assays are based on the amplification of ribosomal genes, which are present in multiple copies in the genome. Those genes offer better results in studying the distribution of *Malassezia* rather than the absolute quantification of the yeasts [57,58].

In animal skin, some qPCR techniques have been developed for *Malassezia* detection and quantification. A study developed a qPCR technique to absolutely quantify the presence of *M. pachydermatis* in the external ear canal of dogs using β-tubulin gene [59]. Also, the multicopy ITS has been used as a target to study the population of *Malassezia* species in samples from dog skin using qPCR [60].

2.2.2.3. Metagenomics

New generation sequencing (NGS) techniques allows the detection of microorganisms in a sample by sequencing millions of DNA fragments in parallel, without the need for prior culture. In this way, it is possible to study microorganisms or microbial diversity without the need to culture the samples, since culturable microorganisms represent a small fraction of all microbial diversity. It allows the study of the abundance and richness of microbial species directly from the matrix of interest and it overcomes the limitation of culture-based methods. It can be applied to study the genetic diversity, the population structures, and the interactions of the communities of microorganisms in their own ecosystem. NGS allows obtaining genomic information on microorganisms without the need to cultivate them with high sensitivity and at a lower cost [61-64].

Two different NGS approaches can be used to detect and identify species within a sample, shotgun metagenomic sequencing and amplicon-based metagenomic sequencing. Shotgun metagenomic sequencing obtains de DNA sequence of an organism's genome and allows the study of the content and diversity of microorganisms in a sample, provides taxonomy information and gene content of a microbial population, without targeting any specific gene [24]. Also, shotgun metagenomic studies can be applied to study the

microbiome of different samples and have been performed on human skin and mucosae [65-67].

In amplicon-based analyses, various regions of fungal ribosomal genes can be used as target to study taxonomy and diversity within samples [24]. Most studies selected the internal transcribed spacer 1-2 (ITS1-2) region of the rRNA [68]. However, the variable length of this regions among different fungal genera and species could lead to preferential amplification and biased assessment of abundance [68]. Due to this, the use of alternative target genes has been promoted, such as portions of the genes coding for 26S (11–13) and 18S (14–16) rRNA [68,69]. This technique made it possible to estimate the relative abundance of the different species of *Malassezia* in skin samples. Studies of the mycobiome have shown that *Malassezia* is one of the most abundant genera on human skin [70-72]. Only a few mycobiome studies have been conducted in dogs and cats. These studies included healthy animals and clinically affected animals with otitis, dermatitis, and/or allergy [60,73-79]. In all these studies, the main phylum identified in healthy animals was *Ascomycota* and the main genera were *Cladosporium* and *Alternaria*, considered as transient mycobiota. In clinically affected animals with dermatitis, otitis and/or allergy, *Basidiomycota* was de main phylum and *Malassezia* de main genus, being *M. pachydermatis* the main species [60,73-79].

NGS techniques have made it possible to detect the presence of *Malassezia* DNA sequences in different ecosystems such as marine corals, seabed sediments, Antarctic sediments, nematode exoskeletons, plant roots or lobster larvae intestines [80].

2.2.3. Identification methods

2.2.3.1. Phenotypic identification

Due to the lipid-dependent nature of *Malassezia*, conventional laboratory techniques used to identify yeasts are not applicable to this genus [25]. The phenotypic identification of the different species of *Malassezia* through biochemical and physiological tests requires obtaining colonies in a culture medium. These tests include catalase activity, β-glucosidase activity, growth at different temperatures (32, 37 and 40ºC) and assimilation of Tweens 20, 40, 60, 80 and Cremophor EL. Furthermore, *M. pachydermatis* is identifiable by its unique ability to grow on SGA [25].

Currently, molecular techniques are the most reliable to identify *Malassezia* species, since the results obtained solely through the phenotypic characteristics require more time, can be ambiguous and do not allow new species to be described [51]. The development of molecular methods allowed the unequivocal separation of the different species of *Malassezia* as well as the identification of new species [25].

2.2.3.2. Molecular methods

2.2.3.2.1. DNA sequencing

DNA sequencing is the most reliable tool for the identification of *Malassezia* species. Ribosomal gene sequencing has made an indispensable contribution to understanding the biodiversity of *Malassezia* by identifying all the different species [51]. Even though the ITS region is considered the universal barcode for fungi [81], the D1/D2 region of the 26S rRNA gene is more suitable for the identification of yeast species, especially *Malassezia* due to its lower variability [82,83]. Several studies demonstrated that sequencing the D1/D2 region is reliable for the identification of basidiomycetous species [84].

Apart from the ribosomal genes, some protein coding genes can be used to identify *Malassezia* species, such as the β-tubulin [82,85] and CHS2 genes [10,86].

2.2.3.2.2. Genome sequencing

Whole genome studies have made it possible to obtain the genomes of different species within the genus *Malassezia*, thus allowing a better understanding of the genus [23]. In 2007, the first genome of a species of the genus *Malassezia*, *M. globosa*, obtained by whole genome shotgun sequencing was published [2]. A short time later, the genome of *M. sympodialis* [28] and *M. pachydermatis* [87] were published following a similar technique. In 2015, a study reported the genomes of 14 *Malassezia* species including several strains of some of the species of relevance in dermatology [23].

Genome sequencing showed the genetic basis of the unique lipid-dependency of *Malassezia* and its adaptation to animal and human skin. Analysis of the existing genomes confirmed that their compact genomes contain only minimal information necessary for their existence and that it might be a consequence of evolutionary adaptation in specific host niches. Whole genome sequencing provided a genomic resource that allowed the investigation of *Malassezia* biology and its ecological distribution. Also, it can be used to perform phylogenetic analysis of *Malassezia* species to identify evolutionary relationships [23].

2.2.3.2.3. Other molecular methods

There are other molecular techniques for the identification and study of the different *Malassezia* species such as karyotyping and other methods based on DNA amplification [51].

Electrophoretic karyotyping using PFGE technique has demonstrated a variability in chromosomal patterns among *Malassezia* species, which is stable within species [9,51]. Thus, PFGE may be used to identify *Malassezia* species, but the method is methodologically demanding and time consuming [51]. Other methods for the identification of *Malassezia* species could be AFLP [12], RFLP [88] and RAPD [9].

Other methods based on PCR have been applied for the identification of *Malassezia* species such as tFLP [53], SSCP [86] and DGGE [89]. However, these methods are not widely used because they require specific equipment and due to the complexity in the interpretation of the results [51].

2.3. Epidemiology and ecology of the genus *Malassezia*

The species of the genus *Malassezia* are part of the microbiota of the skin and mucous membranes of a wide range of warm-blooded animals, including humans. Some *Malassezia* species can be detected in multiple hosts while other are host-specific and have a series of adaptations to the skin ecosystem of an animal species or a group of phylogenetically close animals [90,91].

Despite being part of the microbiota, under certain circumstances the population of these yeasts can proliferate excessively and act as opportunistic pathogens, causing various skin

pathologies [50,92]. The factors involved in the transformation of these yeasts from commensal to pathogenic are still unknown, as are the factors that predispose to it [50].

2.3.1. *Malassezia* in humans

Malassezia yeasts colonize human skin with a predilection for anatomic site with a higher proportion of sebaceous glands such as the face, scalp, or the trunk. *Malassezia* is the predominant fungal genus on the human skin except for the feet [94]. The predominant species, in non-culture-based studies, in human skin are *M. globosa* and *M. restricta* [4,24] but other species such as *M. furfur, M. sympodialis, M. obtusa*, *M. dermatis*, *M. japonica* and *M. arunalokei* have been isolated [16,51] (Table 1).

Colonization of human skin by *Malassezia* begins short after birth and increases in the first weeks of life [95]. Since the distribution of *Malassezia* on human skin is associated with the distribution of sebaceous glands, age is therefore an important factor involved in the proliferation of these yeasts. Sebaceous glands have minimal activity during childhood and are activated at puberty [94,96].

Another relevant factor is gender, with men being the most predisposed [93,94]. Interestingly in humans, the *Malassezia* species present on the skin may be conditioned by geographic distribution. Thus, *M. dermatis* has been isolated mostly in East Asia [4] whereas *M. anuralokei* has been isolated in India [16] and *M. obtusa* has been isolated mostly in Sweden, Canada, Bosnia, and Herzegovina [4].

Malassezia species are associated with different diseases in human skin [94]. The most common ones are pityriasis versicolor, seborrheic dermatitis, atopic dermatitis, and

folliculitis [51]. Cases of iatrogenic fungemias in hospitals caused by *M. pachydermatis, M. furfur* and *M. sympodialis* have been reported. These infections usually occur in neonatal or immunocompromised patients and are related to parenteral nutrition [97-103].

2.3.2. *Malassezia* in animals

Malassezia is part of the normal microbiota of a wide range of warm-blooded vertebrate animals [51,90]. *Malassezia* species have been isolated from almost every domestic animal species [17-20,104,105], but also from wild animals in captivity [106,107] and in their natural habitat [21,90].

Malassezia colonization of animals' skin probably occurs at birth or in the first days of life. It is not completely understood how it occurs but likely involves transfer from the mother's mycobiota and contact with the mother and the environment. There are different factors that determine the proliferation of *Malassezia* on animal skin such as the environment, the animal's morphology, cohabitation with other animals and health status. Also, there is an anatomic predisposition being the perioral region and the interdigital space the sites with higher population of the yeasts [50].

Although a wide variety of *Malassezia* species have been reported in animal skin some studies are solely based on phenotypic identification and not confirmed by rDNA sequencing. Thus, only isolates confirmed by DNA sequencing, can be recognised as part of an animal host's skin [50,51]. *Malassezia* spp. are frequently isolated from domestic carnivores (Table 1), especially from canine skin [51]. *Malassezia pachydermatis* has been isolated from healthy dogs and cats but also from dogs and cats with otitis externa being the predominant species in dogs and cats [51,90]. In dogs, the lipid dependent species *M.*

nana and *M. furfur* have also been isolated [50,108]. In cats, *M. nana* is the most common lipid dependent species but other lipid dependent species such as *M. furfur* and *M. slooffiae* have been isolated [50,108].

Lipid-dependent species, other than *M. pachydermatis* constitute the major population of *Malassezia* in herbivores, such as horses, sheeps, cows and goats (Table 1). The occurrence of traditional lipid-dependent species of *Malassezia* in herbivores is much greater than that of *M. pachydermatis* [51,105]. *Malassezia furfur* has been isolated from cattle, sheep, goat, and horse [108] whilst *M. globosa* and *M. nana* have only been isolated from cattle [17,109]. *Malassezia caprae* and *M. equina* have only been isolated in herbivores [18].

The skin of pigs is colonized by a diversity of *Malassezia* species. *Malassezia pachydermatis, M. furfur, M. sympodialis* and *M. slooffiae* have been isolated from the external ear canal and the skin of healthy pigs [108,110,111]. Also, *M. pachydermatis* has been isolated from the external ear canal of pigs with otitis externa [112,113].

In birds, different *Malassezia* species have been isolated such as *M. brasiliensis, M. psittaci* [20], *M. furfur* and *M. pachydermatis* [108,113].

Malassezia species are rarely isolated from lagomorphs. Only the lipid dependent species *M. cuniculi* has been described in the skin and the external ear canal of healthy rabbits [19].

Recently, a new *Malassezia* species, *M. vespertilionis* was described in bats, and is the only species of the genus isolated from this animal host [21].

Table 1. Summary of *Malassezia* taxonomy and main hosts [16,20,21,50,90,108].

***Malassezia* species**	**Main host/others[a]**
M. furfur, (Robin) Baillon, 1989	Human/pig, elephant, cattle, monkey, sheep, horse, goat, cat, dog, ibis, ostrich, parrot, spoonbill
M. pachydermatis, (Weidman) Dodge, 1925	Dog, cat/carnivores, birds, pig
M. sympodialis, Simmons & Guého, 1990	Human/ pig, sheep, horse
M. globosa, Midgley et al., 1996	Human/cheetah, cattle
M. obtusa, Midgley et al., 1996	Human
M. restricta, Guého et al., 1996	Human
M. slooffiae, Guillot et al., 1996	Human, pig/ cat, sheep, goat
M. dermatis, Sugita et al., 2002	Human
M. japonica, Sugita et al., 2003	Human
M. nana, Hirai et al., 2004	Cat, cattle/ dog
M. yamatoensis, Sugita et al., 2004	Human
M. caprae, Cabañes & Boekhout, 2007	Goat/ horse
M. equina, Cabañes & Boekhout, 2007	Horse/ cattle
M. cuniculi, Cabañes & Castellá, 2011	Rabbit
M. arunalokei, Honnavar et al., 2016	Human
M. brasiliensis, Cabañes et al., 2016	Parrot
M. psittaci, Cabañes et al., 2016	Parrot
M. vespertilionis, Lorch & Vanderwolf, 2018	Bat

[a]Cited only species confirmed by sequencing methods.

2.3.3. *Malassezia* related diseases in animals

Many factors may be associated with an overgrowth of *Malassezia* yeasts in animals and with their transformation from commensal to opportunistic pathogen [51]. The proliferation of *Malassezia* yeasts in animals is dependent upon the metabolic activities of the yeasts and the host's immune response. Interaction with other skin commensals (especially *Staphylococcus* spp.) may also play an important role [50].

The presence of *Malassezia* yeasts within the stratum corneum of the skin, exposes the host to an array of allergens, immunogens, and chemicals such as cell-wall carbohydrates, proteins and lipids, secreted enzymes, and metabolic by-products [91,114,115]. *Malassezia* antigens can stimulate the host's immune response as well as triggering hypersensitivity reactions [93]. Although the activation of the immune response offers a degree of protection against the over proliferation of the yeasts, it may also be harmful causing inflammation the skin leading to pruritus and dermatitis [50].

There are many predisposing factors for the development of *Malassezia* diseases. Some dog breeds have a predilection for *Malassezia* overgrowth such as Dalmatian, German Shepherd, West Highland White Terrier, American Cocker Spaniel, English Setter, Shih Tzu, Australian Silky Terrier, Basset Hound and German Shorthaired Pointer [51]. Also, there are some feline predisposed breeds as Devon Rex and Sphynx that show increased *Malassezia* counts when compared to other breeds [50].

Malassezia diseases in dogs and cats are usually a secondary process associated with an alteration of the skin immune system or alterations of the skin's surface microclimate [50]. In dogs, hypersensitivity disorders (especially in atopic dermatitis), primary cornification

defects of the skin and endocrinopathies are common underlying factors and need to be managed together with the antifungal treatment [93]. Also, *Malassezia* dermatitis is more common in tropical climates and during warm and humid months in temperate areas. Thus, the animal's external environment is an important predisposing factor. In cats, skin folds and allergies are also predisposing factors. Moreover, *Malassezia* dermatitis in older cats is occasionally associated with visceral neoplasia [50].

Malassezia diseases have also been described in other animal species [35]. Exfoliative dermatitis has been described in an Indian rhinoceros [116] and in a Southern White rhinoceros [117]. A dermatitis caused by *M. pachydermatis* has been also reported in a California sea lion [118]. Also, otitis externa caused by *M. pachydermatis* has been described in ferrets, fennecs, pigs, and dromedaries [35].

2.3.3.1. Dermatitis

In *Malassezia* dermatitis, the yeast population can be increased up to 100-10.000 folds the values of healthy skin. However, clinical signs are associated to a hypersensitivity reaction against the yeasts. The over proliferation of *Malassezia* yeasts within the stratum corneum of the skin activates the immune response through the release of antigens. This activation leads to epidermal damage and inflammation. Thus, low yeast counts can potentially produce severe clinical signs and any number of *Malassezia* yeasts may be consider relevant when associated with them [50].

Skin lesions associated with *Malassezia* dermatitis are usually erythematous, with varying degrees of alopecia and keto-sebaceous scale that can be brown. In addition, a greasy exudate is a common feature of lesions and is normally accompanied by a

prominent malodor. Also, hyperpigmentation, lichenificaton and papules are frequently observed in chronic cases. Skin lesions may be localized or generalized and inter-triginous and interdigital areas are commonly affected [50,93]. Pruritus is a common clinical sign, that may vary from mild to very severe [50,93], and it is manifested as head shaking, face rubbing, paw licking and scratching [50].

2.3.3.2. Otitis externa

Otitis externa is the most common disorder of the canine external ear canal, affecting approximately 15% of the dogs presenting at the veterinary clinic [119,120]. This disease is defined as an inflammation of the external ear canal with or without infection. *Malassezia pachydermatis* is the most common etiological agent associated with canine otitis externa and is involved in up to 57% of all infections [119,121]. It is also the most common *Malassezia* yeast isolated from cats' ears, being the frequency of isolation increased in cats with otitis externa [50].

Malassezia overgrowth in the ears typically results in pruritic, erythematous, and ceruminous otitis externa that is usually accompanied by a brownish discharge. In chronic cases, the medial aspect of the pinna may become lichenified [50].

2.3.3.3. Treatment

Current options for the treatment of *Malassezia* diseases include various antiseptics such as chlorhexidine, systemic and topic therapy with several antifungal agents. Those antifungal agents belong to different chemical classes and have different mechanisms of action. Due to the intrinsic resistance of *Malassezia* species to some antifungals, particularly echinocandins, the primary antifungal treatment for *Malassezia*-associated diseases are azole antifungals such as clotrimazole, ketoconazole, itraconazole and

miconazole [93,122]. Azole-containing compounds functions by interacting with the lanosterol-14α-demethylase that is involved in the synthesis of ergosterol [123,124]. Also, polyene macrolides like nystatin and natamycin, that interact with membrane sterols, are also effective against *M. pachydermatis*, but at higher concentrations *in vitro* [93,123]. The use of AMB, a polyene macrolide, though very active against *M. pachydermatis*, is not recommended in dogs due to its cost, difficulty of usage and toxicity [50,124]. Thiabendazole, a benzimidazole, is useful to control *Malassezia* overgrowth and produced the inhibition of microtubule assembly in yeast cells [123,124].

However, since *Malassezia* related diseases are usually secondary problems in most cases, it is of utmost importance to identify and resolve the primary underlying condition [124-126]. On the other hand, in some cases like predisposing breeds, it is not possible to identify the underlying cause and the disease resolves completely after treatment with antifungals [92,124].

2.3.4. Antifungal resistance in *M. pachydermatis*

Antifungal treatments are generally successful in controlling and resolving *Malassezia* yeast overgrowth and disease, but failure or rapid recurrence occasionally occur. Usually, the reason for failure is the lack of identifying and resolving predisposing factors and primary causes [124,125,127]. In fact, most wild-type *Malassezia* yeast are susceptible to the most used azole antifungals such as ITZ, KTZ and MCZ [128,129], although the efficacy of FLZ is variable [130,131].

However, some studies in *M. pachydermatis* showed that strains can become resistant to azoles by inducing resistance *in vitro* [132]. Recently, some sporadic cases of treatment

failure with azoles in canine *M. pachydermatis* dermatitis associated with an increased azole tolerance *in vitro* have been reported [133,134]. Only two *M. pachydermatis* strains have demonstrated, at the present, to be resistant to azoles [135,136].

2.3.4.1. Susceptibility testing

Establishing a set of standardized criteria for *in vitro* susceptibility testing is essential, since different testing variables are known to have an impact on *in vitro* determinations [124]. In 2002, the Clinical and Laboratory Standards Institute (CLSI) developed a reference broth microdilution method for susceptibility testing of *Candida* spp. and *Cryptococcus neoformans* [137]. However, it is universally accepted that these general conditions designed for yeast are not suitable for susceptibility testing of *Malassezia* species due to their unique physiologic features such as their lipid dependency, slower growth rate and tendency to form clusters [124,138]. Therefore, different adjustments have been adopted to susceptibility testing the genus, such as modifying the media, time of incubation and inoculum but official guidelines are not available yet [130,138-140]. Also, other susceptibility testing methods as disk diffusion method and E-test gradient have been applied on *Malassezia* [124,128].

However, officially recognized interpretive criteria of *in vitro* results, clinical breakpoints and epidemiological cut-off values are not available for *Malassezia* spp. While *in vitro* susceptibility results are indicators of the relative susceptibility of a strain to antifungals, they are insufficient to predict the clinical outcome *in vivo* [124].

2.3.4.2. Mechanisms of resistance

Mechanisms of antifungal resistance are related to intrinsic and acquired characteristics of fungal pathogens that interfere with the antifungal mechanism of drugs or lower drug levels [124,141]. In *Malassezia*, reduced azole susceptibility may be caused by a reduced affinity of the target for the azole [136] or by increased expression of the gene (*ERG11*) encoding the target enzyme [142]. Also, an increased efflux by overexpression of genes encoding membrane transporters of the ABC transporter family (CDR1/CDR2) or the major facilitator (MDR1) family have been suggested [50].

2.3.4.2.1. Alterations of the *ERG11* gene

Azole antifungals act by inhibiting a cytochrome P450 enzyme, lanosterol-14-α-demethylase, which participates in the synthesis of ergosterol, a major constituent of the fungal cell membrane [124,143]. This enzyme is encoded by the *ERG11* gene that belongs to the cytochrome P450 monooxygenase (CYP) superfamily [144]. The inactivation of this enzyme causes the demethylation of lanosterol, that inhibits the synthesis of ergosterol and leads to the accumulation of bypass products as toxic methylated sterols in the fungal membrane, defects in fungal plasma membrane integrity, and inhibition of cell growth [143,145]. However, azole antifungals are fungistatic and prolonged treatments offers the opportunity to acquire resistance.

Mutations in the coding region of the *ERG11* gene, produce amino acid substitutions which alter the structure of the enzyme and thus, reduce the affinity of the antifungal drug to their target [146,147]. Some studies have reported the isolation of strains of different *Malassezia* spp. with tolerance to different azoles associated with mutations in the *ERG11* gene [136,148-150].

Also, overexpression of the *ERG11* gene that results in increased levels of the antifungal target protein, can also be a mechanisms of azole resistance [151,152]. A tandem quadruplication of a region in chromosome 4, that includes *ERG11* and *ERG4* genes whose overexpression is associated with azole resistance, was detected in *M. pachydermatis* isolates with a high MIC to KTZ [142].

2.3.4.2.2. Efflux pumps

The increased expression of drug efflux pumps is considered the major cause of azole resistance in *Candida* spp. There are two classes of efflux pumps characterized, the ATP-binding cassette (ABC) transporters (encoded by *CDR1/CDR2* genes) that use as energy source the hydrolysis of ATP, and the major facilitator superfamily (encoded by *MDR1* gene) that use membrane potential to drive the efflux. The up regulation of the genes coding the transports induce resistance in *Candida* spp. [141,153]. It has been reported that efflux pumps, especially the major facilitator superfamily might be a defense mechanism against azoles in *M. restricta* [143], *M. pachydermatis* and *M. furfur* [154].

3. HYPOTHESIS

The hypotheses proposed in this doctoral thesis are the following:

1. It is difficult to detect and isolate *Malassezia* yeasts from lagomorphs. Although *Malassezia cuniculi* is part of their skin it is possible that other species, difficult to isolate, can be found in this location.
2. The population of *M. pachydermatis* may vary among samples obtained from dogs with pododermatitis, dogs after antifungal treatment and healthy dogs. The diversity of the mycobiota may be affected in cases of pododermatitis. Perhaps, there is a correlation between traditional and molecular methods used to detect this variation.
3. The variability of the *ERG11* gene in *M. pachydermatis* strains isolated from animals is unknown. There may be a correlation between *ERG11* gene mutations and a reduced azole susceptibility. Also, there may be a correlation between *ERG11* gene DNA sequences and certain genotypes.

4. OBJECTIVES

The main objective of this thesis is to study *Malassezia* species from the skin of dogs and rabbits using routine laboratory methods but also advanced molecular methods. To this end, different studies were carried out with the following objectives:

1. To study the external ear canal mycobiota of healthy rabbits of different breeds using advanced molecular methods like NGS. Also, to determine whether a predisposition to an increased *Malassezia* population exists associated to the ear morphology.
2. To study the variation of the *Malassezia* load in samples from interdigital fold of healthy dogs, dogs with pododermatitis and dogs after treatment with an antifungal agent using cytology, culture, qPCR and NGS. Also, to study de mycobiota of the interdigital fold of healthy dogs, dogs with pododermatitis and after antifungal treatment and to determine whether a difference exist.
3. To evaluate the *in vitro* antifungal susceptibility of *M. pachydermatis* strains from different origins against the most used antifungals to treat *Malassezia* skin diseases in dogs.
4. To study the correlation between antifungal resistance and mutations in the *ERG11* gene sequence. Also, to study the genetic variability of this gene within strains of *M. pachydermatis* isolated from different origins.

5. PAPERS

5.1. Díaz L, Castellá G, Bragulat MR, et al. External ear canal mycobiome of some rabbit breeds. *Med Mycol*. 2021; 59(7): 683-693. doi:10.1093/mmy/myaa097.

Medical Mycology, 2021, 59, 683–693
doi:10.1093/mmy/myaa097
Advance Access Publication Date: 28 December 2020
Original Article

Original Article

External ear canal mycobiome of some rabbit breeds

Leyna Díaz[1], Gemma Castellá[1,*], M. Rosa Bragulat[1], Jaume Martorell[2], Andreu Paytuví-Gallart[3], Walter Sanseverino[3] and F. Javier Cabañes[1]

[1]Veterinary Mycology Group, Department of Animal Health and Anatomy, Universitat Autònoma de Barcelona, Bellaterra, Catalonia, Spain, [2]Department of Animal Medicine and Surgery, Universitat Autònoma de Barcelona, Bellaterra, Catalonia, Spain and [3]Sequentia Biotech SL, Barcelona, Catalonia, Spain

*To whom correspondence should be addressed. Gemma Castellá. Grup de Micologia Veterinària. Departament de Sanitat i d'Anatomia Animals, Facultat de Veterinària, Universitat Autònoma de Barcelona, E-08193 Bellaterra (Barcelona), Spain. Tel: +34 93 5811089; Fax: +34 93 5812006; E-mail: Gemma.Castella@uab.cat

Received 31 July 2020; Revised 28 September 2020; Accepted 13 November 2020; Editorial Decision 6 November 2020

Abstract

The genus *Malassezia* is part of the normal skin mycobiota of a wide range of warm-blooded animals. In this genus, *M. cuniculi* is the only species described from rabbits. However, *Malassezia* species are rarely studied in lagomorphs. In the present study, the presence of *Malassezia* was assessed in samples from the external ear canal of healthy rabbits of different breeds. Cytological and culture techniques, Sanger sequencing, and Next-generation sequencing (NGS) were used to describe the ear mycobiota in the samples. Although no growth was observed in the cultured plates, cytological examination revealed the presence of round cells similar to those of *Malassezia* yeasts. For metagenomics analysis, the D1/D2 domain of the large subunit of the ribosomal DNA (LSU rDNA) was PCR amplified and the resulting reads were mapped against a custom-made cured database of 26S fungal sequences. NGS analysis revealed that *Basidiomycota* was the most abundant phylum in all the samples followed by *Ascomycota*. *Malassezia* was the most common genus presenting the highest abundance in the external ear canal. *Malassezia* phylotype 131 and *M. cuniculi* were the main sequences detected in the external auditory canal of rabbits. The study included both lop-eared and erect-eared rabbits and no differences were observed in the results when comparing both groups. This is the first attempt to study the external ear canal mycobiome of rabbits of different breeds using NGS.

Lay Summary

In the present study, the presence of *Malassezia* was assessed in samples from the external ear canal of healthy rabbits of different breeds. Cytological and culture techniques, Sanger sequencing, and Next-generation sequencing (NGS) were used to describe the ear mycobiota in the samples.

Key words: *Malassezia*, *M. cuniculi*, phylotype 131, NGS, ear mycobiome, rabbits.

Introduction

Yeasts of the genus *Malassezia* belong to the normal skin microbiota of a wide range of warm-blooded animal species. The genus *Malassezia* includes, at the present, 18 species that have been mostly isolated from different wild and domestic animals but also from human hosts.[1–3] Some *Malassezia* yeasts appear to have a broad host range, whereas others are more host-specific with a close adaptation to the cutaneous ecosystem of a single animal species or a group of phylogenetically related animals like *M. cuniculi* that has only been isolated from rabbits.[4] Although *M. caprae* and *M. equina* are recovered from ruminants and horses,[5] *M. pachydermatis* is the main species in dogs and *M. nana* is commonly isolated from cats.[6,7] In parrots, *M. brasiliensis* and *M. psittaci* have been described[8] and *M. vespertilionis* was recently described in bats.[2,9] However, *Malassezia* species are rarely isolated and studied in lagomorphs.[10] Since the description of *M. cuniculi*,[4] a few more studies have been conducted in rabbits.[11,12] At present, this is the first attempt to

study *Malassezia* and the mycobiome in rabbits using advanced molecular techniques like NGS.

Members of the genus *Malassezia* are lipophilic and lipid-dependent yeasts that require specific media with a specific lipid composition for laboratory growth, such as Leeming & Notman agar (LNA)[13] and modified Dixon agar (mDA).[14] Because of their lack of genes involved in carbohydrate metabolism and the genes encoding for the fatty acid synthase, they are unable to synthesize long-chained fatty acids (C14 or C16) de novo thus, they rely on the host as an exogenous source of fatty acids.[15,16] They have a predilection for seborrheic skin sites such as the external ear canal, the scalp, the trunk, and the perianal, inguinal and submandibular areas.[17] *Malassezia pachydermatis* is the only member that is able to grow on Sabouraud's glucose agar (SGA) even though they lack the same genes, is uniquely able to utilize lipid fractions within the peptone component of SGA.[16,18,19]

These growth requirements could be the result of a host adaptation phenomenon among *Malassezia* species. In healthy conditions, the distribution of *Malassezia* species, the proportion of colonization, and its density may vary according to the animal host and even between anatomic sites on the host. This likely is a result of the cutaneous lipid composition and the competition of different types of microorganisms. Many other factors such as environmental conditions, age, and gender of the host could influence the distribution of *Malassezia* spp.[10] In rabbits, a lop-eared morphology is suggested as a predisposing factor for *Malassezia* overgrowth. This morphology causes a stenosis of the ear canal that produces a reduction of the expulsion of cerumen. Therefore, an accumulation of cerumen at the base of the ear occurs.[11]

Malassezia species are identified based on morphological, ultrastructural, physiological, and molecular analyses.[20] Members of the genus *Malassezia* share similar morphological and biochemical characteristics, so differentiating between species based solely on phenotypic features could cause ambiguity, is time consuming, and cannot be used to describe new species. Molecular techniques are the most reliable for the identification of *Malassezia*.[10] DNA sequencing has become a widely used method to identify *Malassezia*. In particular, sequencing the domains D1/D2 of the large subunit of the ribosomal DNA (LSU rDNA), internal transcribed spacer region 1 and 2 (ITS), β-tubulin, and chitin synthase 2 (CHS2) genes has become a useful method to identify and differentiate species within the genus *Malassezia*.[10,21,22]

Next-generation sequencing (NGS) platforms perform sequencing of millions of small fragments of DNA in parallel, then bioinformatics analyses are needed to piece together these fragments by mapping the individual reads to the reference database. NGS provided a culture-independent method to obtain microorganisms' genome information with high sensitivity at a lower cost. Culturable microorganisms represent only a small fraction of the microbial diversity. NGS represents a useful tool to fully investigate and understand microbial diversity without the need to culture the samples. This method can be used to explore the genetic diversity, population structures, and interactions of microbial communities in their ecosystems.[23–26] Several regions of the fungal rRNA genes have been used in NGS to study fungal taxonomy and diversity, including the small subunit (SSU) and the large subunit (LSU) rRNA genes and the internal transcribed spacer (ITS).[27,28] Few metagenomic studies have been conducted in fungi due to the complexity of their genomes, which are generally larger than the prokaryotic genomes because they contain large amounts of non-coding and repetitive DNA, and the lack of validated databases cataloging enough diversity.[29,30]

The aim of this work was to study the external ear canal *Malassezia* population in different rabbit breeds using culture and non-culture based techniques, including NGS, and to identify the presence of non-culturable *Malassezia* yeasts in the skin of these animals.

Materials and methods

Animals, sampling, and culture media used

A total of 60 rabbits were sampled including pet and farmed rabbits (Table 1, Supplementary Table S1). All pet rabbits (n = 44) presented to the Exotic Pets Service of the Veterinary Teaching Hospital of the Universitat Autònoma de Barcelona (UAB, Cerdanyola del Vallès, Spain) from 2017–2019, were considered for the study regardless of the presenting reason for consultation. Farmed rabbits (n = 16) were housed on the Experimental Farm Service of the UAB. In all cases, the criterion for inclusion was the absence of history and clinical signs of otitis externa, confirmed by otoscopic examination. Both lop-eared breeds such as Belier and Toy, and erect-eared breeds such as Rex and New Zealand were included in this study.

Samples were collected from the external ear canals of both ears by using a swab soaked in the wash fluid, that is, 0.075 mol/L phosphate-buffered physiological saline, pH 7.9 containing 0.1% Tween 80. Samples were obtained following procedures approved by Ethics Committee on Animal and Human Experimentation from UAB and Generalitat de Catalunya (approval CEEAH 4600).

Two samples from each ear were taken by gently inserting a sterile cotton swab into the external part of the ear canal and rotating fully for 30 s. One swab was used for *Malassezia* culture and cytological examination. Briefly, one side of the swab was streaked onto the following media: SGA (Oxoid), mDA (36 g malt extract, 6 g peptone, 20 g desiccated ox-bile, 10 mL Tween 40, 2 mL glycerol, 2 mL oleic acid, and 12 g agar per liter, pH 6.0)[14] and LNA (10 g peptone, 5 g glucose, 0.1 g yeast extract, 4 g desiccated ox-bile, 1 mL glycerol, 0.5 g glycerol monostearate, 0.5 mL Tween 60, 10 mL whole-fat cow's milk, and 12 g agar per liter, pH 6.2).[13] All media contained 0.05% of chloramphenicol and 0.05% of cycloheximide. Plates

Table 1. Animals per breed included in this study and results of the cytological examination.

	Pet rabbits (n = 44)			Farmed rabbits (n = 16)	
Breed	Number of animals	Positive cytology	Breed	Number of animals	Positive cytology
New Zealand	8	6	New Zealand	13	13
Belier	10	5	Belier	-	-
Rex	3	2	Rex	3	1
Toy	21	5	Toy	-	-
Mixed breed	2	1	Mixed breed	-	-
Total	44	19		16	14

were incubated at 32°C and examined daily for 20 days. For the cytological examination smears from the cotton swabs were stained with Diff-Quick stain and the presence of typical *Malassezia* cells was microscopically determined. The other swab was maintained at –20°C and used for PCR and NGS.

DNA extraction

Six ear samples cytologically positive for *Malassezia* yeasts like cells and from different pet rabbit breeds were selected for Sanger sequencing studies (Supplementary Table S1). We used Sanger sequencing in these few samples to obtain the complete sequence of the D1D2 and ITS regions in order to get a good identification with BLAST and a suitable sequence for inferring accurate phylogenetic relationships. DNA was extracted from swabs using the DNeasy PowerSoil Kit (Qiagen, Madrid, Spain) according to manufacturer's instructions with two modifications. At the first lysis step, the swab tip was cut and placed in the bead tube, and an incubation step at 65°C for 10 min following the addition of Solution C1. The final elution step was performed on 50 μL of C6 instead of 100 μL to obtain a higher DNA concentration. A sterile swab was processed under the same conditions as the external ear canal samples in order to control cross-contamination. DNA was stored at −20°C until used as a template in the PCR for Sanger sequencing and NGS.

Sanger sequencing

The variable D1/D2 region of the 26S rRNA gene and the ITS-5.8S rRNA gene were amplified and sequenced, using the primers and the protocols described previously.[21] Sequence alignments were carried out using MUSCLE implemented in MEGA X software.[31] Maximum likelihood analysis of the individual genes was conducted using MEGA 6 software with 1000 bootstrap replicates. A suitable substitution model was determined for each gene. The initial tree for heuristic search was obtained by applying the Neighbour-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. Clades that were supported by bootstrap values (bs) of ≥70% were regarded as strongly supported. Sequences of *Cryptococcus neoformans* CBS 132 were selected as an outgroup for the tree construction.

NGS and data analysis

A total of four ear samples were subjected to metagenomics NGS of fungal 26S rRNA genes. Three samples were previously selected for Sanger sequencing and a sample of a farmed rabbit cytologically positive to *Malassezia* yeasts like cells was also included (Supplementary Table S1). The samples covered animals from four different breeds: New Zealand (farmed rabbit), Rex, Belier, and Toy (pet rabbits). All the animals showed no clinical signs and were free from otitis externa. Both lop (Belier and Toy) and erect-eared (Rex, New Zealand) were included. DNA was extracted as previously described and a positive PCR for D1D2 region was obtained before library preparation.

Quality control was performed at IGA Technology. DNA concentration was evaluated by using a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA). Amplicon-seq libraries of D1/D2 regions of the fungal 26S rRNA gene were obtained from each sample by following 16S Metagenomic Sequencing Library Preparation protocol with minor modifications. Briefly, a composite pair of primers: the forward primer (NL1) 5′-GCATATCAATAAGCGGAGGAAAAG-3′ and the reverse primer (NL4) 5′-GGTCCGTGTTTCAAGACGG-3′[32] containing Illumina overhang sequences necessary for the compatibility with Illumina index and sequencing adapters were used for the first PCR amplification under the following conditions: 95°C for 3 min; 28 cycles of: 95°C for 30 s, 55°C for 30 s, 72°C for 30 s; 72°C for 5 min; hold at 4°C. Upon the clean-up, the second PCR was performed under the following conditions: 95°C for 3 min; nine cycles of: 95°C for 30 s, 55°C for 30 s, 72°C for 30 s; 72°C for 5 min; hold at 4°C. Relevant flow-cell binding domains and unique indices (NexteraXT Index Kit, FC-131-1001/FC-131-1002) were integrated to the amplicon target. Libraries were normalized by Qubit 2.0 Fluorometer, pooled, and sequenced on MiSeq using paired 300-bp reads, and MiSeq v3 reagents (Illumina, San Diego). Sequence reads were analyzed in the cloud bioinformatics platform GAIA (metagenomics.sequentiabiotech.com).[33] Sequencing quality was assessed using

FastQC (bioinformatics.babraham.ac.uk/projects/fastqc/) and BBduk (jgi.doe.gov/data-and-tools/bbtools/), setting a minim length of 35 bp and a minimum Phred-quality score of 25.

The resulting high-quality reads were mapped against a custom-made cured database of 26S fungal sequences from NCBI. To have the dataset as complete as possible for the analysis, all the sequences were downloaded from GenBank with the queries '28S rRNA' or '28S ribosomal RNA' not 'uncultured' not 'fungal endophyte' not 'fungal sp.' Also, our dataset included a sequence for every *Malassezia* species even the nonculturable ones. The database included only fungal sequences due to the aim of this study. For taxonomic classification, the mapping-based approach against the database with the BWA mapper[34] is followed by an in house Lowest Common Ancestor (LCA) algorithm. The minimum identity thresholds applied to classify the reads into different taxonomic levels were species (99%), genus (98%), family (96%), order (94%), class (92%), and phylum (90%) following the limits proposed by GAIA software. Those taxa with an abundance below 0.01% considering its mean across the different samples within an experimental group were filtered out before further analysis. DESeq2 (version 1.26)[35] was used to carry out differential abundance analyses.

GAIA also assesses the diversity within (alpha-diversity) and between (beta-diversity) samples. Alpha-diversity measures the richness (number of OTUs) and evenness (the relative abundance of OTUs) in samples. On the other hand, beta-diversity measures the distance or dissimilarity between pairs of samples. Alpha and beta diversities are calculated using the R package phyloseq.[36]

Results

Culture and cytological examination

The presence of spherical *Malassezia* yeast-like cells was identified on 33 out of the 60 rabbits (Table 1; Supplementary Table S1). The cells observed were spherical with buds in a monopolar pattern on a narrowed base that in some cases were elongated but remained more narrow than the bud (Figure 1). No growth was observed in any of the culture media used after 20 days of incubation at 32°C (Supplementary Table S1).

Sanger sequencing

D1/D2 region was successfully amplified and sequenced for five swabs (Supplementary Table S1) resulting in a product of 603 bp. The sequences were nearly identical showing only a base pair difference.

A BLAST search against the NCBI database revealed that this sequence had a % identity of 100% to an uncultured Basidiomycota clone 131[37] with a query coverage of 74%, and 91% to the sequence GU733708 belonging to *M. cuniculi* CBS 11721 type strain, which was the closest match. Our sequences differed from the sequence of *M. cuniculi* at 56–57 positions (dissimilarity 9%).

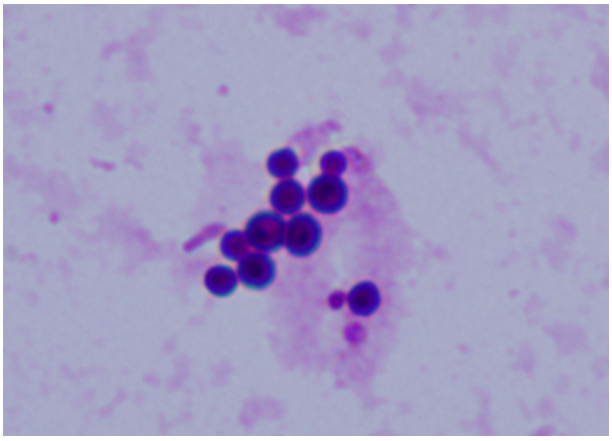

Figure 1. Diff-Quick stain of a smear from an otic swab of a rabbit showing the presence of spherical yeasts cells, possibly of *Malassezia* phylotype 131 (2-2.5 μm in diameter).

ITS-5.8S rRNA was successfully amplified and sequenced for five swabs (Supplementary Table S1), resulting in a product of 807 base pairs. These sequences obtained were identical. A search on GenBank database revealed that this sequence had a percent identity of 80.74% to *M. cuniculi* CBS11721 type material (NR_137752).

The sequences generate have been deposited at the GenBank database under accession numbers MT812469, MT812503, MT812504.

Maximum likelihood analysis of the D1/D2 and ITS-5.8S rRNA sequences are shown in Figures 2 and 3, respectively. With both genes, the sequences obtained clustered close to *M. cuniculi* CBS11721.

NGS data analysis

All samples were correctly sequenced, and the generated fastq files reported an average value of 52,886 reads passing the filter. The number of generated sequence reads of each sample is described in Table 2. The raw sequencing data is available at the NCBI database, SRA accession PRJNA649860.

To characterize the diversity of species in each sample we used the Shannon diversity index (Table 2) which increases as both the richness and the evenness of the community increase. We obtained an average of 120 species and a diversity of 2.43. The sample from Rex rabbit was the most diverse among the group and the sample from New Zealand rabbit showed the least diversity.

We investigated the taxonomic compositions of all samples at various taxonomic levels. Fungi from three different phyla were identified (Figure 4). The most abundant fungal phyla across all samples was *Basidiomycota* with a median relative abundance of 56.29% (range, 43.77-83.49%) followed by *Ascomycota*

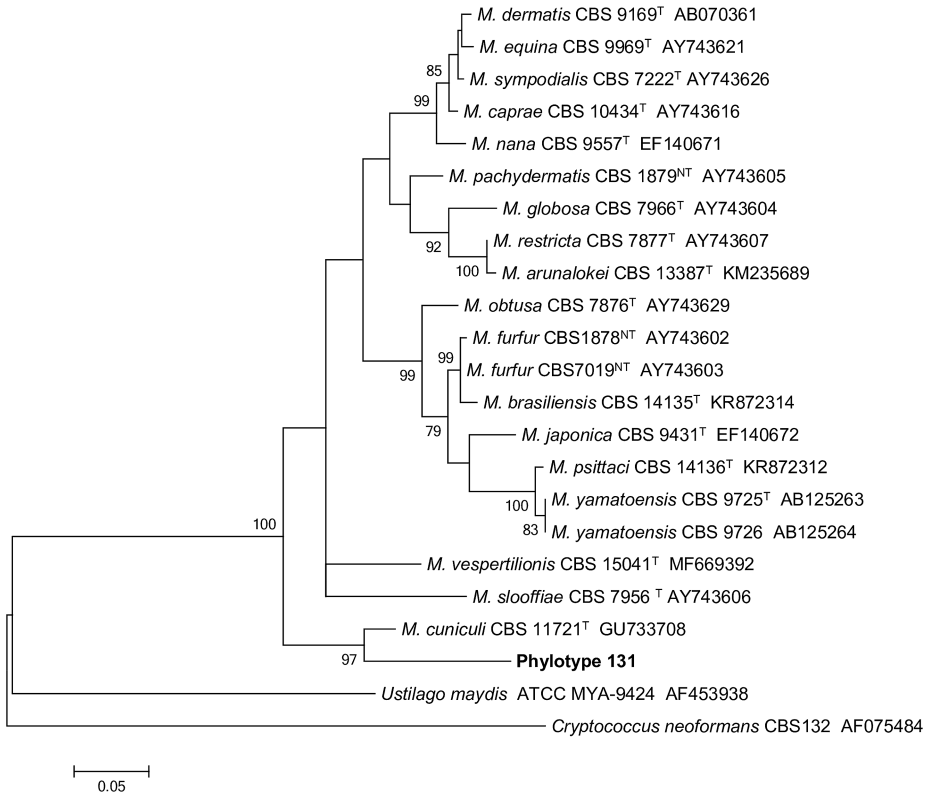

Figure 2. Molecular phylogenetic tree inferred from maximum likelihood analysis of D1D2 sequences of members of the genus *Malassezia*. Bootstrap values > 70% in 1000 replications are shown at the nodes. Sequences of *Ustilago maydis* ATCC MYA-4924 and *Cryptococcus neoformans* CBS 132 were selected as outgroup for the tree construction.

(median = 17.32%; range, 2.68-27.91%) and *Mucoromycota* (median = 0.25%; range, 0.09-0.38%).

When analysed at class (Figure 5), order (Figure 6) and family level (Figure 7), *Malasseziomycetes* (51.89%), *Malasseziales* (51.77%), and *Malasseziaceae* (51.39%) represented the highest abundance. As shown in Figure 8, within the phylum *Basidiomycota*, the most common genus was *Malassezia* (30.56-78.63%; median = 48.55%), followed by *Filobasidium* (0.04-0.79%; median = 0.37%) and *Vishniacozyma* (0.06-1.75%; median = 0.21%) in much smaller percentage. Within the phylum *Ascomycota* the three most common genera were *Cladosporium* (0.65-7.39%; median = 3.25%), *Fusarium* (0.00-1.25%; median = 0.55%) and *Alternaria* (0.21-0.98%; median = 0.40%). Within the phylum *Mucoromycota* not enough reads were classified at the level of genera.

Different fungal taxa were identified in the samples as shown in Figure 9. *Malassezia* phylotype 131 was detected in all samples and it was the predominant taxa (median = 41.45%; range, 26.08-69.13%). This study identified other *Malassezia* species (Figure 10). *Malassezia cuniculi* was also detected from all samples but in lower abundance (median = 0.02%; range, 0.01-0.03%). *Malassezia pachydermatis* and *M. restricta* were only present in two samples corresponding to the Rex and New Zealand rabbits. An average of 10.09% of the *Malassezia* sequences was not identified to species level. Within this percentage, the majority of sequences were marked as unknown because a match with enough coverage and identity was not found in our database for them. Within this group of unknowns, 7.20% were identified to the genus level, 2.50% to the family level, and 0.36% and 0.16% to order and class level, respectively. A small percentage of the sequences (0.036%) were considered ambiguous because their sequence matched with two different *Malassezia* species sequences.

Neither *Malassezia* phylotype 131 nor *M. cuniculi* were present in the sterile cotton swab used as control. In this swab, *M. globosa*, *M. pachydermatis*, and *M. restricta* were detected at a percentage between 0.52% and 1.94%.

No significant differences were observed in the amount of *Malassezia* sequences detected between lop and erect-eared rabbits.

Discussion

The presence of *Malassezia* in the samples was determined by both cytological examination and Sanger sequencing. On direct microscopic examination, the presence of round yeast cells with unipolar budding was detected in more than 50% of the samples. The morphology of the yeast cells observed suggested a *Malassezia* species different from *M. pachydermatis* and similar to *M. cuniculi*. When attempted to recover this organism in

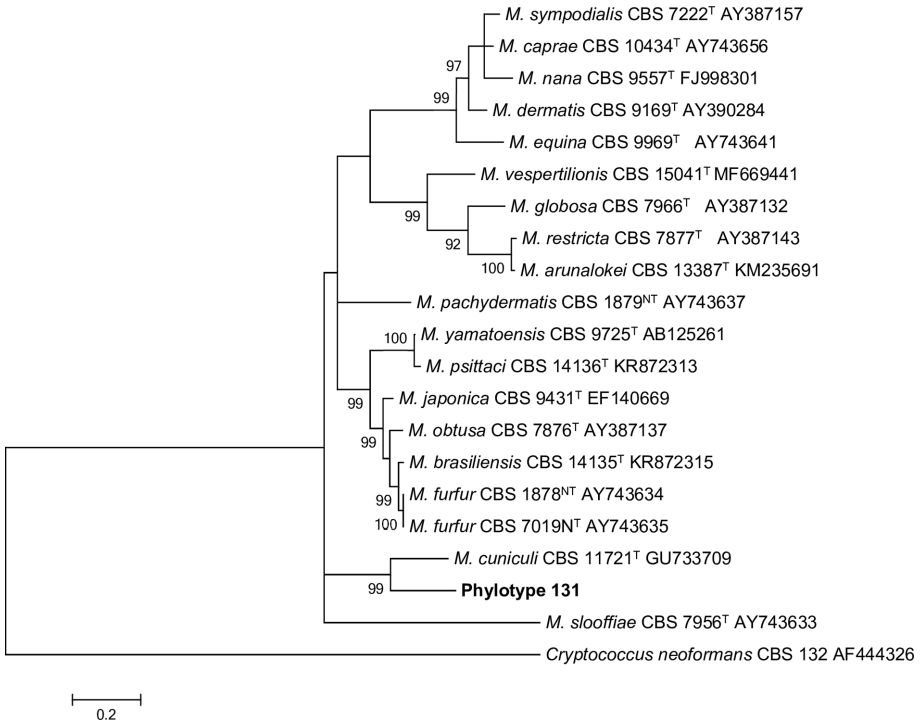

Figure 3. Molecular phylogenetic tree inferred from maximum likelihood analysis of ITS sequences of members of the genus *Malassezia*. Bootstrap values > 70% in 1000 replications are shown at the nodes. Sequence of *Cryptococcus neoformans* CBS 132 was selected as outgroup for the tree construction.

Table 2. NGS reads after filter and biodiversity data obtained from metagenomics analysis.

Sample	Number of reads after quality processing	% Reads classified to genus	Shannon species diversity	Number of species identified
New Zealand	41,913	80.67	1.20	116
Belier	46,162	57.24	2.82	184
Rex	52,886	54.55	3.26	176
Toy	54,285	61.79	1.59	120
Average	52,886	59.51	2.43	120

culture, no growth was observed in any of the culture media used including LNA from which *M. cuniculi* was isolated for the first time.[4] Some *Malassezia* species are fastidious yeasts that have specific nutritional requirements to grow. For example, *M. cuniculi* is only able to grow after 7 days of incubation on LNA and grows better at 37–40°C. At 32°C, the colonies are smaller than the ones cultured between 37 and 40°C.[4]

In this study the sequences D1/D2 of the 26S rRNA gene and the ITS rRNA genes of the samples were sequenced by Sanger sequencing. The main sequence obtained was coincident with that of *Malassezia* phylotype 131, a non-described *Malassezia* yeast detected in the external auditory ear canal of humans.[37] This phylotype has not yet been cultured, and would explain the lack of growth observed in our study.

The phylogenetic trees inferred from the maximum likelihood analysis of both the D1D2 and the ITS sequences obtained in this study show that *Malassezia* phylotype 131 clustered close but with a significant distance to *M. cuniculi*. These results agree with the study conducted by Zhang et al.[37] in which the phylotype 131 was described.

The D1/D2 region of the fungal 26S rRNA gene was selected in this study for the metagenomics analysis of the samples instead of the widely extended ITS region. The 26S rRNA gene is part of the LSU that has been used extensively for fungal phylogeny and taxonomic placement.[38] The ITS region is considered the universal barcode for fungi.[39] However, the extent of the ITS sequence length variability among the different fungal species and genera does not allow for robust sequence alignment[40] and may lead to preferential amplification and sequencing. Also, an incorrect estimation of the abundance of population may occur.[28] Thus, in this study the LSU region was considered the most reliable because this region provides a molecular marker placement of new fungal lineages or for analysis of fungal lineages,[40–42] especially in *Malassezia* genus.[21] In a study comparing NGS results

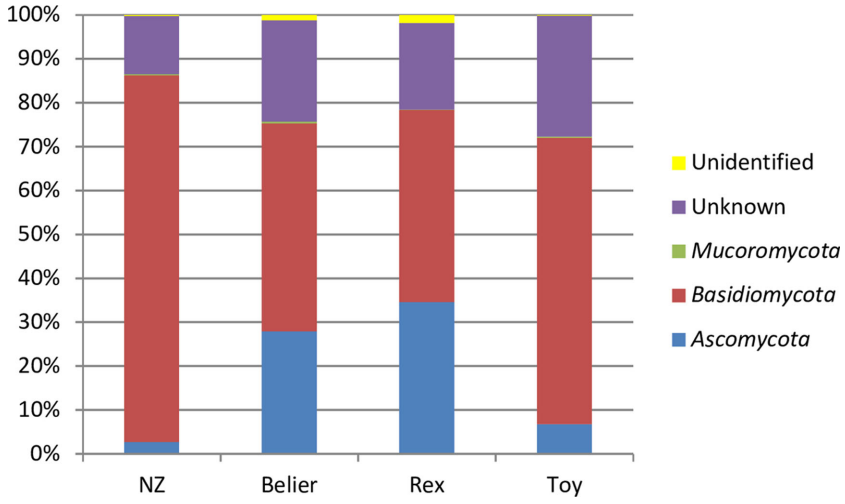

Figure 4. Average relative abundance of fungal phyla across the different rabbit breeds.

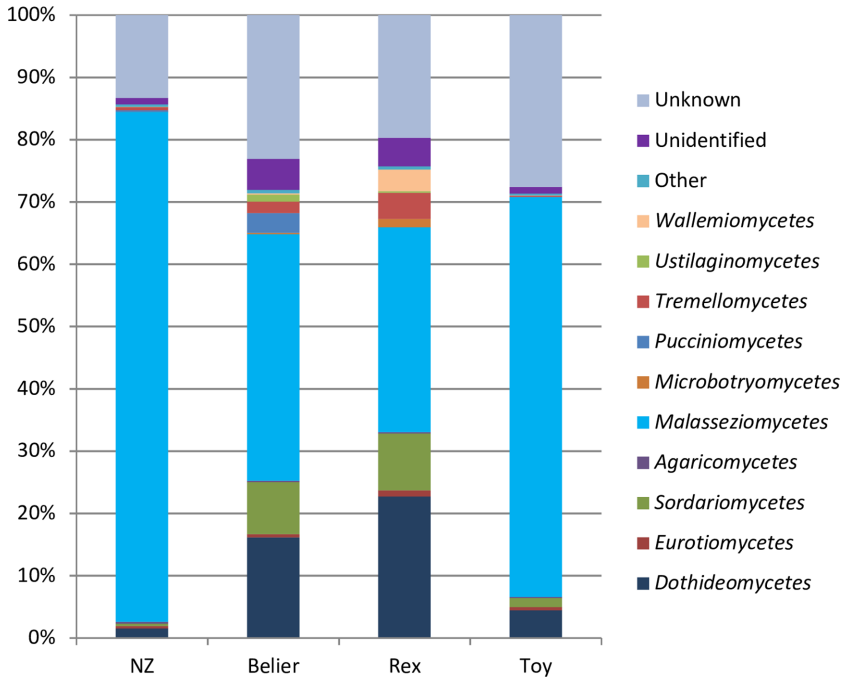

Figure 5. Average relative abundance of fungal classes across the different rabbit breeds.

using both ITS and 26S as targeted genes, Mota-Gutiérrez and co-workers suggested that 26S as a target gene showed a greater biodiversity in biological samples compared with the universal primer ITS.[43] Currently the LSU has shown to work better in species discrimination for yeasts than for filamentous fungi.[44] A study conducted by Vu et al. demonstrated that while ITS worked better in species discrimination in *Ascomycota*,[44] the LSU outperformed in *Basidiomycota*. Finally, a study conducted by Hoggard et al. comparing the results obtained in NGS using three different genes (ITS, LSU, and SSU) demonstrated that *Malassezia* spp. are markedly under-represented using ITS.[45]

It has been demonstrated by previous authors that strains of yeasts species show less than 1% of dissimilarity in LSU regions.[40–42] Therefore, an identity threshold of 99% for taxonomic classification at the level of species was applied in this study.

Basidiomycota was the main phylum identified in the ear samples of healthy rabbits, followed by *Ascomycota*. Within *Ascomycota*, *Cladosporium*, *Fusarium*, and *Alternaria* were the three most common fungal genera detected. These genera are commonly isolated from environmental samples and they are considered transient mycobiota. Within *Basidiomycota*, *Malassezia* was the main genus present in the samples from healthy rabbits as it is a common member of the mycobiota of the skin and the external ear canal of rabbits. These results differ from those obtained in studies of the ear mycobiota of healthy

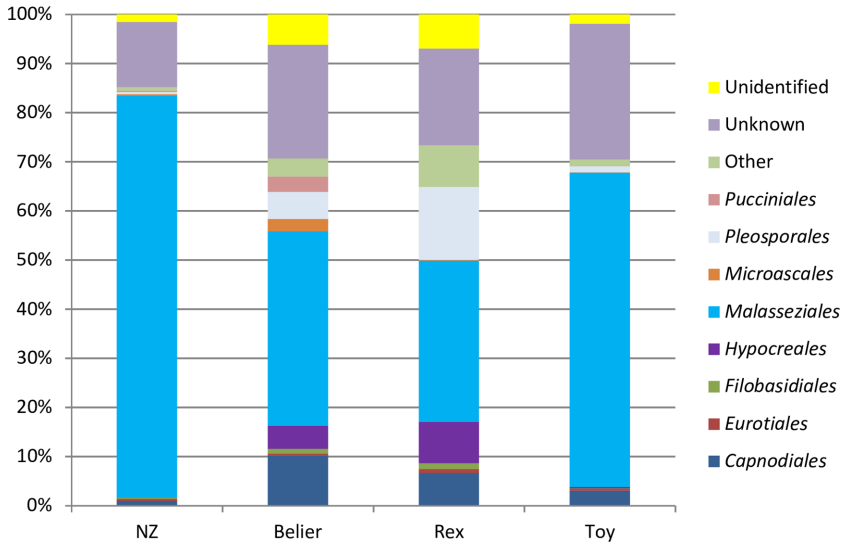

Figure 6. Average relative abundance of fungal orders across the different rabbit breeds.

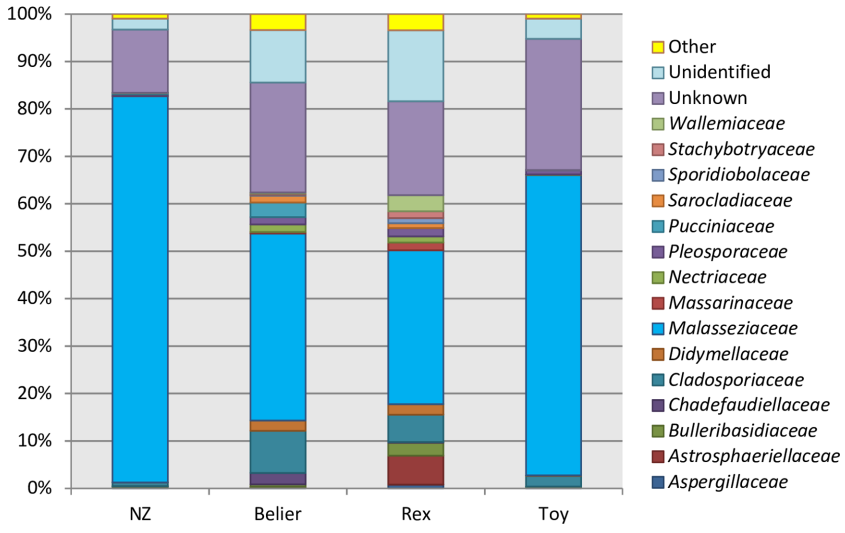

Figure 7. Average relative abundance of fungal families across the different rabbit breeds.

dogs and cats.[46,47] In the ear canal of both dogs and cats the main phylum identified was *Ascomycota*. Within the *Basidiomycota*, *Cryptococcus* was the main genus in dogs and cats, followed by *Malassezia* in dogs.[46,47] Two comparative studies of the ear mycobiota of healthy dogs and dogs with otitis externa conducted by Korbelik et al. and Bradley et al. agreed with the results obtained by previous authors.[48,49] *Ascomycota* was the main phylum followed by *Basidiomycota* in healthy dogs. The samples from allergic dogs and dogs with otitis showed less fungal diversity and richness in both studies.[46,48] In dogs with affected ears *Basidiomycota* was the most abundant phylum and *M. pachydermatis* the most abundant species.[48,49] Neither *M. cuniculi* nor phylotype 131 were detected in the samples of dogs and cats.[46–49] In those studies, the ITS region was selected to be amplified and the Findley et al. and the UNITE fungi databases were used.[50]

In humans, *M. slooffiae* and *M. restricta* were the predominant *Malassezia* species in the external ear canal.[37] Therefore, *Malassezia* was the predominant genus in the external ear canal of humans and thus *Basidiomycota* the main phylum. *Malassezia cuniculi* was not detected in the samples of humans.[37]

The presence of a small amount of *M. pachydermatis* and *M. restricta* was detected in two of the samples corresponding with the New Zealand and Rex breeds. Both *M. pachydermatis* and *M. restricta* were also detected in the sterile swab used as a negative control. The inclusion of control is indispensable in multiple steps of NGS-based studies due to the greater detection ratio compared to traditional technics.[51,52] In our study a sterile swab was included as a negative control and processed in parallel with the other samples. Controls must be treated identically to other samples during the whole process. Currently there is no consensus in how to handle the sequences recovered from the negative

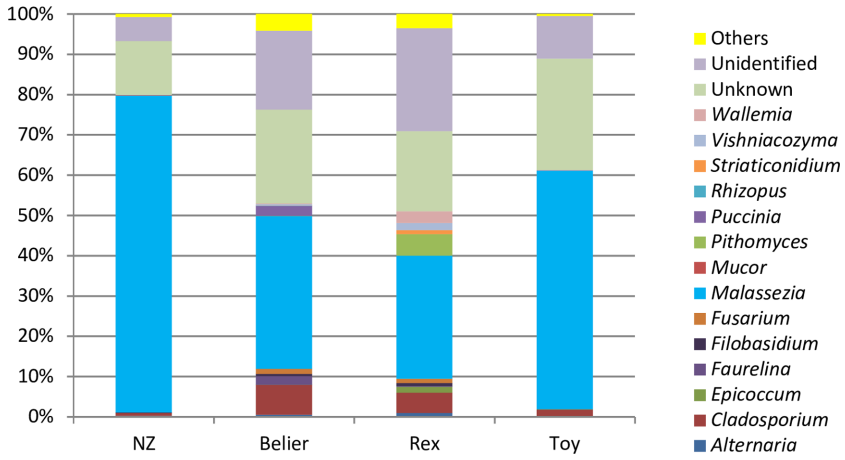

Figure 8. Average relative abundance of fungal genera across the different rabbit breeds.

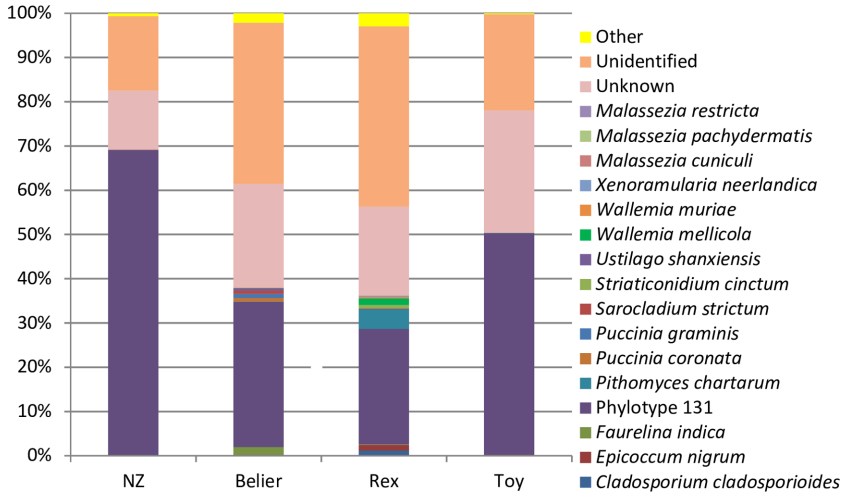

Figure 9. Average relative abundance of fungal species across the different rabbit breeds.

controls. One approach could be to simply eliminate from all the samples any OTUs that appeared in negative controls. This approach could eliminate some of the most abundant OTUs. To avoid eliminating OTUs that could be relevant to the study another approach could be to subtract the number of sequences of each OTU present in the negative control from the abundance of that OTU found in the samples.[51] In this case, neither *M. pachydermatis* nor *M. restricta* would be present in the rabbit samples. The presence of these OTUs in the negative control could be the result of contamination during the manipulation of the samples (sampling, storage, DNA extraction, PCR…) but also it could be the result of primer cross-contamination at any stages from oligonucleotide manufacturing to PCR.[51]

Although the ear morphology has been suggested as a factor that could affect the diversity of the ear mycobiota, our study did not identify a significant difference in the abundance of *Malassezia* between lop and ear-erected rabbits. This lack of significant difference could be as a result of the fairly small sample size in our study. However, our results agree with the findings of previous studies of the increased predisposition of lop-eared rabbit to aural and dental problems and of the external auditory meatus in healthy domestic rabbits.[11,12] *Malassezia* being part of the normal microbiota of the ear canal of rabbits, as it is in other species such as dogs or cats, could explain this lack of statistical difference between lop and erect-eared rabbits.[11,12,53]

Although no growth was observed in any of the culture media used, the presence of *Malassezia* fungal taxa was detected in more than a half of the external ear canal of the rabbits by cytology. *Malassezia* was also detected by Sanger sequencing and NGS in all selected samples. Regarding NGS, the use of the LSU as a target gene allowed us the description of fungal diversity and the taxonomic classification of several *Malassezia* species. *Malassezia* phylotype 131 and *M. cuniculi* were detected in all the samples. Among all the fungal taxa detected in this study, *Malassezia* phylotype 131 showed the highest abundance in all the samples. Further studies would be needed to isolate and characterize the *Malassezia* phylotype 131 and to know its role in the external ear canal mycobiome of rabbits and human beings.

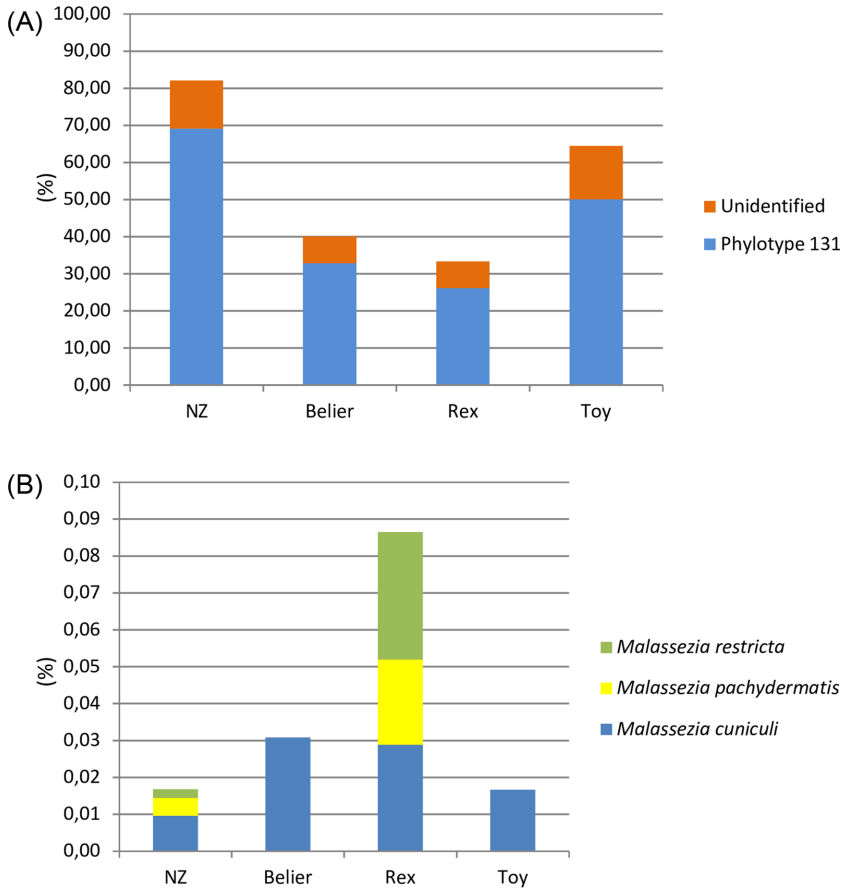

Figure 10. Average relative abundance of *Malassezia* taxa across the different rabbit breeds. (**A**) Taxa with a percentage greater than 1%. (**B**) Taxa with a percentage lower than 1%.

Supplementary material

Supplementary data are available at *MMYCOL* online.

Acknowledgments

The authors thank Carolina Gómez from the Veterinary Mycology Group of Universitat Autònoma de Barcelona (UAB) for valuable technical assistance. We also thank to all the staff of the Exotic Pets Service of the Veterinary Teaching Hospital of UAB.

Funding

Financial support came from Servei Veterinari de Bacteriologia i Micologia from the UAB.

Conflict of interest

Walter Sanseverino and Andreu Paytuví-Gallart were employed by company Sequentia Biotech S.L. All other authors declare no competing interests.

References

1. Cabañes FJ. *Malassezia* yeasts: how many species infect humans and animals?. *PLoS Pathog*. 2014; 10. 10: e1003892. doi:10.1371/journal.ppat. 1003892.
2. Cabañes FJ. Diversity and adaptation within the genus *Malassezia*: Bats already have their species. *Rev Iberoam Micol*. 2020; 37: 37–38.
3. Guillot J, Bond R. *Malassezia* yeasts in veterinary dermatology: an updated overview. *Front. Cell. Infect. Microbiol*. 2020; 10: 79.
4. Cabañes FJ, Vega S, Castellá G. *Malassezia cuniculi* sp. nov., a novel yeast species isolated from rabbit skin. *Med Mycol*. 2011; 49: 40–48.
5. Cabañes FJ. Yeast pathogens of domestic animals. In: Ashbee R Bignell EM, eds. *Pathogenic Yeasts*, 1st edn. Berlin, Heidelberg: Springer, 2009: 253–279.
6. Hirai A, Kano R, Makimura K et al. *Malassezia nana* sp. nov., a novel lipid-dependent yeast species isolated from animals. *Int J Syst Evol Microbiol*. 2004; 54: 623–627.
7. Bond R, Guillot J, Cabañes FJ. *Malassezia* yeasts in animal disease. In: Boekhout T, Guého-Kellerman E Mayser P, eds. *Malassezia And The Skin*, 1st edn. Berlin, Heidelberg: Springer, 2010: 271–299.
8. Cabañes FJ, Coutinho SD, Puig L, Bragulat MR, Castellá G. New lipid-dependent *Malassezia* species from parrots. *Revista Iberoamericana de Micología or the correct form: Rev Iberoam Micol*. 2016; 33: 92–99.
9. Lorch JM, Palmer JM, Vanderwolf KJ et al. *Malassezia vespertilionis* sp. nov.: a new cold-tolerant species of yeast isolated from bats. *Persoonia*. 2018; 41: 56–70.
10. Sugita T, Boekhout T, Velegraki A, Guillot J, Hađina S, Cabañes FJ. Epidemiology of *Malassezia*-related skin diseases. In: Boekhout T, Guého-Kellerman E Mayser P, eds. *Malassezia And The Skin*, 1st edn. Berlin, Heidelberg: Springer, 2010: 65–119.
11. Johnson JC, Burn CC. Lop-eared rabbits have more aural and dental problems than erect-eared rabbits: a rescue population study. *Vet Rec*. 2019; 185: 758.
12. Quinton JF, François M, Laprais A, Prelaud P. Cytology of the external auditory meatus in healthy domestic pet rabbits (*Oryctolagus cuniculus*). *Rev Méd Vét*. 2014; 165: 263–266.
13. Leeming JP, Notman FH. Improved methods for isolation and enumeration of *Malassezia furfur* from human skin. *J Clin Microbiol*. 1987; 25: 2017–2019.
14. Guého E, Midgley G, Guillot J. The genus *Malassezia* with description of four new species. *Antonie Van Leeuwenhoek*. 1996; 69: 337–355.

15. Xu J, Saunders CW, Hu P et al. Dandruff-associated *Malassezia* genomes reveal convergent and divergent virulence traits shared with plant and human fungal pathogens. *Proc Natl Acad Sci U S A*. 2007; 104: 18730–18735.
16. Wu G, Zhao H, Li C et al. Genus-wide comparative genomics of *Malassezia* delineates its phylogeny, physiology, and niche adaptation on human skin. *PLoS Genet*. 2015; 11: e1005614.
17. Hay RJ, Midgley G. Introduction: *Malassezia* yeasts from a historical perspective. In: Boekhout T, Guého-Kellerman E Mayser P, eds. *Malassezia And The Skin*, 1st edn. Berlin, Heidelberg: Springer, 2010: 1–16.
18. Puig L, Bragulat MR, Castellá G, Cabañes FJ. Characterization of the species *Malassezia pachydermatis* and re-evaluation of its lipid dependence using a synthetic agar medium. *PLoS ONE*. 2017; 12: e0179148.
19. Cabañes FJ. *Malassezia pachydermatis*: To be, or not to be lipid-dependent. *Rev Iberoam Micol*. 2020; 37: 3–4.
20. Guého-Kellerman E, Boekhout T, Begerow D. Biodiversity, phylogeny and ultrastructure. In: Boekhout T, Guého-Kellerman E Mayser P, eds. *Malassezia And The Skin*, 1st edn. Berlin, Heidelberg: Springer, 2010:17–63.
21. Castellá G, Coutinho SD, Cabañes FJ. Phylogenetic relationships of *Malassezia* species based on multilocus sequence analysis. *Med Mycol*. 2014; 52: 99–105.
22. Theelen B, Cafarchia C, Gaitanis G, Bassukas ID, Boekhout T, Dawson TL. *Malassezia* ecology, pathophysiology, and treatment. *Med Mycol*. 2018; 56: S10–S25.
23. Forde BM, O'Toole PW. Next-generation sequencing technologies and their impact on microbial genomics. *Brief Funct Genomics*. 2013; 12: 440–453.
24. Kulski J (eds). *Next Generation Sequencing: Advances, Applications And Challenges*, 1st ed. IntechOpen, 2016.
25. Forbes JD, Knox NC, Ronholm J, Pagotto F, Reimer A. Metagenomics: the next culture-independent game changer. *Front Microbiol*. 2017; 8: 1069.
26. Lücking R, Hawksworth DL. Formal description of sequence-based voucherless Fungi: promises and pitfalls, and how to resolve them. *IMA Fungus*. 2018; 9: 143–166.
27. Wilkening S, Tekkedil MM, Lin G et al. Genotyping 1,000 yeast strains by next-generation sequencing. *BMC Genomics*. 2013; 14: 90.
28. De Filippis F, Laiola M, Blaiotta G, Ercolini D. Different amplicon targets for fungal sequencing-based studies of fungal diversity. *Appl Environ Microbiol*. 2017; 83: e00905–17.
29. Mohanta TK, Bae H. The diversity of fungal genome. *Biol Proced Online*. 2015; 17: 8.
30. Galagan JE, Henn MR, Ma LJ, Cuomo CA, Birren B. Genomics of the fungal kingdom: insights into eukaryotic biology. *Genome Res*. 2005; 15: 1620–1631.
31. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. Mega X: molecular evolutionary genetics analysis across computing platforms. *Mol Biol Evol*. 2018; 35: 1547–1549.
32. Reynolds DR, Taylor JW. *The Fungal Holomorph: Mitotic, Meiotic and Pleomorphic Speciation In Fungal Systematics*, 1st edn. CAB international, 1993.
33. Giampaoli S, De Vittori E, Frajese GV et al. A semi-automated protocol for NGS metabarcoding and fungal analysis in forensic. *Forensic Sci Int*. 2020; 306: 110052. doi: 10.1016/j.forsciint.2019.110052.
34. Li H, Durbin R. Fast and accurate long-read alignment with Burrows–Wheeler transform. *Bioinformatics*. 2010; 26: 589–595.
35. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol*. 2014; 15: 550.
36. McMurdie PJ, Holmes S. phyloseq: An R Package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE*. 2013; 8: e61217.
37. Zhang E, Tanaka T, Tsuboi R, Makimura K, Nishikawa A, Sugita T. Characterization of *Malassezia* microbiota in the human external auditory canal and on the sole of the foot. *Microbiol Immunol*. 2012; 56: 238–244.
38. Vu D, Groenewald M, de Vries M et al. Large-scale generation and analysis of filamentous fungal DNA barcodes boosts coverage for kingdom fungi and reveals thresholds for fungal species and higher taxon delimitation. *Stud Mycol*. 2019; 92: 135–154.
39. Schoch CL, Seifert KA, Hunhdorf S et al. Nuclear ribosomal trancribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proc Natl Acad Sci U S A*. 2012; 109: 6241–6246.
40. Scorzetti G, Fell JW, Fonseca A, Statzell-Tallman A. Systematics of basidiomycetous yeasts: a comparison of large subunit D1/D2 and internal transcribed spacer rDNA regions. *FEMS Yeast Res*. 2002; 2: 495–517.
41. Fell JW, Boekhout T, Fonseca A, Scorzetti G, Statzell-Tallman A. Biodiversity and systematics of basidiomycetous yeasts as determined by large-subunit rDNA D1/D2 domain sequence analysis. *Int J Syst Evol Microbiol*. 2000; 50: 1351–1371.
42. Kurtzman CP, Robnett CJ. Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. *Antonie Van Leeuwenhoek*. 1998; 73: 331–371.
43. Mota-Gutierrez J, Ferrocino I, Rantsiou K, Cocolin L. Metataxonomic comparison between internal transcribed spacer and 26S ribosomal large subunit (LSU) rDNA gene. *Int J Food Microbiol*. 2019; 290: 132–140.
44. Vu D, Groenewald M, Szöke S et al. DNA barcoding analysis of more than 9, 000 yeast isolates contributes to quantitative thresholds for yeast species and genera delimitation. *Stud Mycol*. 2016; 85: 91–105.
45. Hoggard M, Vesty A, Wong G et al. Characterizing the Human Mycobiota: A Comparison of Small Subunit rRNA, ITS1, ITS2, and Large Subunit rRNA Genomic Targets. *Front Microbiol*. 2018; 9: 2208.
46. Meason-Smith C, Diesel A, Patterson AP et al. What is living on your dog's skin? Characterization of the canine cutaneous mycobiota and fungal dysbiosis in canine allergic dermatitis. *FEMS Microbiol Ecol*. 2015; 91: fiv139.
47. Meason-Smith C, Diesel A, Patterson AP et al. Characterization of the cutaneous mycobiota in healthy and allergic cats using next generation sequencing. *Vet Dermatol*. 2017; 28: 71.
48. Korbelik J, Singh A, Rosseau J, Weese S. Analysis of the otic mycobiota in dogs with otitis externa compared to healthy individuals. *Vet Dermatol*. 2018; 29: 417 e138.
49. Bradley CW, Lee FF, Rankin SC et al. The otic microbiota and mycobiota in a referral population of dogs in eastern USA with otitis externa. *Vet Dermatol*. 2020; 31: 225–249.
50. Findley K, Oh J, Yang J et al. Topographic diversity of fungal and bacterial communities in human skin. *Nature*. 2013; 498: 367–370.
51. Nguyen NH, Smith D, Peay K, Kennedy P. Parsing ecological signal from noise in next generation amplicon sequencing. *New Phytol*. 2015; 205: 1389–1393.
52. Palmer JM, Jusino MA, Banik MT, Lindner DL. Non-biological synthetic spike-in controls and the AMPtk software pipeline improve mycobiome data. *PeerJ*. 2018; 6: e4925.
53. Campbell JJ, Coyner KS, Rankin SC, Lewis TP, Schick AE, Shumaker AK. Evaluation of fungal flora in normal and diseased canine ears. *Vet Dermatol*. 2010; 21: 619–625.

5.2. Díaz L, Castellá G, Bragulat MR, Paytuví-Gallart A, Sanseverino W, Cabañes FJ. Study of the variation of the *Malassezia* load in the interdigital fold of dogs with pododermatitis. *Vet Res Commun*. 2022; doi:10.1007/s11259-022-09951-2.

Veterinary Research Communications
https://doi.org/10.1007/s11259-022-09951-2

ORIGINAL ARTICLE

Study of the variation of the *Malassezia* load in the interdigital fold of dogs with pododermatitis

Leyna Díaz[1] · Gemma Castellá[1] · M. Rosa Bragulat[1] · Andreu Paytuví-Gallart[2] · Walter Sanseverino[2] · F. Javier Cabañes[1]

Received: 17 November 2021 / Accepted: 4 June 2022

Abstract
The yeast *Malassezia pachydermatis* is a common inhabitant of the skin and mucosae of dogs. However, under certain circumstances this yeast can overgrow and act as an opportunistic pathogen causing otitis and dermatitis in dogs. Canine pododermatitis is a common disorder in dogs in which *M. pachydermatis* acts as an opportunistic pathogen. In the present study, the presence of *Malassezia* yeasts was assessed and quantified in samples collected from the interdigital space of dogs with pododermatitis before and after treatment, and from healthy dogs. The samples were subjected to two different cytological examinations, culture on Sabouraud glucose agar and modified Dixon's agar and a quantitative PCR targeting the internal transcribed spacer (ITS) genomic region. A selection of samples was analyzed by next generation sequencing (NGS) using the D1D2 domain of the large subunit of the ribosomal DNA as target. The pododermatitis samples before treatment showed higher cell counts, colony-forming units and ITS copies than the rest of samples. The NGS analysis revealed that *Ascomycota* was the main phylum in the healthy and post-treatment samples. However, *Basidiomycota* and *M. pachydermatis* was more abundant in the pododermatitis samples before treatment. These results support *M. pachydermatis* as an opportunistic agent in canine pododermatitis by a variety of methods, and demonstrate the correlation between cytologic and molecular methods for quantification.

Keywords Interdigital fold · *Malassezia* · *M. pachydermatis* · Mycobiome · Pododermatitis

Introduction

The yeast *Malassezia pachydermatis* is part of the normal microbiota of the skin and mucosae of healthy dogs (Guillot and Bond 1999; Cabañes 2021). Despite being part of the normal microbiota, under certain circumstances, the population of *M. pachydermatis* can overgrow and this yeast may act as an opportunistic pathogen. Thus *M. pachydermatis* is considered one of the most frequent aetiological agents responsible for otitis and dermatitis in dogs (Bond et al. 2020).

Canine pododermatitis is a common disorder in the general veterinary practice. *Malassezia pachydermatis* commonly acts as an opportunistic pathogen that produces pododermatitis in dogs, especially in atopic animals or animals diagnosed with endocrine disease. It may affect the interdigital spaces, nail folds, and nails, and usually more than one paw is affected (Duclos 2013; Bajwa 2016). In dogs with *Malassezia* pododermatitis, pruritus is a major and constant sign often associated with erythema and/or greasy exudate. In chronic cases hyperpigmentation, alopecia and lichenification may be present (Miller et al. 2000).

Its diagnosis is based on history, physical examination, clinical signs, cytological examination, and response to antifungal therapy (Miller et al. 2000; Bond et al. 2010). Detection of *Malassezia* yeasts by cytology in compatible skin lesions is a key for the diagnosis of *Malassezia* pododermatitis (Duclos 2013). Cultural techniques are primarily used in research rather than routine veterinary practice. However, they have been suggested to have a diagnostic value because

✉ Gemma Castellá
Gemma.Castella@uab.cat

[1] Veterinary Mycology Group, Department of Animal Health and Anatomy, Universitat Autònoma de Barcelona, Bellaterra, Catalonia, Spain

[2] Sequentia Biotech SL, Barcelona, Catalonia, Spain

Published online: 15 June 2022

they have higher sensitivity than cytology (Bond et al. 1994, 2010).

To overcome limitations of culture-based techniques, several molecular methods have been developed. To detect and quantify *Malassezia* yeast in samples, quantitative PCR (qPCR) is a fast, sensitive, and precise technique (Sugita et al. 2006). The internal transcribed spacer (ITS) region and β-tubulin gene have been used as target to study the population of *Malassezia* in samples from dogs by qPCR (Puig et al. 2019; Meason-Smith et al. 2020).

On the other hand, next generation sequencing (NGS) is a high sensitivity tool to investigate the microbial diversity within samples at a lower cost without the need of a culture (Forde and O'Toole 2013; Forbes et al. 2017). Although only few studies have been conducted using NGS in dogs, Tang et al. (2020) highlighted the adequacy of NGS-based methods as a diagnostic tool to diagnose canine skin and ear infections.

The aim of this work was to study the presence of *M. pachydermatis* from samples of dogs with pododermatitis, healthy dogs and dogs after treatment using different techniques including cytologic examination, culture, qPCR and NGS, and to determine if there is a correlation between common cytological techniques used in veterinary practice and molecular methods. Besides, using NGS the mycobiome was compared among a selection of the three different kind of samples studied.

Materials and methods

Animals and sample collection

A total of 13 dogs including five females and seven males were enrolled in this study (Table 1). Eleven dogs were included in the pododermatitis group. All were diagnosed with pruritus in at least one extremity, erythema in the affected interdigital fold, and a positive cytology for the presence of *Malassezia* yeasts. A total of 15 samples were collected from this group. Three dogs initially diagnosed with *Malassezia* pododermatitis were also sampled after treatment with antifungal agents (Table 1). These dogs were included in the post-treatment group and four samples were obtained. Two dogs without erythema, pruritus and skin lesions were assigned to the healthy group, obtaining three samples. A total of 22 samples were obtained following the procedures on Animal and Human Experimentation from UAB and Generalitat de Catalunya approved by Ethics Committee (Approval number, 4600; Approval date, 22Feb2019). Also, the written consent of owners was obtained before sampling the animals.

Two samples were taken from each interdigital fold by simultaneously rotating fully two sterile swabs (Deltalab, Spain) for 30 s. Also, a cell tape (Scotch, Spain) for cytological examination was obtained from each interdigital fold. Tape strip was applied to the skin and then stained with a Diff-Quick staining (Microptic, Spain) of which the first fixative step with methanol was omitted. One swab was used for *Malassezia* culture and cytological examination. This swab was dipped for 10 s into an Eppendorf (Deltalab, Spain) containing 500 µL of a solution of distilled water and 0.04% Tween 80 (ICN Biomedical, USA) to reduce clump formation. This suspension was used to perform cytological examinations and culture. The other swab was maintained at -20 ºC and used for qPCR and NGS.

Culture and cytological examination

Aliquots of 0.1 mL of the suspension were inoculated on Sabouraud glucose agar (SGA; Oxoid, Spain) and modified Dixon agar (mDA) (Guého et al. 1996) plates using the surface-spread method. Both media were supplemented with 0.05% of chloramphenicol (Sigma, Spain) and 0.05% of cycloheximide (Sigma, Spain) (Guého-Kellerman et al. 2010). The plates were incubated at 35 ºC to a maximum of 7 days and colony forming units (CFU) were counted.

A first cytological examination was performed using the tape strip. A second cytological examination was performed from the suspension. An aliquot of 10 µL of the suspension was heat-fixed on a slide and stained with the Diff-Quick stain. The presence of *M. pachydermatis* cells was quantified by counting cells in 10 different random fields at 100 × with immersion oil.

DNA extraction

DNA was extracted from swabs using the DNeasy PowerSoil Kit (Qiagen, Germany) according to manufacturer's instructions with some modifications previously described (Díaz et al. 2021). To control cross-contamination, a sterile swab was processed under the same conditions. The DNA was stored at -20 ºC until used as template for qPCR and NGS.

Malassezia pachydermatis quantification by qPCR

All samples were selected for *M. pachydermatis* quantification. All qPCRs were performed using an Applied Biosystems 7500 real-time system, and SYBR Green chemistry (PowerUp SYBR Green master mix, Applied Biosystems, USA) with the absolute quantification method.

The multicopy ITS region was used as target gene according to Meason-Smith et al. (2020) with some modifications. Primers ITS-ANA-F and PachyR (Vuran et al. 2014) were used at a final concentration of 900 nM each. The runs were

Table 1 Samples included in the study

Sample	Dog	Breed	Sex	Origin	Clinical signs	Treatment
Samples from dogs with pododermatitis						
1	Dog 1	Labrador Retriever	FN	Left thoracic limb	Pruritus, erythema, alopecia, and surface debris	_
2	Dog 2	Yorkshire	F	Left thoracic limb	Pruritus, erythema, alopecia, and surface debris	_
3	Dog 3	Labrador Retriever	ND	Right pelvic limb	Pruritus, erythema, and greasy exudate	_
4	Dog 4	Labrador Retriever	M	Right pelvic limb	Pruritus, erythema, and alopecia	_
5	Dog 5	Bernese Mountain Dog	M	Left thoracic limb	Pruritus, erythema, alopecia, and a small nodule	_
6	Dog 5	Bernese Mountain Dog	M	Right thoracic limb	Pruritus, erythema, and alopecia	_
7	Dog 6	Mixed breed	M	Right thoracic limb	Pruritus, erythema, and greasy exudate	_
8	Dog 7	American Staffordshire	FN	Right pelvic limb	Pruritus, erythema, and alopecia	_
9	Dog 8	Mixed breed	MN	Left thoracic limb	Pruritus and erythema	_
10	Dog 8	Mixed breed	MN	Right thoracic limb	Pruritus and erythema	_
11	Dog 9	West highland white terrier	M	Left thoracic limb	Pruritus, erythema, greasy exudate, and surface debris	_
12	Dog 9	West highland white terrier	M	Right thoracic limb	Pruritus, erythema, greasy exudate, and surface debris	_
13	Dog 10	Labrador Retriever	M	Left thoracic limb	Pruritus, erythema, alopecia, and surface debris	_
17	Dog 13	Labrador Retriever	FN	Right thoracic limb	Pruritus and erythema	_
18	Dog 13	Labrador Retriever	FN	Left thoracic limb	Pruritus and erythema	_
Samples from healthy dogs						
14	Dog 11	Labrador Retriever	MN	Right pelvic limb	None	_
15	Dog 11	Labrador Retriever	MN	Left pelvic limb	None	_
16	Dog 12	Labrador Retriever	FN	Right pelvic limb	None	_
Samples after antifungal treatment						
19	Dog 3	Labrador retriever	ND	Right pelvic limb	None	Topical clotrimazole twice daily, 4 weeks
20	Dog 7	American Staffordshire	FN	Right pelvic limb	None	Topical miconazole, 3 weeks
21	Dog 8	Mixed breed	MN	Left thoracic limb	None	Oral itraconazole once daily,3 weeks
22	Dog 8	Mixed breed	MN	Right thoracic limb	None	Oral itraconazole once daily, 3 weeks

[a]In bold, samples analysed by NGS. F = female, M = male, FN = neutered female, MN = neutered male, ND = no data available

performed following these thermal conditions: 1 cycle of 50 ºC for 2 min, 1 cycle of 98 ºC for 3 min, followed by 40 cycles of 95 ºC for 30 s, and 60 ºC for 1 min. Finally, a melting curve analysis with a gradual increase of temperature from 60 ºC to 95 ºC was performed. To achieve absolute quantification of the samples, the standard curve method was used. A standard curve of genomic DNA extracted from the neotype strain of *M. pachydermatis* CBS1879 was constructed, including 7 ten-fold dilutions, from 18 ng to 0.018 pg of DNA. The quantity and purity of the gDNA were determined by spectrometry (NanoDrop 2000; Thermo Scientific, Spain). The number of copies of the template was calculated considering the amount of a template present and the length of the template. All samples were run in duplicate, including the standards and negative control. The negative control contained all the elements of the reaction mixture and water instead of DNA template spiked.

NGS and data analysis

Due to the high technical cost of the analysis, five samples were selected for the metagenomics NGS analysis of the fungal 26S rRNA gene. These samples were selected based on dog's breed and the results of the cytological examination and culture. The samples belonged to four different Labrador Retriever dogs. One sample from the

healthy group (sample 16) with negative cytology and no growth in the culture media used was included. Three different samples from pododermatitis group (samples 1, 3 and 4) with a positive cytological examination and growth on both culture media used were selected. Also, a sample from the post-treatment group (sample 19) was included.

Quality control was performed at IGA Technology. DNA concentration was evaluated by using a Qubit 2.0 Fluorometer (Invitrogen, USA). Amplicon-seq libraries of D1/D2 regions of the fungal 26S rRNA gene were obtained from each sample by following 16S Metagenomic Sequencing Library Preparation protocol with minor modifications. Briefly, the forward primer (NL1) and the reverse primer (NL4) (Reynolds and Taylor 1993) containing Illumina overhang sequences necessary for the compatibility with Illumina index and sequencing adapters were used for the first PCR amplification under the following conditions: 95 °C for 3 min; 28 cycles of: 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s; 72 °C for 5 min; hold at 4 °C. Upon the clean-up, the second PCR was performed under the following conditions: 95 °C for 3 min; 9 cycles of: 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s; 72 °C for 5 min; hold at 4 °C. Relevant flow-cell binding domains and unique indices (NexteraXT Index Kit, Illumina, USA) were integrated to the amplicon target. Libraries were normalized by Qubit 2.0 Fluorometer, pooled and sequenced on MiSeq using paired 300-bp reads, and MiSeq v3 reagents (Illumina). Sequence reads were analyzed in the cloud bioinformatics platform GAIA (https://metagenomics.sequentiabiotech.com) (Giampaoli et al. 2020). Sequencing quality was assessed using FastQC (bioinformatics.babraham.ac.uk/projects/fastqc/) and BBduk (jgi.doe.gov/data-and-tools/bbtools/), setting a minim length of 35 bp and a minimum Phred-quality score of 25.

The resulting high-quality reads were mapped against a custom-made cured database of 26S fungal sequences from NCBI (Díaz et al. 2021). For the taxonomic classification, the mapping-based approach against the database with the BWA mapper (Li and Durbin 2010) was followed by an in house Lowest Common Ancestor (LCA) algorithm. Following the limits proposed by GAIA software, the minimum identity thresholds applied to classify the reads into different taxonomic levels were species (99%), genus (98%), family (96%), order (94%), class (92%), and phylum (90%). Those taxa with abundance below 0.01% considering its mean across the different samples within an experimental group were filtered out for further analysis. DESeq2 (v1.26) (Love et al. 2014) was used to carry out differential abundance analyses. GAIA also assesses the diversity within (alpha-diversity) and between (beta-diversity) samples. Alpha and beta diversities were calculated using the R package phyloseq (McMurdie and Holmes 2013). A Principal Coordinates Analysis (PCoA) was also carried out using Bray–Curtis dissimilarities; cluster significance was assessed with PERMANOVA and ANOSIM (R package vegan).

Statistical analysis

Other statistical analyses were conducted by Minitab 17 statistical software (Minitab). The Ryan-Joiner normality test was applied to determine whether data followed a normal distribution. Differences in both cytologic examinations, CFU in SGA and mDA, and qPCR quantification values between samples were tested by Kruskal–Wallis test. Student's t test was applied in values of CFU in both culture media. A multiple regression analysis was used to study the relationship between the qPCR quantification values and values obtained in the rest of variables. The significance level for all statistical analyses was set at $P \leq 0.05$.

Results

Culture and cytological examination

The results of the two cytological examinations performed (tape strip and heat-fixed slide) and the results obtained in both SGA and mDA culture media are summarized in Table 2.

The 15 samples from the pododermatitis group had a positive cytological evaluation for *Malassezia* yeast cells, with a range of 0.1–45.9 cells/field (mean = 7.37) on the tape strip. All samples had growth on SGA plates ranging from 1 to 468 CFU/plate (mean = 83.8) (Tables 2 and 3).

The three samples from the healthy group had a negative cytological evaluation for the presence of *Malassezia* yeasts and no growth was observed in any of the culture media used.

In the post-treatment group, no growth of *M. pachydermatis* was obtained. All samples had a negative cytological evaluation except for sample 19 which showed a low cell count in the first cytological examination.

Although in almost all samples the SGA medium gave higher CFU counts than mDA, no significant differences ($P = 0.382$) were obtained. Statistically significant differences in tape-strip, heat-fixed slide, plate counts in SGA and mDA media values were found between samples from dogs with pododermatitis signs and healthy and post-treatment dogs (Table 3).

Quantification of *M. pachydermatis* by qPCR

The standard curve generated was linear over 7 ten-fold dilutions of *M. pachydermatis* DNA, from 1.2×10^{12} to 1.2×10^{5} copies. The qPCR amplifications of each standard showed

Table 2 Results obtained in samples, including the two cytological examinations performed presented in counted cells per field, the colony forming units (CFU) per plate in both Sabouraud's glucose agar (SGA) and modified Dixon agar (mDA), and qPCR quantification values

Sample	Dog	Cytological examination (cells/field)		Culture CFU		qPCR
		Tape strip	Heat-fixed slide	SGA	mDA	ITS nº of copies
Samples from dogs with pododermatitis						
1	Dog 1	1.3	1.1	79	66	2.6×10^7
2	Dog 2	0.2	0	1	0	1.4×10^6
3	Dog 3	12.6	0.4	218	1	1.2×10^7
4	Dog 4	1.4	0.5	76	14	7.5×10^6
5	Dog 5	6.2	2.2	2	9	2.1×10^8
6	Dog 5	11.4	0.6	120	311	8.1×10^7
7	Dog 6	8.4	0.2	4	0	1.7×10^7
8	Dog 7	45.9	0.7	468	153	2.7×10^8
9	Dog 8	6.8	0.3	172	91	1.3×10^6
10	Dog 8	1.3	0.3	10	17	1.6×10^6
11	Dog 9	4.7	0.2	38	15	1.6×10^8
12	Dog 9	2.3	0	3	1	1.9×10^7
13	Dog 10	4.7	1.3	59	24	2.8×10^8
17	Dog 13	3.2	0	2	1	2.2×10^6
18	Dog 13	0.1	0	5	1	6.5×10^6
Samples from healthy dogs						
14	Dog 11	0	0	0	0	4.9×10^6
15	Dog 11	0	0	0	0	1.1×10^5
16	Dog 12	0	0	0	0	1.2×10^6
Samples after antifungal treatment						
19	Dog 3	0.2	0	0	0	1.3×10^7
20	Dog 7	0	0	0	0	3.7×10^5
21	Dog 8	0	0	0	0	6.5×10^5
22	Dog 8	0	0	0	0	1.7×10^5

[a]In bold, samples analysed by NGS

Table 3 Mean values, standard deviation (SD), and range of values for plate counts (CFU) in Sabouraud's glucose agar (SGA) and modified Dixon agar (mDA), cytologic examination (cells/field) and qPCR quantification values between samples from dogs with sings of pododermatitis and samples from healthy and post-treatment dogs

	Dogs with pododermatitis ($n=15$)		Healthy dogs ($n=3$)		Dogs after treatment ($n=4$)		*P* value
	mean value ± SD	range	mean value ± SD	range	mean value ± SD	range	
CFU in SGA	83.8 ± 126.0	1.0–468.0	0 ± 0	0–0	0 ± 0	0–0	0.001
CFU in mDA	46.9 ± 85.0	0–311.0	0 ± 0	0–0	0 ± 0	0–0	0.004
Tape-strip	7.37 ± 11.34	0.1–45.9	0 ± 0	0–0	0.05 ± 0.10	0–0.2	0.001
Heat-fixed slide	0.52 ± 0.61	0–2.2	0 ± 0	0–0	0 ± 0	0–0	0.015
qPCR	$7.2\times10^7\pm1.0\times10^8$	1.3×10^6–2.810^8	$2.0\times10^6\pm2.5\times10^6$	1.1×10^5–4.9×10^6	$3.6\times10^6\pm6.4\times10^6$	1.7×10^5–1.3×10^7	0.014

amplification plots corresponding to mean Cq values of 14.36 to 34.33. The standard curve yielded r^2 values > 0.99, and slope values of − 3.44. Amplification was obtained in all samples with a Cq value lower than 35. The Cq values of samples are detailed in Supplementary Table 1.

As shown in Table 2, in the pododermatitis group, quantification values of 1.3×10^6 to 2.8×10^8 copies were obtained whereas values from 1.1×10^5 to 4.9×10^6 copies were obtained from the healthy group. Samples from the post-treatment group showed quantifications values of 1.7×10^5 to 1.3×10^7 copies. Statistically significant differences in qPCR values were found between samples from the pododermatitis group and samples from the healthy and after treatment groups (Table 3).

Multiple regression analysis showed that the relationship ($r^2=70.7\%$) between qPCR quantification and both

cytological examinations in the model was statistically significant ($P < 0.001$).

NGS data analysis

The five samples were correctly sequenced, and the generated fastq files reported an average value of 62,178 LSU reads passing filter. The number of generated sequences reads of each sample is described in Table 4. The raw sequencing data is available at the NCBI database (SRA accession number: PRJNA742914).

The Shannon diversity index was selected to measure alpha diversity. The sample of the healthy dog was the most diverse with an index of 3.32 (Table 4). Samples from the pododermatitis group showed less diversity, on average, than the healthy and post-treatment samples.

The Bray Curtis index was used to measure beta diversity. Samples from the healthy and post-treatment group with an index of 0.323 were more similar compared to the pododermatitis samples (average of 0.716 between the healthy sample and average of 0.496 between the post-treatment sample). Also, pododermatitis samples were more similar among them with an index ranging from 0.112 to 0.196. Although in the PCoA plots, the samples clustered in two different groups, one including the healthy and post-treatment samples and one including all three pododermatitis samples, there was no significant difference between the two different categories based on PERMANOVA ($F = 18.9$, $P = 0.1$) and ANOSIM ($R = 1$, $P = 0.1$) tests probably due to the small sample size.

The taxonomic composition of the samples was investigated at various taxonomic levels. Within the pododermatitis group, *Basidiomycota* with a median of abundance of 22.18% was the main phylum (Fig. 1). At the level of class (Fig. 2), *Malasseziomycetes* showed the highest abundance (16.57%) being *Malasseziales* (16.33%) the main order (Fig. 3). With a median of abundance of 15.94%, *Malasseziaceae* was the main family (Fig. 4). *Malassezia* was the main genus with a median of abundance of 14.26% (Fig. 5) and, *M. pachydermatis* was the predominant species (10.64%). To a lesser extent, other *Malassezia* species were identified in this group of samples like *M. restricta* (0.04%), *M. globosa* (0.02%), and *M. sympodialis* (0.01%). An average of 6.29% of the *Malassezia* sequences could not be identified to the species level (Fig. 6). This percentage includes sequences marked as unknown (6.04%) because a match with enough

Table 4 NGS reads after filter and biodiversity data obtained from metagenomics analysis

Sample	Number reads after quality processing	% Reads classified to genus	Shannon species diversity	Number of species identified
16 (healthy)	52,488	52.19	3.32	220
1 (pododermatitis)	73,732	26.15	2.37	247
3 (pododermatitis)	60,227	21.58	1.46	201
4 (pododermatitis)	62,178	26.23	2.35	219
19 (post-treatment)	106,095	45.07	2.48	248

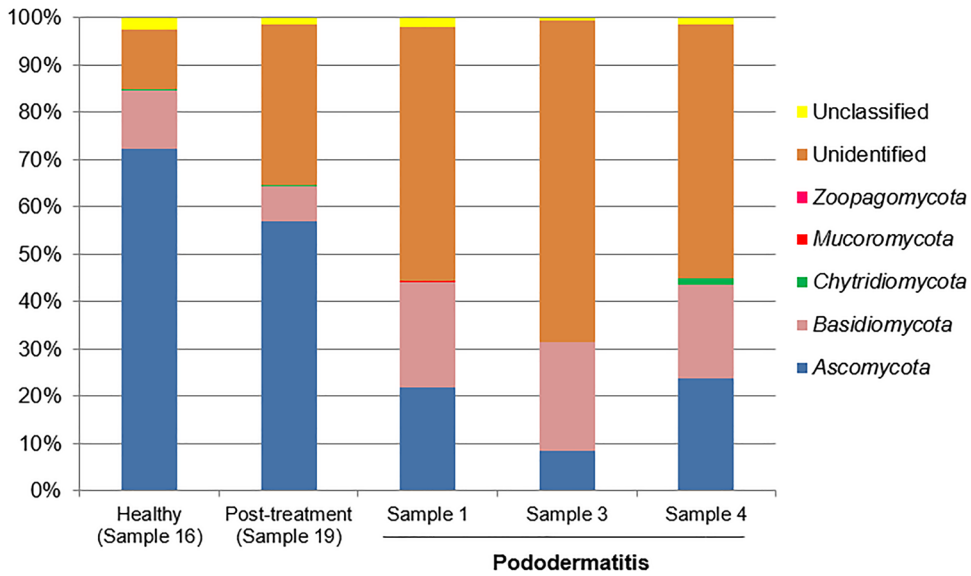

Fig. 1 Relative abundance of fungal phyla across different interdigital fold samples

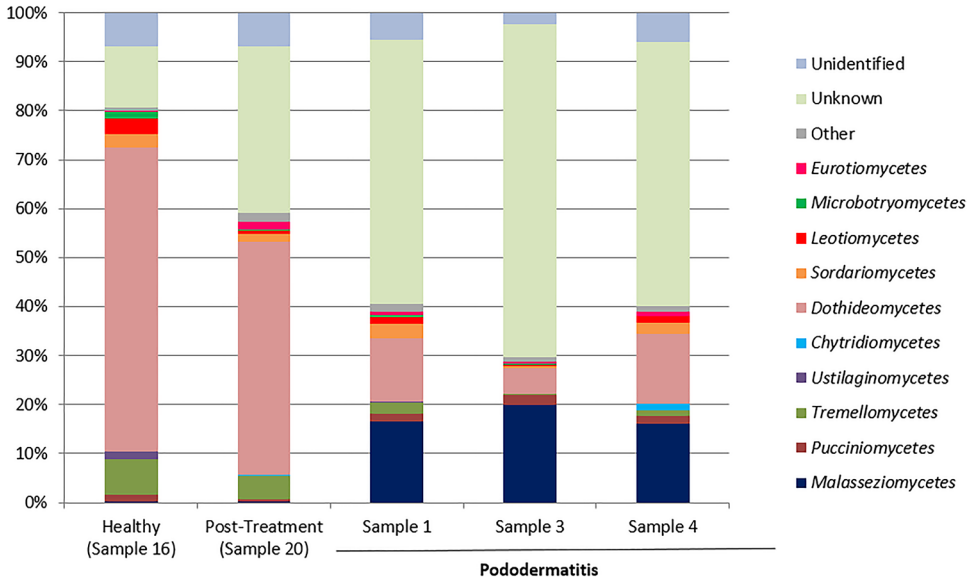

Fig. 2 Relative abundance of fungal classes across the different interdigital fold samples

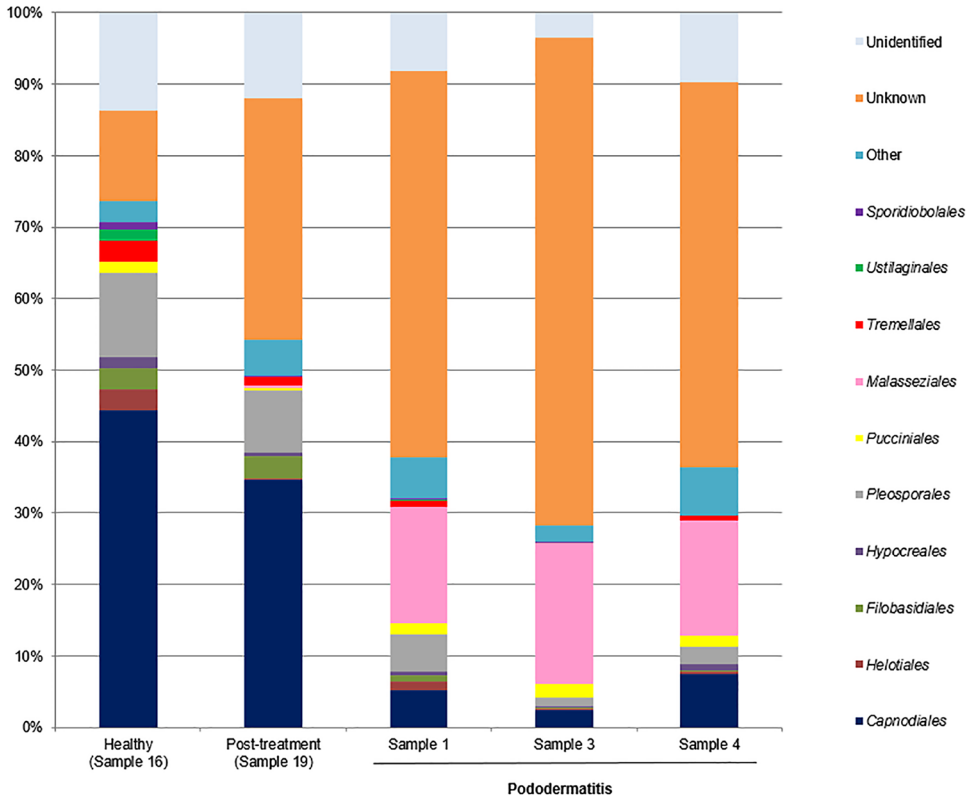

Fig. 3 Relative abundance of fungal orders across the different interdigital fold samples

coverage and identity was not found in our database. Also, the percentage includes ambiguous sequences (0.21%) because they matched with two different sequences from the database but could not discriminate between them. *Malassezia pachydermatis* and *M. sympodialis* showed a higher abundance in the pododermatitis group ($P < 0.05$) while no differences were observed in the abundance of *M. restricta* ($P = 0.024$), and *M. globosa* ($P = 0.56$) between both groups.

Within the healthy and post-treatment samples, *Ascomycota* was the predominant phylum with an abundance of 72.42% and 56.97% respectively (Fig. 1), and *Dothideomycetes* was the main class (Fig. 2). *Capnodiales* with an abundance of 44.54% and 34.64% respectively, was the predominant order (Fig. 3). At the family level (Fig. 4), *Cladosporiaceae* showed the highest abundance

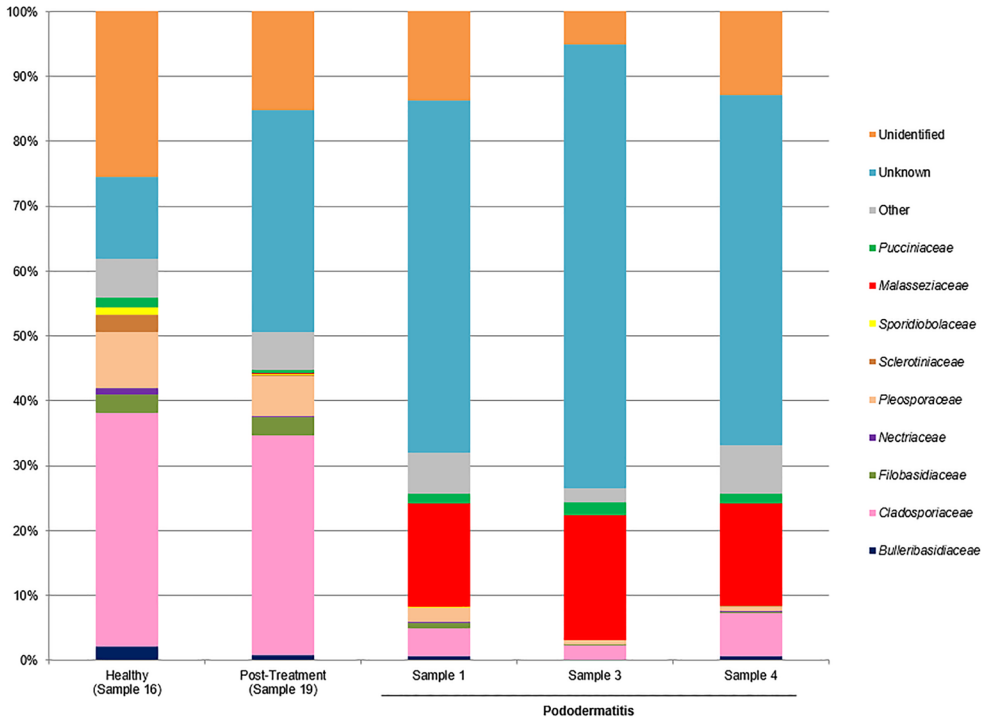

Fig. 4 Relative abundance of fungal families across the different interdigital fold samples

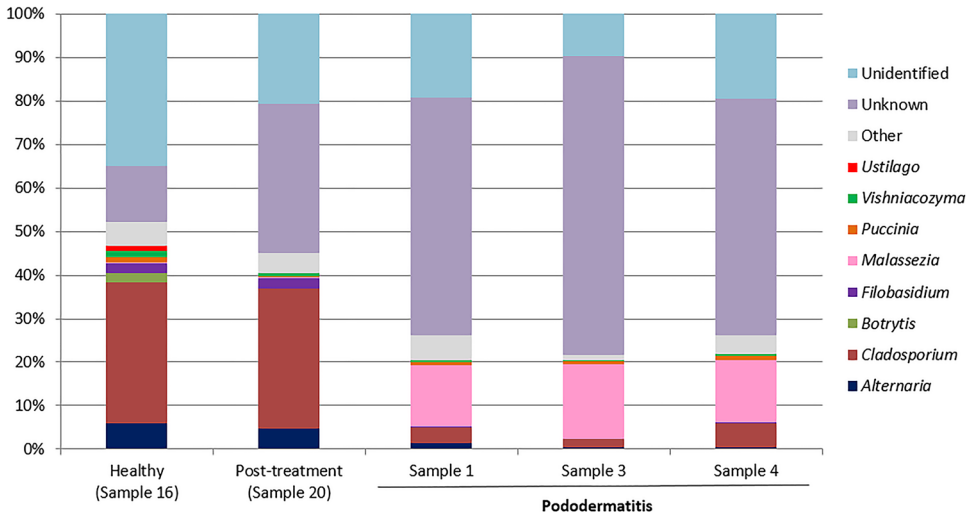

Fig. 5 Relative abundance of fungal genera across the different interdigital fold samples

in both samples (36.02 and 33.81% respectively) and *Cladosporium* was the main genus with an abundance of 32.40% and 32.15% respectively (Fig. 5). *Malassezia pachydermatis* (Fig. 6) was also detected but with low abundance (0.06% and 0.03% respectively). Other *Malassezia* species were identified in these samples. *Malassezia restricta* (0.02%) was identified in both samples, and *M. globosa* (0.04%) was only present in the post-treatment sample. The percentage of *Malassezia* sequences that could not be identified to the species level in healthy samples decreased to 0.03% whereas in post-treatment samples was 0.08%.

Discussion

Samples of the pododermatitis group showed the highest number of yeast cells in the two cytological examinations and the highest number of CFU in the plates. Whereas samples from the healthy and after treatment groups showed much less counted cells in both cytological examinations and no growth was observed in the plates. Clinical signs are associated to a hypersensitivity reaction against the yeast. The proliferation of *Malassezia* within the stratum corneum produces numerous antigens and allergens that activate an immune response resulting in epidermal

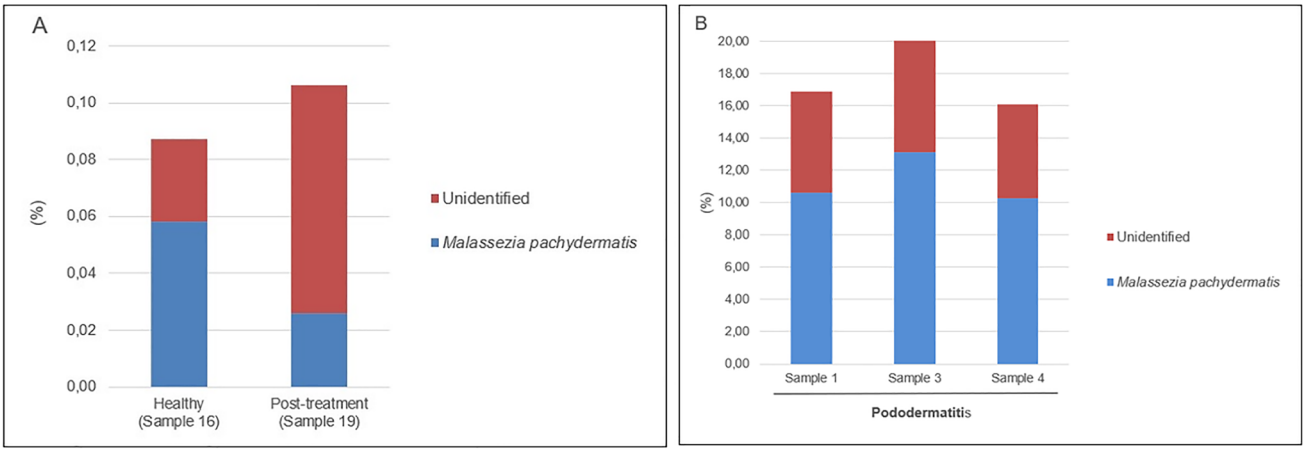

Fig. 6 Relative abundance of *M. pachydermatis* and unidentified taxa across the different samples. (**A**) Healthy and post-treatment samples. (**B**) Pododermatitis samples

damage and inflammation (Bond et al. 2020). Thus, low yeast counts can produce severe clinical sings and any number of the yeast may be considered significant (Bond et al. 2020). However, in the veterinary practice more than 3–5 cells or more per high power field in the skin or a number higher than 70 CFU could be indicative of infection by *Malassezia* spp. (Cafarchia et al. 2005b; Bajwa 2017).

All samples with a positive cytological examination had growth, except the sample 19 that showed a low cell count on the tape strip but had no growth. All the samples with a negative cytology showed no growth in any of the culture media used. Thus, cytological examination is a useful technique for diagnosing *Malassezia* pododermatitis (Cafarchia et al. 2005a; Duclos 2013). Modified Dixon's agar has been suggested as the preferred media to culture *M. pachydermatis* due to the rapid growth of the colonies and its potential to support the lipid-dependent isolates that exist (Puig et al. 2017; Bond et al. 2020). However, in our study no significant differences were obtained in terms of CFU between SGA and mDA.

In our study, two cytological examinations were done using completely different techniques. In all cases, the tape strip method showed the highest abundance of cells, in agreement with other studies (Bond et al. 2020). A study on dogs with *Malassezia* pododermatitis showed that the number of yeasts recovered with swabs were significantly lower than those obtained by using other techniques as tape stripping, superficial scraping, and direct impressions (Miller et al. 2000).

Although a low number of samples has been analyzed, ITS copies of *M. pachydermatis* were increased on most of the samples of the pododermatitis group (73.3%) compared to that of healthy and post-treatment groups. Besides, the qPCR values dramatically decreased in samples from dogs after effective treatment. Due to the higher sensitivity of the qPCR method, DNA of *M. pachydermatis* was detected in all samples. As cytological examination is considered a useful technique for diagnosing *Malassezia* pododermatitis, the results of both cytological examinations were compared to those obtained by qPCR. A good correlation was observed between both diagnostic methods. These results showed that this qPCR method could be a fast useful technique to reliably detect *Malassezia pachydermatis* pododermatitis in dogs.

The fungal diversity studied using NGS was reduced in samples of the pododermatitis group compared with the healthy and post-treatment samples. These results are in accordance with other studies where a reduction of fungal species diversity and richness was observed in samples from dogs with otitis when compared to healthy dogs (Korbelik et al. 2018) and after treatment (Puigdemont et al. 2021). Also, atopic dogs usually present lower diversity in their microbiome when compared to healthy animals (Meason-Smith et al. 2015).

The results obtained in the metagenomic analysis were different between the pododermatitis samples and the healthy and post-treatment samples. *Basidiomycota* was the main phylum identified on pododermatitis samples while *Ascomycota* was the main one on healthy and post-treatment samples. Within the pododermatitis group, *Malassezia* was the majority genus identified in all three samples, and *M. pachydermatis* the main species. The genus *Malassezia* was also present in the healthy and post-treatment samples in a low abundance as it is a common member of the skin of dogs. Within the healthy and post-treatment samples, *Cladosporium* was the main genus followed by *Alternaria*. These genera are considered transient mycobiota as they are commonly isolated from environmental samples.

Our results agree with those obtained in studies of the ear and skin mycobiota of healthy dogs and dogs diagnosed with

otitis or skin pathologies. In all these studies the main phylum identified in healthy dogs was *Ascomycota* and the main genus was part of the transient mycobiota. The second main genus identified in healthy dogs was *Malassezia*. In dogs diagnosed with otitis or dermatitis, *Basidiomycota* was the main phylum and *Malassezia* the main genus identified. *Malassezia pachydermatis* was the main species identified in dogs with otitis and atopic dermatitis (Korbelik et al. 2018; Bradley et al. 2020; Puigdemont et al. 2021). A study conducted by Tang et al. (2020) identified *M. pachydermatis* as the only yeast species present in the samples of both healthy dogs and dogs with otitis/dermatitis. These results differ from those obtained by Meason-Smith et al. (2020) where *M. restricta* was the main species detected in healthy and allergic canine skin. However, in this paper only molecular techniques were used, so this species was not isolated from those animals.

Focusing only on *Malassezia*, *M. pachydermatis* was the main species detected in all the samples, but *M. restricta, M. globosa, M. sympodialis* were also detected. *Malassezia restricta* and *M. globosa* have never been isolated from dogs (Bond et al. 2010). Although *M. sympodialis* has been previously cited from dogs with dermatitis and/or otitis, its identification was based solely on phenotypic characteristics (Raabe et al. 1998; Nardoni et al. 2004; Cafarchia et al. 2005a). Thus, the presence of small amounts of these three species in our samples could be a result of cross-contamination. These species are commonly found on the skin of healthy humans (Sugita et al. 2010) and thus, the contamination may have occurred during the sample extraction or contamination of the kit reagents, or they may be on the dog's skin as a result from interaction with humans or with the indoor environment (Frau et al. 2019; Díaz et al. 2021).

Metagenomic studies on dog skin selected the ITS region as target gene. Although the ITS region is considered the universal barcode for fungi (Schoch et al. 2012), its variability among fungal species could lead to an incorrect estimation of fungal populations (Scorzetti et al. 2002; De Filippis et al. 2017). The LSU selected in this study as target gene has shown greater biodiversity in biological samples and works better for species discrimination in *Basidiomycota* and yeasts, especially within *Malassezia* genus (Hoggard et al. 2018; Mota-Gutiérrez et al. 2019). The use of this region allowed us the description of fungal diversity and the taxonomic classification of several *Malassezia* species. There was a percentage of unidentified *Malassezia* species within the pododermatitis (6.29%) and the healthy/post-treatment (0.05%) group. However, all the sequences belonged to the class *Malasseziomycetes* and could represent new taxa that have not been yet described.

The major limitation of this study was the sample size. Due to the high cost of the method, a total of five samples were selected for metagenomic analysis. Future studies with a larger sample size would be needed to clarify some of the results obtained in this study. Also, further studies including more breeds would be of interest to study the genetic predisposition of some breeds to *Malassezia* pododermatitis.

The NGS analysis allowed a better understanding of dynamics of *M. pachydemartis* pododermatitis. There is a difference in the results obtained in terms of fungal species identified and richness of fungal populations between healthy and post-treatment samples and pododermatitis samples. In the pododermatitis group *Malassezia* is predominant and in the healthy and post-treatment samples transient mycobiota predominates. Also, statistically significant differences were observed between dogs with pododermatitis before and after treatment and healthy dogs in values of cytological examination, CFU and qPCR. The results obtained showed a good correlation between cytologic and molecular methods and its value as a diagnostic tool.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11259-022-09951-2.

Acknowledgements The authors thanks Mar Bardagí for the samples kindly provided for our work. We also thank Carolina Gómez from the Veterinary Mycology Group of Universitat Autònoma de Barcelona (UAB) for valuable technical assistance.

Authors' contributions Gemma Castellá, M. Rosa Bragulat, and F. Javier Cabañes contributed to the study conception and design. Material preparation, data collection and analysis were performed by Leyna Díaz, Gemma Castellá, M. Rosa Bragulat, and F. Javier Cabañes. Leyna Díaz, Gemma Castellá, Walter Sanseverino, and Andreu Paytuví-Gallart performed bioinformatics analyses. The first draft of the manuscript was written by Leyna Díaz and all authors commented and contributed to the preparation of the final manuscript. All authors read and approved the final manuscript.

Funding Open Access Funding provided by Universitat Autònoma de Barcelona. Financial support came from Servei Veterinari de Bacteriologia i Micologia from the UAB.

Data availability The raw sequencing data is available at the NCBI database (SRA accession number: PRJNA742914).

Declarations

Ethics approval All samples were obtained following the procedures on Animal and Human Experimentation from UAB and Generalitat de Catalunya approved by Ethics Committee (Approval number, 4600; Approval date, 22Feb2019). Also, the written consent of owners was obtained before sampling the animals.

Consent to participate Written informed consent was obtained from the dog-owners.

Consent for publication Dog-owners signed informed consent regarding publishing their dogs' data.

Conflict of interest Walter Sanseverino and Andreu Paytuví-Gallart were employed by company Sequentia Biotech S.L. All other authors declare no competing interests.

References

Bajwa J (2016) Canine pododermatitis. Can Vet J 57:991–993

Bajwa J (2017) Cutaneous cytology and the dermatology patient. Can Vet J 58:625–627

Bond R, Collin NS, Lloyd DH (1994) Use of contact plates for the quantitative culture of *Malassezia pachydermatis* from canine skin. J Small Anim Prac 35:68–72

Bond R, Guillot J, Cabañes FJ (2010) *Malassezia* yeasts in animal disease. *Malassezia* and The Skin, 1st edn. Springer, Berlin, pp 271–299

Bond R, Morris DO, Guillot J, Bensignor EJ, Robson D, Mason KV, Kano R, Hill PB (2020) Biology, diagnosis and treatment of *Malassezia* dermatitis in dogs and cats Clinical Consensus Guidelines of the World Association for Veterinary Dermatology. Vet Dermatol 31:28–74. https://doi.org/10.1111/vde.12809

Bradley CW, Lee FF, Rankin SC, Kalan LR, Horwinski J, Morris DO, Grice EA, Cain CL (2020) The otic microbiota and mycobiota in a referral population of dogs in eastern USA with otitis externa. Vet Dermatol 31:225–249. https://doi.org/10.1111/vde.12826

Cabañes FJ (2021) Diagnosis of *Malassezia* dermatitis and otitis in dogs and cats, is it just a matter of counting? Rev Iberoam Micol 38:3–4. https://doi.org/10.1016/j.riam.2020.03.001

Cafarchia C, Gallo S, Capelli G, Otranto D (2005a) Occurrence and population size of *Malassezia* spp. in the external ear canal of dogs and cats both healthy and with otitis. Mycopathologia 160:143–149. https://doi.org/10.1007/s11046-005-0151-x

Cafarchia C, Gallo S, Romito D, Capelli G, Chermette R, Guillot J, Otranto D (2005b) Frequency, body distribution, and population size of *Malassezia* species in healthy dogs and in dogs with localized cutaneous lesions. J Vet Diagn Invest 17:316–322. https://doi.org/10.1177/104063870501700403

De Filippis F, Laiola M, Blaiotta G, Ercolini D (2017) Different amplicon targets for fungal sequencing-based studies of fungal diversity. Appl Environ Microbiol 83:e00905-e917. https://doi.org/10.1128/AEM.00905-17

Díaz L, Castellá G, Bragulat MR, Martorell J, Paytuví-Gallart A, Sanseverino W, Cabañes FJ (2021) External ear canal mycobiome of some rabbit breeds. Med Mycol 59:683–693. https://doi.org/10.1093/mmy/myaa097

Duclos D (2013) Canine pododermatitis. Vet Clin North Am Small Anim Pract 43:69–70. https://doi.org/10.1016/j.cvsm.2012.09.012

Forbes JD, Knox NC, Ronholm J, Pagotto F, Reimer A (2017) Metagenomics: the next culture-independent game changer. Front Microbiol 8:1069. https://doi.org/10.3389/fmicb.2017.01069

Forde BM, O'Toole PW (2013) Next-generation sequencing technologies and their impact on microbial genomics. Brief Funct Genomics 12:440. https://doi.org/10.1093/bfgp/els062

Frau A, Kenny JG, Lenzi L, Campbell BJ, Ijaz UZ, Duckworth CA, Burkitt MD, Hall N, Anson J, Darby AC et al (2019) DNA extraction and amplicon production strategies deeply inf luence the outcome of gut mycobiome studies. Sci Rep 9:9328. https://doi.org/10.1038/s41598-019-44974-x

Giampaoli S, De Vittori E, Frajese GV, Paytuví A, Sanseverino W, Anselmo A, Barni F, Berti A (2020) A semi-automated protocol for NGS metabarcoding and fungal analysis in forensic. Forensic Sci Int 306:110052. https://doi.org/10.1016/j.forsciint.2019.110052

Guého E, Midgley G, Guillot J (1996) The genus *Malassezia* with description of four new species. Antonie Van Leeuw 69:337–355. https://doi.org/10.1007/BF00399623

Guého-Kellerman E, Boekhout T, Begerow D (2010) Biodiversity, phylogeny and ultrastructure. *Malassezia* and The Skin, 1st edn. Springer, Berlin, pp 17–63

Guillot J, Bond R (1999) *Malassezia pachydermatis*: a review. Med Mycol 37:295–306. https://doi.org/10.1046/j.1365-280x.1999.00237.x

Hoggard M, Vesty A, Wong G, Montgomery JM, Fourie C, Douglas RG, Biswas K, Taylor MW (2018) Characterizing the Human Mycobiota: A Comparison of Small Subunit rRNA, ITS1, ITS2, and Large Subunit rRNA Genomic Targets. Front Microbiol 9:2208. https://doi.org/10.3389/fmicb.2018.02208

Korbelik J, Singh A, Rosseau J, Weese S (2018) Analysis of the otic mycobiota in dogs with otitis externa compared to healthy individuals. Vet Dermatol 29:417e138. https://doi.org/10.1111/vde.12665.

Li H, Durbin R (2010) Fast and accurate long-read alignment with Burrows-Wheeler transform. Bioinformatics 26:589–595. https://doi.org/10.1093/bioinformatics/btp698

Love MI, Huber W, Anders S (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 15:550. https://doi.org/10.1186/s13059-014-0550-8

McMurdie PJ, Holmes S (2013) phyloseq: An R Package for reproducible interactive analysis and graphics of microbiome census data. PLoS ONE 8:e61217. https://doi.org/10.1371/journal.pone.0061217

Meason-Smith C, Olivry T, Lawhon SD, Hoffmann AR (2020) *Malassezia* species dysbiosis in natural and allergen-induced atopic dermatitis in dogs. Med Mycol 58:756–765. https://doi.org/10.1093/mmy/myz118

Meason-Smith C, Diesel A, Patterson AP, Older CE, Mansell JM, Suchodolski JS, Rodrigues Hoffmann A (2015) What is living on your dog's skin? Characterization of the canine cutaneous mycobiota and fungal dysbiosis in canine allergic dermatitis. FEMS Microbiol Ecol 91:fiv139. https://doi.org/10.1093/femsec/fiv139

Miller WH, Griffin CE, Campbell KL (2000) Fungal skin diseases. Muller & Kirk's Small Animal Dermatology, 6th edn. Saunders, Philadelphia, pp 336–422

Mota-Gutiérrez J, Ferrocino I, Rantsiou K, Cocolin L (2019) Metataxomic comparison between internal transcribed spacer and 26S ribosomal large subunit (LSU) rDNA gene. Int J Food Microbiol 290:132–140. https://doi.org/10.1016/j.ijfoodmicro.2018.10.010

Nardoni S, Mancianti F, Corazza M, Rum A (2004) Occurrence of *Malassezia* species in healthy and dermatologically diseased dogs. Mycopathologia 157:383–388. https://doi.org/10.1023/b:myco.0000030416.36743.dd

Puig L, Bragulat MR, Castellá G, Cabañes FJ (2017) Characterization of the species *Malassezia pachydermatis* and re-evaluation of its lipid dependence using a synthetic agar medium. PLosONE 12:e0179148. https://doi.org/10.1371/journal.pone.0179148

Puig L, Castellá G, Cabañes FJ (2019) Quantification of *Malassezia pachydermatis* by real-time PCR in swabs from the external ear canal of dogs. J Vet Diagn Invest 31:440–447. https://doi.org/10.1177/1040638719840686

Puigdemont A, D'Andreano S, Ramió-Lluch L, Cuscó A, Francino O, Brazis P (2021) Effect of an anti-inflammatory pomegranate otic

treatment on the clinical evolution and microbiota profile of dogs with otitis externa. Vet Dermatol 32:158-e37. https://doi.org/10.1111/vde.12930

Raabe P, Mayser P, Weiss R (1998) Demonstration of *Malassezia furfur* and *M. sympodialis* together with *M. pachydermatis* in veterinary specimens. Mycoses 41:493–500. https://doi.org/10.1111/j.1439-0507.1998.tb00712.x

Reynolds DR, Taylor JW (1993) The Fungal Holomorph: Mitotic, Meiotic and Pleomorphic Speciation in Fungal Systematics, 1st edn, CAB international, Surrey, UK

Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA, Chen W et al (2012) Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Proc Natl Acad Sci U S A 109:6241–6246. https://doi.org/10.1073/pnas.1117018109

Scorzetti G, Fell JW, Fonseca A, Statzell-Tallman A (2002) Systematics of basiodiomycetous yeasts: a comparison of large subunit D1/D2 and internal transcribed spacer rDNA regions. FEMS Yeast Res 2:495–517. https://doi.org/10.1111/j.1567-1364.2002.tb00117.x

Sugita T, Tajima M, Tsubuku H, Tsuboi R, Nishikawa A (2006) Quantitative analysis of cutaneous *Malassezia* in atopic dermatitis patients using real-time PCR. Microbiol Immunol 50:549–552. https://doi.org/10.1111/j.1348-0421.2006.tb03825.x

Sugita T, Boekhout T, Velegraki A, Guillot J, Hađina S, Cabañes FJ (2010) Epidemiology of *Malassezia*-related skin diseases. *Malassezia* and The Skin, 1st edn. Springer, Berlin, pp 65–119

Tang S, Prem A, Tjokrosurjo J, Sary M, Van Bel MA, Rodrigues-Hoffmann A, Kavanagh M, Wu G, Van Eden ME, Krumbeck JA (2020) The canine skin and ear microbiome: A comprehensive survey of pathogens implicated in canine skin and ear infections using a novel next-generation-sequencing-based assay. Vet Microbiol 247:108764. https://doi.org/10.1016/j.vetmic.2020.108764

Vuran E, Karaarslan A, Karasartova D, Turegun B, Sahin F (2014) Identification of *Malassezia* species from pityriasis versicolor lesions with a new multiplex PCR method. Mycopathologia 177:41–49. https://doi.org/10.1007/s11046-013-9704-6

5.3. Díaz L, Castellá G, Bragulat MR, Cabañes FJ. *ERG11* gene variability and azole susceptibility in *Malassezia pachydermatis. Mycopathologia.* 2022; doi:10.1007/s11046-022-00696-9.

Mycopathologia
https://doi.org/10.1007/s11046-022-00696-9

ORIGINAL ARTICLE

ERG11 Gene Variability and Azole Susceptibility in *Malassezia pachydermatis*

Leyna Díaz · Gemma Castellá · M. Rosa Bragulat · F. Javier Cabañes

Received: 15 September 2022 / Accepted: 18 November 2022

Abstract *Malassezia pachydermatis* is part of the normal skin microbiota of various animal species but under certain circumstances becomes an opportunistic pathogen producing otitis and dermatitis. Commonly these *Malassezia* diseases are effectively treated using azoles. However, some cases of treatment failure have been reported. Alterations in the *ERG11* gene have been associated with in vitro azole resistance in *M. pachydermatis*. In the present study, in vitro antifungal susceptibility of 89 different strains of *M. pachydermatis* isolated from different animal species and health status was studied. The susceptibility to fluconazole (FLZ), itraconazole (ITZ), ketoconazole and amphotericin B was tested by a disk diffusion method and 17 strains were also subjected to an ITZ E-test. Mueller–Hinton supplemented with 2% glucose and methylene blue was used as culture medium in both susceptibility assays. Multilocus sequence typing was performed in 30 selected strains using D1D2, ITS, CHS2 and β-tubulin genes. Also, *ERG11* gene was sequenced. The four antifungals tested were highly effective against most of the strains. Only two strains showed no inhibition zone to antifungals and a strain showed an increased MIC to ITZ. The study of the *ERG11* sequences revealed a high diversity of DNA sequences and a total of 23 amino acid substitutions, from which only two have been previously described. Also, three deleterious substitutions (A302T, G459D and G461D) previously associated with azole resistance in this yeast were recovered. A correlation between certain genotypes and *ERG11* mutations was observed. Some of the *ERG11* mutations recovered were correlated with a reduced susceptibility to azoles.

Keywords Antifungal susceptibility · Azole drugs · *ERG11* mutations · *Malassezia pachydermatis*

Handling Editor: Takashi Sugita.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11046-022-00696-9.

L. Díaz · G. Castellá (✉) · M. R. Bragulat · F. J. Cabañes
Veterinary Mycology Group, Department of Animal Health and Anatomy, Universitat Autònoma de Barcelona, Bellaterra, Catalonia, Spain
e-mail: Gemma.Castella@uab.cat

G. Castellá
Grup de Micologia Veterinària, Departament de Sanitat i d'Anatomia Animals, Facultat de Veterinària, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain

Introduction

Malassezia pachydermatis is a lipophilic yeast that belongs to the normal skin microbiota of various animal species and colonizes the skin and mucosal

Published online: 10 December 2022

sites of healthy dogs. Although *M. pachydermatis* has been considered classically the only non-lipid-dependent species of the genus *Malassezia,* they also lack the genes coding for a fatty acid synthase and thus, are unable to synthetize long-chained fatty acids (C14 or C16) *de novo* [1, 2]. *Malassezia pachydermatis* can utilize lipid fractions within the peptone component of SGA, thus they also need a few lipids to be able to grow [2–4].

Despite being part of the microbiota, *Malassezia* may act as an opportunistic pathogen under certain circumstances that allow the overgrowth of the yeast population. *Malassezia pachydermatis* is a common responsible agent of otitis and dermatitis in dogs [5, 6]. In humans, it has also been reported to produce systemic infections particularly in neonates and immunocompromised patients receiving parenteral nutrition [7]. The treatment of *Malassezia* diseases is currently based on the use of topical and systemic antifungal therapy. Topical agents include chlorhexidine, clotrimazole, enilconazole, ketoconazole, miconazole and nystatin that can be used in various forms such as shampoos, sprays, or ointments [8]. Azoles like ketoconazole (KTZ) or itraconazole (ITZ) are commonly used as systemic agents for the treatment of *Malassezia* dermatitis and otitis. Fluconazole (FLZ) can be used for the treatment of *Malassezia* skin diseases like seborrheic dermatitis [9, 10]. Amphotericin B (AMB) and liposomal amphotericin B are indicated in the treatment of systemic cases of *Malassezia* in humans [8, 11].

The Clinical and Laboratory Standards Institute (CLSI) developed in 2002 a reference broth microdilution method for evaluating the susceptibility of *Candida* spp. and *Cryptococcus neoformans* [12]. This method is inapplicable to the genus *Malassezia* because of their lipid-dependency, their slower growth rate and tendency to form clusters [13, 14]. Thus, it has been subsequently adapted to *Malassezia* by various researchers modifying the media, time of incubation and inoculum but it has not been yet standardized [13, 15–17]. Also, disk diffusion method and the E-test gradient have been adapted to evaluate the susceptibility of *Malassezia* against antifungal compounds [14, 18].

Although antimicrobial resistance is a global serious threat to human and animal health, recent studies have showed that most wild-type *Malassezia* yeast remain susceptible to the most used azoles such as KTZ, ITZ and miconazole but the efficacy of FLZ is variable [8, 11, 19]. Some sporadic reports of therapeutic failure with azoles in canine *M. pachydermatis* dermatitis associated with increased tolerance to azoles in vitro might reflect the chronic and relapsing course of *Malassezia* diseases that often need frequent and lengthy treatments [16, 20–23].

There are various mechanisms of azole antifungal resistance in *Malassezia* species. The drug efflux pump is associated to azole resistance in *M. pachydermatis* [14, 25]. Mutations in the coding region of the *ERG11* gene, encoding a lanosterol 14α-demethylase, which is the target enzyme of azole antifungals, are considered a mechanism of resistance [26–28]. Kim et al. [29] demonstrated that tandem quadruplication of the genomic region of the *ERG11* gene contributes to azole resistance in *M. pachydermatis*. At the present, only two *M. pachydermatis* strains have demonstrated to be resistant to azoles. The strains were isolated in Japan [23] and Italy [22]. Both isolates showed high minimum inhibitory concentrations (MICs) against azoles but only in one case this resistance was linked to *ERG11* mutations [23]. Although some mutations have been detected in this gene, its intrinsic diversity in *M. pachydermatis* yeasts, that are mainly susceptible to antifungals, is yet unknown.

Even though there is not a reference method for susceptibility testing of *M. pachydermatis* available yet, on this study we evaluate the susceptibility of different strains of this yeast against three different azoles (FLZ, ITZ, and KTZ) and AMB. We included a wide variety of strains recovered from different animal species with different health status and collected over different years. Also, we study the genetic variability of *ERG11* gene in these strains. Gene mutations and antifungal susceptibility testing results was investigated.

Materials and Methods

Strains

A total of 89 strains of *M. pachydermatis* (Table S1) were selected from our culture collection for susceptibility testing. Strains were isolated between 1994 and 2021 and included different animal species: dog (n = 73), cat (n = 12), pig (n = 1), cow (n = 1), goat

Table 1 Interpretative criterion of zones of inhibition

Inhibitory zone diameter (mm)						
	Rosco©			Bio-Rad©		
	S	SDD	R	S	SDD	R
Amphotericin B	≥ 15	14–10	≤ 10	> 10	–	≤ 10
Fluconazole	≥ 19	18–15	≤ 14	≥ 19	18–15	≤ 14
Itraconazole	≥ 23	22–14	≤ 13	–	–	–
Ketoconazole	≥ 28	27–21	≤ 20	≥ 20	20–10	≤ 10

S, susceptible; SDD, susceptible dose dependent; R, resistant

(–) No Data

(n = 1), and horse (n = 1). The *M. pachydermatis* neotype strain CBS1879 was also included. The strains were stored at − 80 °C [24].

The 73 strains of dogs included four strains isolated from healthy dogs, eight strains from dogs with dermatitis, 59 from dogs with otitis externa and two from otitis media. According to the data provided by the clinicians when sending the samples, the strains from otitis externa were classified into chronic otitis externa (lasting more than three months, n = 25), acute otitis externa (lasting less than six weeks, n = 17), purulent otitis externa (n = 6) and recurrent otitis externa (n = 4). The strains isolated from cats included five strains recovered from cats with dermatitis and six strains from animals with otitis, from which two were otitis media and four otitis externa. The strains from otitis externa were classified into acute otitis externa (n = 2), chronic otitis externa (n = 1) and purulent otitis externa (n = 1). One strain from a healthy cat was also included. The strains from cow, horse, pig, and goat were all obtained from healthy animals.

Susceptibility Testing

Four different antifungals were selected for susceptibility testing: amphotericin B (AMB), fluconazole (FLZ), itraconazole (ITZ) and ketoconazole (KTZ). Susceptibility of the strains was tested by a disk diffusion method. For the disk diffusion method, two stock inoculum suspensions were prepared from 3-day old cultures on SGA (Oxoid) at 35°C from each strain. The stock inoculum suspensions were performed in 3 mL of distilled water supplemented with 0.004% Tween 80 to reduce clump formation and were adjusted to a density of 1 McFarland standards (3×10^8 CFU/ml). Mueller–Hinton supplemented with 2% glucose and 0.5 mg/l methylene blue (MH-GM) was the media selected for the disk diffusion method [13, 30]. For each suspension tube, 5 plates of the media were streaked using a sterile cotton swab dipped into the inoculum suspension. One of the plates was a growth control and on the remaining four, one disk of antifungal per plate was placed. Two commercial antifungal disks were used comprising AMB, FLZ, ITZ and KTZ (Neo-Sensitabs, Rosco) at concentrations of 10, 25, 10 and 15 µg/disk respectively and FLZ, KTZ and AMB (Bio-Rad) at concentrations of 25, 50 and 100 µg/disk, respectively. Plates were then incubated at 35°C, and inhibition zone sizes were measured at 48, 72 and 96 h. The inhibition zone diameter was determined after incubating at 35°C for 72 h. *Candida parapsilopsis* ATCC 90,028 and *Pichia kundriavzevii* ATCC 6258 were included as quality control strains in both disk diffusion tests.

The interpretative criterion was according to the manufacturer's guidelines for yeasts, as shown in Table 1.

Susceptibility against ITZ was also studied using the E-test method on 17 out of the 89 strains. The 17 strains were selected based on their diverse response to antifungals by disk diffusion method, but also different animal species were included. The strain selection included strains obtained from healthy animals (n = 7), from animals with otitis externa (n = 8) and animals with dermatitis (n = 2). Also, the *M. pachydermatis* neotype strain CBS1879 was included. E-test was performed under the same conditions that the disk diffusion method. One E-test gradient stripe for ITZ (Biomérieux) was placed on each plate. The plates were then incubated at 35°C and examined at 48, 72 and 96 h. The MICs were determined after 72 h of incubation. Also, some strains were evaluated using SGA supplemented with 1% Tween 80 [23] to compare the results obtained.

ERG11 Gene Amplification and Sequencing

A total of 31 strains were selected to sequence their *ERG11* gene, including the neotype strain of *M. pachydermatis* CBS1879 and the 17 strains evaluated by E-test. The strains selected included different animal species, dog (n = 23), cat (n = 4), pig (n = 1), horse (n = 1), goat (n = 1) and cow (n = 1). These strains were recovered from nine healthy animals, 17 animals with otitis externa, one animal with otitis media and four animals with dermatitis. The strains were selected based on their susceptibility results against the four antifungals tested. Strains without an inhibition zone, a reduced inhibition zone in the disk diffusion method and a high MIC to ITZ were included. Also, strains with wider inhibition zone diameters were included.

The DNA was extracted directly from 5-day-old cultures grown in SGA according to the FastDNA Spin kit protocol (MP Bio-medicals, Biolink, Barcelona, Spain). The *ERG11* gene was amplified using the primers pairs MALAERG1S/R, MALAERG2S/R, MALAERG3S/R, MALAERG4S/R described by Kano et al. [23]. Reaction mixtures contained 5.0 µl of template DNA, 5.0 µl of 10 × PCR buffer, 0.2 mM of each dNTP, 1.5 mM MgCl2, 0.2 µM each primer and 1.25 U of *Taq* polymerase in a final volume of 50 µl. The amplification process consisted of a pre-denaturation step at 95 °C for 10 min, followed by 35 cycles of denaturation at 95 °C /15 s, annealing at 55 °C /30 s and extension at 72 °C /1 min, and a final extension of 5 min at 72 °C. Both strands of purified gene fragments were sequenced with BigDye Terminator v3.1. cycle sequencing kit (Applied Biosystems) on an Applied Biosystems 3730 DNA Analyzer (Applied Biosystems). Sequence alignments were carried out using Clustal X v2.0.12. software [31]. The PROVEAN software [32] was used to study the effect on the protein of the amino acid substitutions.

Maximum likelihood analysis of the *ERG11* sequences was conducted using MEGA 11 software [33] with 1,000 bootstrap replicates. Clades that were supported by bootstrap values of ≥ 70% were regarded as strongly supported.

Multilocus Sequences Typing

The strains selected for *ERG11* gene sequencing were also chosen for a multilocus sequence typing system based on four target regions (D1/D2 region of the 26S rRNA gene, the ITS-5.8S rRNA gene, the β-tubulin gene and the CHS2 gene).

Sequences of the four genes of 16 strains had been characterized previously [34]. The four genes of 15 remaining strains were amplified and sequenced as described previously [35]. To illustrate the phylogenetic relatedness between our strains a minimum spanning tree was constructed using the PHYLOViZ 2.0 software [36]. Each genotype cluster was identified by using the goeBURST algorithm version.

Statistical Analysis

Statistical analyses were conducted by Minitab 17 Statistical software (Minitab). The results of both dilutions of each strain were compared using the Student's t-test. To compare the means of inhibition zone diameter between strains obtained from healthy animals and strains obtained from animals with dermatitis/otitis the ANOVA test was used.

Results

Susceptibility Testing

All the strains tested showed growth on the media selected (MH-GM). Susceptibility results were determined after 72 h of incubation at 35°C, since it was the optimal time of growth for all the strains included. At 48 h not all the strains had growth and no differences were observed between inhibition zone diameters at 72 and 96 h. The results of both disk diffusion methods at 72 h are summarized in Table 2. No significant differences were observed between both dilutions of each strain ($P > 0.05$).

In general, according to the manufacturer's breakpoints for yeasts, the four antifungals tested were active against most of the strains regardless of their origin. However, a few exceptions were observed. With Rosco antifungal disks, five strains were classified as susceptible dose dependent (SDD) and five as resistant (R) to FLZ. One strain was classified as R to KTZ, one as SDD to ITZ, and four as SDD and one as R to AMB. One strain (MA165) was classified as R to ITZ, KTZ and AMB, and one strain (MA1386) was classified as R to FLZ and SDD to AMB. With Bio-Rad antifungal disks, four strains were classified as

Table 2 Mean (± SD) inhibition diameters at 72 h and range to fluconazole (FLZ), itraconazole (ITZ), ketoconazole (KTZ) and amphotericin B (AMB) by both disk diffusion tests performed in strains from healthy animals, animals with otitis and animals with dermatitis

	HEALTHY (n = 9)			OTITIS (n = 67)			DERMATITIS (n = 13)			TOTAL (n = 89)			*p* value
	n	X ± SD	range	n	X ± SD	range	n	X ± SD	range	n	X ± SD	range	
FLZ													
R 25 μg	4	32.25 ± 2.90	29.00–36.00	67	30.50 ± 10.31	7.50–56.50	12	30.38 ± 11.76	0- 42.00	83	30.57 ± 10.22	0–56.50	0.945
B 25 μg	8	35.19 ± 3.94	28.00–39.00	66	33.68 ± 12.82	5–73.00	12	35.75 ± 17.06	0- 68.00	86	34.11 ± 12.49	0–73.00	0.775
ITZ													
R 10 μg	8	36.44 ± 3.97	29.50–42.00	67	39.43 ± 6.80	19.0–58.00	12	39.88 ± 5.41	28.00–47.50	87	39.22 ± 6.43	19.00–58.00	0.433
KTZ													
R 15 μg	8	48.94 ± 2.80	44.00–53.00	67	53.78 ± 7.88	20.00–73.00	12	53.71 ± 3.86	45.50–59.00	87	53.32 ± 7.22	20.00–73.00	0.199
B 50 μg	8	56.94 ± 3.45	53.00–63.00	67	62.79 ± 6.45	38.50–78.00	13	66.85 ± 5.12	57.50–77.00	88	62.86 ± 6.46	38.50–78.00	0.002
AMB													
R 10 μg	4	16.63 ± 0.75	15.50–17.00	67	17.33 ± 2.99	0–22.00	12	17.88 ± 1.55	15.00–20.00	83	17.37 ± 2.76	0–22.00	0.706
B 100 μg	8	19.88 ± 1.22	18.00–22.00	67	19.88 ± 2.73	13.00–27.50	13	19.46 ± 2.20	16.00–23.00	88	19.82 ± 2.54	13.00–27.50	0.530

R, Rosco©; B, Bio-Rad©

Table 3 Strains of *Malassezia pachydermatis* used, including animal host, health status, corresponding type of *ERG11* sequence, amino acid substitutions, mean inhibition diameters of the disk diffusion method and itraconazole minimum inhibitory concentration (MIC) using E-test method at 72 h

Strain	Host	Health status	*ERG11* DNA Sequence type	Amino acid substitutions	Mean inhibition diameters (mm) values at 72 h					MIC (μg/ml)
					FLZ (R)	FLZ (B)	ITZ (R)	KTZ (R)	KTZ (B)	E-test ITZ
CBS1879	Dog	OE	I[a]	–	28	36.5	37.5	49	54.5	0.002
CBS6535	Dog	H	I[a]	–	26	36.5	34	50	55	0.003
MA13	Dog	H	I[a]	–	36	36	40	53	63	0.003
MA52	Dog	H	I[a]	–	34	37	38.5	51.5	58.5	0.002
MA56	Dog	H	I[a]	–	35	35.5	38	47.5	59.5	0.003
MA195	Dog	OE	I[a]	–	56.5	47	57	64	63.5	0.002
MA1595	Cow	H	I[a]	–	36	39	35	49.5	57	–
CBS1884	Dog	OE	II[a]	E181Q	35	42.5	42	51.5	69.5	0.002
MA94	Horse	H	II[a]	E181Q	31.5	39	34.5	49	53	0.002
MA312	Cat	OE	XI[a]	E181Q	36	38.5	37.5	48.5	62	0.003
MA587	Cat	OM	II[a]	E181Q	37.5	54.5	36.5	46.5	38.5	–
MA579	Cat	D	XVIII[a]	I25V, E181Q	31.5	41	40	54	67	0.002
MA1716	Dog	D	XXI[a]	**A302T, G459D**	–	6.5	–	–	59	0.125
MA10	Dog	OE (C)	VIII[a]	I25V, E181Q, T354I	31	28	58	47	49	–
MA140	Cat	H	IV[a]	I25V, E181Q, T354I	32.5	28	42	47	56	0.002
MA1401	Dog	OE (R)	XVI[a]	V33I, E181Q, **R202H**	23	30	34	45	60	–
MA475	Pig	H	V[b]	R84K, D166E, E181Q, D405N	29	30.5	29.5	44	53	0.002
MA7	Dog	OE (R)	VI[b]	A17T, R84K, R175H, Q178R, E181Q	21	17.5	30	50	61	–
MA8	Dog	OE (C)	VII[b]	A17T, R84K, R175H, Q178R, E181Q	40.5	41.5	45.5	59	60	–
MA856	Dog	OE (A)	XIV[b]	A17T, R84K, R175H, Q178R, E181Q	20	24	33	39.5	63.5	–
MA944	Dog	OE (C)	XIV[b]	A17T, R84K, R175H, Q178R, E181Q	15.5	27.5	29	45	59	–
MA968	Dog	OE (P)	XIV[b]	A17T, R84K, R175H, Q178R, E181Q	25	25	25	44.5	68.5	–
MA1382	Dog	OE (C)	XV[b]	A17T, R84K, R175H, Q178R, E181Q	16	18.5	41	54	59.5	0.002
MA107	Goat	H	III[c]	E181Q, N212S, E290D, **Y352F**, H399R	–	–	–	–	–	–
MA165	Dog	OE (C)	IX[b]	A17T, R84K, **F143S**, R175H, Q178R, E181Q	21	33.5	19	20	66.5	0.002
MA356	Dog	O	XII[b]	A17T, R84K, Q178R, E181Q, **Y352F**, H399R	23.5	27.5	39	51	53.5	0.002
MA280	Dog	OE (C)	X[c]	I25S, **W52L**, R84K, L86F, E181Q, N212S, E290D, **Y352F**, H399R	24.5	9.5	46	62.5	64	0.002
MA361	Dog	OE (C)	XIII[c]	I25S, **W52L**, R84K, L86F, E181Q, N212S, E290D, **Y352F**, H399R	18	20	34	51	63	–
MA1289	Dog	D	XIX[c]	I25S, **W52L**, R84K, L86F, E181Q, N212S, E290D, **Y352F**, H399R	19	19	40	52.5	65	–
MA1478	Dog	OE (A)	XVII[c]	I25S, **W52L**, R84K, L86F, E181Q, N212S, **S226L**, E290D, **Y352F**, H399R	8	5.5	32	59	58.5	–

Table 3 continued

Strain	Host	Health status	*ERG11* DNA Sequence type	Amino acid substitutions	Mean inhibition diameters (mm) values at 72 h					MIC (μg/ml)
					FLZ (R)	FLZ (B)	ITZ (R)	KTZ (R)	KTZ (B)	E-test ITZ
MA1429	Dog	D	XX[c]	I25S, **W52L**, R84K, L86F, E181Q, N212S, E290D, **A306S, Y352F**, H399R, **G461D**	0	0	32.5	47	64.5	–

In bold, deleterious mutations

OE, otitis externa; OM, otitis media; A, acute; C, chronic; P, purulent; R, recurrent; H, healthy; D, dermatitis; FLZ, fluconazole; ITZ, itraconazole; KTZ, ketoconazole; R, Rosco©; B, Bio-Rad©

Subclades from the phylogenetic analysis of *ERG11* gene: [a]subclade I; [b]subclade III; [c]subclade II

– Not tested

SDD and seven as R to FLZ. These strains were recovered from animals with chronic otitis externa or dermatitis. All strains recovered from healthy animals were classified as susceptible according to the manufacturer's breakpoints for yeasts. Strain MA1429, isolated from a dog with dermatitis, showed the complete absence of inhibition zone to FLZ and strain MA165, isolated from a dog with chronic otitis externa, showed no inhibition zone to AMB.

No differences were observed in the inhibition zone diameters between strains recovered from healthy animals and strains from animals with otitis or dermatitis except for the KTZ Bio-Rad antifungal disks (Table 2). Strains recovered from chronic otitis externa and otitis media showed smaller inhibition diameters to KTZ than strains from purulent and acute otitis externa ($P = 0.010$).

Most of the strains selected for the E-test method using MH-GM as culture medium, showed MICs between 0.002 and 0.003 μg/ml to ITZ (Table 3). The only exception was strain MA1716 that showed a MIC of 0.125 μg/ml (Fig. 1). This strain was also tested using SGA supplemented with 1% Tween 80 and a MIC of 8-12 μg/ml was obtained. The neotype strain CBS 1879 showed a MIC of 0.002 μg/ml and 0.38 μg/ml in MH-GM and SGA + 1% Tween 80, respectively.

ERG11 Sequence

Amplification of the complete *ERG11* gene and screening for amino acid substitutions was performed for each of the 31 strains (Table 3). The *ERG11* sequence included 1623 bp. The sequences of the strains shared 96.0–100% sequence similarity to *M. pachydermatis* neotype strain CBS1879 and were clustered into 21 different genetic types. Representative nucleotide sequences of the different genetic types determined in this study have been deposited at the GenBank database under accession numbers ON814677- ON814697.

Of the 31 strains of *M. pachydermatis*, seven of them belonged to the genetic type I, including the neotype strain CBS1879. All strains of genetic type I were isolated from healthy animals except two (MA195 and CBS1879). Genetic type II included three strains recovered from different animal species and genetic type XIV included three strains recovered from dogs with otitis. The rest of genetic types were unique for each strain.

The predicted *ERG11* amino acid sequences consisted of 540 amino acids. Some silent polymorphisms were identified, and comparison of the deduced amino acid sequences revealed 14 unique amino acid sequence types. Strains belonging to genetic type I had no amino acid substitution. Strains from genetic type II and XI showed the same amino acid sequence with one amino acid substitution (E181Q). Strains from genetic type IV and VIII showed the same amino acid sequence with three substitutions (I25V, E181Q, and T354I). Strains from genetic type X, XIII and XIX showed the same amino acid sequence with nine substitutions (I25S, W52L, R84K, L86F, E181Q, N212S, S226L, E290D, Y352F, H399R). Strains from

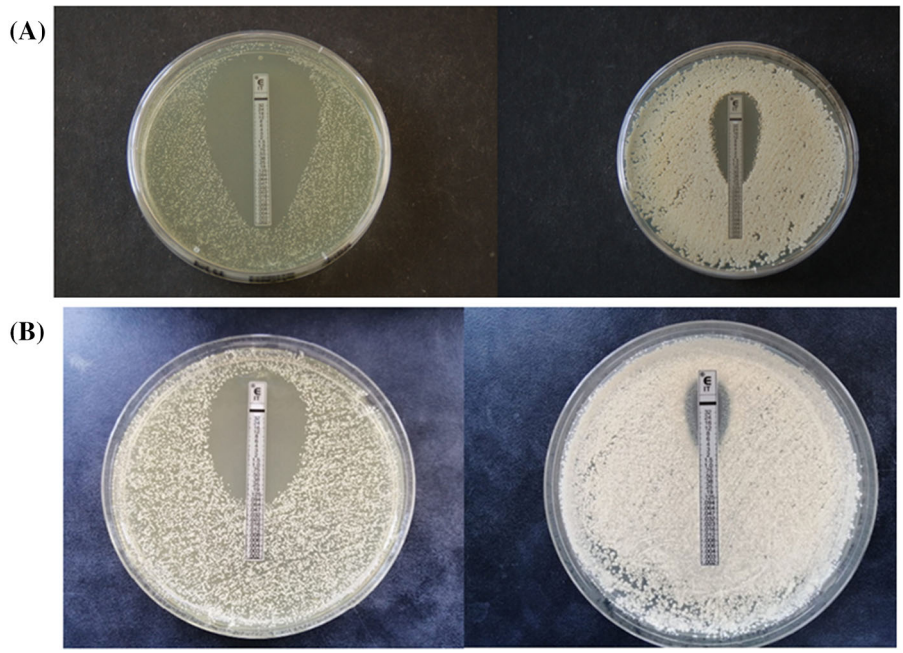

Fig. 1 E-test assay of two isolates of *M. pachydermatis* against itraconazole. A) Reference strain CBS1879 in MH-GM (left) and SGA + Tween 80 (right) culture media. B) Strain MA1716 in MH-GM (left) and SGA + Tween 80 (right) culture media

genetic type VI, VII, XIV and XV showed the same amino acid sequence with five substitutions (A17T, R84K, R175H, Q178R, and E181Q). The number of amino acid substitutions of the rest of genetic types varied between the strains and ranged from 2 to 11 substitutions per strain. A total of 23 amino acid substitutions were identified. Fourteen amino acid substitutions were considered as neutral: A17T, I25V, I25S, V33I, R84K, L86F, D166E, R175H, Q178R, E181Q, N212S, E290D, T354I, H399R, D405N. All these mutations were recovered from strains isolated from different animal species and health status. Nine amino acid substitutions were considered as deleterious substitutions (W52L, F143S, R202H, S226L, A302T, A306S, Y352F, G459D, G461D) and were recovered from strains isolated mainly from dogs with otitis or dermatitis. Mutations A302T, G459D were detected in strain MA1716 with a MIC of 0.125 µg/ml and mutations W52L, A306S, Y352F, and G461D were detected in strain MA1429 with no inhibition zone to FLZ.

Maximum likelihood analysis of the *ERG11* gene is shown in Fig. 2. All *M. pachydermatis* sequences grouped in a supported clade. The strains were grouped in three different subclades. Subclade I (94% bootstrap) grouped strains from healthy animals and animals with otitis or dermatitis. Subclade II (96% bootstrap) grouped mainly strains isolated from dogs with otitis and dermatitis and one strain from a healthy goat. A third subclade, subclade III (81% bootstrap) grouped strains isolated from dogs with otitis and one strain from a healthy pig.

Multilocus Sequence Typing

Sequence types obtained for each gene are listed in Supplementary Table S2. Six different sequence types of D1/D2 region were recovered, five from them were previously described and one is new from this study (VI). Eleven out of the 30 strains (36.66%) had sequence type I which was the most abundant. Strains from healthy animals only showed sequence types I or II.

The ITS 5.8S rRNA was successfully amplified and sequenced. Fifteen different sequence types were recovered from which four were new (XIV, XV, XVI and XVII). Ten strains had one of the new ITS sequence types. The sequence type I was the majority (n = 5; 16.6%) and was recovered from strains from dogs.

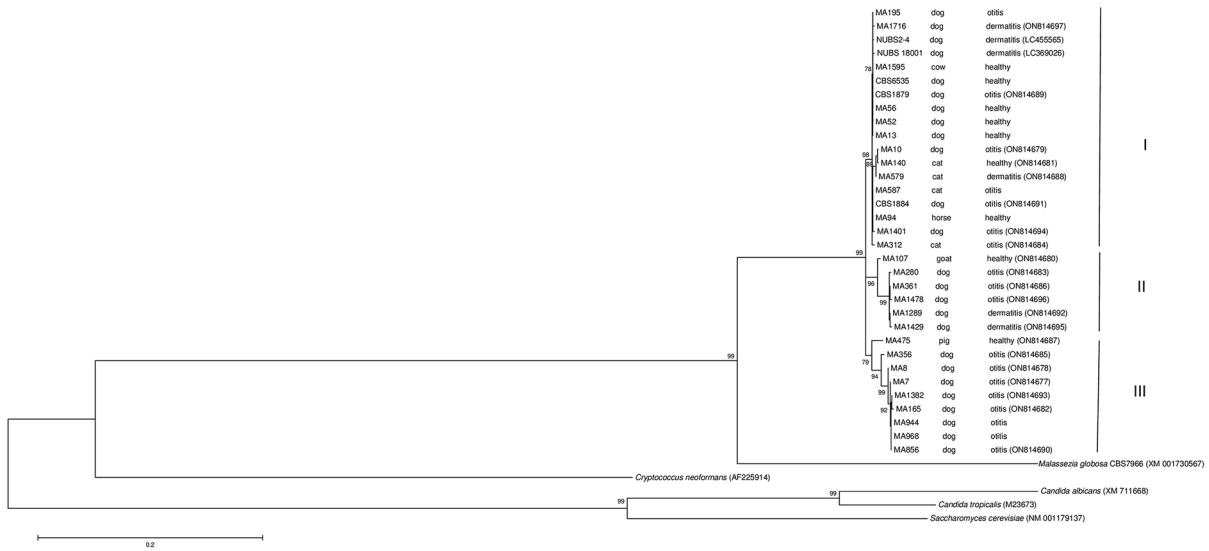

Fig. 2 Molecular phylogenetic tree inferred from maximum likelihood analysis of *ERG11* sequences of *M. pachydermatis* strains. Bootstrap values > 70% in 1,000 replications are shown at the nodes. Sequences of *Cryptococcus neoformans*, *Candida albicans*, *Candida tropicalis* and *Saccharomyces cerevisiae* were selected as outgroup for the tree construction

Ten different sequence types of β-tubulin were recovered from which two were new ones (X and XI). Sequence type VIII was the most abundant and was recovered from eight strains (26%) isolated from dogs with otitis or dermatitis.

Regarding CHS2 gene, nine different sequence types were recovered in total, and all of them have been described previously. Sequence type I was majority and was obtained from a total of nine strains (30%) of dogs (n = 6), horse (n = 1), cat (n = 1) and cow (n = 1).

The sequences obtained in this study have been deposited at the GenBank database under accession numbers ON787824 (D1D2), ON791562-ON791565 (ITS), and ON814675- ON814676 (β-tubulin).

When the four loci (D1D2, ITS, CHS2 and β-tubulin) were combined, a total of 25 genotypes were identified. Among all genotypes, 22 were only found once. Two genotypes were shared by two strains, and one genotype was shared by five strains. When the presence of amino acid substitutions was considered, the graphing algorithm analysis revealed small clusters of *ERG11*-mutated isolates. As shown in Fig. 3, a cluster of 12 strains of 10 different genotypes (I) with a few amino acid substitutions (0–3) was observed. All the strains within this group showed wide inhibition diameters to azoles except for one strain (MA1716) with a small inhibition diameter to FLZ and a higher MIC to ITZ. A second cluster (II) included seven strains of seven different genotypes with five to 11 amino acid mutations. One strain within this cluster (MA1429) showed no inhibition zone to FLZ. Also, a third cluster (III) of seven strains of three different genotypes was obtained with five to six amino acid substitutions. One strain was considered as R to KTZ and SDD to ITZ, and two strains were considered SDD to FLZ.

Discussion

Antifungal susceptibility testing for *M. pachydermatis* must be interpreted with caution neither breakpoints nor a reference method have been yet stablished for this yeast species. This means that any classification of the strains into susceptible, intermediate, and resistant remains speculative. However, according to the manufacturer's breakpoints available for yeasts the four antifungals tested in our study were active against most of the strains selected. Only one strain in our study showed an increased MIC to ITZ. As reported by some authors [8, 16, 37, 38], *M. pachydermatis* is highly susceptible to KTZ and ITZ which is consistent with the results observed in our study. Since now, only two *M. pachydermatis* isolates have demonstrated to be resistant to both ITZ and KTZ [22, 23]. In our study,

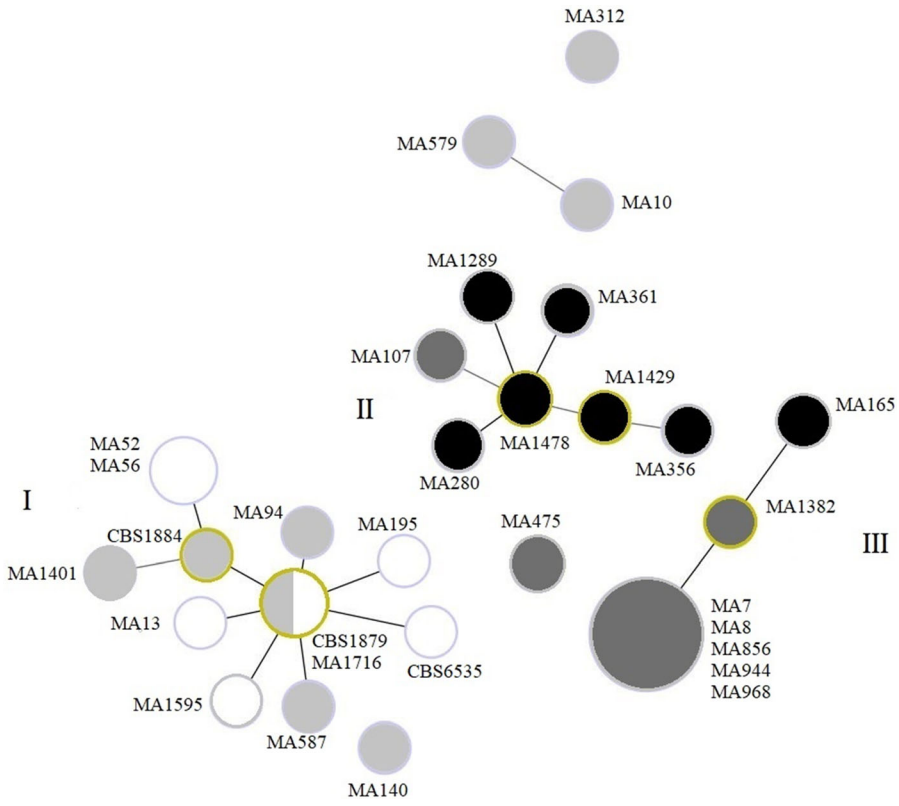

Fig. 3 Minimum spanning trees using the goeBURST algorithm of 31 *Malassezia pachydermatis* strains based on analysis of the four loci used for genotyping. Each circle represents a unique genotype. The diameter of each circle corresponds to the number of isolates sharing the same genotype and the numbers within circles represent strain number. The color of the circle is related to the presence of amino acid substitutions in *ERG11*: black for strains with 6 to 11 amino acid substitutions, dark gray for strains with 4 to 5 amino acid substitutions, light gray for strains with 1 to 3 amino acid substitutions and white for strains without any amino acid substitution

only one strain showed no inhibition zone to FLZ. Fluconazole consistently returns significantly higher MICs when compared to other antifungals tested among studies with *M. pachydermatis* [17, 39, 40]. It has been demonstrated that *M. pachydermatis* isolates can become resistant during treatment with FLZ by inducing resistance in vitro to this antifungal [41]. Thus, the clinical utility of this azole in dogs and cats is questionable [8, 42]. Bernardo et al. [43] found a *M. pachydermatis* isolate resistant to AMB. However, due to the potential toxicity of AMB its use in veterinary medicine is limited to serious progressive or disseminated systemic mycoses [8].

In our study, all the strains with a reduced inhibition zone or no inhibition zone were recovered from animals with otitis and/or dermatitis. These results agree with the ones obtained in three different studies where the MICs of various antifungals agents were higher for isolates from animals with otitis/dermatitis [21, 38, 44]. Also, in our study, differences were observed in the inhibition diameters between strains from chronic otitis externa and otitis media, and strains from purulent and acute otitis externa. The mean inhibition diameters to KTZ were higher in the strains from acute and purulent otitis externa. A study by Chiavassa et al. [20] compared the MIC values of two different antifungal agents between isolates of *M. pachydermatis* from chronic otitis and acute otitis externa. The results showed that the isolates from chronic otitis externa had MIC values higher than the isolates from acute otitis externa. It was hypothesized that those increased MICs were a result to the exposure of isolates to antifungal agents. In our study, strains MA1716, with an increased MIC to ITZ, and MA1429, with complete absence of inhibition zone to FLZ, were recovered from dogs with previous

antifungal treatments with ITZ and miconazole, respectively.

In the E-test method, only one strain (MA1716) showed higher MICs in MH-GM and SGA + 1% Tween 80 and thus, could be considered to have a reduced susceptibility against ITZ. An increase of 64-fold of the MIC was observed when the media used was SGA with lipid supplementation. This increase in MIC values when the culture medium has a lipid supplementation was also observed in *M. pachydermatis* CBS1879 (Fig. 1). This medium was also used to assess susceptibility to ITZ and KTZ by the E-test technique [23]. Different testing variables are known to have an impact on in vitro determinations as lipid supplementation enhancing the yeast growth [8, 45]. Thus, the isolates could appear to be susceptible or resistant only by modifying test conditions. Due to this, it is essential to establish a set of standardized criteria for in vitro susceptibility testing of *Malassezia* spp. [14, 46].

Mutations of the *ERG11* gene could reduce the susceptibility of fungi to azoles [26–28, 47–49]. In our study, a high variability of this gene was observed. Sequence differences in the *ERG11* gene among strains ranged from 0.1 to 4.0%, which is greater than those described with CHS2 (1.9 to 3.4%) or β-tubulin (0.3 to 3.4%) [34]. Indeed, only 6 of the strains displayed the sequence of the *M. pachydermatis* neotype strain CBS1879. This strain has been previously used as comparison to detect possible amino acid mutations [23]. The analysis of the *ERG11* gene grouped *M. pachydermatis* strains in three different clades. This aggrupation correlates quite well with the groups defined by multilocus sequencing.

Even though many polymorphisms were observed, not all lead into amino acid substitutions. Our data clearly show that in *M. pachydermatis* point mutations leading to amino acid changes are a frequent event in *ERG11* gene. A total of 23 different amino acid substitutions were recovered from which nine were deleterious substitutions. The analysis of multilocus groups using *ERG11* amino acid substitutions as discriminant parameter revealed some closely related genotypes carrying more amino acid substitutions. This suggested a correlation between certain genotypes of *M. pachydermatis* and in vitro susceptibility results, in agreement with other authors [38, 50].

Of the amino acid substitutions identified, only two (G459D, G461D) have been reported previously in *M. pachydermatis* associated with azole resistance [51, 52]. One of the mutations present in our study, G461D, recovered from a strain with no inhibition zone to FLZ, was also observed in a clinical isolate with in vitro resistance to ravuconazole [51].

The other mutation, G459D, was reported in miconazole tolerant clones of the CBS1879, selected by serial passage on miconazole supplemented media [52]. This mutation was also present in a strain (MA1716) with a higher MIC to ITZ and with a reduced inhibition zone to FLZ in our study. This strain also showed a mutation at point A302T. This point mutation but with a different amino acid change (A302V) has been described by Kano et al. [23] in an isolate with proven in vitro resistance to ITZ and KTZ. The different amino acid change could explain the results obtained in susceptibility testing of our strain. Another point mutation described by Kano et al. [23] and associated with azole resistance, M138V, was not detected in our study.

Point mutations of the *ERG11* gene leading to amino acid substitutions that induce antifungal resistance have also been observed in other *Malassezia* species [47, 53]. In *M. furfur* a point mutation Y67F (130 in *M. pachydermatis*) is associated with fluconazole resistance [53]. In *M. globosa* three-point mutations Y127F, A169S and K176N (synonymous with 130, 172 and 179 in *M. pachydermatis*) are associated with azole resistance [47]. However, none of these point mutations were observed in the strains of this study.

Some of the amino acid substitutions observed in our study had been described in other fungal species associated to a reduced antifungal susceptibility. In *Candida albicans*, point mutations at positions 54, 145, 226 and 307 (synonymous with 52, 143, 202, and 226 in *M. pachydermatis*, respectively) were associated with a reduced azole susceptibility [54–57]. These point mutations are deleterious in *M. pachydermatis* and were observed in our study in some strains. Also, the presence of two amino acid substitutions in combination G307S + G450E in *C. albicans* was reported to increase by 16-fold the MICs to FLZ [56]. This combination is synonymous with the combination A306S + G461D in *M. pachydermatis* observed in one strain of our study (MA1429) with a disk diffusion with no inhibition zone to FLZ.

The relationship between *ERG11* amino acid mutations and drug resistance has been reported in

many pathogenic fungi such as *Aspergillus fumigatus*, *Candida* spp. and *Cryptococcus* spp. [58]. This is not the case of *M. pachydermatis*. Since now, few strains of *M. pachydermatis* have demonstrated to be resistant to azoles and only four amino acid mutations in *ERG11* have been described [23, 51, 52]. None of these substitutions have been confirmed to cause azole resistance using in vitro experiments and it's not known which part of the protein is involved probably because there is no experimentally determined three-dimensional structure of the *ERG11* protein available. Based on the three-dimensional structure of *C. albicans ERG11*, the mutations G448D and G450D in *C. albicans* (synonymous with G459D and G461D in *M. pachydermatis*) are located near the heme-binding site and near the end of helix I of the protein, respectively [59, 60]. Also, mutation F145L (synonymous with F143S in *M. pachydermatis*) is located near the substrate channel [60]. Based on the three-dimensional structure of *C. neoformans* CYP51, residues A317 and F158 in *C. neoformans* (synonymous with A306S and F143S in *M. pachydermatis*, respectively) are in the heme binding site and mutation A313 in *C. neoformans* (A302T in *M. pachydermatis*) is part of one of the active site cavities [61]. However, the possibility that other deleterious substitutions, which are located outside of the active side, may also contribute to fungal resistance via other structural changes cannot be ruled out.

The presence or absence of amino acid substitutions may not be the solely cause of antifungal resistance [25, 55]. Other mechanisms of resistance against azoles have been suggested in *M. pachydermatis* as efflux pumps [14, 25]. Other possibility for these reduced susceptibilities is a chromosomal rearrangement that leads to an overexpression of the *ERG11* and *ERG4* genes. Kim et al. [29] found a tandemly quadruplicated region in chromosome 4 of two *M. pachydermatis* isolates with high in vitro MICs of KTZ. Thus, an overexpression of this region was observed that could be responsible of the higher MIC.

In conclusion, this study highlights the high diversity of sequences in *ERG11* gene, the primary target of azole antifungal drugs. This high diversity could be part of the high intrinsic variability of this gene in *M. pachydermatis*. Even though we found three mutations that were already reported, we also found some new mutations. Also, genotyping revealed small clusters of *ERG11*-mutate isolates. Although some mutant strains showed a reduced susceptibility to some antifungals, further studies would be necessary to completely understand the role of these mutations in the susceptibility against antifungal agents.

Acknowledgements The authors thanks Laura Ordeix for a sample from a dog with recurrent dermatitis kindly provided for our work. The authors thank Carolina Gómez from the Veterinary Mycology Group of Universitat Autònoma de Barcelona (UAB) for valuable technical assistance.

Author Contributions GC, MRB, and FJC contributed to the study conception and design. Material preparation, data collection and analysis were performed by LD, GC, MRB, and FJ C. The first draft of the manuscript was written by LD, and all authors commented and contributed to the preparation of the final manuscript. All authors read and approved the final manuscript.

Funding Open Access Funding provided by Universitat Autonoma de Barcelona. Financial support came from Servei Veterinari de Bacteriologia i Micologia from the UAB.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Xu J, Saunders CW, Hu P, et al. Dandruff-associated *Malassezia* genomes reveal convergent and divergent virulence traits shared with plant and human fungal pathogens. Proc Natl Acad Sci USA. 2007;104:18730–5.
2. Wu G, Zhao H, Li C, et al. Genus-wide comparative genomics of *Malassezia* delineates its phylogeny, physiology, and niche adaptation on human skin. PLoS Genet. 2015;11:1–26.
3. Puig L, Bragulat MR, Castellá G, Cabañes FJ. Characterization of the species *Malassezia pachydermatis* and re-

evaluation of its lipid dependence using a synthetic agar medium. PLoS ONE. 2017;12: e0179148.
4. Cabañes FJ. *Malassezia pachydermatis*: to be, or not to be lipid-dependent. Rev Iberoam Micol. 2020;37:3–4.
5. Chen TA, Hill PB. The biology of *Malassezia* organisms and their ability to induce immune responses and skin disease. Vet Dermatol. 2005;16:4–26.
6. Bond R. Superficial veterinary mycoses. Clin Dermatol. 2010;28:226–36.
7. Velegraki A, Cafarchia C, Gaitanis G, Iatta R, Boekhout T. *Malassezia* infections in humans and animals: pathophysiology, detection, and treatment. PLoS Pathog. 2015;11: e1004523.
8. Bond R, Morris DO, Guillot J, et al. Biology, diagnosis and treatment of *Malassezia* dermatitis in dogs and cats Clinical Consensus Guidelines of the World Association for Veterinary Dermatology. Vet Dermatol. 2020;31:28–74.
9. Zisova LG. Fluconazole and its place in the treatment of seborrheic dermatitis, new therapeutic possibilities. Folia Med (Plovdiv). 2006;48:39–45.
10. Alizadeh N, Monadi Nori H, Golchi J, Eshkevari SS, Kazemnejad E, Darjani A. Comparison the efficacy of fluconazole and terbinafine in patients with moderate to severe seborrheic dermatitis. Dermatol Res Pract. 2014;2014: 705402.
11. Schlemmer KB, de Jesus FPK, Loreto ES, Farias JB, Alves SH, Ferreiro L, Santurio JM. *In vitro* combination of antifungal agents against *Malassezia pachydermatis*. Med Mycol. 2019;57:324–7.
12. Clinical Laboratory and Standards Institute. Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standards, 3rd edn, document M27-A3. Wayne, PA: Clinical Laboratory and Standards Institute. 2008.
13. Peano A, Pasquetti M, Tizzani P, Chiavassa E, Guillot J, Johnson E. Methodological issues in antifungal susceptibility testing of *Malassezia pachydermatis*. J Fungi. 2017;3:37.
14. Peano A, Johnson E, Chiavassa E, Tizzani P, Guillot J, Pasquetti M. Antifungal resistance regarding *Malassezia pachydermatis*: where are we now? J Fungi. 2020;6:93.
15. Velegraki A, Alexopoulos EC, Kritikou S, Gaitanis G. Use of fatty acid RPMI 1640 media for testing susceptibilities of eight *Malassezia* species to the new triazole posaconazole and to six established antifungal agents by a modified NCCLS M27–A2 microdilution method and Etest. J Clin Microbiol. 2004;242:3589–93.
16. Nijima M, Kano R, Nagata M, Hasegawa A, Kamata H. An azole-resistant isolate of *Malassezia pachydermatis*. Vet Microbiol. 2011;149:288–90.
17. Cafarchia C, Figueredo LA, Favuzzi V, et al. Assessment of the antifungal susceptibility of *Malassezia pachydermatis* in various media using a CLSI protocol. Vet Microbiol. 2012;159:536–40.
18. Tenover FC. Antibiotic susceptibility testing. In: Schaechter M, editor. Encyclopedia of microbiology. 3rd ed. San Diego: San Diego State University; 2009. p. 67–77.
19. Brilhante RSN, Rocha MGD, Guedes GMM, et al. *Malassezia pachydermatis* from animals: planktonic and biofilm antifungal susceptibility and its virulence arsenal. Vet Microbiol. 2018;220:47–52.
20. Chiavassa E, Tizzani P, Peano A. *In vitro* antifungal susceptibility of *Malassezia pachydermatis* strains isolated from dogs with chronic and acute otitis externa. Mycopathologia. 2014;178:315–9.
21. Watanabe S, Koike A, Kano R, Nagata M, Chen C, Hwang CY, et al. *In vitro* susceptibility of *Malassezia pachydermatis* isolates from canine skin with atopic dermatitis to ketoconazole and itraconazole in East Asia. J Vet Med Sci. 2014;76:579–81.
22. Angileri M, Pasquetti M, De Lucia M, Peano A. Azole resistance of *Malassezia pachydermatis* causing treatment failure in a dog. Med Mycol Case Rep. 2019;23:58–61.
23. Kano R, Yokoi S, Kariya N, Oshimo K, Kamata H. Multiazole-resistant strain of *Malassezia pachydermatis* isolated from a canine *Malassezia* dermatitis. Med Mycol. 2019;57:346–50.
24. Crespo MJ, Abarca ML, Cabañes FJ. Evaluation of different preservation and storage methods for *Malassezia* spp. J Clin Microbiol. 2000;38:3872–5.
25. Iatta R, Puttilli MR, Immediato D, Otranto D, Cafarchia C. The role of drug efflux pumps in *Malassezia pachydermatis* and *Malassezia furfur* defence against azoles. Mycoses. 2017;60:178–82.
26. Joseph-Horne T, Hollomon DW. Molecular mechanisms of azole resistance in fungi. FEMS Microbiol Lett. 1997;149:141–9.
27. Feng W, Yang J, Xi Z, Qiao Z, et al. Mutations and/or overexpressions of *ERG4* and *ERG11* genes in clinical azoles-resistant isolates of *Candida albicans*. Microb Drug Resist. 2017;23:563–70.
28. Park M, Cho Y, Lee YW, Jung WH. Genomic multiplication and drug efflux influence ketoconazole resistance in *Malassezia restricta*. Front Cell Infect Microbiol. 2020;10:191.
29. Kim M, Cho Y, Park M, Choi Y, Hwang SY, Jung WH. Genomic tandem quadruplication is associated with ketoconazole resistance in *Malassezia pachydermatis*. J Microbiol Biotechnol. 2018;28:1937–45.
30. Pasquetti M, Chiavassa E, Tizzani P, Danesi P, Peano A. Agar diffusion procedures for susceptibility testing of *Malassezia pachydermatis*: Evaluation of Mueller-Hinton agar plus 2 % glucose and 0.5 μg/ml methylene blue as the test medium. Mycopathologia. 2015;180:153–8.
31. Larkin MA, Blackshields G, Brown NP, et al. Clustal W and Clustal X version 2.0. Bioinformatics. 2007;23:2947–8.
32. Choi Y, Chan AP. PROVEAN web server: a tool to predict the functional effect of amino acid substitutions and indels. Bioinformatics. 2015;31:2745–7.
33. Tamura K, Stecher G, Kumar S. MEGA11: molecular evolutionary genetics analysis version 11. Mol Biol Evol. 2021;38:3022–7.
34. Puig L, Castellá G, Cabañes FJ. Cryptic diversity of *Malassezia pachydermatis* from healthy and diseased domestic animals. Mycopathologia. 2016;181:681–8.
35. Castellá G, Coutinho SD, Cabañes FJ. Phylogenetic relationships of *Malassezia* species based on multilocus sequence analysis. Med Mycol. 2014;52:99–105.
36. Nascimento M, Sousa A, Ramirez M, Francisco AP, Carriço JA, Vaz C. PHYLOViZ 2.0: providing scalable data integration and visualization for multiple phylogenetic inference methods. Bioinformatics. 2017;33:128–9.

37. Gupta AK, Kohli Y, Li A, Faergemann J, Summerbell RC. *In vitro* susceptibility of the seven *Malassezia* species to ketoconazole, voriconazole, itraconazole and terbinafine. Br J Dermatol. 2000;142:758–65.
38. Cafarchia C, Figueredo LA, Iatta R, Montagna MT, Otranto D. *In vitro* antifungal susceptibility of *Malassezia pachydermatis* from dogs with and without skin lesions. Vet Microbiol. 2012;155:395–8.
39. Carrillo-Muñoz AJ, Rojas F, Tur-Tur C, et al. *In vitro* antifungal activity of topical and systemic antifungal drugs against *Malassezia* species. Mycoses. 2013;56:571–5.
40. Álvarez-Pérez S, García ME, Peláez T, Blanco JL. Genotyping and antifungal susceptibility testing of multiple *Malassezia pachydermatis* isolates from otitis and dermatitis cases in pets: is it really worth the effort? Med Mycol. 2016;54:72–9.
41. Jesus FP, Lautert C, Zanette RA, et al. *In vitro* susceptibility of fluconazole-susceptible and -resistant isolates of *Malassezia pachydermatis* against azoles. Vet Microbiol. 2011;152:161–4.
42. Sickafoose L, Hosgood G, Snook T, Westermeyer R, Merchant S. A noninferiority clinical trial comparing fluconazole and ketoconazole in combination with cephalexin for the treatment of dogs with *Malassezia* dermatitis. Vet Ther. 2010;11:E1–13.
43. Bernardo FM, Martins HM, Martins ML. A survey of mycotic otitis externa of dogs in Lisbon. Rev Iberoam Micol. 1998;15:163–5.
44. Weiler CB, de Jesus FP, Nardi GH, et al. Susceptibility variation of *Malassezia pachydermatis* to antifungal agents according to isolate source. Braz J Microbiol. 2013;44:174–8.
45. Lorenzini R, de Bernardis F. Studies on the isolation, growth and maintenance of *Malassezia pachydermatis*. Mycopathologia. 1987;99:129–31.
46. Arikan S. Current status of antifungal susceptibility testing methods. Med Mycol. 2007;45:569–87.
47. Kim D, Lim YR, Ohk SO, Kim BJ, Chun YJ. Functional expression and characterization of CYP51 from dandruff-causing *Malassezia globosa*. FEMS Yeast Res. 2011;11:80–7.
48. Vazquez JA, Manavathu EK. Molecular characterization of a voriconazole-resistant, posaconazole-susceptible *Aspergillus fumigatus* isolate in a lung transplant recipient in the United States. Antimicrob Agents Chemother. 2015;60:1129–33.
49. Selb R, Fuchs V, Graf B, et al. Molecular typing and in vitro resistance of *Cryptococcus neoformans* clinical isolates obtained in Germany between 2011 and 2017. Int J Med Microbiol. 2019;309: 151336.
50. Cafarchia C, Figueredo LA, Iatta R, Colao V, Montagna MT, Otranto D. *In vitro* evaluation of *Malassezia pachydermatis* susceptibility to azole compounds using E-test and CLSI microdilution methods. Med Mycol. 2012;50:795–801.
51. Kano R, Aramaki C, Murayama N, et al. High multi-azole-resistant *Malassezia pachydermatis* clinical isolates from canine *Malassezia* dermatitis. Med Mycol. 2020;58:197–200.
52. Kano R, Kamata H. Miconazole-tolerant strains of *Malassezia pachydermatis* generated by culture in medium containing miconazole. Vet Dermatol. 2020;31:97–101.
53. Leong C, Kit JCW, Lee SM, et al. Azole resistance mechanisms in pathogenic *M. furfur*. Antimicrob Agents Chemother. 2021;65:e01975-e2020.
54. Cernicka J, Subik J. Resistance mechanisms in fluconazole-resistant *Candida albicans* isolates from vaginal candidiasis. Int J Antimicrob Agents. 2006;27:403–8.
55. Morio F, Loge C, Besse B, Hennequin C, Le Pape P. Screening for amino acid substitutions in the *Candida albicans Erg11* protein of azole-susceptible and azole-resistant clinical isolates: new substitutions and a review of the literature. Diagn Microbiol Infect Dis. 2010;66:373–84.
56. Flowers SA, Colón B, Whaley SG, Schuler MA, Rogers PD. Contribution of clinically derived mutations in *ERG11* to azole resistance in *Candida albicans*. Antimicrob Agents Chemother. 2015;59:450–60.
57. Berkow EL, Lockhart SR. Fluconazole resistance in *Candida* species: a current perspective. Infect Drug Resist. 2017;10:237–45.
58. Zhang J, Li L, Lv Q, Yan L, Wang Y, Jiang Y. The fungal CYP51s: their functions, structures, related drug resistance, and inhibitors. Front Microbiol. 2019;10:691.
59. Xiang MJ, Liu JY, Ni PH, Wang S, Shi C, Wei B, Ni YX, Ge HL. Erg11 mutations associated with azole resistance in clinical isolates of *Candida albicans*. FEMS Yeast Res. 2013;13:386–93.
60. Marichal P, Koymans L, Willemsens S, Bellens D, Verhasselt P, Luyten W, Borgers M, Ramaekers FCS, Odds FC, Vanden BH. Contribution of mutations in the cytochrome P450 14alpha-demethylase (Erg11p, Cyp51p) to azole resistance in *Candida albicans*. Microbiology. 1999;145:2701–13.
61. Sheng C, Miao Z, Ji H, Yao J, Wang W, Che X, Dong G, Lü J, Guo W, Zhang W. Three-dimensional model of lanosterol 14 alpha-demethylase from *Cryptococcus neoformans*: active-site characterization and insights into azole binding. Antimicrob Agents Chemother. 2009;53:3487–95.

6. GENERAL DISCUSSION

In this Doctoral Thesis dissertation, the *Malassezia* species from the skin of rabbits and dogs was studied using routine methods and advanced molecular methods. The correlation between the results obtained by routine methods and molecular methods was evaluated. Also, antifungal susceptibility of *M. pachydermatis* strains obtained from different animal species and health status, and the possible correlation with *ERG11* gene mutations was evaluated.

The first paper **(5.1.)** was focused on studying the external ear canal mycobiota of different breeds of healthy rabbits using different methods such as cytology, culture, DNA sequencing and NGS. A total of 60 rabbits were sampled, including pet and farmed rabbits and both erect and lop-eared animals. Although no grow was obtained in any of the culture media used, the presence of round cells like those of *Malassezia cuniculi*, was revealed by cytology in 33 out of the 60 rabbits. The sequences of the D1/D2 region of five swabs was obtained by DNA sequencing, and the sequences obtained were identical between them and had a 100% identity score to an uncultured *Basidiomycota* clone 131 (*Malassezia* phylotype 131). Also, the ITS 5.8S rRNA gene was sequenced by DNA sequencing and the sequences obtained were identical between them and had 80.74% identity score to *M. cuniculi*. Maximum likelihood analysis of D1/D2 and ITS 5.8s sequences showed that they clustered close to *M. cuniculi*. These results agree with a previous study conducted on human skin which described the phylotype 131 [70]. For metagenomic analysis, the D1/D2 domain of the LSU was used as target. The results revealed that *Basidiomycota* was the main phylum and *Malassezia* the predominant genus in all samples. Although *M. cuniculi* was present in all samples in low abundance, the main taxa detected in all samples was the *Malassezia* phylotype 131. These results

differed from those obtained in studies of the ear mycobiota on healthy dogs and cats where *Ascomycota* was the main phylum [74,75]. Further studies would be needed to isolate and characterize this *Malassezia* phylotype 131 and to understand its role in the external ear canal of rabbits. Also, NGS results showed that the use of the LSU as target gene allows the description of fungal diversity and the taxonomic identification of several fungal species in agreement with previous researchers [155,156]. Although the ear morphology was suggested as a factor that could alter the ear mycobiota, in this study no significant differences in the abundance of *Malassezia* between lop and erect-eared rabbits were observed. This lack of significant differences could be a result of the fairly small sample size in our study. However, our results agree with the ones obtained in previous studies [157,158]. *Malassezia* being part of the normal microbiota of the ear canal of rabbits, as it is in other species such as dogs or cats, could explain this lack of statistical differences between lop and erect-eared rabbits [157-159].

The second study **(5.2.)** was focused on the variation of the *M. pachydermatis* load in samples from dogs with pododermatitis, healthy dogs and dogs after treatment with antifungal drugs. Samples were obtained from the interdigital fold and subjected to two cytological examinations, culture on SGA and mDA, qPCR targeting the ITS region and NGS targeting the LSU. Samples from dogs with pododermatitis showed higher cell counts, CFU and ITS copies than the rest of the samples. NGS results revealed that *Ascomycota* was the main phylum in healthy and post-treatment samples while *Basidiomycota* predominates in pododermatitis samples. *Malassezia pachydermatis* was the predominant species in pododermatitis samples but it was also present in healthy and post-treatment samples in low abundance. In healthy and post-treatment samples, the

predominant species belonged to genera considered as transient mycobiota. These results support the role of *M. pachydermatis* as opportunistic pathogen in canine pododermatitis and agree with previous studies in healthy dogs and dogs with otitis [76,160]. The study revealed the differences in terms of fungal diversity and richness between samples obtained from dogs with pododermatitis and healthy and after-treatment dogs. Also, a good correlation between cytologic and molecular methods was observed.

In the third study **(5.3.)**, the susceptibility of *M. pachydermatis* strains against KTZ, ITZ, FLZ and AMB was evaluated. Strain selection included different animal species, different health status including healthy animals but also cases of otitis and dermatitis. The susceptibility to the four antifungals was evaluated by a disk diffusion method using MH-GM as culture medium. The susceptibility against ITZ was tested on a few strains by E-test using the same culture medium. Multilocus sequence typing was performed in 30 selected strains using D1/D2, ITS, CHS2 and β-tubulin genes. Also, *ERG11* gene was sequenced. The susceptibility results revealed that the four antifungals were highly effective against most of the strains tested. However, as neither a standardized method nor breakpoints are available for this yeast species, the results must be interpreted with caution [50]. In this study, only one strain had a higher MIC to ITZ and only one strain had no inhibition zone to FLZ. Resistance to ITZ and KTZ has been reported in only two *M. pachydermatis* strains [135,136]. A reduced susceptibility to FLZ has been previously reported in *M. pachydermatis* [161,162], and it has also been demonstrated that isolates can become resistant during treatment with FLZ [132]. In our study, strains with reduced susceptibility to the antifungals tested were isolated from animals with otitis or dermatitis, which agrees with the results reported by other authors [131,134,140]. Mutations in the *ERG11* gene could reduce the susceptibility of *Malassezia* to azoles [124,150]. In our study, a high

variability in this gene was observed. The analysis of this gene grouped the strains in three clades that correlate quite well with the groups defined by the multilocus sequencing. The analysis of the multilocus groups using the *ERG11* amino acid substitutions as a parameter to discriminate, revealed a correlation between certain genotypes and *in vitro* susceptibility results. Although many polymorphisms were observed in the *ERG11* sequences, not all lead to amino acid mutations. A total of 23 different amino acid substitutions were observed, from which nine were deleterious. Only two amino acid substitutions (G459D, G461D) had been previously reported in *M. pachydermatis*. One of them, G459D, recovered from a strain with no inhibition zone to FLZ, had been associated with ravuconazole resistance [148]. The other one, G461D, was previously associated with MCZ tolerant clones [163] but in our study was recovered from a strain with a higher MIC to ITZ and a reduced susceptibility to FLZ. That strain had also a mutation at point A302T. A mutation at this point with a different amino acid change (A302V), was associated with ITZ resistance [136]. The different amino acid change could explain the different results obtained. None of the point mutations described in other *Malassezia* studies were recovered in our study. Some of the amino acid substitutions observed in our study had been described in *Candida albicans* associated to a reduced antifungal susceptibility [151,164-166]. The presence of amino acid substitutions may not be the solely cause of antifungal resistance in *M. pachydermatis* and other mechanisms may be involved as efflux pumps [124,154] or chromosomal rearrangement [142].

7. CONCLUSIONS

As a summary of the results obtained and as conclusions, we can highlight that:

1. Although *Malassezia* cells were detected in samples from the external ear canal of rabbits, no growth was obtained. A fact that confirms that they are fastidious or non-culturable yeasts.
2. NGS results revealed that *Basidiomycota* is the most abundant phylum in the external ear canal of healthy rabbits.
3. *Malassezia* is the most common fungal genus in the external ear canal of healthy rabbits.
4. The *Malassezia* phylotype 131 is the most abundant taxa present in the external ear canal of healthy rabbits.
5. *Malassezia cuniculi* is present in low abundance in the external ear canal of healthy rabbits.
6. There are no significant differences in the *Malassezia* population in the external ear canal associated with the ear morphology.
7. The D1/D2 domain of the LSU allowed the study of fungal diversity and the taxonomic identification of several *Malassezia* species in skin samples from rabbits and dogs.
8. NGS results revealed that *Ascomycota* is the main phylum in healthy and post-treatment dogs while *Basidiomycota* predominates in dogs with pododermatitis.
9. *Malassezia pachydermatis* is the predominant species in pododermatitis samples but it is also present in low abundance in healthy and post-treatment samples.
10. In healthy and post-treatment samples the predominant species belonged to genera considered as transient mycobiota.

11. Samples from dogs with pododermatitis have a higher *Malassezia* load than samples from healthy dogs or dogs after antifungal treatment.
12. There is a reduction in the quantity of species identified and richness of fungal populations in samples from dogs with pododermatitis compared with samples from healthy dogs and dogs after treatment.
13. There is a good correlation between cytology and molecular methods for the diagnosis of *Malassezia* overgrowth.
14. Most *M. pachydermatis* strains are highly susceptible to KTZ, ITZ, FLZ and AMB.
15. There is a high variability of *ERG11* gene sequences within *M. pachydermatis* strains.
16. There is a correlation between certain *M. pachydermatis* genotypes and *ERG11* mutations.
17. Some *ERG11* mutations are correlated with a reduced azole susceptibility in *M. pachydermatis*.

8. REFERENCES

1. Ashbee HR. Update on the genus *Malassezia*. *Med Mycol*. 2007; 45(4): 287-303.
2. Xu J, Saunders CW, Hu P, et al. Dandruff-associated *Malassezia* genomes reveal convergent and divergent virulence traits shared with plant and human fungal pathogens. *Proc Natl Acad Sci USA.* 2007; 104(47): 18730-18735.
3. Hay RJ, Midgley G. Introduction: *Malassezia* yeasts from a historical perspective. In: Boekhout T, Gueho E, Mayser P, eds. *Malassezia* and the skin: science and clinical practice. Berlin: Springer; 2010: 1-16.
4. Gaitanis G, Magiatis P, Hantschke M, Bassukas ID, Velegraki A. The *Malassezia* genus in skin and systemic diseases. *Clin Microbiol Rev*. 2012; 25(1): 106-141.
5. Simmons RB, Gueho E. A new species of *Malassezia*. *Mycol Res.* 1990; 94(8): 1146-1149.
6. Guillot J, Guého E. The diversity of *Malassezia* yeasts confirmed by rRNA sequence and nuclear DNA comparisons. *Antonie Van Leeuwenhoek.* 1995; 67(3): 297-314.
7. Guého E, Midgley G, Guillot J. The genus *Malassezia* with description of four new species. *Antonie Van Leeuwenhoek*. 1996; 69(4): 337-355.
8. Guillot J, Guého E, Lesourd M, Midgley G, Chevrier G, Dupont B. Identification of *Malassezia* species. A practical approach. *J Mycol Med*. 1996; 6: 103-110.
9. Boekhout T, Kamp M, Gueho E. Molecular typing of *Malassezia* species with PFGE and RAPD. *Med Mycol.* 1998; 36(6): 365-372.
10. Kano R, Aizawa T, Nakamura Y, Watanabe S, Hasegawa A. Chitin synthase 2 gene sequence of *Malassezia* species. *Microbiol Immunol*. 1999; 43(8): 813-815.

11. Guillot J, Deville M, Berthelemy M, Provost F, Guého E. A single PCR-restriction endonuclease analysis for rapid identification of *Malassezia* species. *Lett Appl Microbiol.* 2000; 31(5): 400-403.
12. Gupta AK, Boekhout T, Theelen B, Summerbell R, Batra R. Identification and typing of *Malassezia* species by amplified fragment length polymorphism and sequence analyses of the internal transcribed spacer and large-subunit regions of ribosomal DNA. *J Clin Microbiol*. 2004; 42(9): 4253-4260.
13. Sugita T, Takashima M, Shinoda T, et al. New yeast species, *Malassezia dermatis*, isolated from patients with atopic dermatitis. *J Clin Microbiol.* 2002; 40 (4): 1363-1367.
14. Sugita T, Takashima M, Kodama M, et al. Description of a new yeast species, *Malassezia japonica*, and its detection in patients with atopic dermatitis and healthy subjects. *J Clin Microbiol.* 2003; 41: 4695-4699.
15. Sugita T, Tajima M, Takashima M, et al. A new yeast, *Malassezia yamatoensis*, isolated from a patient with seborrheic dermatitis, and its distribution in patients and healthy subjects. *Microbiol Immunol.* 2004; 48(8): 579-583.
16. Honnavar P, Prasad GS, Ghosh A, et al. *Malassezia arunalokei* sp. nov., a novel yeast species isolated from seborrheic dermatitis patients and healthy individuals from India. *J Clin Microbiol*. 2016; 54(7): 1826-1834.
17. Hirai A, Kano R, Makimura K, et al. *Malassezia nana* sp. nov., a novel lipid-dependent yeast species isolated from animals. *Int J Syst Evol Microbiol*. 2004; 54 (Pt 2): 623-627.
18. Cabañes FJ, Theelen B, Castellá G, Boekhout T. Two new lipid-dependent *Malassezia* species from domestic animals. *FEMS Yeast Res.* 2007; 7(6): 1064-1076.

19. Cabañes FJ, Vega S, Castellá G. *Malassezia cuniculi* sp. nov., a novel yeast species isolated from rabbit skin. *Med Mycol.* 2011; 49(1): 40-48.
20. Cabañes FJ, Coutinho SD, Puig L, Bragulat MR, Castellá G. New lipid-dependent *Malassezia* species from parrots. *Rev Iberoam Micol*. 2016; 33(2): 92-99.
21. Lorch JM, Palmer JM, Vanderwolf KJ, et al. *Malassezia vespertilionis* sp. nov.: a new cold-tolerant species of yeast isolated from bats. *Persoonia*. 2018; 41: 56-70.
22. Wang QM, Theelen B, Groenewald M, Bai FY, Boekhout T. *Moniliellomycetes* and *Malasseziomycetes*, two new classes in *Ustilaginomycotina*. *Persoonia*. 2014; 33: 41-47.
23. Wu G, Zhao H, Li C, et al. Genus-wide comparative genomics of *Malassezi*a delineates its phylogeny, physiology, and niche adaptation on human skin. *PLoS Genet*. 2015; 11(11): e1005614.
24. Ianiri G, LeibundGut-Landmann S, Dawson TL Jr. *Malassezia:* a commensal, pathogen, and mutualist of human and animal skin. *Annu Rev Microbiol*. 2022; 76: 757-782.
25. Gueho-Kellerman E, Boekhout T, Begerow D et al. Biodiversity, phylogeny and ultrastructure. In: Boekhout T, Gueho-Kellerman E, Mayser P et al., eds. *Malassezia* and the skin. Science and clinical practice. Berlin: Springer; 2010: 17-63.
26. Mayser P, Gaitanis G. Physiology and biochemistry. In: Boekhout T, Guého-Kellermann E, Mayser P, Velegraki A, eds. *Malassezia* and the Skin. Berlin: Springer, 2010: 271-299.
27. Stalhberger T, Simenel C, Clavaud C, et al. Chemical organization of the cell wall polysaccharide core of *Malassezia restricta*. *J Biol Chem*. 2014; 289(18): 12647-12656

28. Gioti A, Nystedt B, Li W, et al. Genomic insights into the atopic eczema-associated skin commensal yeast *Malassezia sympodialis*. *MBio.* 2013; 4(1): e00572-00512.
29. Nielsen K, Heitman J. Sex and virulence of human pathogenic fungi. *Adv Genet.* 2007; 57:143-173.
30. Coelho MA, Sampaio JP, Gonçalves P. Living and thriving on the skin: *Malassezia* genomes tell the story. *mBio*. 2013; 4(2): e00117-13.
31. Coelho MA, Sampaio JP, Goncalves P. A deviation from the bipolar-tetrapolar mating paradigm in an early diverged basidiomycete. *PLoS Genet.* 2010; 6(8): e1001052.
32. Heitman J, Carter DA, Dyer PS, Soll DR. Sexual reproduction of human fungal pathogens. *Cold Spring Harb Perspect Med*. 2014; 4(8): a019281.
33. Shifrine M, Marr AG. The requirement of fatty acids by *Pityrosporum ovale*. *J Gen Microbiol*. 1963; 32: 263-70.
34. Cabañes FJ. *Malassezia pachydermatis*: To be, or not to be lipid-dependent. *Rev Iberoam Micol.* 2020; 37(1): 3-4.
35. Guillot J, Bond R. *Malassezia pachydermatis*: a review. *Med Mycol*. 1999; 37(5): 295-306.
36. Bond R, Anthony RM, Dodd M, et al. Isolation of *Malassezia sympodialis* from feline skin. *J Med Vet Mycol.* 1996; 34(2): 145-147.
37. Duarte ER, Lachance MA, Hamdan JS. Identification of atypical strains of *Malassezia* spp. from cattle and dog. *Can J Microbiol*. 2002; 48(8): 749-752.
38. Kaneko T, Makimura K, Abe M, et al. Revised culture-based system for identification of *Malassezia* species. *J Clin. Microbiol*. 2007; 45(11): 3737-3742.

39. Puig L, Bragulat MR, Castellá G, Cabañes FJ. Characterization of the species *Malassezia pachydermatis* and re-evaluation of its lipid dependence using a synthetic agar medium. *PLoS One*. 2017;12(6): e0179148.
40. Sankaranarayanan SR, IaniriG, Coelho MA, et al. Loss of centromere function drives karyotype evolution in closely related *Malassezia* species. *ELife*. 2020; 9: e53944.
41. Saunders CW, Scheynius A, Heitman J. *Malassezia* fungi are specialized to live on skin and associated with dandruff, eczema, and other skin diseases. *PLOS Pathog.* 2012; 8(6): e1002701.
42. White TC, Findley K, Dawson TL Jr, et al. Fungi on the skin: dermatophytes and *Malassezia*. *Cold Spring Harb Perspect Med*. 2014; 4(8): 019802.
43. Poh SE, Goh JPZ, Fan C, et al. Identification of *Malassezia furfur* secreted aspartyl protease 1 (MfSAP1) and its role in extracellular matrix degradation. *Front Cell Infect Microbiol*. 2020; 10: 148.
44. Ianiri G, Averette AF, Kingsbury JM, Heitman J, Idnurm A. Gene function analysis in the ubiquitous human commensal and pathogen *Malassezia* genus. *mBio*. 2016; 7(6): e01853-16.
45. Park M, Cho YJ, Kim D, et al. A novel totivirus alters gene expression and vacuolar morphology in *Malassezia* cells and induces a TLR3-mediated inflammatory immune response. *mBio*. 2020; 11(5): e01521-20.
46. Applen Clancey S, Ruchti F, LeibundGut-Landmann S, Heitman J, Ianiri G. A novel mycovirus evokes transcriptional rewiring in the fungus *Malassezia* and stimulates beta interferon production in macrophages. *mBio*. 2020; 11(5): e01534-20.

47. Ianiri G, Coelho MA, Ruchti F, et al. HGT in the human and skin commensal *Malassezia*: A bacterially derived flavohemoglobin is required for NO resistance and host interaction. *PNAS*. 2020; 117(27): 15884-15894.
48. Leeming JP, Notman FH. Improved methods for isolation and enumeration of *Malassezia furfur* from human skin. *J Clin Microbiol.* 1987; 25(10): 2017-2019.
49. Cafarchia C, Gallo S, Romito D, et al. Frequency, body distribution, and population size of *Malassezia* species in healthy dogs and in dogs with localized cutaneous lesions. *J Vet Diagn Invest*. 2005; 17(4): 316-322.
50. Bond R, Morris DO, Guillot J, et al. Biology, diagnosis and treatment of *Malassezia* dermatitis in dogs and cats Clinical Consensus Guidelines of the World Association for Veterinary Dermatology. *Vet Dermatol*. 2020; 31(1): 28-74.
51. Sugita T, Boekhout T, Velegraki A, et al. Epidemiology of *Malassezia*-related skin diseases. In: Boekhout T, Gueho-Kellerman E, Mayser P, et al., eds. *Malassezia* and the skin. Science and clinical practice. Heidelberg: Springer; 2010: 65–119.
52. Sugita T, Suto H, Unno T, et al. Molecular analysis of *Malassezia* microflora on the skin of atopic dermatitis patients and healthy subjects. *J Clin Microbiol*. 2001; 39: 3486-3490.
53. Gemmer CM, DeAngelis YM, Theelen B, Boekhout T, Dawson TL Jr. Fast, noninvasive method for molecular detection and differentiation of *Malassezia* yeast species on human skin and application of the method to dandruff microbiology. *J Clin Microbiol*. 2002; 40(9): 3350-3357.
54. Amaya M, Tajima M, Okubo Y, Sugita T, Nishikawa A, Tsuboi R. Molecular analysis of *Malassezia* microflora in the lesional skin of psoriasis patients. *J Dermatol*. 2007; 34(9): 619-624.

55. Vuran E, Karaarslan A, Karasartova D, Turegun B, Sahin F. Identification of *Malassezia* species from pityriasis versicolor lesions with a new multiplex PCR method. *Mycopathologia*. 2014; 177(1-2): 41-49.
56. Akaza N, Akamatsu H, Sasaki Y, et al. Cutaneous *Malassezia* microbiota of healthy subjects differ by sex, body part and season. *J Dermatol*. 2010; 37(9): 786-792.
57. Sugita T, Tajima M, Tsubuku H, Tsuboi R, Nishikawa A. Quantitative analysis of cutaneous *Malassezia* in atopic dermatitis patients using real-time PCR. *Microbiol Immunol*. 2006; 50(7): 549-552.
58. Saad M, Sugita T, Saeed H, Ahmed A. Molecular epidemiology of *Malassezia globosa* and *Malassezia restricta* in Sudanese patients with pityriasis versicolor. *Mycopathologia*. 2013; 175(1-2): 69-74.
59. Puig L, Castellá G, Cabañes FJ. Quantification of *Malassezia* pachydermatis by real-time PCR un swabs from the external ear canal of dogs. *J Vet Diagn Invest*. 2019; 31(3): 440-447.
60. Meason-Smith C, Olivry T, Lawhon SD, Hoffmann AR. *Malassezia* species dysbiosis in natural and allergen-induced atopic dermatitis in dogs. *Med Mycol*. 2020; 58(6): 756-765.
61. Forde BM, O'Toole PW. Next-generation sequencing technologies and their impact on microbial genomics. *Brief Funct Genomics*. 2013; 12(5): 440-453.
62. Kulski J (eds). Next generation sequencing: advances, applications and challenges, 1st ed. IntechOpen, 2016.
63. Forbes JD, Knox NC, Ronholm J, Pagotto F, Reimer A. Metagenomics: the next culture-independent game changer. *Front Microbiol*. 2017; 8: 1069.

64. Lücking R, Hawksworth DL. Formal description of sequence-based voucherless Fungi: promises and pitfalls, and how to resolve them. *IMA Fungus*. 2018; 9(1): 143-166.

65. Chng KR, Tay AS, Li C, et al. Whole metagenome profiling reveals skin microbiome-dependent susceptibility to atopic dermatitis flare. *Nat Microbiol*. 2016; 1(9): 16106.

66. Kong G, Lê Ca KA, Hannan AJ. Alterations in the Gut Fungal Community in a Mouse Model of Hunting-ton's Disease. *Microbiol Spectr*. 2022; 10(2): e0219221.

67. Tong L, Constancias F, Hou A, et al. Shotgun metagenomic sequencing analysis of ocular surface microbiome in Singapore residents with mild dry eye. *Front Med (Lausanne)*. 2022; 9: 1034131.

68. De Filippis F, Laiola M, Blaiotta G, Ercolini D. Different amplicon targets for fungal sequencing-based studies of fungal diversity. *Appl Environ Microbiol*. 2017; 83(17): e00905-17.

69. Wilkening S, Tekkedil MM, Lin G, et al. Genotyping 1,000 yeast strains by nextgeneration sequencing. *BMC Genomics*. 2013; 14: 90.

70. Zhang E, Tanaka T, Tsuboi R, Makimura K, Nishikawa A, Sugita T. Characterization of *Malassezia* microbiota in the human external auditory canal and on the sole of the foot. *Microbiol Immunol*. 2012; 56(4): 238-244.

71. Findley K, Oh J, Yang J et al. Topographic diversity of fungal and bacterial communities in human skin. *Nature*. 2013; 498(7454): 367-370.

72. Takemoto A, Cho O, Morohoshi Y, Sugita T, Muto M. Molecular characterization of the skin fungal microbiome in patients with psoriasis. *J Dermatol*. 2015; 42(2): 166-170.

73. Rodrigues Hoffmann A, Patterson AP, Diesel A, et al. The skin microbiome in healthy and allergic dogs. *PLoS One.* 2014; 9(1): e83197.
74. Meason-Smith C, Diesel A, Patterson AP, et al. What is living on your dog's skin? Characterization of the canine cutaneous mycobiota and fungal dysbiosis in canine allergic dermatitis. *FEMS Microbiol Ecol*. 2015; 91(12): fiv139.
75. Meason-Smith C, Diesel A, Patterson AP, et al. Characterization of the cutaneous mycobiota in healthy and allergic cats using next generation sequencing. *Vet Dermatol.* 2017; 28(1): 71e-17.
76. Korbelik J, Singh A, Rosseau J, Weese JS. Analysis of the otic mycobiota in dogs with otitis externa compared to healthy individuals. *Vet Dermatol*. 2018; 29(5): 417-e138.
77. Chermprapai S, Ederveen THA, Broere F, et al. The bacterial and fungal microbiome of the skin of healthy dogs and dogs with atopic dermatitis and the impact of topical antimicrobial therapy, an exploratory study. *Vet Microbiol*. 2019; 229: 90-99.
78. Bradley CW, Lee FF, Rankin SC, et al. The otic microbiota and mycobiota in a referral population of dogs in eastern USA with otitis externa. *Vet Dermatol*. 2020; 31(3): 225-249.
79. Tang S, Prem A, Tjokrosurjo J, et al. The canine skin and ear microbiome: A comprehensive survey of pathogens implicated in canine skin and ear infections using a novel next-generation-sequencing-based assay. *Vet Microbiol*. 2020; 247: 108764.
80. Amend A. From dandruff to deep-sea vents: *Malassezia*-like fungi are ecologically hyper-diverse. *PLoS Pathog.* 2014; 10(8): e1004277.

81. Schoch CL, Seifert KA, Hunhdorf S, et al. Nuclear ribosomal trancribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proc Natl Acad Sci U S A.* 2012; 109(16): 6241-6246.
82. Castellá G, Coutinho SD, Cabañes FJ. Phylogenetic relationships of *Malassezia* species based on multilocus sequence analysis. *Med Mycol*. 2014; 52(1): 99-105.
83. Vu D, Groenewald M, de Vries M, et al. Large-scale generation and analysis of filamentous fungal DNA barcodes boosts coverage for kingdom fungi and reveals thresholds for fungal species and higher taxon delimitation. *Stud Mycol.* 2019; 92: 135-154.
84. Scorzetti G, Fell JW, Fonseca A, Statzell-Tallman A. Systematics of basidiomycetous yeasts: a comparison of large subunit D1/D2 and internal transcribed spacer rDNA regions. *FEMS Yeast Res*. 2002; 2(4): 495-517.
85. Castellá G, De Bellis F, Bond R, Cabañes FJ. Molecular characterization of *Malassezia nana* isolates from cats. *Vet Microbiol*. 2011; 148(2-4): 363-367.
86. Cafarchia C, Stefania Latrofa M, Testini G, et al. Molecular characterization of *Malassezia* isolates from dogs using three distinct genetic markers in nuclear DNA. *Mol Cell Probes*. 2007; 21(3): 229-238.
87. Triana S, Gonzalez A, Ohm RA, et al. Draft genome sequence of the animal and human pathogen *Malassezia pachydermatis* Strain CBS 1879. *Genome Announc.* 2015; 3(5): e01197-15.
88. Gaitanis G, Velegraki A, Frangoulis E, et al. Identification of *Malassezia* species from patient skin scales by PCR-RFLP. *Clin Microbiol Infect.* 2002, 8(3):162-173.
89. Theelen B, Silvestri M, Guého E, van Belkum A, Boekhout T. Identification and typing of *Malassezia* yeasts using amplified fragment length polymorphism

(AFLP), random amplified polymorphic DNA (RAPD) and denaturing gradient gel electrophoresis (DGGE). *FEMS Yeast Res*. 2001; 1(2): 79-86.

90. Cabañes FJ. *Malassezia* yeasts: how many species infect humans and animals? *PLOS Pathog*. 2014; 10(2): e1003892.
91. Guillot J, Bond R. *Malassezia* yeasts in veterinary dermatology: an updated overview. *Front Cell Infect Microbiol*. 2020; 10: 79.
92. Chen TA, Hill PB. The biology of *Malassezia* organisms and their ability to induce immune responses and skin disease. *Vet Dermatol.* 2005; 16(1): 4-26.
93. Bond R, Guillot J, Cabañes FJ. *Malassezia* yeast in animal disease. In: Boekhout T, Guého-Kellermann E, Mayser P, Velegraki A, eds. *Malassezia* and the Skin. Berlin: Springer, 2010: 271-299.
94. Vijaya Chandra SH, Srinivas R, Dawson TL Jr, Common JE. Cutaneous *Malassezia*: Commensal, Pathogen, or Protector?. *Front Cell Infect Microbiol*. 2021; 10: 614446.
95. Bernier V, Weill FX, Hirigoyen V, et al. Skin colonization by *Malassezia* species in neonates: a prospective study and relationship with neonatal cephalic pustulosis. *Arch Dermatol.* 2002; 138(2): 215-218.
96. Gupta AK, Kohli Y. Prevalence of *Malassezia* species on various body sites in clinically healthy subjects representing different age groups. *Med Mycol*. 2004; 42(1): 35-42.
97. Chryssanthou E, Broberger U, Petrini B. *Malassezia pachydermatis* fungaemia in a neonatal intensive care unit. *Acta Paediatr*. 2001; 90(3): 323-327.
98. Chow NA, Chinn R, Pong A, et al. Use of whole-genome sequencing to detect an outbreak of *Malassezia pachydermatis* infection and colonization in a neonatal

intensive care unit-California, 2015-2016. *Infect Control Hosp Epidemiol*. 2020; 41(7): 851-853.

99. Rhimi W, Theelen B, Boekhout T, Otranto D, Cafarchia C. *Malassezia* spp. Yeasts of Emerging Concern in Fungemia. *Front Cell Infect Microbiol*. 2020; 10: 370.
100. Teoh Z, Mortensen J, Schaffzin JK. Invasive *Malassezia pachydermatis* infection in an 8-year-old child on lipid parenteral nutrition. *Case Rep Infect Dis*. 2022: 8636582.
101. Iatta R, Cafarchia C, Cuna T, et al. Bloodstream infections by *Malassezia* and *Candida* species in critical care patients. *Med Mycol*. 2014; 52(3): 264-269.
102. Aguirre C, Euliarte C, Finquelievich J, Sosa Mde L, Giusiano G. Fungemia and interstitial lung compromise caused by *Malassezia sympodialis* in a pediatric patient. *Rev Iberoam Micol*. 2015; 32(2): 118-121.
103. Velegraki A, Cafarchia C, Gaitanis G, Iatta R, Boekhout T. *Malassezia* infections in humans and animals: pathophysiology, detection, and treatment. *PLoS Pathog*. 2015; 11(1): e1004523.
104. Crespo MJ, Abarca ML, Cabañes FJ. Occurrence of *Malassezia* spp. in the external ear canals of dogs and cats with and without otitis externa. *Med Mycol*. 2002; 40(2): 115-121.
105. Crespo MJ, Abarca ML, Cabañes FJ. Occurrence of *Malassezia* spp. in horses and domestic ruminants. *Mycoses*. 2002; 45(8): 333-337.
106. Nakagaki K, Hata K, Iwata E, Takeo K. *Malassezia* pachydermatis isolated from a South American sea lion (*Otaria byronia*) with dermatitis. *J Vet Med Sci*. 2000; 62(8): 901-903.
107. Coutinho SDA, Fedullo JD, Corrêa SH. Isolation of *Malassezia* spp. from cerumen of wild felids. *Med Mycol*. 2006; 44(4): 383-387.

108. Puig L, Bragulat MR, Castellá G, Cabañes FJ. Phenotypic and genetic diversity of *Malassezia furfur* from domestic and zoo animals. *Med Mycol*. 2018; 56(8): 941-949.

109. Zia M, Mirhendi H, Toghyani M. Detection and identification of *Malassezia* species in domestic animals and aquatic birds by PCR-RFLP. *Iran J Vet Res*. 2015; 16(1): 36-41.

110. Garau M, del Palacio A, García J. Prevalence of *Malassezia* spp. in healthy pigs. *Mycoses.* 2005; 48(1): 17-20.

111. Nardoni S, Merildi V, Frangioni S, et al. F. Isolation and characterization of *Malassezia* spp. in healthy swine of different breeds. *Vet Microbiol*. 2010; 141(1-2): 155-158.

112. Pinter L, Anthony RM, Glumac N, Hajsig D, Pogacnik M, Drobnic-Kosorok M. Apparent cross-infection with a single strain of *Malassezia pachydermatis* on a pig farm. *Acta Vet. Hung*. 2002; 50(2): 151-156.

113. Hobi S, Cafarchia C, Romano V, Barrs VR. *Malassezia*: Zoonotic Implications, Parallels and Differences in Colonization and Disease in Humans and Animals. *J Fungi (Basel)*. 2022; 8(7): 708.

114. Ashbee HR, Bond R. *Malassezia* species and immunity: host-pathogen interactions. In: Boekhout T, Gueho E, Mayser P et al., eds. *Malassezia* and the skin. Science and clinical practice. Heildelberg: Springer, 2010; 139-174.

115. Sparber F, LeibundGut-Landmann S. Host Responses to *Malassezia* spp. in the mammalian skin. *Front Immunol.* 2017; 8: 1614.

116. Weidman FD. Exfoliative dermatitis in the Indian Rhinoceros (*Rhinoceros unicornis*) with description of a new yeast species, *Pityrosporum pachydermatis*.

Report of the Laboratory Museum Comparative Pathology Zoological Society, Philadelphia, 1925; 36-45.

117. Bauwens L, De Yroey C, De Mcurichy W. A case of exfoliative dermatitis in a captive southern white rhinoceros (*Ceratotherium simum simum*). *J Zoo Wildl Med* 1996; 27(2): 271-274.

118. Guillot J, Petit T, Rubiai-Degorce F, Guého E, Chermette R. Dermatitis caused by *Malassezia pachydermatis* in a California sea lion (*Zalophus californianus*). *Vet Rec.* 1998; 142(12): 311-312.

119. Scott DW, Miller WH, Griffin CE. Muller & Kirk's Small Animal Dermatology Philadelphia, PA: WB Saunders; 2001: 1203-1231.

120. Tater KC, Scott DW, Miller WH, Erb HN. The cytology of the external ear canal in the normal dog and cat. *J Vet Med*. 2003; 50(7): 370–374.

121. Cafarchia C, Gallo S, Capelli G, Otranto D. Occurrence and population size of *Malassezia* spp. in the external ear canal of dogs and cats both healthy and with otitis. *Mycopathologia*. 2005; 160(2): 143-149.

122. Leong C, Buttafuoco A, Glatz M, Bosshard PP. Antifungal susceptibility testing of *Malassezia* spp. with an optimized colorimetric broth microdilution method. *J Clin Microbiol.* 2017; 55(6): 1883-1893.

123. Vanden Bossche H, Engelen M, Rochette F. Antifungal agents of use in animal health–chemical, biochemical and pharmacological aspects. *J Vet Pharmacol Ther*. 2003; 26(1): 5-29.

124. Peano A, Johnson E, Chiavassa E, Tizzani P, Guillot J, Pasquetti M. Antifungal resistance regarding *Malassezia pachydermatis*: where are we now?. *J Fungi.* 2020; 6(2): 93.

125. Scott DW, Miller WH, Grin CE. Otitis externa. In: Miller WH, ed. Muller and Kirk's Small Animal Dermatology. 7th ed. Philadelphia, PA: Saunders WB; 2013: 741-773.

126. Scott DW, Miller WH, Grin CE. *Malassezia* dermatitis. In: Miller WH, ed. Muller and Kirk's Small Animal Dermatology. 7th ed. Philadelphia, PA: Saunders WB; 2013; 243-249.

127. Scott DW, Miller WH, Grin CE. Antifungal Therapy. In: Miller WH, ed. Muller and Kirk's Small Animal Dermatology. 7th ed. Philadelphia, PA: Saunders WB; 2013; 225-231.

128. Brilhante RSN, Rocha MGD, Guedes GMM, et al. *Malassezia pachydermatis* from animals: Planktonic and biofilm antifungal susceptibility and its virulence arsenal. *Vet Microbiol.* 2018; 220: 47-52.

129. Schlemmer KB, de Jesus FPK, Loreto ES, et al. *In vitro* combination of antifungal agents against *Malassezia pachydermatis*. *Med Mycol.* 2019; 57(3): 324-327.

130. Velegraki A, Alexopoulos EC, Kritikou S, Gaitanis G. Use of fatty acid RPMI 1640 media for testing susceptibilities of eight *Malassezia* species to the new triazole Posaconazole and to six established antifungal agents by a modified NCCLS M27–A2 microdilution method and Etest. *J Clin Microbiol*. 2004; 42(8): 3589-3593.

131. Weiler CB, de Jesus FP, Nardi GH, et al. Susceptibility variation of *Malassezia pachydermatis* to antifungal agents according to isolate source. *Braz JMicrobiol* 2013; 44(1): 174-178.

132. Jesus FP, Lautert C, Zanette RA, et al. *In vitro* susceptibility of fluconazole-susceptible and -resistant isolates of *Malassezia pachydermatis* against azoles. *Vet Microbiol.* 2011; 152(1-2): 161-164.

133. Chiavassa E, Tizzani P, Peano A. *In vitro* antifungal susceptibility of *Malassezia pachydermatis* strains isolated from dogs with chronic and acute otitis externa. *Mycopathologia*. 2014; 178(3-4): 315-319.

134. Watanabe S, Koike A, Kano R, et al. In vitro susceptibility of *Malassezia pachydermatis* isolates from canine skin with atopic dermatitis to ketoconazole and itraconazole in East Asia. *J Vet Med Sci*. 2014; 76(4): 579-81.

135. Angileri M, Pasquetti M, De Lucia M, Peano A. Azole resistance of *Malassezia pachydermatis* causing treatment failure in a dog. *Med Mycol Case Rep.* 2019; 23: 58–61.

136. Kano R, Yokoi S, Kariya N, Oshimo K, Kamata H. Multi-azole-resistant strain of *Malassezia pachydermatis* isolated from a canine *Malassezia* dermatitis. *Med Mycol.* 2019; 57(3): 346-350.

137. Clinical Laboratory and Standards Institute. Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standards, 3rd edn, document M27-A3. Wayne, PA: Clinical Laboratory and Standards Institute. 2008.

138. Peano A, Pasquetti M, Tizzani P, Chiavassa E, Guillot J, Johnson E. Methodological issues in antifungal susceptibility testing of *Malassezia pachydermatis*. *J Fungi*. 2017; 3(3): 37.

139. Nijima M, Kano R, Nagata M, Hasegawa A, Kamata H. An azole-resistant isolate of *Malassezia pachydermatis*. *Vet Microbiol*. 2011; 149(1-2): 288-290.

140. Cafarchia C, Figueredo LA, Iatta R, Montagna MT, Otranto D. *In vitro* antifungal susceptibility of *Malassezia pachydermatis* from dogs with and without skin lesions. *Vet Microbiol*. 2012; 155(2-4): 395-398.b

141. Pfaller MA. Antifungal drug resistance: Mechanisms, epidemiology, and consequences for treatment. *Am J Med*. 2012; 125 (Suppl 1): S3-S13.

142.Kim M, Cho Y, Park M, Choi Y, Hwang SY, Jung WH. Genomic tandem quadruplication is associated with ketoconazole resistance in *Malassezia pachydermatis*. *J Microbiol Biotechnol*. 2018; 28(11): 1937-1945.´

143.Park M, Cho Y, Lee YW, Jung WH. Genomic multiplication and drug efflux influence ketoconazole resistance in *Malassezia restricta*. *Front Cell Infect Microbiol.* 2020; 10: 191.

144.Zhang J, Li L, Lv Q, Yan L, Wang Y, Jiang Y. The fungal CYP51s: their functions, structures, related drug resistance, and inhibitors. *Front Microbiol*. 2019; 10: 691.

145.Heimark L, Shipkova P, Greene J, et al. Mechanism of azole antifungal activity as determined by liquid chromatographic/mass spectrometric monitoring of ergosterol biosynthesis. *J Mass Spectrom*. 2002; 37(3): 265-269.

146.Sanglard D, Ischer F, Koymans L, Bille J. Amino acid substitutions in the cytochrome P-450 lanosterol 14alpha-demethylase (CYP51A1) from azole-resistant *Candida albicans* clinical isolates contribute to resistance to azole antifungal agents. *Antimicrob Agents Chemother*. 1998; 42(2): 241-253.

147.Warrilow AG, Martel CM, Parker JE, et al. Azole binding properties of *Candida albicans* sterol 14-a demethylase (CaCYP51). *Antimicrob Agents Chemother*. 2010; 54(10): 4235-4245.

148.Kano R, Aramaki C, Murayama N, et al. High multi-azoleresistant *Malassezia pachydermatis* clinical isolates from canine *Malassezia* dermatitis. *Med Mycol*. 2020; 58(2): 197-200.

149.Kim D, Lim YR, Ohk SO, Kim BJ, Chun YJ. Functional expression and characterization of CYP51 from dandruff-causing *Malassezia globosa*. *FEMS Yeast Res*. 2011; 11(1): 80-87.

150.Leong C, Kit JCW, Lee SM, et al. Azole resistance mechanisms in pathogenic *M. furfur*. *Antimicrob Agents Chemother*. 2021; 65(5): e01975-e2020.

151.Flowers SA, Colo´n B, Whaley SG, Schuler MA, Rogers PD. Contribution of clinically derived mutations in *ERG11* to azole resistance in *Candida albicans*. *Antimicrob Agents Chemother*. 2015; 59(1): 450-460.

152.Feng W, Yang J, Xi Z, et al. Mutations and/or overexpressions of *ERG4* and *ERG11* genes in clinical azoles-resistant isolates of *Candida albicans*. *Microb Drug Resist*. 2017; 23(5): 563-570.

153.Cannon RD, Lamping E, Holmes AR, et al. Efflux-mediated antifungal drug resistance. *Clin Microbiol Rev*. 2009; 22(2): 291-321.

154.Iatta R, Puttilli MR, Immediato D, Otranto D, Cafarchia C. The role of drug efflux pumps in *Malassezia pachydermatis* and *Malassezia furfur* defense against azoles. *Mycoses*. 2017; 60(3): 178-182.

155.Vu D, Groenewald M, Szöke S, et al. DNA barcoding analysis of more than 9, 000 yeast isolates contributes to quantitative thresholds for yeast species and genera delimitation. *Stud Mycol*. 2016; 85: 91-105.

156.Mota-Gutierrez J, Ferrocino I, Rantsiou K, Cocolin L. Metataxonomic comparison between internal transcribed spacer and 26S ribosomal large subunit (LSU) rDNA gene. *Int J Food Microbiol*. 2019; 290: 132-140.

157.Quinton JF, François M, Laprais A, Prelaud P. Cytology of the external auditory meatus in healthy domestic pet rabbits (*Oryctolagus cuniculus*). *Rev Méd Vét*. 2014; 165: 263-266.

158.Johnson JC, Burn CC. Lop-eared rabbits have more aural and dental problems than erect-eared rabbits: a rescue population study. *Vet Rec*. 2019; 185(24): 758.

159.Campbell JJ, Coyner KS, Rankin SC, Lewis TP, Schick AE, Shumaker AK. Evaluation of fungal flora in normal and diseased canine ears. *Vet Dermatol*. 2010; 21(6): 619-625.

160.Puigdemont A, D'Andreano S, Ramió-Lluch L, Cuscó A, Francino O, Brazis P. Effect of an anti-inflammatory pomegranate otic treatment on the clinical evolution and microbiota profile of dogs with otitis externa. *Vet Dermatol*. 2021; 32(2): 158-e37.

161.Carrillo-Muñoz AJ, Rojas F, Tur-Tur C, et al. *In vitro* antifungal activity of topical and systemic antifungal drugs against *Malassezia* species. *Mycoses*. 2013; 56(5): 571-575.

162.Álvarez-Pérez S, García ME, Peláez T, Blanco JL. Genotyping and antifungal susceptibility testing of multiple *Malassezia pachydermatis* isolates from otitis and dermatitis cases in pets: is it really worth the effort? *Med Mycol*. 2016; 54(1): 72-79.

163.Kano R, Kamata H. Miconazole-tolerant strains of *Malassezia pachydermatis* generated by culture in medium containing miconazole. *Vet Dermatol*. 2020; 31(2): 97-101.

164.Cernicka J, Subik J. Resistance mechanisms in fluconazole resistant *Candida albicans* isolates from vaginal candidiasis. *Int J Antimicrob Agents*. 2006; 27(5): 403-408.

165.Morio F, Loge C, Besse B, Hennequin C, Le Pape P. Screening for amino acid substitutions in the *Candida albicans Erg11* protein of azole-susceptible and azole-

resistant clinical isolates: new substitutions and a review of the literature. *Diagn Microbiol Infect Dis*. 2010; 66(4): 373-384.

166. Berkow EL, Lockhart SR. Fluconazole resistance in *Candida* species: a current perspective. *Infect Drug Resist*. 2017; 10: 237-245.