Universitat Autònoma de Barcelona

THE FORGOTTEN WORLD OF MYCOBIOME IN THE SKIN OF DOGS

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Thesis submitted for the degree of Doctor (PhD)

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CERTIFIQUEN

Que la Tesi Doctoral amb títol **"The forgotten world of mycobiome in the skin of dogs"**, presentada per a Sara D'Andreano, s'ha dut a terme sota les seves direccions i s'ha realitzat al Departament de Ciència Animal i dels Aliments de la Facultat Veterinària de la Universitat Autònoma de Barcelona (UAB).

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Armand Sanchez Bonastre

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“La frode, ond'ogne coscïenza è morsa,
può l'omo usare in colui che 'n lui fida
e in quel che fidanza non imborsa”

Dante Alighieri, Canto XXVI dell’Inferno

“Science, my lad, is made up of mistakes,
but they are mistakes which it is useful to make,
because they lead little by little to the truth”

Jules Verne

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RESUM

La paraula microbioma inclou el conjunt de microbis que habiten un microhàbitat comú. Els mètodes de seqüenciació de nova generació van permetre la caracterització de les comunitats microbianes que viuen en les diferents parts del cos, tant humà com animals, la seva diversitat, les interaccions amb l'hoste (cèl·lula o immunitat), l'ambient i també entre microbis.

El primer objectiu és caracteritzar les comunitats bacterianes i fúngiques de la pell dels gossos amb otitis externa, mitjançant seqüenciació massiva amb una avaluació longitudinal després d'un tractament antibiòtic i no-antibiòtic. El gossos amb símptomes clínics es van dividiren dos grups depenent del tractament rebut. Les mostres de pell de les orelles es van recollir en cinc punts de temps. El perfil bacterià i fúngic es va caracteritzar en funció del tractament i del temps de recollida de les mostres. Es va analitzar la regió hipervariables V4 del gen 16S rRNA per a les comunitats bacterianes. Es van avaluar les regions hipervariables ITS1 i ITS2 de l'operó ribosòmic de fongs per comprendre quina regió fos la millor per a la classificació de la taxonomia. Es va verificar que la millor discriminació taxonòmica per a les comunitats de fongs s'obté amb la ITS2 i la base de dades UNITE. Després de l'amplificació per PCR, es van seqüenciar els amplicons amb la plataforma Ion Torrent PGM i amb xips 318^{TM} i es va analitzar amb bioinformàtica mitjançant en QIIME2. Els resultats van demostrar que un tractament amb corticoides tòpics més un agent antimicrobian natural, la magrana, va ser adequat pel control del sobrecreixement bacterià i fúngic. Concretament es va observar un canvi dràstic amb el fong *Malassezia*, que va disminuir durant el tractament per ser després substituït per altres comunitats de fongs. Les anàlisis amb seqüenciació de nova generació van permetre comprendre millor la dinàmica de l'otitis canina i van demostrar que el compost natural de la magrana seria una bona alternativa antimicrobiana per a una infecció lleu.

El segon objectiu és identificar els fongs a nivell d'espècie mitjançant el desenvolupament i l'optimització d'un assaig de seqüenciació basat en fragments llargs, per captar la màxima variabilitat i informació taxonòmica de l'operó ribosòmic

de fongs sencer. Es va avaluar la seqüenciació de tercera generació per nanopors amb el dispositiu MinION (Oxford Nanopore Technologies), per poder seqüenciar l'operó rRNA de fongs sencer (18S-ITS1-5.8S-ITS2-28S). Es van comparar els amplicons de les dues regions ITS1 i ITS2 juntes (3.5 Kb de longitud) amb els amplicons de l'operó sencer (6 Kb de longitud). Es va validar l'estratègia amb sis cultius microbiològics i quatre mostres d'otitis. La seqüenciació amb MinION va detectar la majoria dels cultius de fongs microbiològics i va confirmar la caracterització fenotípica de les dues mostres d'otitis. Els amplicons de 6 Kb van proporcionar una millor tasca taxonòmica pels fongs, tot i que per alguns d'ells, com *Malassezia*, *Saccharomyces* i *Cryptococcus* totes dues les mides dels amplicons van ser adequades. Aquesta tecnologia va ser un bon enfocament per a la caracterització de les comunitats de fongs en mostres complexes, i arribant al nivell d'espècie per al fong més abundant; *Malassezia* spp.

El tercer objectiu és avaluar la seqüenciació de nanopors per a l'assemblatge dels genomes de fongs. Malassezia és un fong comensal de la pell en mamífers, i la *Malassezia pachydermatis* és l'espècie de més importància veterinària. El seu sobrecreixement està correlacionat amb algunes malalties com l'otitis o la dermatitis. L'ADN es va extreure d'un cultiu microbiològic i es va seqüenciar amb nanopors mitjançant el dispositiu MinION. Les anàlisis de bioinformàtica van incloure la ultima versió de basecalling i correcció de seqüencies consensus. El genoma complet (8.6 Mb) en 10 *contigs* (N50 de 1,4 Mb i N90 de 1,1 Mb), va demostrar un assemblatge millor respecte al genoma de referencia (64 *contigs*) i amb una *completeness* similar (76.6%).

RESUMEN

La palabra microbioma incluye el conjunto de microbios que habitan un microhábitat común. Los métodos de secuenciación de nueva generación permitieron la caracterización de las comunidades microbianas que viven en las diferentes partes del cuerpo, tanto humano como animales, su diversidad, las interacciones con el huésped (célula o inmunidad), el ambiente y también entre microbios.

El primer objetivo es caracterizar las comunidades bacterianas y fúngicas de la piel de los perros con otitis externa, mediante secuenciación masiva con una evaluación longitudinal tras un tratamiento antibiótico y no-antibiótico. Los perros con síntomas clínicos se dividieron en dos grupos dependiendo del tratamiento recibido. Las muestras de piel de las orejas se recogieron en cinco puntos de tiempo. El perfil bacteriano y fúngico se caracterizó en función del tratamiento y del tiempo de recogida de las muestras. Se analizó la región hipervariables V4 del gen 16S rRNA para las comunidades bacterianas. Se evaluaron las regiones hipervariables ITS1 y ITS2 del operón ribosómico de hongos para comprender qué región fuera la mejor para la clasificación de la taxonomía. Se verificó que la mejor discriminación taxonómica para las comunidades de hongos se obtiene con la ITS2 y la base de datos UNITE. Después de la amplificación por PCR, se secuenciaron los amplicones con la plataforma Ion Torrent PGM y con chips 318^{TM} y se analizó con bioinformática mediante el QIIME2. Los resultados demostraron que un tratamiento tópico con corticoides más un agente antimicrobiano natural, la granada, fue adecuado para el control del sobrecrecimiento bacteriano y fúngico. Concretamente se observó un cambio drástico con el hongo *Malassezia,* que disminuyó durante el tratamiento para ser luego sustituido por otras comunidades de hongos. Los análisis con secuenciación de nueva generación permitieron comprender mejor la dinámica de la otitis canina y demostraron que el compuesto natural de la granada sería una buena alternativa antimicrobiana para una infección leve.

El segundo objetivo es identificar los hongos a nivel de especie mediante el desarrollo y la optimización de un ensayo de secuenciación basado en fragmentos largos, para captar la máxima variabilidad e información taxonómica del operón

ribosómico de hongos entero. Se evaluó la secuenciación de tercera generación para nanopors con el dispositivo MinION (Oxford Nanopore Technologies), para poder secuenciar el operón rRNA de hongos entero (18S-ITS1-5.8S-ITS2-28S). Se compararon los amplicones de las dos regiones ITS1 y ITS2 juntas (3.5 Kb de longitud) con los amplicones del operón entero (6 Kb de longitud). Se validó la estrategia con seis cultivos microbiológicos y cuatro muestras de otitis. La secuenciación con MinION detectó la mayoría de los cultivos de hongos microbiológicos y confirmó la caracterización fenotípica de las dos muestras de otitis. Los amplicones de 6 Kb proporcionaron una mejor labor taxonómica por los hongos, aunque para algunos de ellos, como *Malassezia*, *Saccharomyces* y *Cryptococcus* fueron adecuados ambos tamaños de los amplicones. Esta tecnología fue un buen enfoque para la caracterización de las comunidades de hongos en muestras complejas, y llegando al nivel de especie para el hongo más abundante: *Malassezia* spp.

El tercer objetivo es evaluar la secuenciación de nanopores para el ensamblaje de los genomas de hongos. Malassezia es un hongo comensal de la piel en mamíferos, y la *Malassezia pachydermatis* es la especie de mayor importancia veterinaria. Su sobrecrecimiento está correlacionado con algunas enfermedades como la otitis o la dermatitis. L'ADN se extrajo de un cultivo microbiológico y se secuenció con nanopores mediante el dispositivo MinION. Los análisis de bioinformática incluyeron la última versión de basecalling y corrección de secuencias consenso. El genoma completo (8.6 Mb) en 10 *contigs* (N50 de 1,4 Mb y N90 de 1,1 Mb), demostró un ensamblaje mejor respecto al genoma de referencia (64 *contigs*) y con una *completeness* similar (76.6%).

SUMMARY

The word microbiome includes the set of microbes that inhabit a common microhabitat. It refers not only to the bacterial community but to fungi and viruses too. Next-generation sequencing methods allowed the characterization of the microbial communities living in different part of the body either human or animal, their diversity, the interactions with the host (cell or immunity), the environment and the microbes.

The first objective is to characterize the skin bacterial and fungal communities in otitis externa in dogs by massive sequencing, with a longitudinal evaluation after antibiotic and non-antibiotic treatment. The dogs with clinical signs were classified depending on the treatment received. Skin samples from the ears were collected at five time-points. The bacterial and fungal profiles were characterized depending on treatment and sample collection time. We analysed the V4 hypervariable region of the 16S rRNA gene for bacterial communities with an optimized workflow. We evaluated the ITS1 and ITS2 hypervariable regions from the fungal ribosomal operon to understand which region is better for taxonomy classification. We verified the highest taxonomical discrimination for fungal communities with ITS2 and the UNITE database. After PCR amplification, amplicons were sequenced with the Ion Torrent PGM platform with 318^{TM} chips and analysed with the QIIME2 bioinformatics workflow. Results showed that a treatment of topical corticosteroid plus a natural antimicrobial agent, pomegranate, was suitable for the control of bacterial and fungal overgrowth. Specifically, a drastic change was observed with the fungus *Malassezia*, which decreased during the treatment to be then replaced by other fungal communities. Next generation sequencing analyses permitted to better understand the dynamics of the canine otitis and showed that the pomegranate natural compound would be a good antimicrobial alternative for a mild infection.

The second objective is to identify fungi at the species level by the development and optimization of a sequencing assay based on long-fragments, to capture the maximum variability and taxonomical information from the whole fungal ribosomal operon. We evaluated the third-generation sequencing by nanopores with the

MinION device to be able to sequence the whole fungal rRNA operon (18S-ITS1-5.8S-ITS2-28S). We compared amplicons targeting ITS1 and ITS2 together (3.5 Kb length) with amplicons targeting the whole ribosomal operon (6 Kb length). We validated the strategy with six microbiological fungal cultures and four otitis samples. MinION sequencing detected most of the microbiological fungal cultures and confirmed the phenotypic characterization of the two otitis samples. The amplicons targeting the whole ribosomal operon (6 Kb) provided a better taxonomic assignment for fungi, although for some of them, such as *Malassezia, Saccharomyces* and *Cryptococcus* (correctly detected at 100%), both amplicons sizes were suitable. Finally, Nanopore Technologies was a good approach for the characterization of the fungal communities in complex samples, reaching the species level for the most abundant fungus found: *Malassezia* spp.

The third objective is to evaluate nanopore sequencing for the assembly of fungal genomes. Malassezia is a skin commensal fungus in mammals, and *Malassezia pachydermatis* is the species of more significant veterinary concern. Its overgrowth is correlated with diseases like otitis or dermatitis. The DNA was extracted from a microbiological culture and nanopore sequenced with MinION. The bioinformatics analyses included the last high accuracy basecalling and consensus correction workflows. The genome assembly of 8.6 Mbp in 10 *contigs* (N50 1.4 Mbp; N90 1.1 Mbp) improved the assembly and the scaffolding of the reference genome (64 *contigs*), with similar completeness (76.6%).

1. INTRODUCTION

1.1) Homeostasis, a balance in skin: microbiota and mycobiota in the dog

The word *homeostasis* derives from Greek, with *homeo* meaning “similar,” and *stasis*, meaning “stable”. It means the ability of a living organism to keep the same state of internal balance despite changes that may occur inside and outside.

These changes in mammals are related to internal and external balance: the first include autoregulation in temperature, sweat glands, cell immunity, the concentration of blood or tissue fluid, and the exchange of gas carbon dioxide and oxygen; the second is related to the environment. An organism is constantly pushed away from its balance point, so the homeostasis aim is to oppose to those changes. Both the gut and the skin are complex immune and neuroendocrine organs relevant to homeostasis. They establish the first contact and communication with the environment (O’Neill et al. 2016; Salem et al. 2018).

Microbes colonize mammals during birth and throughout its life. Post-natal colonization is an ongoing process, due to complex interactions as lifestyle, diet, host genotype, diseases and use of antibiotics. In 2008 the NIH Human Microbiome Project (HMP) was established by the National Institute for Health in the USA. The aim was to generate resources that would enable to understand the characterization of the human microbiome and the analysis of its role in human health and disease. The term “microbiome” refers to the bacteria, archaea, fungi and viruses and their genomes, that live in an ecological niche. The number of host cells and microbiota in an individual are nearly the same: 3×10^{13} host cells and 3.9×10^{13} colonizing microorganisms (Prescott 2017; Thomas et al. 2017). The gut, the skin, and the oral cavity harbour the largest concentrations of microbes. They are present in every environmental niche and are essential for health: they provide protection against foreign invaders, stimulate the immune response, produce antimicrobials, aid in digestion and produce vitamins (Findley et al. 2013). In mammals, understanding the complex interaction among communities is essential to figure out the possible roles in diseases (Sotiropoulou and Blanpain 2012; Cénit et al. 2014; Kotas and Medzhitov 2016; O’Neill et al. 2016).

The skin. The skin is a stratified and cornified epithelium of basal stem cells that after birth become enucleated, cross-linked sacs of proteins and sealed together by lipids. In humans, its surface shows 10^{10} of bacterial cells and a wide range of micro-environments characterized by pH, temperature, moisture, sebum and topography (Findley et al. 2013; Byrd et al. 2018).

It is composed of three membrane layers: the epidermis, the dermis, and the subcutaneous tissue. The epidermis is the outermost one, and its role against infections and disease is high. It contains three specialized cells: melanocytes for the production of the pigment (melanin), the Langerhans cells that act as the first line of defence in the skin's immune system, and the Merkel cells, which function is not entirely understood yet. The dermis is the middle layer and it contains connective tissue, blood capillaries, oil and sweat glands, nerve endings, and hair follicles. At last, we found the subcutaneous tissue, the deepest and innermost layer, which connects the skin to bones and muscles and it controls the temperature (Figure 1).

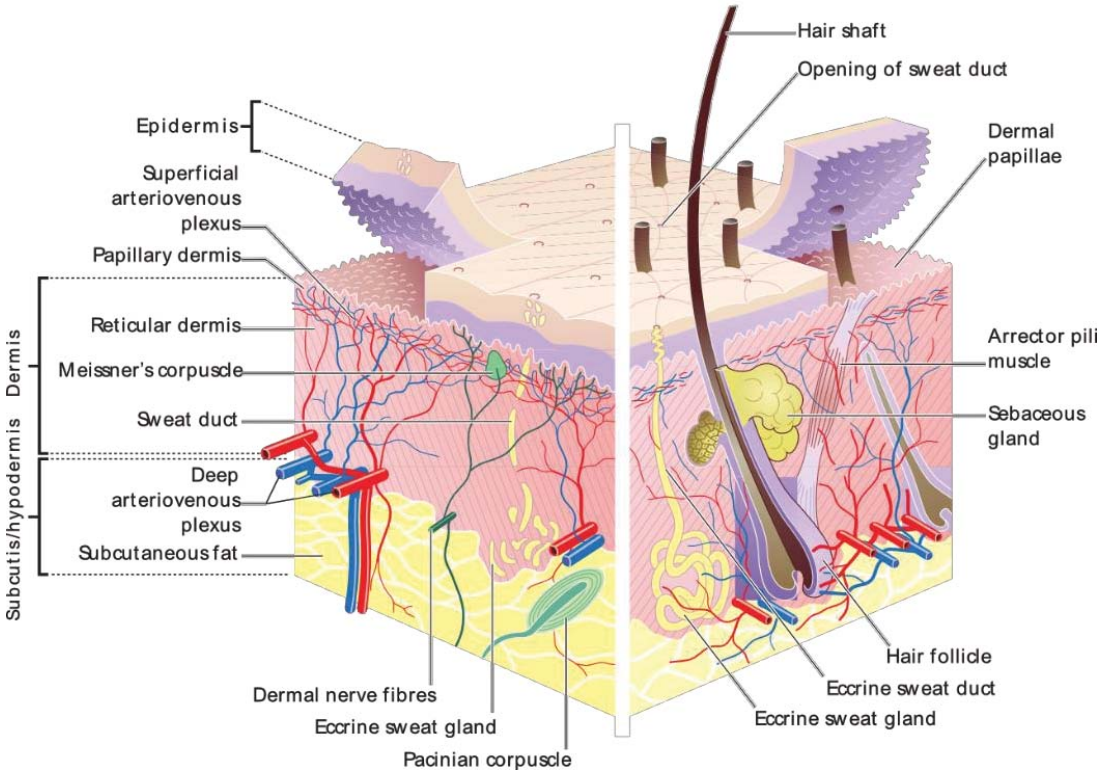

Figure 1. Skin layers details (https://enacademic.com/dic.nsf/enwiki/17181).

Skin is the outermost compartment of the body and together with the gut, it is the first contact to the external environment and a physical barrier to prevent the invasion of pathogens. Beneficial microorganisms colonize this barrier that is exposed to endogenous and exogenous factors that may impact the balance between commensals microbes, leading to inflammatory conditions: infections, allergies or autoimmune diseases (Byrd et al. 2018). In humans, the microbiome composition id different per each anatomical sites: sebaceous, moist and dry (Findley et al. 2013; Byrd et al. 2018). Different types of bacteria, fungi, and viruses that colonize each niche show a specific immunological network, due to the microorganisms in it (Grice and Segre 2011; Schommer and Gallo 2013; Belkaid and Tamoutounour 2016). First and foremost, the ecological system under normal physiological conditions must maintain homeostasis within and between microbial species and the host. However, but the mechanisms of this balance are not clearly understood yet (Weyrich et al. 2015; Prescott et al. 2017). The mother influences the first colonization of microbes at birth: microbes from the vagina are the first found

on the skin, and the diversity of the communities is low. These communities evolve to form the “core” microbiota (commensal): the microbial community that is generally found in the skin and it re-established after disorders (Zaura et al. 2009; Tremaroli and Bäckhed 2012; Cuscó et al. 2017b).

This process is necessary to establish immune tolerance to commensal microorganisms: the T-cells are the main ones involved for the adapted immune response (Dréno et al. 2016). The immune system, particularly the adaptive one, helps to acquire a microbiota that evolves its complexity and maintains a symbiotic relationship with the host. Thanks to the immune-microbial combination it is possible to preserve the tissue integrity, and when there are damages, the microbiota can help to repair it (Belkaid and Tamoutounour 2016). The skin microorganisms can control the expression of antimicrobial peptides and proteins (AMPs), promote the expression of components of the complementary system, and participate in the expression of interleukin-1 (IL-1). However, when massive changes happen, the immune system is contributing to increase inflammatory and autoimmune disorders. The dysregulation of the immune response is apparent in several disorders like psoriasis, atopic dermatitis, or eczema. How those dysregulations affect or result from changes in the microbiota remains unclear (Grice and Segre 2011). The skin microbial community is stable and persists for one year or more, suggesting that this stability is due the re-acquisition of common species from the environment. So, we can find almost the same main strains in the same individual along time (Byrd et al. 2018).

Several studies on human skin microbiome analyse the communities found in health and disease, during different treatments, and during different life periods: the early-life exposures can help the development of allergic or inflammatory diseases (Abrahamsson et al. 2012; Prescott et al. 2017).

Four main phyla represent the most common bacterial communities of the skin: Actinobacteria, Firmicutes, Proteobacteria and Bacteroides, while the most common genera are Corynebacteria, Propionibacteria and Staphylococci (Grice and Segre 2011; Dréno et al. 2016). For the fungi, *Malassezia* yeast is the most common found in healthy skin, but alteration of it may be associated to disorders (Gaitanis et al. 2012). The primary bacterial colonizers were the coagulase-negative staphylococci

and in particular *Staphylococcus epidermidis*. Then we have the coryneform bacteria as *Corynebacterium, Propionibacterium* and *Brevibacterium* all belonging to Actinobacteria phylum. The *Malassezia* spp., prevalent in sebaceous areas, is the most common fungi found in the skin plus the microscopic arthropod *Demodex* mites. *Demodex* is commonly found in the sebaceous areas of the face because it feeds on sebum, as *Propionibacterium* (Grice and Segre 2011). Microbes and their hosts have co-evolved: advantages for the host about the evolution of its microbiota include the quick adaptation to any environmental changes; also, microbes profit from the host-derived nutrients and stable ecological niches (Schommer and Gallo 2013).

Until now we described the communities of microbes found in the skin by using the term “microbiome”. Antonie van Leeuwenhoek first started to study microorganisms in the 17th century but the real definition of “microbiota” was described for the first time by Lederberg and McCray in 2001 (Marchesi and Ravel 2015; Prescott 2017) referring to microorganisms (bacteria, archaea, eukaryotes, and viruses) and their genomes. In 2000, Casadevall and Pirofski described the differences among terms that describe the “status” of microbes (Casadevall and Pirofski 2000). Even if there are different microbes’ communities in the skin, only few ones are related to some diseases. Besides, not all of them cause the same disease in different hosts; microbes classified as pathogens in a host can be part of the healthy communities in others. Depending on the interaction between the host and the microbe, there are different damages or benefits. A description of what could happen on the skin is reviewed in Casadevall and Pirofski (Casadevall and Pirofski 2000).

The most important terms used for the microbes classification are commensalism, colonization persistence, and disease. Commensalism refers to a functional interaction between host and microbes without ongoing/apparent host damage; despite this definition, a commensal microbe can cause damages depending on the host. Microbes found on the surface of the skin that are only very infrequently associated with disease, are typically referred to as commensal. This term implies that the microbe lives in peaceful coexistence with the host while benefiting from the sheltered ecological niche (Cogen et al. 2009). Persistence associates with a

prolonged state of colonization that can induce damage in the host. Finally, the disease is the damage cause by the interaction of host and microbe (Casadevall and Pirofski 2000). Mutualism describes the interaction host/microbes. In the skin, the host provides the nutrients, and the microbes promote the epithelial and immune homeostasis (Chen et al. 2018).

Now, talking about microbes, the word “microbiome” is usually referring only to bacteria, but it also includes fungi and viruses. For the fungi, the term is “mycobiome” (a combination of the words ‘mycology’ and ‘microbiome’). Introduced in 2010, it refers to the overall fungal habitat, the genomes and the surrounding environmental conditions (Cui et al. 2013).

With new technologies, the mycobiome started to be in-depth investigated to understand its relation to health and disease condition in humans and animals (Findley et al. 2013; Underhill and Iliev 2015; Limon et al. 2017). Researches also investigated the interactions between bacterial and fungal communities that are important to maintain the homeostasis but also crucial in various skin disorders such as seborrheic dermatitis, atopic dermatitis, and dermatophytosis (Peleg et al. 2010). Findley and collaborators (Findley et al. 2013) characterized the “basal” skin mycobiome in humans and highlighted a few essential points:

1. Bacterial and fungal diversities grouped into clusters, with the same core-body site having similar bacterial and fungal richness.
2. Skin topography and tissue structure are the key determinants of the microbiome composition.
3. The skin mycobiome is highly constant in humans, except for the feet (Figure 2).

Malassezia is the most common fungal genus in all the skin sites, except for the foot site analysed (plantar heel, toenail, and toe web) that exhibited a higher fungal diversity.

Sample analysis from arm sites revealed higher bacterial diversity and lower fungal diversity. The plantar heel was the most diverse site, with prevalence of *Malassezia, Aspergillus, Cryptococcus, Rhodotorula, Epicoccum*, and others.

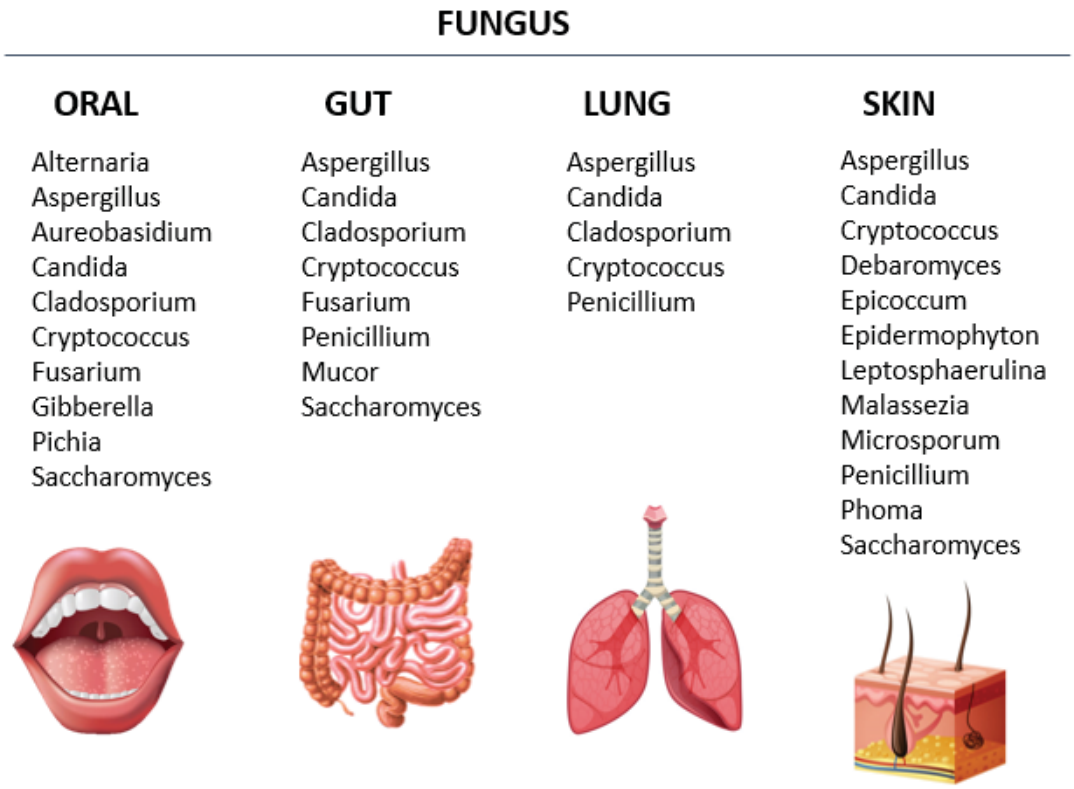

Figure 2. The most common fungi found in different body zone.

Skin in dogs. Information about healthy and diseased skin in humans is increasing, but the knowledge in healthy dogs comes primarily from the study of Hoffmann et al. 2014. In dogs, as in all mammals, the skin is the first barrier among host, and environmental changes.

The main skin difference between dogs and humans is the epidermis thickness (3-5 cells in dogs and 10-15 cells in humans), and by the hair (hair grows in bundles in dogs, while solitary in humans). However, a real comparison between microbes that are commonly living on the skin of both is not so easy: dogs share lifestyle and environment with us. The three factors that currently influence the exposure of the dog skin to environment are land use in the residential environment, contact with different land-use types during exercise, and lifestyle of the dog owner. Hoffmann

(Hoffmann et al. 2014) did the first study on skin microbiota in healthy and allergic dogs. Results showed that the main phyla found were Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes and Cyanobacteria, and their distribution on the skin site was changing (Hoffmann et al. 2014). At genus level *Ralstonia* spp., *Bacillus* spp., *Corynebacterium* spp., *Pseudomonas* spp., and *Staphylococcus pseudintermedius* were the most abundant found (although *Ralstonia* spp. is considered an artefact due to kits contamination). They also reported that the variability of the communities found was high among the different skin regions in the same dog. In 2017, Cuscó et al. analysed the composition and variability of the microbiota in a cohort of healthy dogs from three pure breeds, founding that the critical factor of the microbiota variability is the individual rather than the breed or the skin site or hair coat (Cuscó et al. 2017b). In a second work, Cuscó et al. analysed a well-controlled cohort of dogs from the same breed, age and sharing the environment (Cuscó et al. 2017a). The results confirmed that the individual shapes the microbiota composition in dog's skin but also reported the environmental effect in driving its composition (Figure 3).

In the same year, Torres et al. (2017) investigated on the high diversity and variability of the microbial communities in the same dog, finding results in agreement with the previously reported (Torres et al. 2017).

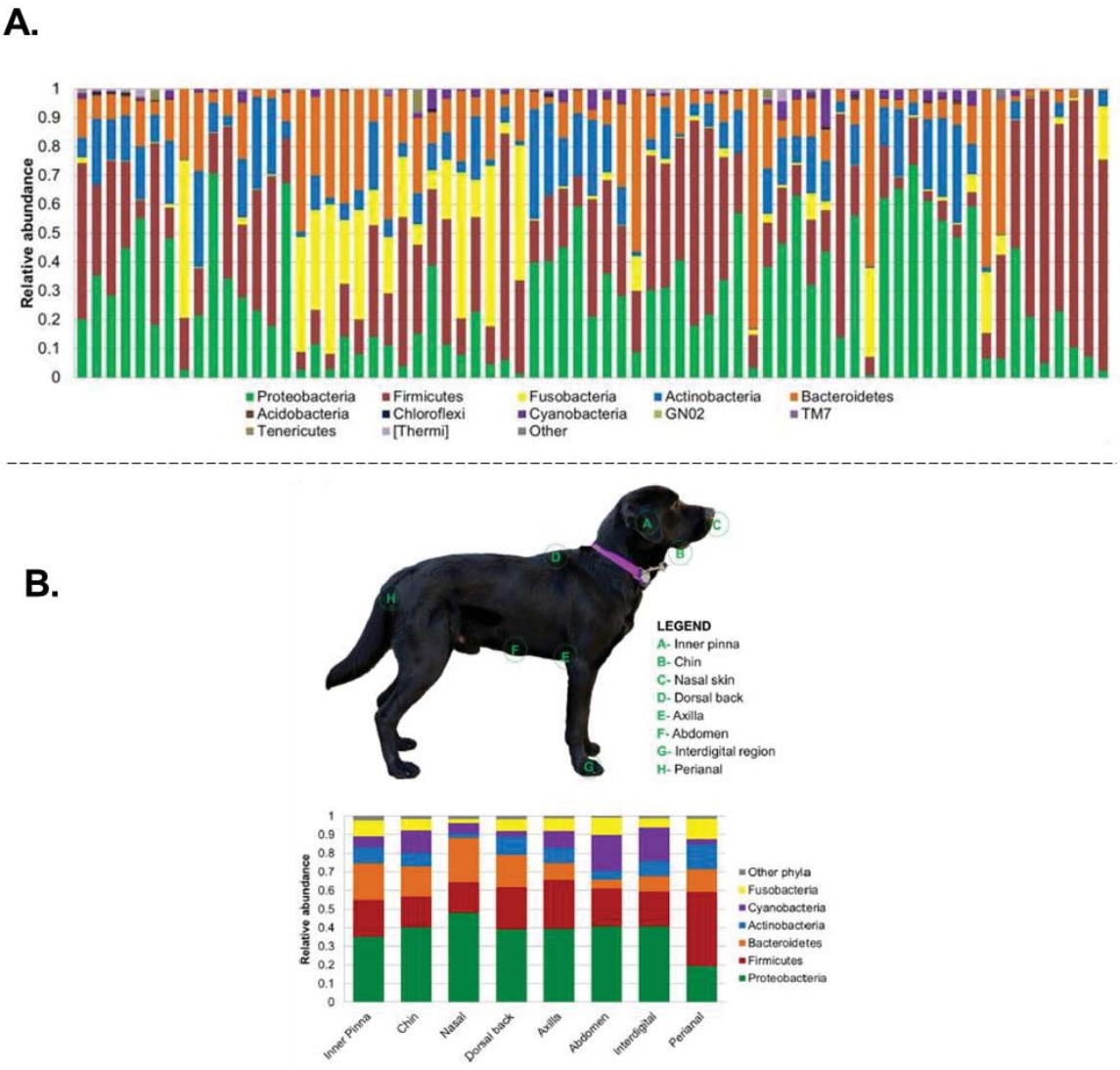

Figure 3. composition and variability of the skin microbiota in healthy dogs at the phylum level. (A) Each individual presents a different microbiota profile. (B) A detailed bacterial distribution in each skin site at phylum level (Cuscó et al. 2017a; Cuscó et al. 2017b).

Regarding the fungal communities in healthy dogs, the main phyla are Ascomycota and Basidiomycota, and the main genera are *Alternaria, Cladosporium*, *Malassezia* and *Cryptococcus* (Meason-smith et al. 2015).

Hoffmann was also investigating the allergic skin condition when atopic dermatitis (AD) occurred. The AD is a common, genetically predisposed, inflammatory, and pruritic disease, with many variations that sometimes may not permit a quick diagnosis because it does not have pathognomonic clinical signs. Therefore, a complete guideline is available to help on the diagnosis (Hensel et al. 2015). It is

associated to the IgE antibodies reaction with environmental allergens, and the possible causal agent associated with it usually are fleas, ectoparasites, or bacterial and fungi overgrowth, such as Staphylococcal and Malassezia (Hensel et al. 2015). Compared to humans, few studies are focused on the canine AD (Hoffmann et al. 2014; Bradley et al. 2016; Chermprapai et al. 2019).

Studies on the skin surface of humans revealed that *Propionibacterium* spp., *Staphylococcus* spp., and *Corynebacterium* spp. predominate in sebaceous and moist areas. In contrast, the gram-negative colonize the dry ones (Hoffmann et al. 2014). In the healthy dog skin, Proteobacteria phylum is the most abundant one. However, when allergic dogs were analysed, other families such as Firmicutes and Actinobacteria in the axilla and interdigital regions, or Firmicutes and Proteobacteria in the nostril, were detected. They also observed that the variability was changing within the same dog, between different areas of the body that included the haired ones (axilla, groin, periocular, pinna, dorsal nose, interdigital, lumbar) and the mucosa (lips, nose, and conjunctiva) (Hoffmann et al. 2014).

Staphylococci are common commensal on canine skin, and the genera better associated with AD are *S. pseudintermedius* and *S. aureus* (Williams and Gallo 2015; Bradley et al. 2016). It was showed that the variability of the microbial communities is low during the disease (Bradley et al. 2016).

In fungal communities, the diversity and the richness of communities are lower in AD (Meason-smith et al. 2015) and Malassezia is the most representative genus (Chermprapai et al. 2019). In dogs with AD, otitis is a common weakness (Ngo et al. 2018) and it is classified in otitis media (OM) and otitis externa (OE). The canine OE is a common multifactorial cutaneous disorder. The causal factors are classified in predisposing, primary, secondary, and perpetuating. The causal factors are classified in predisposing, primary, secondary, and perpetuating. Predisposing indicates the ear canal conformation and primary describes the first symptoms that evolve later in the secondary characterized by bacterial or yeast infection. The perpetuating refers to the progressive pathological changes of the ear canal (Saridomichelakis et al. 2007). The primary causes are correlated to atopic dermatitis and adverse food reactions. However, due to many other agents and the emergence of antibiotic resistance among the microorganisms, it remains

challenging to treat (Oliveira et al. 2008). That is the reason to apply regular ear washes and correct antimicrobial therapy in routine of pets.

To date, only few studies have analysed the otic microbiota in health and allergic condition. In the past, this was not easy because culture-independents methods could not analyse many species living on the skin. Thanks to next generation sequencing (NGS), there was a revolution in the analysis of bacterial and fungal communities. In the healthy ear, the main bacterial phyla were Proteobacteria, Actinobacteria, Firmicutes, Bacteroidetes and Fusobacteria while the most abundant genera were *Escherichia, Conchiformibus, Corynebacterium* and *Staphylococcus*. In the atopic dogs, a decrease of Proteobacteria leaves space to Firmicutes and *Staphylococcus* genus followed by *Corynebacterium, Propionibacterium* and *Escherichia* (Ngo et al. 2018; Korbelik et al. 2019).

About fungi, the main genus was *Malassezia* that may play a key role in otitis in dogs with allergic conditions.

The genus *Malassezia*: an overview. The fungus *Malassezia* belong to the class Malasseziomycetes, in the subphylum Ustilaginomycotina. It is a basidiomycete yeast that colonizes the skin of homoeothermic animals, including humans, dogs, horses, pigs, goats, cats and lambs (Wu et al. 2015). It may play a role in dermatopathology, since it is associated with skin disorders, such as dandruff, atopic eczema/dermatitis, pityriasis versicolor, seborrheic dermatitis and other systemic diseases (Coelho et al. 2013; Cabañes 2014).

The genus *Malassezia* was described in 1846, and it was considered as a non-culturable yeast until 1939. In the ‘80s, Midgley started culturing it, and in 1996 the genus *Malassezia* was classified in the first seven species: *M. furfur*, *M. pachydermatis*, *M. sympodialis*, *M. globosa*, *M. obtuse*, *M.restricta* and *M. slooffiae* (Gueho et al. 1996; Gaitanis et al. 2012).

Thanks to the conventional and modern techniques, three more species (*M. dermatis*, *M. japonica* and *M. yamatoensis*) have been isolated since 2004 from the skin from both healthy and seborrheic dermatitis diseased patients (Gaitanis et al. 2012). Then, from 2004 to 2011, new lipid-dependent species such as *M. nana*, *M. caprae*, *M. equina*, and *M. cuniculi*, were found in animals’ skin, raising the number of currently recognized *Malassezia* species to 14 (Gaitanis et al. 2012; Harada et al.

2015). In 2016, three new species were described, namely *M. brasiliensis* and *M. psittaci* from parrots and *M. arunalokei* from human skin (Cabañes et al. 2016; Honnavar et al. 2016). *M. vespertilionis*, a new cold-tolerant species, was recently isolated from bats; thus the genus comprises 18 species (Lorch et al. 2018). The number of currently described species is likely limited to a sampling bias towards humans and domestic animals (Table 1). However, this number may increase when the skin microbiota of a broader range of wild animals is investigated (Guillot and Bond 2020).

Table 1. Very recently, Guillot and Bond listed the currently accepted Malassezia species and the hosts in which they can be found (Guillot and Bond 2020).

***Malassezia* species**	***Synonymous***	**Presence on healthy skin**	**Presence in lesion**
M. furfur	*Pityrosporum ovale*	Humans / animals	In humans (PV, FG)
M. pachydermatis	*P. pachydermatis* *P. canis*	Dogs / cats / others Humans (dog contact)	In dogs, cats, others (SD, OT) Sometimes in humans (FG)
M. sympodialis	*M. furfur serovar A*	Humans / animals	In humans (AD, SD) Sometimes in cats (OT)
M. globosa	*P. orbiculare* *M. furfur serovar B*	Humans / animals	In humans (PV, SD, AD) sometimes in cats (OT)
M. obtusa		Humans	In humans
M. slooffiae		Pigs / cats / humans	In humans
M. restricta	*M. furfur serovar C*	Humans	In humans (SD)
M. dermatis		Humans	In humans (AD)
M. japonica		Humans	In humans (AD, SD)
M. nana		Cats / horses	In cats and cattle (OT)
M. yamatoensis		Humans	In humans (SD)
M. caprae		Goats	
M. equina	*M. equi*	Horses	In horses
M. cuniculi		Rabbit	
M. arunalokei		Humans	In humans
M. brasiliensis		Parrots	
M. psittaci		Parrots	
M. vespertolionis		Hibernating bats	

The genetic basis of the lipophilic status of Malassezia and the adaptation to animal skin is still under study. At the beginning, the identification of the *Malassezia* species relied on the morphological characterization and the ability to grow in a specific SGA agar (Sabouraud glucose agar). Nowadays the culture-dependent methods are not yet the best one for all species identification, even if the physiological characterization of some species as *M. pachydermatis* is based mainly on the evaluation to grow in SGA agar (Puig et al. 2017).

The genome of this fungus is one of the smallest in the fungal kingdom. It contains only the minimal complement of information necessary for the existence in its specific ecological niche (Wu et al. 2015). Compared to other Basidiomycota, *Malassezia* showed gene sets lost with an increase in others involved in carbohydrate metabolic process, hydrolysis activity, and adaptation to the environment. As it lacks the gene encoding the fatty acid synthase (FAS), it indicates that the genus is lipid-dependent (Wu et al. 2015). Among all the species, *M. pachydermatis* was the only one considered as non-lipid dependent, a property that might help for its identification (Gaitanis et al. 2012). However, this concept relied on a subset of *M. pachydermatis* growing in Sabouraud-dextrose agar media without added lipids. A re-investigation on the content of this media, showed that the fungus was able to grow only in the presence of added lipids, confirming the unique lipid-dependent nature of all *Malassezia* species (Wu et al. 2015). Another study on *M. pachydermatis* from skin of dogs showed that the use of complex culture media containing a variety of fatty acids required for the growth of this fungus species, helped in the characterization of its strains. *M. pachydermatis* was classified as the less lipid-demanding species of the genus because it needs a component from the Sabouraud glucose agar (SGA) that provide fatty acids that are essential for its growth (Puig et al. 2017).
These observations explained its failure to grow on lipid-free media and they suggest so that *M. pachydermatis* should be regarded as “lipid-dependent” (Guillot and Bond 2020).

Studies on dogs are usually based on *M. pachydermatis* because it is recognized as a normal yeast living on the surface of most animals; also, it may be related to

diseases that occur in the skin when hypersensitivity reaction to the organisms or cutaneous overgrowth are presented (Hnilica and Patterson 2017). *Malassezia* overgrowth is almost always associated with an underlying cause, such as atopy, food allergy, endocrinopathy, keratinization disorder, metabolic disease, or prolonged therapy with corticosteroids. Its pathogenic role has been a matter of controversy: disorders in which *Malassezia* is associated regards the host immune system and the cells of the epidermis interaction (Cabañes 2014). In dermatitis, the main sites affected are lips, ear canals, moist folds axillae, groin, ventral neck, interdigital skin, perivulvar skin, and perianal skin (Bajawa 2017). Predisposition to a *Malassezia* overgrowth is reported in the basset hound, West Highland white terrier, dachshund, American cocker spaniel, English springer spaniel, and German shepherd dog, possibly because they have significantly more yeast on their skin (Hnilica and Patterson 2017).

1.2) Microbiome and its biomarker: 16S rRNA gene

At the begin, microorganisms were studied because of their capacity to cause disease in the host. The methods used to understand the causal agent of the disease rely on the cultivation of microbial colonies, isolation of the possible causal agent, and use of postulates to describe and confirm the characteristics (Garza and Dutilh 2015). Disadvantages of culture methods were related to a proper growth and isolation of microorganisms: when a microbe can be cultured, it is easier to study it. The reproducibility of the results is another common problem with culture methods: most microbes cannot be cultured or the small colonies that grown give poor results to understand the microbe nature.
These early studies were not able to classify the microbial communities in samples but only focus on one specific microbe at a time. The knowledge of extent microbial diversity grew in time, thanks to culture-free methods (Garza and Dutilh 2015): new sequences based methods were developed for microbes classification. The study of the ribosomal rRNA gene, in specific the small subunit 16S rRNA for bacteria/Archaea and the 18S for Eukaryota, became the most used for taxonomic identification (Woese et al. 1990; Chakravorty et al. 2007; Wang et al. 2014).

Between 1970s and 1980s, the analysis of molecular sequences through phylogenetic approaches based on the ribosomal RNA gene, 16S rRNA, became the standard for reconstructing genealogies and the backbone for a new prokaryotic taxonomy. The advent of DNA amplification and sequencing techniques facilitated the classification of the microorganisms. Even if the use of a single gene has disadvantages, the 16S rRNA gene is currently widely used as taxonomic marker because: (i) it is present in almost all bacteria or operons; (ii) its function over time has not changed; and (iii) it is long enough (1.500 bp) for a correct taxonomic identification and application in clinical diagnosis (Figure 4) (Janda and Abbott 2007; Yarza et al. 2014).

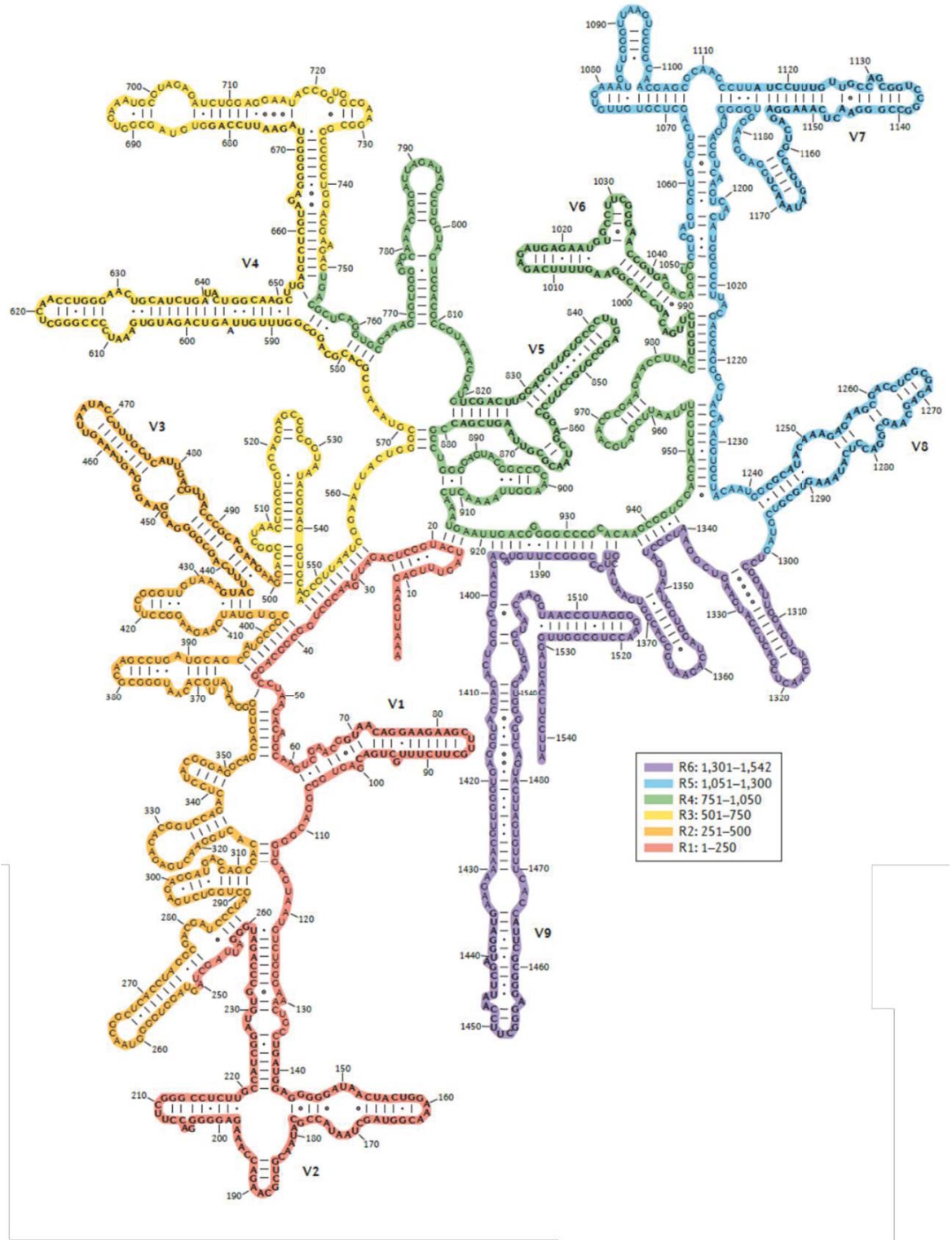

Figure 4. Structure of the 16S rRNA gene (Yarza et al. 2014).

The 16S rRNA gene is a multi-copy gene, which increases the detection sensitivity. It is ubiquitous because ribosomes cannot translate mRNA without their 16S rRNA component, so all bacteria have it. It is highly conserved because these genes are essential but with hypervariable regions for each genus/species, meaning that universal primers can amplify it from divergent bacteria but also that a tree of life for all bacteria can be built.

Sequence analysis of the 16S ribosomal RNA (rRNA) gene has been extensively used to identify bacterial species and to perform taxonomic studies (Baker et al. 2003; Bukin et al. 2019).

Bacterial 16S rRNA gene contains nine “hypervariable regions” suitable for species identification (Figure 5). Hypervariable regions are flanked by conserved stretches in most bacteria, enabling PCR amplification of target sequences using universal primers (Chakravorty et al. 2007) to identify a single bacteria species. A disadvantage of the 16S rRNA hypervariable regions is that one cannot be enough to distinguish among all bacteria. The nine hypervariable regions have different size: 69–99, 137–242, 433–497, 576–682, 822–879, 986–1043, 1117–1173, 1243–1294 and 1435–1465 for V1 through V9 respectively.

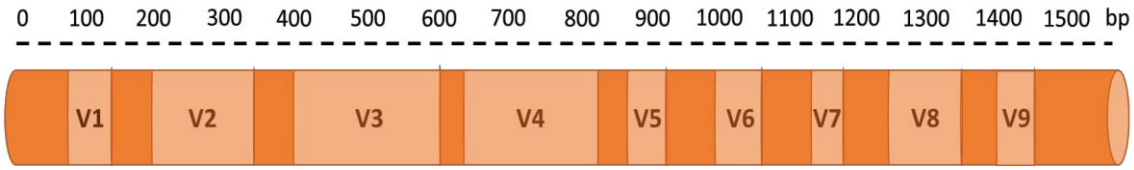

Figure 5. Structure of the 16S rRNA gene.

Taxonomy at low level such genus and species, demonstrates that 16S rRNA gene sequencing achieves genus classification (90%). However, it is less valuable at for species identification due to a less discriminatory power (65% - 83%) when only choosing one hypervariable region, because it can significantly affect the estimates of taxonomic diversity (Janda and Abbott 2007; Bukin et al. 2019).

Also, these regions reveal to be able to detect different types of bacteria (Chakravorty et al. 2007):

- V1 targets common pathogenic *Streptococcus* sp., *Staphylococcus aureus* and coagulase-negative *Staphylococcus* species.
- V2 targets the *Staphylococcal* and *Streptococcal* pathogens, but also *Haemophilus*, *Clostridium* and *Neisseria* species. It appears to be a good target also for *Mycobacterial* species. It is not considered the best target for *Escherichia* sp., *Shigella* sp., *Klebsiella pneumoniae* and *Enterobacter aerogenes*.
- V3 region is considered almost similar to the V2 one but it is not the best choice for *Mycobacterium fortuitum*, *Nocardia* sp., *Propionibacterium acnes* and *Rhodococcus equi*. It seems to be better than the V2 for distinguish *Klebsiella pneumoniae* and *Enterobacter aerogenes*, and the SNP variation among different *Haemophilus* species.
- V6 region is only 58 bp, it is used as the best target region for detecting *Bacillus anthracis* and *Bacillus cereus, Burkholderia mallei, Burkholderia pseudomallei, Coxiella burnetti, Clostridium botulinum, Clostridium perfringens*, *E. coli* O157H7, *Francisella tularensis, Rickettsia prowazekii, Rickettsia rickettsii and Y. pestis.* It is not the best option for the analysis of closely related Enterobacteriaceae *Escherichia* sp., *Shigella* sp. and *Salmonella sp*.
- Other regions: V4, V5, V7 and V8 have higher degree of sequence conservation. The V4 hypervariable region covers a broader range of phyla when compared to other regions of the 16S rRNA gene (Kuczynski et al. 2012). Degenerated primers are used to avoid bias and underrepresentation of some bacterial species such as *Staphylococcus epidermidis* and *Propionibacterium* spp. (Kuczynski et al. 2012; Meisel et al. 2016). The V8 region was used in studies of Arctic bacterioplankton (Comeau et al. 2012).

It is known that only one region of the 16S rRNA gene may not be enough for the classification of bacteria because it does not provide enough information to discern among related bacterial at the species level. So the hypervariable regions can be combined to provide enough sequence diversity for the identification of bacteria

(The Human Project Microbiome 2012) as it is reviewed in Bukin et al. 2019. But the use of different primer sequences (for example V4-V5 or V1-V2 degenerate primers) resulted in differences in the genera and species detected (Fouhy et al. 2016). Some studies were considering the ITS region of the ribosomal operon because it may provide more information for the identification until species level: a first example is a study about genera and species of Cyanobacteria (Boyer et al. 2001). There are also studies of the full length 16S sequences generation for expanding microbial characterization (Schloss et al. 2016; Wagner et al. 2016). Analysis of the whole operon with long amplicons provides a better classification at the species level (Benítez-Páez et al. 2016; Cuscó et al. 2017c).

We will focus on the microbiota analysed with 16S in skin and next-generation sequencing (NGS) as a culture-free method that enables analysis of the entire microbial community within a sample. With the ability to combine many samples in a sequencing run, microbiology researchers can use NGS-based 16S rRNA gene and the ITS region sequencing as a cost-effective technique to identify genera that may not be found using traditional methods.

For the classification of the sequences in bacteria, there are specialized 16S rRNA databases and the frequently used ones are RDP (Maidak et al. 1996), SILVA (Pruesse et al. 2007) and Greengenes (Desantis et al. 2006). Debates about which one should be used as the best for analysis of the sequences are still ongoing. These databases are large, containing mostly environment sequences predicted by Naïve Bayesian Classifier (NCB) for the RDP and by a predicted phylogenetic tree derived from multiple sequence alignments (Edgar 2018). The SILVA database provides a curated taxonomy of bacteria Archaea and Eukaryota for the three-domain life using the small and large subunit rRNA genes (Yilmaz et al. 2014). Greengenes database “offers annotated, chimera-checked, full-length 16S rRNA gene sequences in standard alignment format” (Desantis et al. 2006). In this thesis the databases considered were Greengenes and SILVA.

Greengenes predicts fewer genera respect the correct number of genera in the sample, while SILVA resulted in many false-positive. The problem is due to the

number of sequences included (190.000 in SILVA and 99.000 in Greengenes). The more sequences contained in the database, the higher probability of assigning the wrong classification or not even classify the microbe at all. Also, the species level shows serious classification problems when comparing only some of the hypervariable regions because of missing taxonomic information. We have not to forget the importance of the released version: Greengenes for example is not actualized from 2013 and it means that it lacks many of the last sequences of novel bacteria (Park and Won 2018).

1.3) Mycobiome and its ribosomal operon: 18S-ITS1-5.8S-ITS2-28S

The three-domain system was introduced by Carl Woese to distinguish archaea, bacteria and eukaryote (Woese et al. 1990). Archaea and bacteria are made up entirely of microorganisms, and they are grouped together as Prokaryotes because they lack in membrane-bound nucleus and internal organelles. Archaea and bacteria can be distinguished depending on (i) their capacity to live in extreme environmental conditions thanks to their high adaption skills; (ii) the composition of their cell wall that does not contain peptidoglycan, (iii) their less sensitive condition to antibiotics and at the end (iv) for their rRNA sequences. The Eukarya domain gather all eukaryote organisms with a membrane-bound nucleus as the protista, fungi, plantae, and animalia kingdoms.

Eukaryotes form cooperative colonies such as multi-cellular organisms, and different cells types make their structure. Looking at this kingdom, the fungi group is one of the most diverse of life earth (Hawksworth and Lücking 2017) and it is considered the second largest kingdom of eukaryotic organisms. It is estimated that the range is between 1.5 and 5.1 million species, present an immense diversity of life forms, nutritional strategies, and associations among organisms and can occupy a broad natural or artificial niche. The mycologists have traditionally used the morphology (phenotypic character) of fruit-bodies or pure cultures in agar medium to distinguish among the fungi species. Also they used the spore-producing structures, formed as a result of asexual (mitosis) or sexual (meiosis) reproduction (Raja et al. 2017). The morphology can help to species identification and understand the evolution of the morphological characters, but it may be contentious or problematic, even for trained mycologists. Mycologist may not always provide accurate groupings within an evolutionary framework, and morphological characters can often be misleading due to hybridization, cryptic speciation, and of course evolution. This problem is affecting mainly the recognition of species level; that is why mycologist used to recognize the fungi until family level and not at lower level (Raja et al. 2017).

Due to those difficulties in fungi recognition only by morphology, and due to difficulties in isolation of fungi in culture, molecular methods helped the taxonomical classification.

During the years, other methods for classifying fungi that cannot be cultured were introduced: restriction fragment length polymorphism (RFLP) analysis, oligonucleotide fingerprinting of rRNA genes (OFRG), denaturing gradient gel electrophoresis (DGGE), and in-situ hybridization. These techniques compare fungal diversities, but they lack the specificity necessary to identify the different fungal species in a large-scale study (Underhill and Iliev 2014). The limitations of culture-dependent methods for mycobiome studies finished with the introduction of culture-independent approaches. In early 1990s the introduction of the PCR technique (polymerase chain reaction) was the beginning of a revolution for the detection of fungi. Furthermore, with the development of next-generation sequencing (NGS) platforms, direct sequencing has become more cost-effective than was the case when only classical Sanger sequencing was available (Cui et al. 2013).

Molecular identification through DNA barcoding of fungi has become, during the last 15-20 years, an integrated and essential part of fungal ecology research. It has provided new insights into the diversity and ecology of many fungi (Bellemain et al. 2010). The internal transcribed spacer (ITS) is the preferred DNA barcoding marker for the identification of single taxa. It has recently been proposed as the official primary barcoding marker for fungi (Deliberation of 37 mycologists from 12 countries at the Smithsonian's Conservation and Research Centre, Front Royal, Virginia, May 2007). In 1989, White et al. (1989) described the first PCR amplification for fungi, proposing different sets of primers, to best analyse the taxonomy (White et al. 1989). As in bacteria, the ribosomal operon is also the region chosen in fungi. This operon contains three conserved regions (18S, 5.8S and 28S rRNA genes) and two hypervariable ones (ITS1 and ITS2) as it is showed in Figure 6.

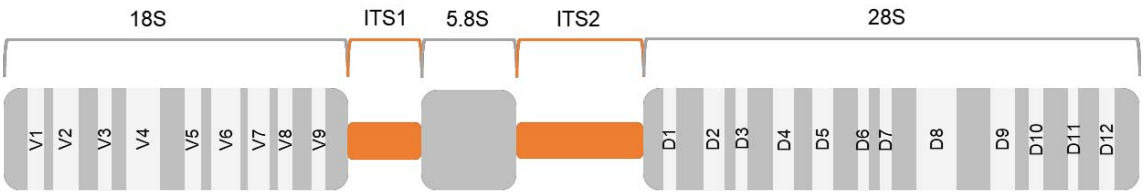

Figure 6. Fungal ribosomal operon: two hypervariable internal transcribed spacers regions (ITS1 and ITS2, marked in orange) and three conserved one (18S, 5.8S and 28S rRNA, marked in grey) that contain variables domains, nine for the 18S and twelve for the 28S rRNA genes.

For fungi and oomycetes, the internal transcribed spacer region in the ribosomal RNA (rRNA) operon has been recognized as the formal DNA barcoding region. The entire ITS region has commonly been targeted with traditional Sanger sequencing approaches and typically ranges between 450 and 700 bp. Its length is of 500 and 600 bp for ascomycetes and basidiomycetes, respectively, and of 600 bp across all fungal lineages (Beeck et al. 2014). Sequences from the hypervariable ITS regions generated by conventional Sanger sequencing are deposited in public databases. Both regions are considered good candidates for the fungal communities' characterization, even if debates on which is the best one for a complete mycobiome study are not solved yet (Bazzicalupo et al. 2012; Blaalid et al. 2013). Comparison between the two ITS regions showed that: ITS is evolving fast, and inside it, the ITS1 is faster than ITS2; the length of the ITS changes among different phyla as Basidiomycota, Chytridiomycota and Zygomycota, Ascomycota and Glomerulomycota (from 260 to 1794 bp) (Yang et al. 2018); taxonomic resolution is not equal and the ITS2 seems more applicable for the richness of fungi in a sample (Yang et al. 2018). indeed, the standardization of the ITS region for sequencing requires further studies.

Due to the high variation and fast evolution of the ITS regions, the analysis should include a part of the conserved region. Inside the conserved subunits there are hypervariable regions, which are important for fungal taxonomy assignment: for example, in species level identification it is recommended to include the 28S region (LSU) because it contain two variable domains (D1 and D2) that help in taxonomic assignment (Petti 2007; Tedersoo et al. 2015). Hoffmann et al. (2014) and Meason-Smith et al. (2015) were studying the fungal population in healthy dogs and during

AD in the skin using both ITS regions to have a high taxonomic resolution of fungal communities in samples (Hoffmann et al. 2014; Meason-smith et al. 2015).
The implication in diseases was primarily studied only for bacteria, while fungi were later discovered to play a role in immunomodulation and to influence inflammatory disease (Seed 2015; Hoggard et al. 2018). The molecular identification of fungi relied on next generation sequencing of rRNA genic regions, and two of them, the 18S rRNA and ITS, were frequently used (Seed 2015). There are many primer sets to amplify different parts of the fungal operon. However, there is not a standard marker to obtain best identification of the fungal communities in different samples (Yang et al. 2018).

Depending on the fungi, The ITS1 or ITS2 region is chosen for its taxonomic identification, even if it could be ambiguous for the results (Brien et al. 2005; Nilsson et al. 2008; Yang et al. 2018). Considering the ITS region, it has a high probability of the correct taxonomic classification of broad lineages, but it cannot always reach the genera level (Badotti et al. 2017; Yang et al. 2018). The advantage of using the ITS region is that most of the fungal species were identified using this genomic region. However, high-throughput technologies have a limitation for the length of the reads and for this reason, either the ITS1 or the ITS2 is used (Badotti et al. 2017).
The specific database for fungal sequences, UNITE (https://unite.ut.ee/), is widely used to correct taxonomic annotation (Badotti et al. 2017). This database was released the first time in 2003, to facilitate the mycological progress in assembling and disassembling metadata of fungi known by the ITS region (Nilsson et al. 2018). For taxonomy classification, the UNITE uses the NCBI Taxonomy classification as taxonomic backbone, supplemented with modifications from Index Fungorum (http://www.indexfungorum.org) and MycoBank (Nilsson et al. 2018). As the efficiency of ITS1 and ITS2 is different, there are different primer sets for their amplification; taxonomic power classification changes depending on the fungus we are looking for, and it is not easy to reach the species level. In this thesis, the whole ribosomal operon amplification was considered. The main reasons were: reducing the classification error at lower taxonomic level, avoid the problem of which could be the best region in complex samples analysis and classification until species level.

1.4) Sequencing: from second (massive parallel sequencing) to third (single-molecule sequencing) generation sequencing

In 1975 Sanger found a method to obtain long fragments of DNA by using a polymerase on radiolabelled nucleotides that were the key to produce fragments longer than before in a polymerization reaction. During the following years the method developed by Sanger was more and more investigated on sequence accuracy and the use of dideoxy chain termination, ending with the introduction of semi-authomated DNA sequencers. This is the “first generation” technology. It sequences DNA fragments (500 - 1000 bp) with high quality, and it was the gold standard between 1975 and 2005 (Besser et al. 2018). In 2005, the pyrosequencing technique was developed, gathering methods that require a DNA polymerase to obtain reads around 400–500 base pairs (bp) long. The Next Generation Sequencing (NGS) was licensed to 454 Life Science. The term of “second-generation sequencing” was introduced about all the methods characterized by the need to prepare amplified sequencing libraries followed by a massive generation of short reads in parallel (massive parallel sequencing) which needed to be assembled by different algorithms (Miyamoto et al. 2014). This is referred to 454 sequencing (used in the 454 Genome Sequencers, Roche Applied Science; Basel), Solexa technology (used in the Illumina (San Diego) Genome Analyzer based on reversible terminator sequencing), the SOLiD platform (Applied Biosystems which became Life Technologies following a merger with Invitrogen; Foster City, USA,), the Polonator (Dover/Harvard) and the HeliScope Single Molecule Sequencer technology (Helicos; Cambridge, MA, USA) and the Ion Torrent, the first “post-light sequencing” technology that used neither fluorescence nor luminescence.

The advantages of the second-generation sequencing were the introduction of new era for a lower cost genome sequencing, the accessibility to many laboratories, the ability to sequence thousands of organisms in parallel (Caporaso et al. 2012) , a vast amount of DNA data output with high quality, and the possibility of sequence in only one run. Disadvantages were more related to “logistical difficulties” such as the operation of the instrument, the high requirement for computer hardware, the generation of fragmented genome assemblies, and the sequencing of the repeated regions. The use of short reads, also could be a problem for (i) the accurate analysis

of repetitive regions or large structural variations, (ii) the highly fragmented sequences that can introduce biases when a reference genome is created by short reads sequences and (iii) the lack in taxonomy reliably at genus and species level in microbiome studies. About their assembly, the datasets could include the biases that occur during library preparation and may exclude some genomic regions from the final sequencing library (Goldstein et al. 2019).
New requirements were needed for the genome analysis such as higher coverage for the assembly of the contigs, the possibility to fix the gaps among them to finally reach the completeness of the genome (Goldstein et al. 2019). In the years, more and more genomes were published in still multiple contigs with a standard set for the quality score (Chain et al. 2009). Since 2015, a large percentage of genomes is still in draft status with an average of 190 contigs, which led to questions about the essential genomic characteristics of the draft genomes such as size, the number of predicted genes, or GC content (Land et al. 2015).

So, a new technology came out as “third-generation sequencing”. This term was discussed and explained by Heather and Chain in 2016 (Heather and Chain 2016). The third-generation sequencing refers to methods that include single-molecule sequencing and real-time sequencing without the requirement for DNA amplification shared by all previous technologies (Heather and Chain 2016; Besser et al. 2018). The expected result was the production of cost-effective longer reads, and the elimination of draft genome concept due to the possibility to create nearly complete genome assemblies of individual microbes directly from the environmental samples without needed of culture methods (Goldstein et al. 2019; Land et al. 2015).

Pacific Bioscience first implemented single-molecule real-time (SMRT) sequencing. This technology allows obtaining long reads (up to 10 Kb), suitable for *de novo* genome assemblies and has significant implications for metagenomic analysis. Starting from 2012, nanopore sequencing from Oxford Nanopore Technologies was coming up. It uses nanopores for the detection and quantification of biological and chemical molecules (Figure 7). Single-stranded RNA or DNA is driven across a lipid bilayer through a large alpha-hemolysin ion channel by electrophoresis. The passage to the channels blocks ion flow and decrease the current for a length of

time, which is proportional to the nucleic acid (Kono and Arakawa 2019). Also, there are no limits for the DNA length sequenced: reads exceeding 1 Mbp have been reported, demonstrating the capabilities of nanopore sequencing extremely long stretches of DNA molecules (Jain et al. 2018; Kono and Arakawa 2019).

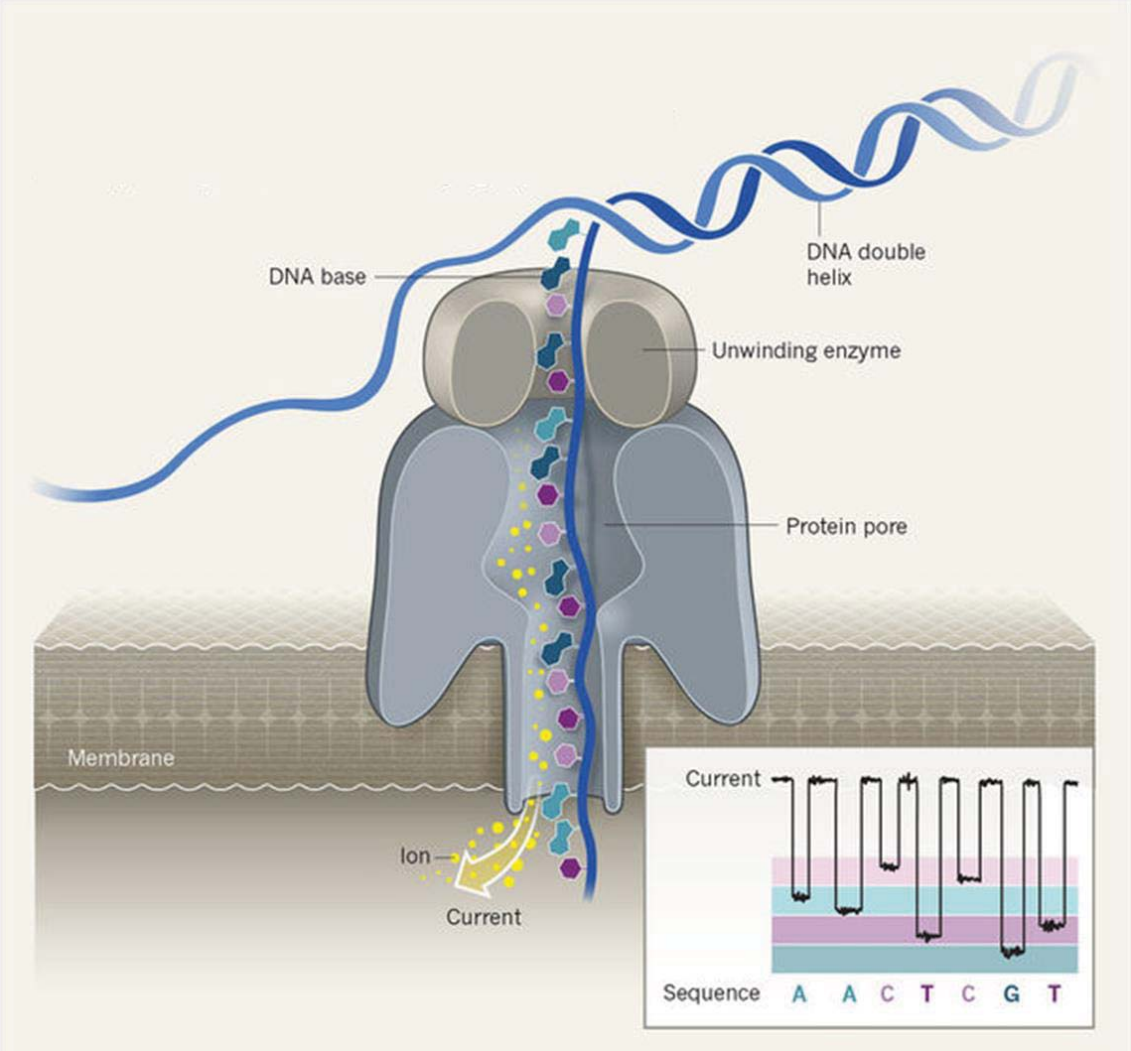

Figure 7. An image of the sequencing pore in MinION device (Eisenstein 2017).

Long reads pave the way to de novo genome assembly with less ambiguity and error, and to close the gaps (Tyler et al. 2018; Kono and Arakawa 2019). The disadvantages, instead, are related to the error rates: 88-94% accuracy in Nanopore, depending on the type of molecules and library preparation methods (99.99% for consensus with high-accuracy basecalling), and 99.8% accuracy for

PacBio with the optimization of circular consensus sequencing (CCS) (Kono and Arakawa 2019; Pearman et al. 2019; Wenger et al. 2019).
The development of new sequencers caused consequentely new requirements for the analysis of the data amount generated by the sequencing. Data of studies are more complex, due to their variety of sources not related to only one gene/genome, or one sequence per genome. They also include thousands of phenotypes or growth conditions and the integration of multiple “omics” data (Land et al. 2015). In the 1990s, the rise of the internet helped to increase the data sharing and analysis by new online programs. The critical change was in the mid-2000s with cloud computing and NGS: data increased and needed a new storage structure (Muir et al. 2016). Computers can store and process 10,000 times as much information than they could when the first bacterial genome was sequenced (Land et al. 2015). Sequencers evolved faster: storing, computing, and visualisation tools or application need updates for performing the best workflow for each type of data.

Data storage systems have increased in capacity and decreased in cost by orders of magnitude as the technology has transitioned from magnetic tapes and disks to distributed cloud storage spanning hundreds or thousands of physical devices. Dramatically reduced storage costs have facilitated and encouraged the collection of massive amounts of data across many scientific disciplines, including those in the life sciences (Land et al. 2015). With reduced costs have come increases in the volume of archived sequence data and joint efforts to develop scalable data models that provide fast, flexible access. GenBank (http://www.ncbi.nlm.nih.gov) is a database created to provide access within the scientific community to DNA sequence information, and it had grown more than 260.000-fold from when it was first built, in 1982 (Benson et al. 2013). The sequences are primarily from individual laboratory submissions or large-scale sequencing projects (Benson et al. 2013). The organization and storage of high-throughput sequencing data related to the introduction of the European Nucleotide Archive (Leinonen et al. 2011a) and The Sequence Read Archive (SRA) (Leinonen et al. 2011b). Built as repositories for sequence reads in 2007, they are about 2000 times as large as GenBank (Land et al. 2015; Muir et al. 2016).

1.5) Long reads and genome assemblies with nanopore technologies: working on *Malassezia pachydermatis*

The first step in any long-read analysis is the basecalling, meaning the conversion from raw data to nucleic acid sequences: the algorithms are quickly evolving, they are available in different versions, and the leading example is Guppy. Following the basecalling there is the correction and polishing of the sequences. The nanopore reads quality is independent of the DNA length. However, it depends on the speed of the nucleic acid through the pore that typically decrease in the late stages of sequencing runs and it affects the quality negatively (Amarasinghe et al. 2020). Protocols to increase the quality were introduced with the sequencing of the complementary strand, generating a more accurate consensus sequence, but are now discontinued. The new generation of pores (R10) increases the accuracy, and four different pore versions were introduced in the last three years: R9.4, R9.4.1, R9.5.1 and R10 with a 12 Guppy releases only in 2019 (Amarasinghe et al. 2020).

The introduction of two groups of methods was developed for the error correction of long reads and successively assembly or other applications: one only for long reads and another that helps the accuracy with the introduction of short-read data. The main strategies for the assembly of bacteria genome using Nanopore technologies regard the capacity of enhancing genome assemblies generated by short-read data and capable of generating an initial genome assembly further polished using MinION or Illumina reads (Goldstein et al. 2019). A total of 62 tools that can carry out the error correction were recently listed by Amarasinghe et al. 2020 while Canu (https://canu.readthedocs.io/en/latest/) was considered the best assembler for short and long reads, but it may be running too long for large genomes. That's why faster assemblers as wtdbg2 (Ruan and Li 2019), Flye (https://github.com/fenderglass/Flye) or Shasta (https://github.com/chanzuckerberg/shasta) were adopted. For the assembly and correction, Nanopolish (https://github.com/jts/nanopolish) is the most popular tool but it is still slow for a large data set. As Nanopore technology is evolving quickly, and all the tools available for data analysis with long reads are listed at long-read-tools.org (Amarasinghe et al. 2020).

With the third generation sequencing and the used of MinION device, analysis about the full-length 16S rRNA were performed as summarized in Cuscó et al 2018: characterization of a mock community, complex microbiota samples from mouse gut, wastewater, algae and dog skin (Cuscó et al. 2017c), characterization of pathogenic agent in clinical (Mitsuhashi et al. 2017). In fungal studies, there are first approaches with MinION device and PacBio from fungal herbarium specimens (Wurzbacher et al. 2018a) and a sequencing of the ITS region compared with the full ribosomal operon for some of the common fungi found in the skin of dogs (D'Andreano et al. 2020, this thesis).

Malassezia species have compact genomes of less than 9 Mb (Triana et al. 2015; Wu et al. 2015) and code for a compact proteome of ~4,000 genes (Wu et al. 2015). Their karyotype ranges from six to nine chromosomes, and this difference depends on the centromeres. These genomic loci ensure that an equal number of chromosomes is passing on to their offspring, but it is not clear how the chromosome number changes have been driven to the evolution of the new species (Sankaranarayanan et al. 2020). A first theory leaded by Sankaranarayanan et al. 2020 proposes that, since nine species had centromeres sharing common features, (for example the richness of adenine and thymine nucleotides) this make it more fragile and occasionally may cause a break and the loss of the chromosomes, resulting then in new species. The 18 accepted species of *Malassezia* are divided in three clades (Lorch et al. 2018; Sankaranarayanan et al. 2020):

- Clade A includes *M. furfur, M. yamatoensis*, *M. obtusa* and *M. japonica*. Also, the recently found *M. vespertilionis* species is a new cluster of this group.
- Clade B includes common inhabitants of human skin that are phylogenetically clustered into two subgroups. The B1 contains *M. globosa* and *M. restricta* while the B2 contains *M. sympodialis*, *M. dermatis*, *M. caprae*, *M. equina*, *M. nana* and *M. pachydermatis*.
- Clade C includes *M. slooffiae* and *M. cuniculi*.

The *M. pachydermatis* species has 6 chromosomes, with molecular sizes estimated of 800 Kbp, 1250 Kbp, 1450 Kbp, 1500 Kbp, 1850 Kbp and 1900 Kbp for 99 out of 104 isolates (Kiuchi et al. 1992). Genome assembly sizes range from 7.2 Mbp for *M. restricta* to 9.0 Mbp for *M. globosa*, with 8.2 Mpb for *M. pachydermatis*.

As described before, *M. pachydermatis* is a commensal in the skin of dogs, but it can be also associated with some diseases. Whole Genome Sequencing (WGS) is a powerful approach to study the genomic background and reveal the genomic features of yeast pathogens. Up to date, there are only two assembled genomes for *M. pachydermatis,* and both correspond to the collection strain CBS 1879: GCF_001278385.1 (Triana et al. 2015) and GCA_001264975.1 (Wu et al. 2015). Both genome assemblies were obtained with short reads. There is a need to gather genomic information from Malassezia isolates from clinical samples and associated with skin disorders, to compare with the Malassezia communities occurring on humans. Thanks to the Nanopore technologies, high-accuracy bioinformatics tools for data analysis and fast updates, we started a first approach for the analysis of *M. pachydermatis* whole genome with long reads to improve the assembly.

1.6) Considerations for a microbiome sudy design: the need of positive and negative controls

Multiple potential standard protocols exist for microbiome studies. Each step, from sampling to data analysis, can introduce biases: type of samples, collection methods, environment, amplification by PCR, sequencing, database and bioinformatics tools for the analysis of the data are examples of the biases introduction.

The sampling methods depend on the sample type and how they are collected (Pollock et al. 2018). It is essential to underline the storage step because it is not often possible to extract the DNA from the fresh samples: rapid freezing to -80 degrees or using a validated preserving solutions is the best method to preserve the integrity of the sample and reduce the possible changes in the microbes communities.

Different extraction kits are available to obtain the best quantity and quality of extracted DNA after rigorous testing. On the other hand, positive or negative controls from the sampling and the DNA extraction steps must be considered. The lack of controls in microbiome research has been overlooked for a long time: their presence helps to ensure that the procedures were correctly performed, and avoid false positive or false negative results (Hornung et al. 2019). Of the 265 publications that report a high-throughput community sequencing such as 16S, metagenomics, 18S, ITS, PacBio or Nanopore, 30% of them showed the use of negative controls and only 10% a positive control (Hornung et al. 2019).

The positive controls were not used in microbiome studies at the beginning because they were unavailable as validated standards. Depending on the microbiome investigation, it is possible to find a variety of them containing only bacteria; or together with fungi (Hornung et al. 2019). Even when the manufacturers took care to select the broad representative species, the standard controls need to be evaluated for each case study. New bacterial or fungal species are discovered, there is not enough information about their physiology or resistance to the current DNA extraction methods, so it is difficult to find a positive control that includes all the genera or species that we want to investigate in a sample. In case that the standard

controls are not suitable for the study, a custom designed positive control might be needed (Hornung et al. 2019).
About the negative controls, they should be included at the sampling step: it exists the possibility that either the researcher, the sampling equipment, or the environment could contaminate the samples. So, including an appropriate negative control is not so easy. When a large number of samples are processed, we cannot assume that the potential contamination will be equally distributed in all of them (Hornung et al. 2019). One of the reasons to include a negative control is the discovery the “kitome” or rests of bacterial DNA that comes from the kit. Various extraction kits contain their unique microbiome (Bhatt et al. 2013; Salter et al. 2014; Hornung et al. 2019) and studies about it have recognized specific organisms as a common contaminant from the kit.

If the approach of the study includes a PCR amplification, this step can also introduce bias for an accurate determination of the microbes’ communities. DNA fragments with a high or low GC content are not amplified in the same rate as fragments with an average GC content (Hornung et al. 2019). The positive control during the amplification helps to distinguish the amplification bias that belongs to the DNA extraction step.

Another significant bias is due to the target chosen for the amplification. Since the 16S rRNA gene cannot be entirely sequenced with second-generation sequencing platforms, a small region or a combination of two or three is chosen for the amplification. The choice of the hypervariable region depends on its taxonomic skill, on the study, and the bacteria we expect. The design of universal primers sets on the conserved regions helped for the amplification step. However, it also affects the phylogenetic resolution, because some of them are not amplifying the relevant bacteria, or it is not possible to achieve the genus or species level (e.g., when they are too closely related such as *Escherichia* and *Shigella*). So, there is not a “truly universal set” (Pollock et al. 2018).
The varying amounts of DNA for the amplification can lead to sequencing errors. It is also crucial to choose a high-fidelity polymerase and keep the lower number of PCR cycles needed to avoid chimeras. There are different biases introduced during

the sequencing, as the batch-effect or de sequencing depth. Sequencing too many samples result in low coverage, and the diversity of the microbes can be underrepresented. A mock community allows quantification of sequencing errors, library preparation, and sampling(Park and Won 2018; Pollock et al. 2018).

Commonly the analysis of short fragments is done with Illumina or Ion Torrent platforms, and the choice depends on the cost, the error rate generated from the sequencing, and on the purpose of the study. The new technologies that sequence longer fragments or the full genome are compelling, but the error rate is the main problem. The bioinformatics processing of the sequencing data also contributes to the issue of accurately determining community composition. The use of the positive controls may help the researcher in taxonomic assignment, but the public databases contain various errors that include contaminations, and sequences with incorrectly assigned taxonomy or names. The negative controls might contain the same profile of sample after sequencing. So, it would be easier to look into the amount of reads obtained to distinguish when there is a contamination or not (Pollock et al. 2018).

To sum up then, the study of the microbial communities allows to understand the diversity in each biological system and the impact of the changes in different conditions on the host and in the environment. It is not possible to eliminate the biases for each step of a microbiome study, but a good experiment design help to reduce them.

1.7) Objectives

The goals of this thesis were:

- Characterization of the dogs' skin microbiome and mycobiome in health and disease status (otitis externa), and longitudinal evaluation after antibiotic and non-antibiotic treatment.
- Identification of fungi at the species level by the development and optimization of a sequencing assay based on long-fragments to capture the maximum variability and taxonomical information from the whole fungal ribosomal operon.
- Whole genome sequencing and *de novo* assembly of *Malassezia pachydermatis* isolated from a dog with otitis using nanopore long reads.

2. MATERIALS AND METHODS

Thanks to the collaboration with LETI laboratories (LETI Animal Health) and the Department of Pharmacology, Therapeutics and Toxicology, of the Veterinary School at *Universitat Autònoma de Barcelona*, we aimed to collect skin swab samples from healthy ears and ears with otitis from different animals; also, they kindly provide microbiological fungal cultures. We characterize the bacterial and fungal communities of the ear skin in dogs with external otitis, and we performed workflows for data analysis of short (400 bp) and long DNA fragments (3.500 and 6.000 bp) to compare between second and third generation sequencing skills for taxonomy analysis

2.1) Skin samples from dogs

Fifteen dogs with a clinical diagnosis of non-purulent or non/low ceruminous otitis externa, and visible tympanic membrane to determine its non-damaged status were enrolled. Other requirements were the absence of antibiotics, anti-inflammatory or immunosuppressive drugs for at least one month before sample collection, or ear canal cleanser for at least seven days before sample collection. Weight, age, breed (with pendant ears), sex, and other relevant data were recorded.
Sample collection was done every 15 days, from May 2017 until February 2018, for a total of five time-point collection: T_0, T_{15}, T_{30}, T_{45} and T_{60}. The sample were collected by Catch-All^{TM} swabs, poured in physiological serum and then rubbed for 30 seconds in the internal side of the ear. After this, the swabs were stored at 4 °C until DNA extraction, within the following 48h.

Dogs were divided in two groups depending on the treatment applied: treatment A (prednisolone (5 mg/ml) + pomegranate otic cleanser) and treatment B

(prednisolone (5 mg/ml) + gentamycin sulphate (10 mg/ml) + ketoconazole (10 mg/ml) + saline otic cleanser). At T_0, microbiological cultures of otitis-affected samples were performed to detect the possible causal agent of the disease. Then, treatment A or B was applied for 15 days (T_{15}) to each group, and from days 15 to 30 (T_{30}) the dogs received only the ear cleanser (pomegranate or placebo). During sample collection, three dogs were excluded due to secondary otitis caused by parasitic, endocrine, or autoimmune diseases, which needed to be treated with other therapies. No treatment was applied from T_{30} to T_{45} and from T_{45} to T_{60}.
Pomegranate is a polyphenol-rich fruit, and in the past decade, it was tested for its antimicrobial activity: it was used as a treatment for diarrhea, helminthiasis and respiratory pathology. It was also identified also as an antimicrobial compound against *Staphylococcus epidermidis* that, together with *S. aureus*, is high resistant to antibiotics (Betanzos-Cabrera et al. 2015).

Four canine otitis samples were further analysed with nanopore sequencing. Two were collected from a Petri dish, divided in two halves parts to culture fungi from the ear of an otitis affected dog. The other two samples were collected by swabbing the inner pinna of the affected ear, from two different dogs, using Sterile Catch-All™ Sample Collection.

2.2) Microbiological fungal cultures

LETI laboratories (LETI Animal Health) kindly provided a total of eight microbiological fungal cultures in petri dish; four cultures were classified until genus level, that included *Alternaria* spp., *Aspergillus* spp., *Candida* spp. and *Malassezia* spp. Four other microbiological cultures were classified until species level: three of *Malassezia pachydermatis* and one of *Microsporum canis.*

The ZymoBIOMICS™ Microbial community DNA standard containing eight bacteria species (*Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella enterica*, *Lactobacillus fermentum*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Listeria monocytogenes* and *Bacillus subtilis*) and two fungal ones (*Saccharomyces cerevisiae* and *Cryptococcus neoformans*) was considered as fungal control for the sequencing step.

2.3) DNA extraction for bacteria and fungi

The bacterial and fungal DNA for the otitis study was extracted from swabs using the MoBIO-PowerSoil® DNA isolation Kit and the QIAGEN-DNA easy PowerSoil Kit. A swab without sample was included as a blank sample in each extraction batch to control for the possible contamination of the kit reagents. DNA quality control was checked by Nanodrop, Agilent Bioanalyser 2100 and Qubit™ Fluorometer (Life Technologies, Carlsbad, CA) for each sample.

The ZymoBIOMICS™ Miniprep kit was used to extract the DNA from all the fungal microbial cultures and the mock community. DNA quality control was checked by Nanodrop and Qubit™ Fluorometer (Life Technologies, Carlsbad, CA).

2.4) PCR and massive sequencing: pilot test for canine otitis samples

We performed a first pilot test to compare taxonomic results obtained for bacteria with short reads, using two different sets of primers targeting different hypervariable regions of the 16S rRNA gene: V1-V2 and V4.

Similarly, ITS1 and ITS2 from the fungal ribosomal operon were evaluated for the taxonomical discrimination power of each region on the microbiological fungal cultures.

- V1-V2 and V4 regions of 16S. The bacterial hypervariable region V1-V2 was amplified by pair F27 (5´-AGAGTTTGATCCTGGCTCAG-3´) and R338 (5´-TGCTGCCTCCCGTAGGAGT-3´), while the V4 was amplified with primer pair F_515 (5'- GTGYCAGCMGCCGCGGTAA-3') and R_806rB (5'-GGACTACNVGGGTWTCTAAT-3'). Primers included sequencing adaptors at the 5′ end and forward primers were tagged with different barcodes to multiplex samples in the sequencing run. The PCR mixtures (25 µl) contained 2.5 µl of DNA template, 5 µl of 5x Phusion Buffer High Fidelity, 0.5 µM of each primer, and 0.02 U/ µl of Phusion Hot Start II High-Fidelity (Thermo Fisher Scientific). The PCR thermal profile was 30s at 98 °C, followed by 30 cycles of 15s at 98 °C, 15s at 55 °C, 20s at 72 °C, and a final extension of 7 min at 72 °C.
- Internal transcribed spacer regions (ITS1 and ITS2). The primers sets tested were three: one for the ITS1 region, and two for the ITS2 because of two possible primers reverse that were both considered good candidates from the literature.

  The first set for the ITS1 amplified with primer pair 18S_F (5'-GTAAAAGTCGTAACAAGGTTTC-3') and 5.8S_1R (5'-GTTCAAAGAYTCGATGATTCAC-3'). The sets for the ITS2 included the same forward primer combined with two possible reverse: forward F_ITS3 (5'-GCATCGATGAAGAACGCAGC-3') with reverse R_ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), and F_ITS3 with the reverse 28S1 (5'-ATGCTTAAGTTCAGCGGGTA-3'). Gradient PCR conditions were applied to determine the best thermal profile for amplification. At the end the profile chosen was a mix of total 25 µl, and contained 2.5 µl of DNA template, 5 µl

of 5x Phusion Buffer High Fidelity, 0.5 µM of each primer, and 0.02 U/ µl of Phusion Hot Start II High-Fidelity (Thermo Fisher Scientific). The PCR thermal profile was 30s at 98 °C, followed by 34 cycles of 15s at 98 °C, 15s at 57 °C, 20s at 72 °C, and a final extension of 7 min at 72 °C.

After PCR amplification, the quality and quantity of bacterial and fungal amplicons were verified by Agilent Bioanalyser 2100 and Qubit Fluorometer. Sequencing was done at CRAG (Centre for Research in Agricultural Genomics) using Ion chip 318™ and Ion Torrent PGM platform (Thermofisher) according with the manufacturer's instruction. This platform has different chip types, and they are chosen based on the number of samples and study requirement (Figure 8).

Chip Type	314	316	318
Wells per chip	1.262.528	6.348.216	11.302.473
Number of reads per chip	400/550 thousand	2/3 M	4/5.5 M
Output	30/100 Mb	300 Mb /1.0 Gb	600 Mb /2.0 Gb
Run Time	2-4 hours	3-5 hours	4-7 hours

Figure 8. Ion Torrent PGM platform chip types with the main characteristics (www.thermofisher.com).

Each 318™ chip contains 11 million of wells, and the reads output is around 4/5.5 million for 400-base sequencing: it is possible to load 96 barcodes at the same time and for our analysis a maximum of 32 samples were loaded per chip, as suggested from the CRAG. A total of 8 Ion chip 318™ were used, four for bacterial amplicons, and four for the fungal ones.

2.5) Bioinformatics analysis: QIIME2 workflow for the pilot test

For data analysis of short fragments, we used QIIME2 software, Quantitative Insights Into Microbial Ecology (https://qiime2.org). Like many others, this software, is releasing new versions to integrate new skills for taxonomy assignment and diversity analysis of microorganism communities. In the new version released in 2018, QIIME2, new workflows and options were included. Most of the analysis are for short fragments sequenced by Illumina technology, but they can be adapted to Ion Torrent sequencer. The first test was to prepare sequences to be processed in QIIME2. Following the tutorial, we tested the quality of the sequences, sample metadata, generation of taxonomy plots and alpha/beta diversity were tested.

DADA2 and Deblur pipelines were the most common methods used for the Illumina quality control and suggested in QIIME2 analysis:

- DADA2 (Callahan et al. 2016), is a pipeline option to correct sequence data and filter for possible chimeras that may be generated during sequencing process. It generates a parametric error model that is successively used to correct the sequence errors into amplicon sequence variants (ASVs). That is an advantage model because every run has its unique error model.
- Deblur (Amir et al. 2017), is another pipeline option that does a quality filtering process depending on a quality score given, and its works similarly as DADA2.

These pipelines were introduced and tested in QIIME2 on Illumina sequences, but they can be applied to Ion Torrent data too, even if a specific tutorial is still not available. Looking at discussion forums for next generation sequencing such as Biostars (https://www.biostars.org/) or SEQanswers (http://seqanswers.com/), DADA2 was suggested as the “best one” for analysis of Ion Torrent data while Deblur was still not evaluated. To test the skills of both, we applied them on the same data set to investigate on the taxonomy output.

Two of the most common databases used for bacterial communities’ assignment were compared: Greengenes and SILVA. For the fungal taxonomy assignment, the UNITE database (https://unite.ut.ee/) is the most common and complete one for analysis of short fragment (Nilsson et al. 2018).

2.6) Final workflow: PCR condition, sequencing and data analysis

The selection of bacterial and fungal regions to amplify, quality control pipeline, and database to analyse samples of otitis externa was based on the results obtained from the pilot test, as shown in Figure 9.

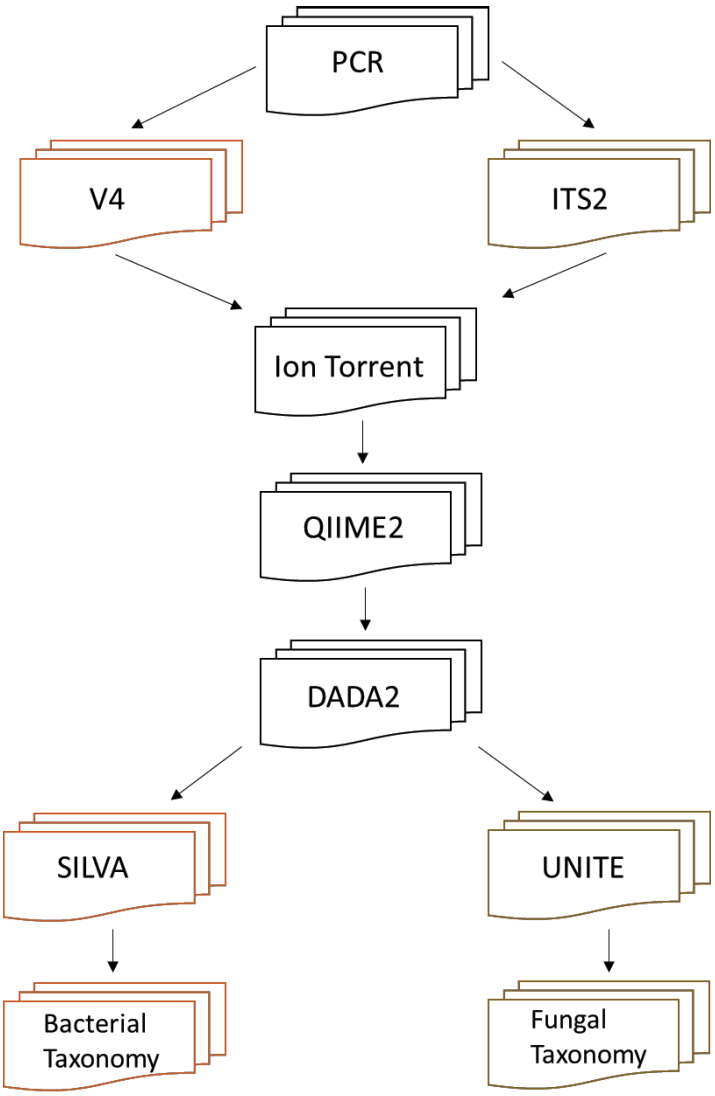

Figure 9. Final workflow followed for all otitis samples analysis. The hypervariable ribosomal regions amplified are V4 for bacterial 16S rRNA gene, and ITS2 for fungal operon (with 28S1 reverse primer). DADA2 is the pipeline selected for quality control, while SILVA and UNITE the databases for bacterial and fungal analysis respectively.

The final PCRs profiles were: mixtures (25 µl) that contained 2.5 µl of DNA template, 5 µl of 5x Phusion Buffer High Fidelity, 0.5 µM of each primer, and 0.02 U/ µl of

Phusion Hot Start II High-Fidelity (Thermo Fisher Scientific). The PCR thermal profile was 30s at 98 °C, followed by (i) 30 cycles of 15s at 98 °C, 15s at 55 °C, 20s at 72 °C, and a final extension of 7 min at 72 °C for 16S-V4 and (ii) 34 cycles of 15s at 98 °C, 15s at 57 °C, 20s at 72 °C, and a final extension of 7 min at 72 °C for ITS2. Primers forward contain the adapter linker, the Key, the barcode that is different per each sample, the spacer, and the conserved bacterial or fungal forward primer, while the Reverse primer contains the adapter linker and the bacterial or fungal reverse primer. We designed 96 barcodes for the forward primer, and we checked their CG content, the annealing temperature, and the secondary structure to select 32 of them.

The quality and quantity of the amplicons were verified by Agilent Bioanalyser 2100 and Qubit Fluorometer. Not all samples amplified for bacterial and fungal PCR, so those with low DNA quantity were excluded from the study. A total of 8 Ion chip 318^{TM} (four for bacterial and four for fungal amplicons) were sequenced by Ion torrent PGM platform following the manufacturer's instruction: a maximum of 32 samples was loaded in each chip.

Reads obtained from sequencing process were demultiplexed and separated by barcode. Quantitative Insight Into Microbial Ecology 2 (QIIME 2, https://qiime2.org) was used for bioinformatics analysis. Within the pipeline, DADA2 (Callahan et al. 2016) was used as quality filtering method to denoise and dereplicate single-end sequences, and to remove chimeras.

For bacterial data, sequences were filtered at a length of 255 bp, the primer sequences were trimmed, and the resulting sequences were classified into amplicon sequence variants (ASV). So, the ASVs were used to classify and assign taxonomy by SILVA v128 database at 99% identity, to reduce redundancy. Chloroplast sequences were excluded from the study.

For fungal data, we proceeded using the same steps but with a change in filtered steps and database used: no length filter was applied to sequences due to different size of fungal amplicons. For taxonomy assignment we used UNITE v8 18.11.2018 database at 99% identity to reduce redundancy.

Taxonomy analysis was performed at different taxonomic levels, and alpha diversity and beta diversity were calculated to analyse differences between and among samples. The alpha diversity shows differences within a sample: Shannon index (evenness or the relative abundance of features) and observed ASVs index (richness or the total number of features) were considered for this study. Finally, we performed the beta diversity, which assesses similarities among samples of the same group, using the weighted UniFrac distance matrix (Lozupone et al. 2011), which considers the phylogeny, the abundances and the composition of the microbial community (either bacterial or fungal).

To assess which taxa was significantly different among time points within each treatment, we used a Wilcoxon test corrected with false discovery rate (FDR), considering paired samples. To assess statistical significance of both alpha and beta diversity, we applied the q2-longitudinal plugin of QIIME2, which includes bioinformatics tools for paired and longitudinal microbiome analyses (Bokulich et al. 2018). We tested the alpha diversity statistical significance using a non-parametric test (Wilcoxon signed-rank test) with FDR correction. For beta diversity, we assessed the statistical significance of clustering by time-point within a treatment for the first three principal coordinates using the longitudinal pairwise differences of the q2-longitudinal plugin of QIIME2

The taxonomy assignment for some of the most abundant bacterial families like *Staphylococcus* spp., *Corynebacterium* spp. and *Proteobacterium* spp. resulted in “undetermined” at species level.

Some of the samples at T_0 and T_{30} were PCR amplified with *Staphylococcus* specific primers. A PCR was done for 20 samples: the final volume per sample was 25 µl, in which 1 ul of the DNA template, 5 µl of 1X Phusion® High Fidelity Buffer, 1 µl of primer mix sets (10 uM), 2.5 µl of dNTPs (0.2 mM) and 0.25 µl (0.02 U/µl) of Phusion® Hot Start II Taq Polymerase. PCR profile: initial denaturation of 30 s at 98 °C, 40 cycles of 10 s at 98 °C, 30 s at 56 °C, 30 s at 72 °C and final step of 7 min at 72 °C. The amplicons were analysed by agarose gel. The expected band lengths were 359 bp for *S. aureus*, 430 bp for *S. intermedius* and 926 for *S. pseudintermedius*.

2.7) Third generation sequencing: fungal ribosomal operon

In fungi, sequencing of short fragments may cause misclassification in taxonomy assignment due to database and current taxonomy names classification. As a second objective, we wonder whether accuracy improves, and misclassification reduces, by sequencing the whole ribosomal operon. We intend to sequence partial (≈3.500 bp) and the whole (≈ 6.000bp) fungal operon (18S-ITS1-5.8S-ITS2-28S). As this is new and a "world to discover", many applications can be applied for data analysis. So, we started with a mock community and then with complex samples as a first approach for long-read sequencing.

Two set of primers were chosen (Table 2): the first set can amplify the operon from V3 region of 18S rRNA gene to D3 region of 28S rRNA gene (≈3.500 bp), while the second one amplifies the complete ribosomal operon from V1 region of 18S rRNA gene to D12 region of 28S rRNA gene (≈6,000 bp). The primers included the Nanopore Universal Tag.

Table 2. Primers targeting the full ITS region (3.5 Kb) and the full fungal operon (6 Kb). The Nanopore Universal Tag is shown in bold type.

NAME	SEQUENCE (5'-3')	TARGET	AMPLICON	REFERENCE
SSU515Fngs-F	**TTTCTGTTGGTGCTGATATTGC** GCCAGCAACCGCGGTAA	18S-V3	3.5 Kb	(Tedersoo et al. 2015)
LR5-R	**ACTTGCCTGTCGCTCTATCTTC** TCCTGAGGGAAACTTCG	28S-D3	3.5 Kb	(Tedersoo et al. 2015)
SR1R-Fw	**TTTCTGTTGGTGCTGATATTGC** TACCTGGTTGATQCTGCCAGT	18S-V1	6 Kb	(Vilgalys lab 1992)
LR12-R	**ACTTGCCTGTCGCTCTATCTTC** GACTTAGAGGCGTTCAG	28S-D12	6 Kb	(Vilgalys lab 1992)

Two PCR were performed: the first for the amplification of the fragment, and the second one to add the specific barcode to each sample. PCR final volume was 50 µl and contained: 5 ng DNA, 10 µl of Phusion® High Fidelity Buffer (5x), 5 µl of dNTPs (2 mM), 0.5 µM of primer forward and reverse, and 0.02 U/µl of Phusion®

Hot Start II Taq Polymerase (Thermo Scientific). PCR profile: initial denaturation of 30 s at 98 °C, 25 cycles of 10 s at 98 °C, 30 s at 62 °C, 80 s at 72 °C, and a final extension of 10 min at 72 °C. Amplicons obtained were purified with Agencourt AMPure XP beads, at 0.4X ratio for the fungal amplicon; then, they were quantified by Qubit™ fluorometer (Life Technologies, Carlsbad, CA).

Following the 1D PCR barcoding amplicons (SQK-LSK109) protocol, 0.5 nM per each sample were required for the second PCR, in which barcodes of PCR barcoding kit (EXP-PBC001) will be added. The final volume of second PCR is 100 µl, in which 20 of DNA template at 0.5 nM, 2 µl of specific barcode and 78 µl of mixture that includes: 20 µl of 5 × Phusion® High Fidelity Buffer, 10 µl of dNTPs (2 mM) and 2 U/µl of Phusion® Hot Start II Taq Polymerase. PCR profile: initial denaturation of 30 s at 98 °C, 15 cycles of 10 s at 98 °C, 30 s at 62 °C, 80 at 72 °C and final step of 10 min at 72 °C. The amplicon obtained were purified again with Agencourt AMPure XP beads, at 0.4X ratio and quantified by Qubit™ fluorometer (Life Technologies, Carlsbad, CA).

We proceeded then to the library preparation for nanopore sequencing, following the manufacturer conditions of 1D PCR barcoding amplicons (SQK-LSK109). protocol. Barcoded samples (1.5 ng) were pooled in 47 µl of nuclease-free water and mixture of 60 µl final volume was prepared.

With a final step of Agencourt AMPure XP beads 0.4X, the DNA library was cleaned and ready to be loaded into the flow cell. We used two SpotON Flow Cells (FLO-MIN106) for three MinION runs, primed with a mixture of sequencing buffer and Flush buffer according to the manufacturer’s instructions. Quality control of sequencing pores was done before each run. The library was mixed with Sequencing Buffer and Loading Beads in a final volume of 75 µl. The final mix was added, by dropping, in the SpotON sample port.

Sequencing runs were between 16h and 19h, using the MinKNOWN 2.2 v18.07.2 and the MinKNOWN v18.12.9.

2.8) Bioinformatics and data analysis for nanopore long amplicons

The analysis of fragments started in April 2018 with the analysis of the 3.5 Kb bp amplicon size and ended in April 2019 with the amplification of the 6 Kb one. The first analysis for 3.5 Kb generated fast5 that were basecalled and demultiplexed by Albacore v2.3.3. we used Porechop (https://github.com/rrwick/Porechop) to remove barcodes and adapters from our sequences. For the taxonomy assignment, we applied EPI2ME v2.55.1578626 platform for cloud-based analysis.

We applied *What's in my pot* (WIMP) application, based in Centrifuge software (https://ccb.jhu.edu/software/centrifuge/manual.shtml) to analyse the long amplicons data. The analysis for the 6 Kb amplicons generated fast5 that were basecalled and demultiplexed by Guppy 2.3.5. Then, Porechop was applied to remove barcodes and adapters. Again, we used EPI2ME v2.59.1896509 and WIMP to analyse the taxonomy of each samples.

2.9) Whole Genome Sequencing of *Malassezia pachydermatis*

LETI Animal Health kindly provided a *Malassezia pachydermatis* isolate after microbiological culture of a swab sample collected from a dog with otitis in Barcelona (Spain). After the DNA extraction the quality and quantity were checked with Nanodrop and Qubit fluorometer (Invitrogen). The library for Nanopore sequencing included two samples, *M. pachydermatis* and another fungal spp, and it was prepared by ligation using the 1D Native barcoding genomic DNA (EXP-NBD103 and SQK-LSK109; Oxford Nanopore Technologies), according to the manufacturer's instructions. The starting material was 1 µg of genomic DNA in 48 µl of nuclease free water. With a final step of Agencourt AMPure XP beads 0.4X, the DNA library was cleaned and ready to be loaded into the flow cell (recovery ≈430 ng). A SpotON Flow Cell (FLO-MIN106 v9.4), primed with a mixture of sequencing buffer and Flush buffer was used according to the manufacturer's instructions.

Quality control of sequencing pores was done before the run. The library was mixed with Sequencing Buffer and Loading Beads in a final volume of 75 µl. The final mix was added, by dropping, in the SpotON sample port. The sequencing run was around 21h, using the MinKNOWN v18.12.9 software.

The fast5 files generated were basecalled with Guppy v2.3.7 including the recommended flip-flop algorithm for improved accuracy of the data. The fastq files obtained were demultiplexed with Deepbinner (Wick et al. 2018), considering only the one of *M. pachydermatis* for the trimming. Results were first analysed with EPI2ME v2.59.1896509 plus *What's in my pot (WIMP)* to confirm the species: 233.185 reads were classified as *M. pachydermatis* with an identity of 99.8%.
We further assembled the genome *de novo* with Flye 2.6 (Kolmogorov et al. 2019), and corrected the reads with racon 1.4.10 https://github.com/lbcb-sci/racon (Vaser et al. 2017) and Medaka 0.11.4 (https://github.com/nanoporetech/medaka). The quantitative assessment of genome assembly and completeness was performed with BUSCO v4.0.1 (Waterhouse et al. 2017). We used Bandage v0.8.1 (Wick et al. 2015) for contigs visualization and genome comparison with the CBS 1879 *M. pachydermatis* reference genome (Triana et al. 2015).

3. RESULTS

3.1) Pilot test for canine otitis samples

For the pilot test of bacteria, we analysed ten samples from two dogs affected by otitis. We compared the taxonomic results with QIIME2 after amplification and sequencing of the V1-V2 and the V4hypervariable regions of the 16S rRNA gene. We compared two pipelines, DADA2 and Deblur, and also two databases, SILVA and Greengenes to analyse the taxonomy. Deblur results showed lower skills in taxonomy assignment respect of DADA2, when comparing the databases and the 16S rRNA gene regions. We chose the V4 region and SILVA database with DADA2 as the most suitable for microbiome analysis due to a better capture of the genus level in bacteria. Many differences between SILVA and Greengenes databases are on the debate. SILVA is the largest between the two 16S taxonomy databases, and it shares the most taxonomic units with the NCBI (Huson 2017).

For the fungi pilot test, we analysed the ITS1 region on one sample of a dog with otitis. The same sample was analysed for the ITS2 region, but it was a double test: the ITS2 region showed two good reverse primer candidates, so both were tested. Once the results for the ITS2 region were analysed and the primer reverse 28S1 was considered the best for fungal analysis in complex samples, the two ITS regions were finally compared. Results showed in Figure 10 found that ITS2 presented better resolution than ITS1 for working with mycobiome complex samples.

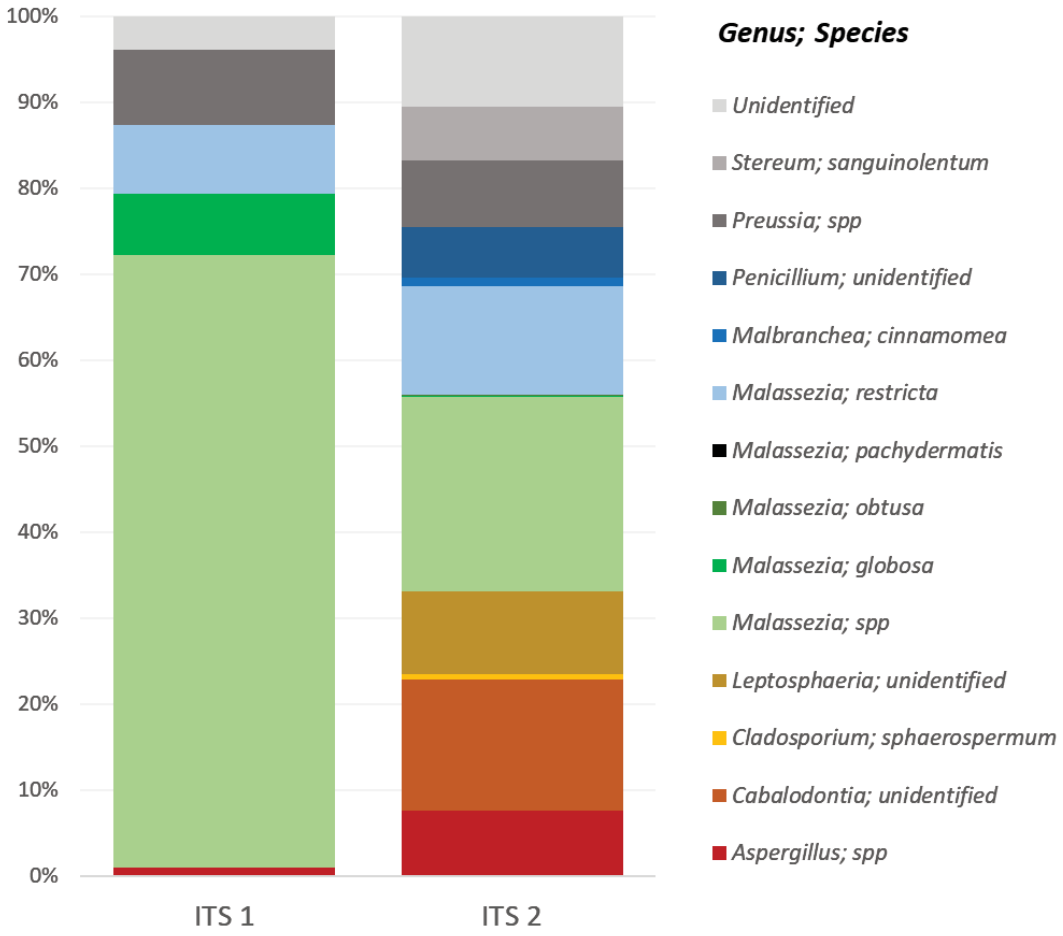

Figure 10. Comparison of taxonomical skills between the two ITS regions for fungal taxonomic classification.

3.2) Bacterial and fungal changes during canine otitis externa

Thanks to the pilot test the final workflows for the analysis of bacterial and fungal microbiota were established. For bacteria, we amplified the V4 region and analysed it with QIIME2 and the DADA2 pipeline combined with the SILVA database; for the fungi, the ITS2 was amplified and analysed by QIIME2 and pipeline of DADA2 combined with UNITE database.

All dogs included in the study showed one or both ears affected by otitis externa with erythema, oedema, and/or non-purulent exudate. Cytology was carried out and microbiological culture was performed in both ears of each dog at T_0 (30 samples) independently of whether or not they presented clinical signs. Microbiological cultures were positive for 21 of the samples (70%) and negative for 9 of them (30%) as it is showed in Figure 11. From the positive cultures, ten samples evidenced the growth of *Malassezia* spp. only; seven samples were positive for *Malassezia* plus *Staphylococcus* and/or *Corynebacterium*; two samples -from the same dog- were positive for *Pseudomonas* and one also showed *Malassezia* spp.; and one sample for *Staphylococcus*. From the nine negative cultures, only the samples from ears with clinical signs and a complete sample collection at each time-point were included in the study. The remaining ones presented no clinical signs of otitis and were consequently excluded from the study (Materials ad Methods 2.1).

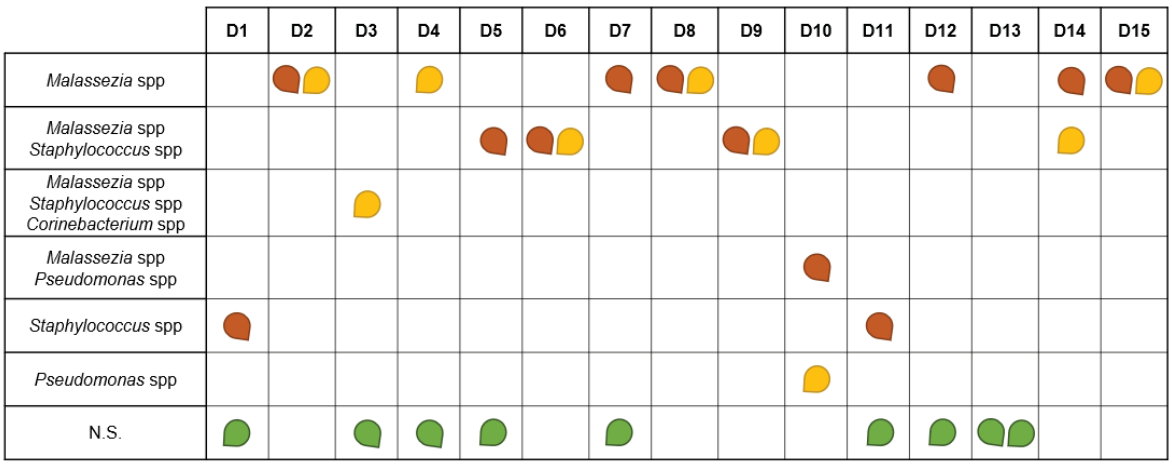

Figure 11. Detailed results from the microbiological cultures at T_0. Different colours were used to distinguish the left (brown) and right (yellow) ear of each dogs. The "N.S." (not specified) indicates the microbiological cultures from right and left ears that showed a negative result and they were marked in green.

Bacterial and fungal communities from each affected ear were investigated by amplification and massive sequencing of the bacterial V4 region of the bacterial 16S rRNA gene and the ITS2 region of the 18S-28S rRNA fungal operon. Taxonomic changes in the microbiota and mycobiota profiles were analysed at three time points (T_0 - enrolment; T_{15} – pomegranate or antibiotic treatment; T_{30} – pomegranate or saline otic cleanser), to assess the effects of both treatments. The taxonomic profiles obtained at time of enrolment (T_0) and after the treatments (T_{15} and T_{30}) are shown for bacteria and fungi (Figure 12 and Figure 13), respectively.

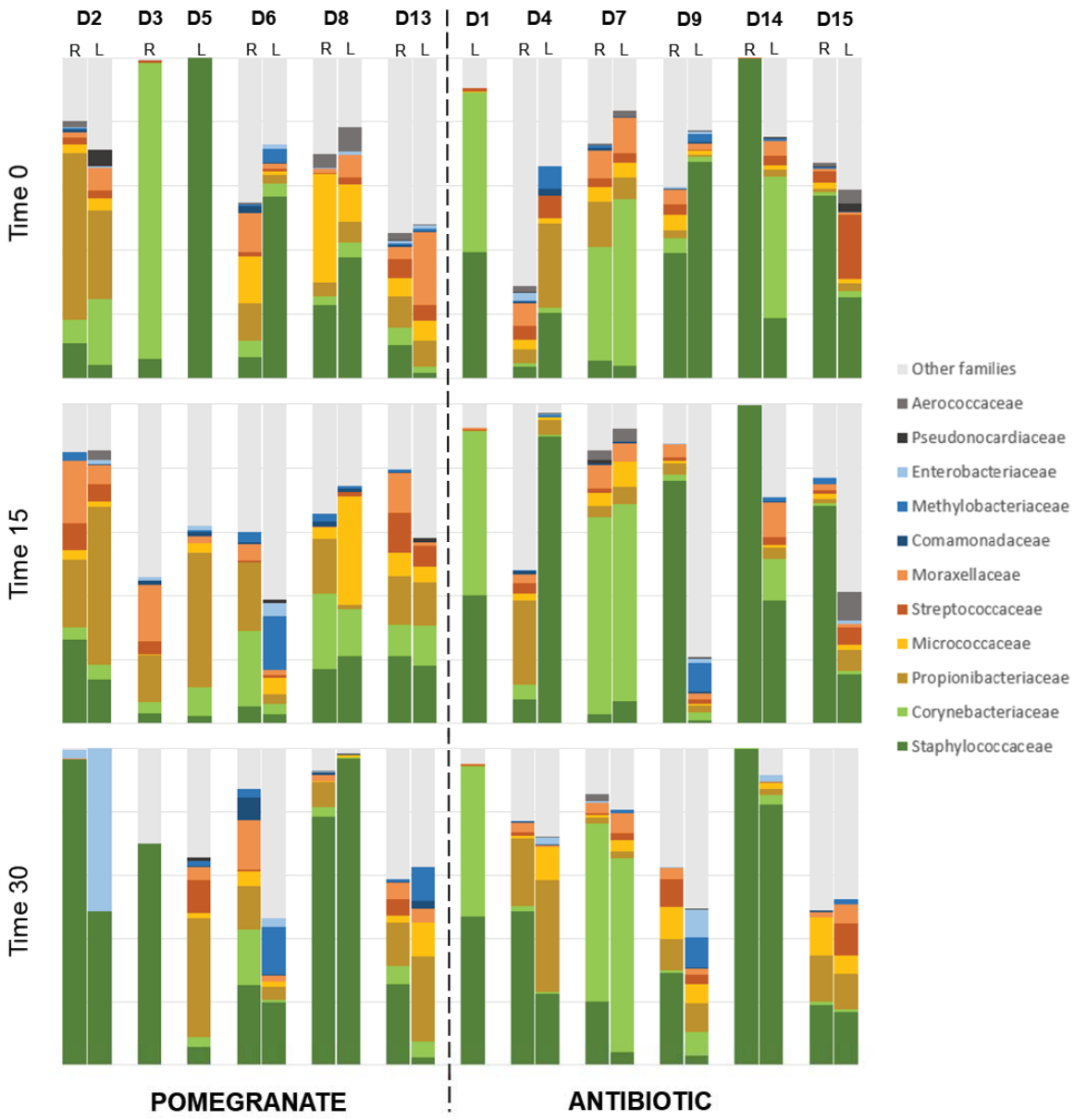

Figure 12. Bacterial composition at family level of the otitis samples at time of enrolment (Time 0), after 15 days of each treatment (Time 15) and after 15 days of ear cleanser (Time 30). The samples are divided in two groups depending on the treatment: Pomegranate or Antibiotic. The samples code is composed by a "D" plus a number and the ear for each individual is specified with R (Right) or L (Left). Only bacterial families that account for >5% of the total bacterial abundance in at least one sample are represented in the bar plots. "Other families" grouped those families that are less than 5%.

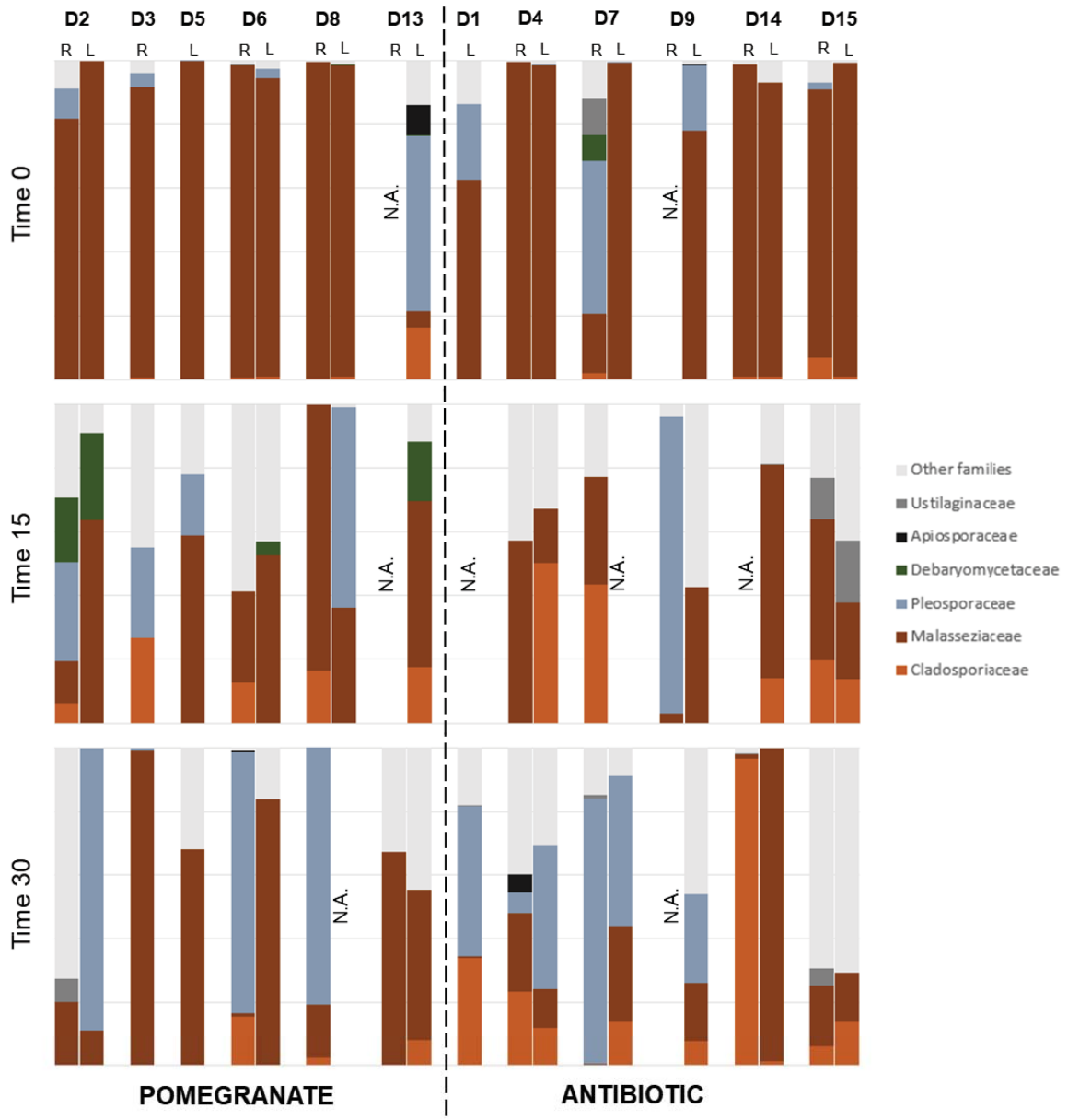

Figure 13. Fungal composition at family level of the otitis samples at time of enrolment (Time 0), after 15 days of each treatment (Time 15) and after 15 days of ear cleanser (Time 30). The samples are divided in two groups depending on the treatment: Pomegranate or Antibiotic. The samples code is composed by a "D" plus a number and the ear for each individual is specified with R (Right) or L (Left). N.A.: no amplification for fungi. Only fungal families that account for >5% of the total bacterial abundance in at least one sample are represented in the bar plots. "Other families" grouped those families that are less than 5%.

Our cohort of dogs with otitis at T_0 presented a Malassezia-rich mycobiome accompanied by Staphylococcaceae, Corynebacteriaceae, Propionibacteriaceae, Micrococcaceae, Streptococcaceae, and Moraxellaceae as the main bacterial

representatives (Figure 12 and 13). This profile was common for almost all the otitis samples, independently of the microbiological cultures were positive for fungi, for fungi and bacteria, or bacteria. The only exceptions were two samples with a negative microbiological culture (D13-L and D7-R) that presented a more diverse pattern with *Malassezia* spp. together with other fungi families such as Pleosporaceae and Cladosporiaceae (Figure 13).
In the two other collection points (T_{15} and T_{30}) after the administration of the treatment and the cleanser, the bacterial profile in almost all samples show an increase of the group “Other families” that gathers bacteria present in a percentage lower than 5%. Staphylococcaceae and Corynebacteriaceae are the families always present in all the samples. Some samples showed almost the same bacterial profile even after administrating the treatment (D1-L and D14-R).

The differences in taxonomy are more evident in fungal communities: at T_0 *Malassezia* is the most abundant genus, but after the treatment, its relative abundance changes in all samples. The growth of Cladosporiaceae and Pleosporaceae is apparent, starting from T_{15}, and more fungal families gathered in “Other families” group are detected. Among them, sometimes, the taxonomy assigned corresponds to only one fungus: at T_{15} Aspergillaceae is the most abundant among all the families detected in “Other families” group (D3-R). At T_{30} the most abundant families found in “Other families” group in D2-R, D5-L, D5-R and D13-L” were Filobasidiaceae, Vuillemiaceae, Erysiphaceae and Aspergillaceae, respectively.

After the treatment, taxonomy was analysed for T_{45} and T_{60} to check the evolution of the microbiome and mycobiome profiles (Figure 14 and Figure 15).

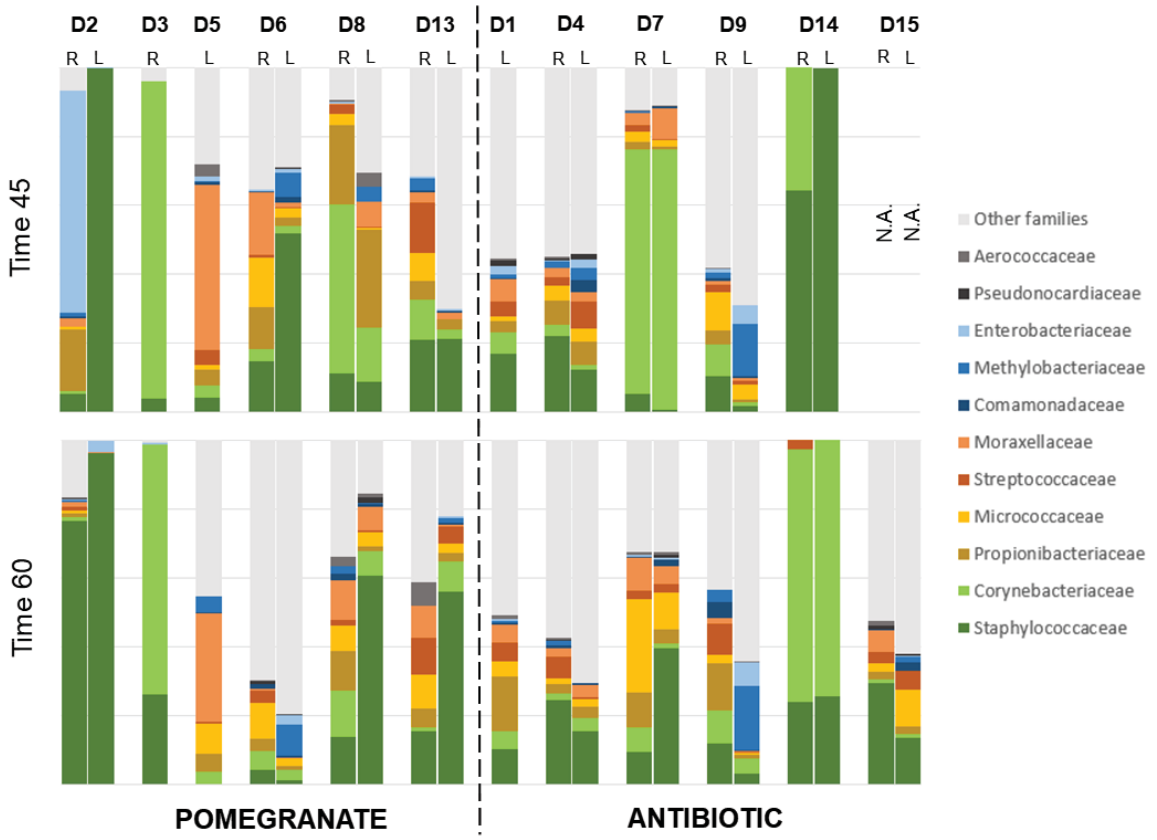

Figure 14. Bacterial profile in each affected ear of dogs after the treatment. The samples are divided in two groups depending on the treatment: Pomegranate or Antibiotic. The samples code is composed by a "D" plus a number and the ear for each individual is specified with R (Right) or L (Left). "Other families" grouped families that are less than 5%. "N. A.": Not Amplified samples.

As in the previous three time-points, Staphylococcaceae and Corynebacteriaceae are always detected. At T_{60}, the relative abundances of "Other families" group is evident for D4, D6 and D15, but there was not a most abundant family that characterizes the group.

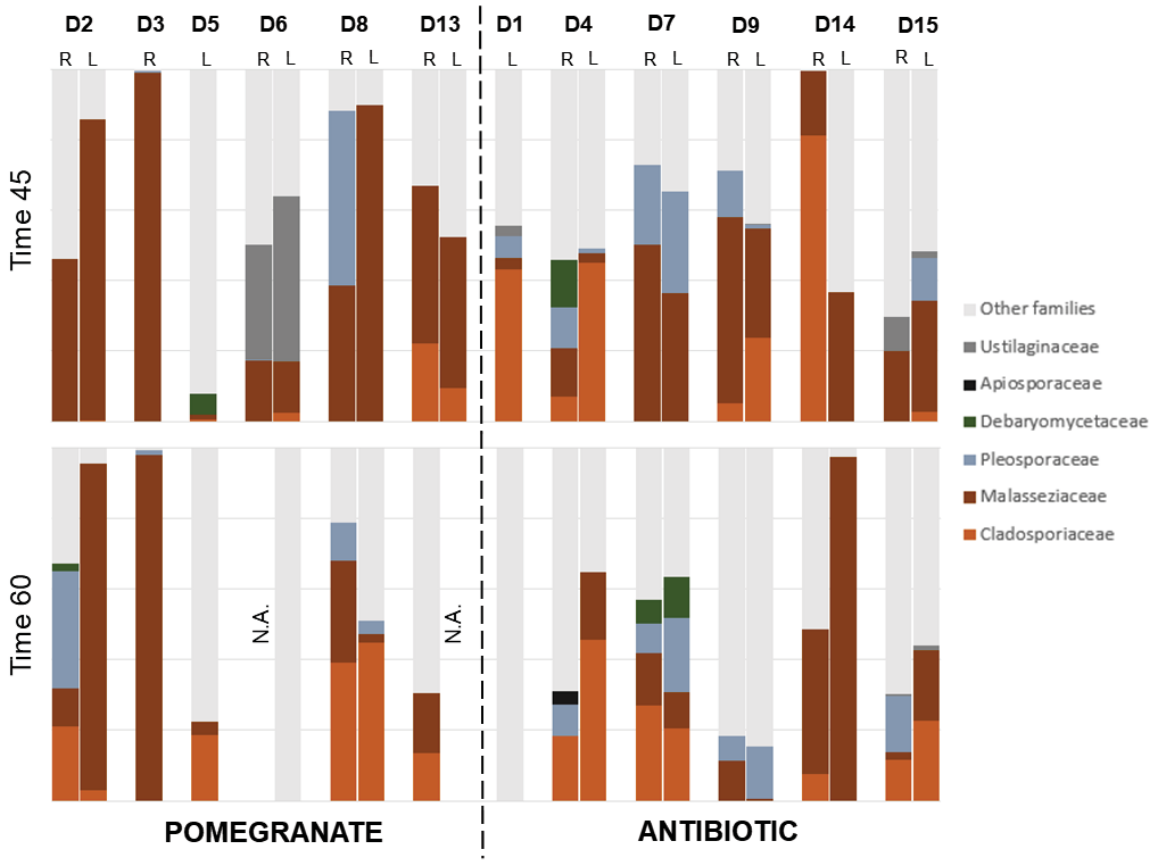

Figure 15. Fungal profile in each affected ear of dogs after the treatment. The samples are divided in two groups depending on the treatment: Pomegranate or Antibiotic. The samples code is composed by a "D" plus a number and the ear for each individual is specified with R (Right) or L (Left). "Other families" grouped families that are less than 5%. "N. A.": Not Amplified samples.

For fungal communities, the main changes belong to Malasseziaceae family (Figure 13):

- Its relative abundance decreased at each time-point until Malasseziaceae is not detected, or its percentage is less than 1% at T_{60} (D1-L, D6-L and D9-L). The most abundant in these three samples were: "Unidentified" for the first dog, Aureobasidiaceae plus Lycoperdaceae for the second and Didymellaceae for the third one.
- Its relative abundance is decreasing after the administration of the treatment, but then it grew up again being the most abundant in the sample (D2-L and D3-R).
- It is the most abundant among all time-collection points (D14-L).

Looking at the “Other families” group of fungi, the most abundant families detected at T_{45} and T_{60} were:

- Saccharomycetae (D6-R), Didymellaceae (both sample of D9), Herpotrichiellaceae (D7-L), Mycosphaerellaceae (D2-R) and Saccharomycetales (D15-R) at T_{45}.
- Didymellaceae (D7-R), Saccharomycetaceae (D5-L and 13-R) and Aspergillaceae (13-R) at T_{60}.

The complete taxonomy of the fungal and bacterial communities at family and genus level can be found at the link: https://www.dropbox.com/sh/t4p9j4715wmesfg/AACvRkZsZN1qeJWmLlFYFjoia?dl=0. Each annex contains the relative abundances at family and genus level for the bacteria and the fungi at each collection point of the samples. Bacterial families are showed in the “Annex 1_Bacteria_families”, while the bacterial genera in the “Annex 2_Bacteria_genus”. Fungal families are showed in the “Annex 3_Fungi_families” while the fungal genera in the “Annex 4_Fungi_genera”.

3.3) Comparison between two treatments for the canine otitis externa

After the characterization of the ear microbiome in dogs with non-purulent otitis externa, due to a bacterial and/or fungal overgrowth, we wanted to examine the efficacy of standard treatment with anti-inflammatory, antibiotic, and antifungal drugs versus anti-inflammatory agents combined with pomegranate extracts.

Dogs were divided in two groups depending on the treatment administered and samples until time 30 were considered.

We assessed if specific bacterial and fungal families differed within a treatment and found that most significant changes are of few bacterial and fungal families (p-value < 0.05), as seen in Table 3.

The common result found in both cohorts after either pomegranate and antibiotic treatment (between T_0 and T_{15}) was a significant decrease on Malasseziaceae.

In the cohort receiving the pomegranate treatment, Methylobacteriaceae increased between T_0 - T_{15} and Staphylococcaceae between T_{15} - T_{30}, whereas Corynebacteriaceae decreased between T_{15} - T_{30}. In the cohort receiving the antibiotic treatment, Streptococcaceae and Pleosporaceae decreased between T_0 and T_{15}, whereas Malasseziaceae decreased but Pleosporaceae and Cladosporiaceae increased between T_0 and T_{30}.

Table 3. Bacterial and fungal families with significant p-value (< 0.05) found at each time point are showed. "Increase (+) / decrease (-)": plus and minus symbols are used to underline if there is an increase/decrease of the family.

	Pomegranate					
	Family	**p-value**	**T0**	**T15**	**T30**	**Increase (+) / decrease (-)**
T_0-T_{15}	Methylobacteriaceae	0,033	0.8%	2.1%		(+)
	Malasseziaceae	0,044	86.6%	40.5%		(-)
T_{15}-T_{30}	Corynebacteriaceae	0,003		9.6%	3.0%	(-)
	Staphylococcaceae	0,005		13.4%	39.0%	(+)
T_0-T_{30}						

	Antibiotic					
	Family	**p-value**	**T0**	**T15**	**T30**	**Increase (+) / decrease (-)**
T_0-T_{15}	Streptococcaceae	0,009	4.4%	1.4%		(-)
	Malasseziaceae	0,035	81.3%	40.9%		(-)
	Pleosporaceae	0,036	10.1%	0.0%		(-)
T_{15}-T_{30}						
T_0-T_{30}	Malasseziaceae	0,008	82.9%		22.1%	(-)
	Pleosporaceae	0,042	9.5%		25.8%	(+)
	Cladosporiaceae	0,025	1.2%		20.9%	(+)

Linked to the decrease of Malassezia with both treatments, we detected an increase on alpha diversity in mycobiome analyses after both treatments from T_0 to T_{15} (Shannon Index p-value = 0.018 for each treatment; see Figure 16).

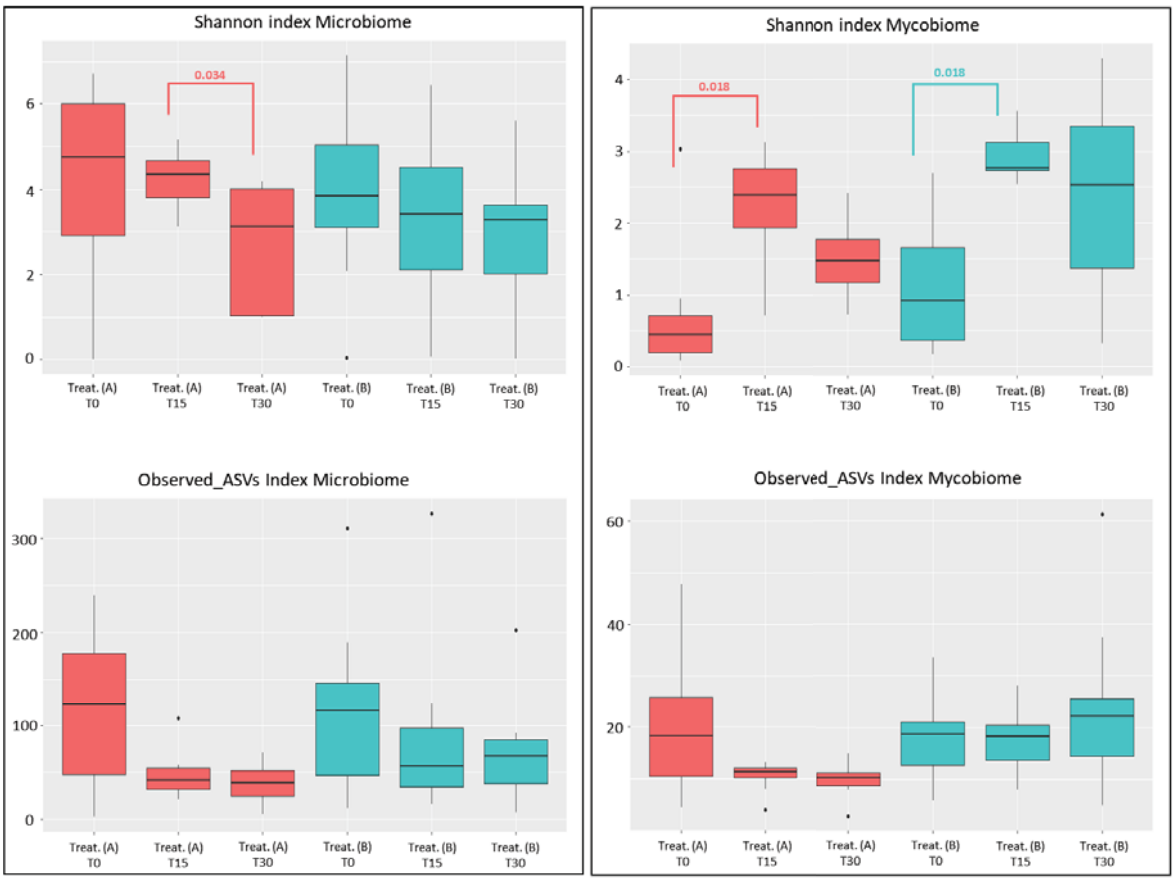

Figure 16. Shannon and Observed_ASVs boxplot in bacterial (left) and fungal (right) microbial communities. Statistical analysis comparing the time-points within each treatment (A=pomegranate and B=antibiotic) and the treatments at each time-point. Significant p-value < 0.01.

In beta diversity, we also detected significant differences between clustering of fungi at pomegranate treatment (A): PC2 p-value=0.035; antibiotic treatment (B): PC1 p-value=0.027 and PC2 p-value=0.017.

Figure 17 shows the clusters of fungal and bacterial samples, depending on the treatment. In the upper part, the fungi samples tend to cluster together at T_0 in pomegranate (Figure 17A) and antibiotic (Figure 17B) treatment while they spread at T_{15}. There is one sample at T_0 for each treatment that did not cluster together with the others: D13-L in pomegranate treatment and D7-R in antibiotic treatment, which

presented a more diverse fungi pattern (*Malassezia* spp. together with Pleosporaceae and Cladosporiaceae). Switching to bacterial communities in the down part of the Figure 17, the beta diversity clustering pattern observed for fungi at T_0 was not present for bacteria (Figure 17C and 17D).

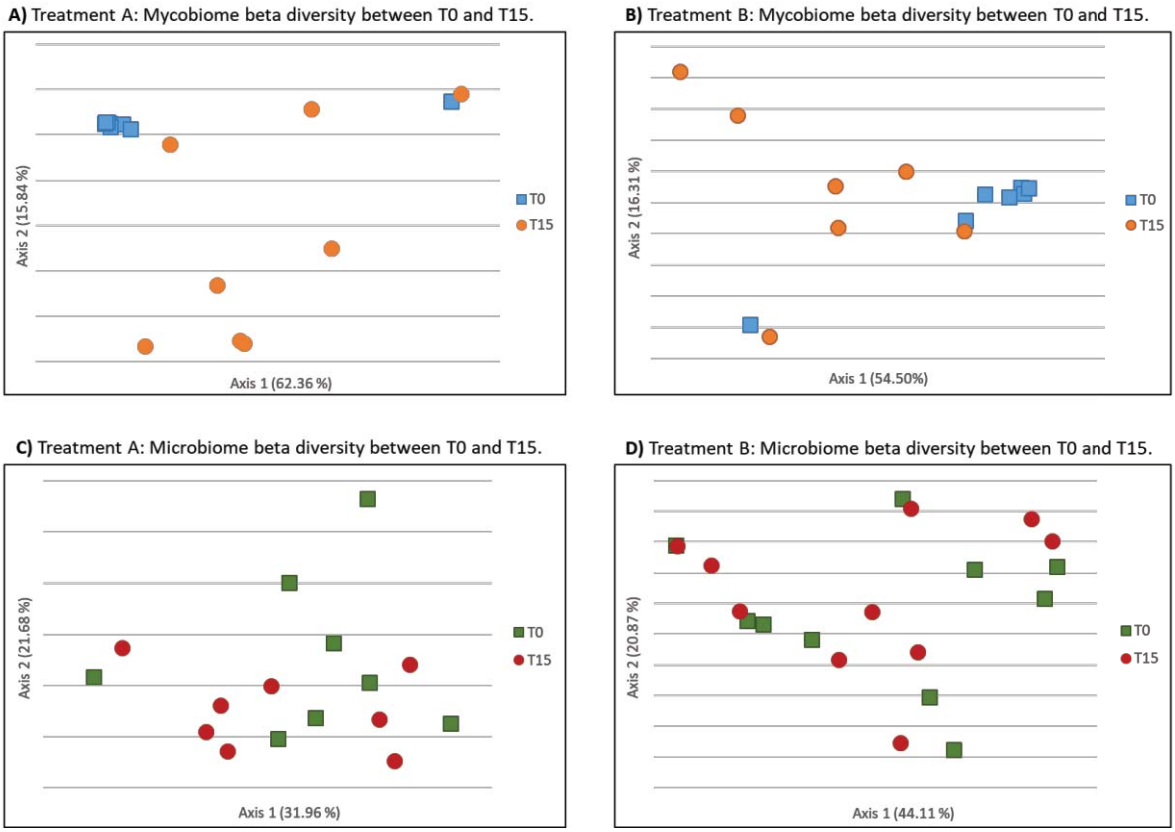

Figure 17. Weighted UniFrac distance matrix plots of fungal (panel A and B) and bacterial (panel C and D) communities within each treatment (Treatment A=pomegranate; Treatment B=Antibiotic).

Taxonomical assignment at genus level and species level. Since Staphylococcaceae and Malasseziaceae are common bacteria and fungi on the skin, and they may be associated with its disorders, we wanted to assess the genus and species level. We were able to identify the genus *Malassezia* and four species in our cohort of dogs with otitis (Figure 18): *M. pachydermatis*, *M. globosa*, *M. restricta*, *M. sympodialis* as well as other species that could not be identified at the species level ("*M.* spp."). *M. pachydermatis* was the prevalent species at T_0 then was replaced at T_{15} and T_{30} by *M. restricta*, *M. globosa*, and other fungal families such as Cladosporiaceae, Pleosporaceae, and Aspergillaceae (Figure 13 and 18).
At T_{45} *M. pachydermatis* and *M. restricta* are the most abundant species found among samples while at T_{60} only three samples showed *M. pachydermatis* as the main one (D2-L, D3-L and D14-L) while in the other the presence of *Malassezia* species is low.

About Staphylococcaceae the most abundant genus found was *Staphylococcus* in all samples at T_0 and T_{15} with different relative abundances. In pomegranate treatment, we observed changes in samples profiles: D2-L and D2-R detected at T_0 the presence of *Propionibacterium* but from T_{30} *Staphylococcus* genus is the main one detected. D5-L showed a high relative abundance of *Staphylococcus* that almost disappears during the other sample collection points. In samples as D8 and D13 (both right and left ears) we observed changes in *Staphylococcus* genus relative abundance: from T_0 until T_{60}, the relative abundances registered are increasing.
In antibiotic treatment the most relevant results obtained is the presence of *Staphylococcus* as the most abundant genus from T_0 until T_{30}, while from T_{45} to T_{60} the relatives abundances changed: *Propionibacterium* and *Corynebacterium* were also detected as the most abundant genera. In sample D14-R and D14-L is interesting to observe that *Staphylococcus* genus remains the most abundant one found in all collection points, except at T_{60}. Here, *Corynebacterium* was classified also as one of the most abundant genus in the sample (Figure 19).
In all samples, other genera were detected but the major part were classified as "Unidentified".

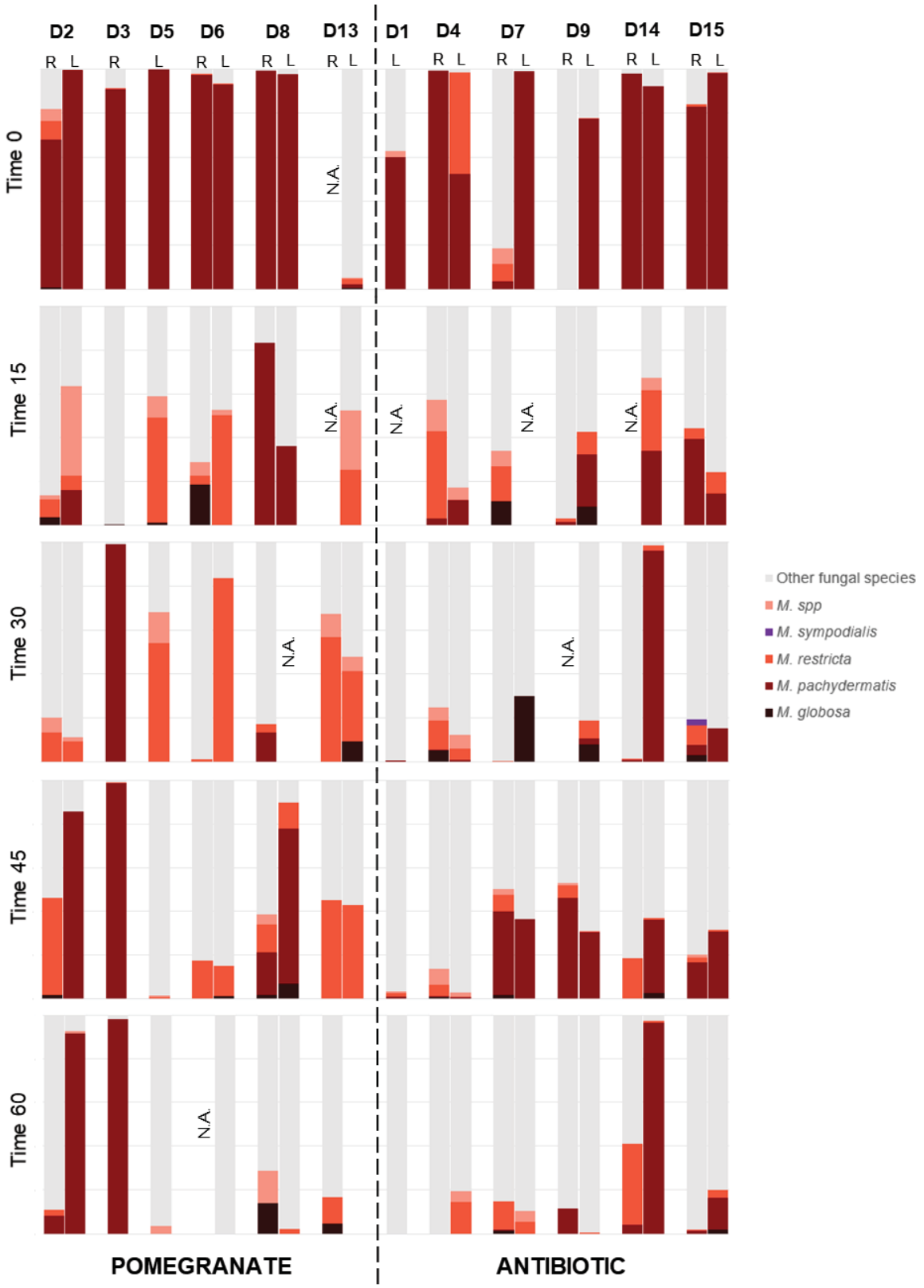

Figure 18. Taxonomy and relative abundance of *Malassezia* species of the otitis samples for pomegranate and antibiotic treatment at the time of enrolment (Time 0), after 15 days of each treatment (Time 15), after 15 days of ear cleanser (Time 30) and at two other collection point in which no treatment was applied (Time 45 and Time 60) . N.A.: no amplification for fungi.

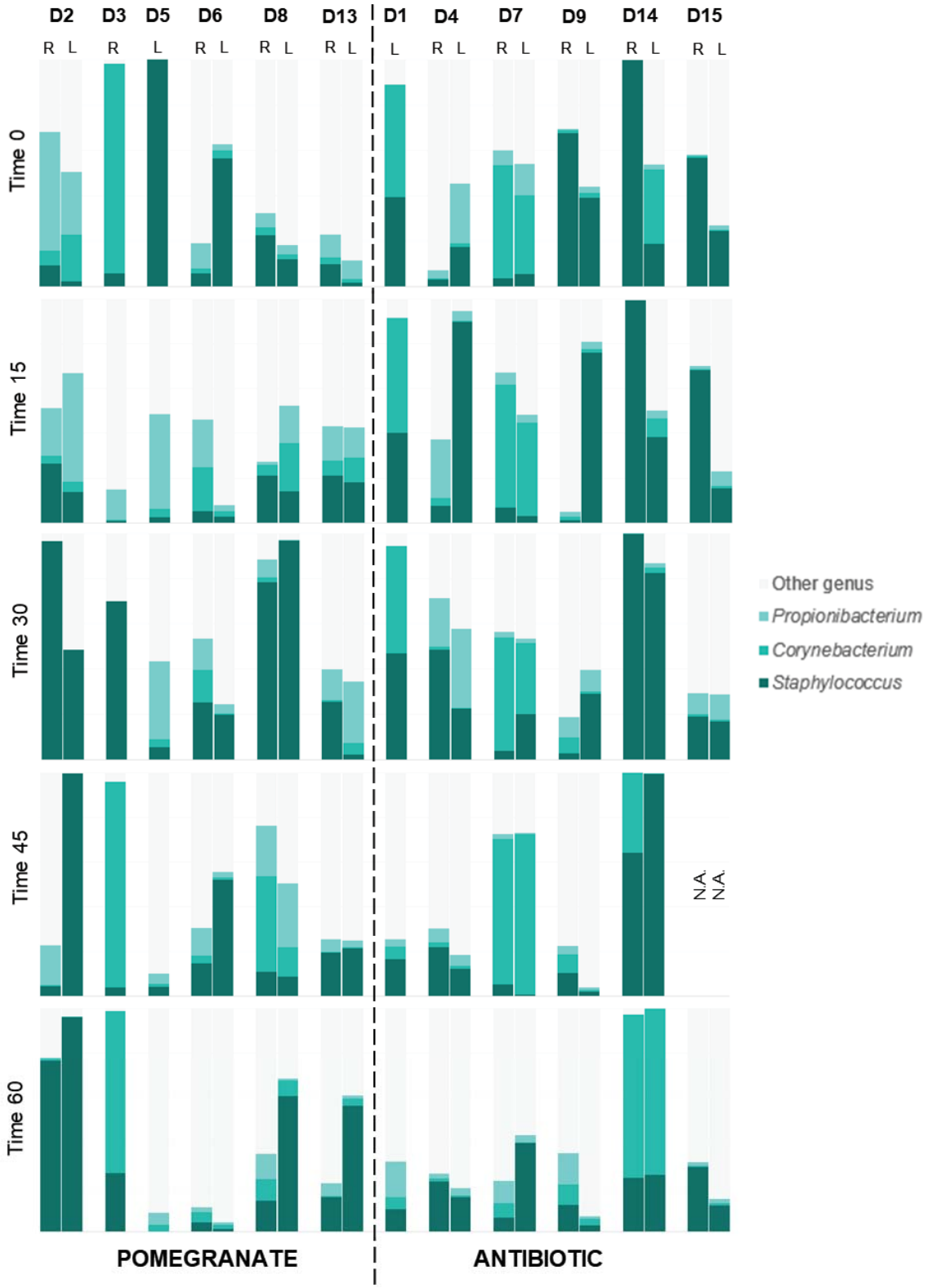

Figure 19. Taxonomy and relative abundance of the main bacterial genus found in the otitis samples for pomegranate and antibiotic treatment at the time of enrolment (Time 0), after 15 days of each treatment (Time 15), after 15 days of ear cleanser (Time 30) and at two other collection points in which no treatment was applied (Time 45 and Time 60). N.A.: no amplification for fungi.

Due to the region amplified, the database and informatic tools used, we were unable to reach the species level for all the genera found. As it was not possible to reach the species level for all the *Staphylococcus* taxa with QIIME2, a test including few samples at T_0 and T_{30} was performed. The criteria to include the samples were: *Staphylococcus* genus (i) showed low changes in relative abundances from T_0 to T_{30}, as in case of D1 and D14; (ii) there was an evident difference between the relative abundances at T_0 and at T_{30}, as in samples D2, D4 and D8; (iii) *Staphylococcus* was the most abundant genus found at T_{30} while at T_0 the most abundant was *Corynebacterium*, as for sample D3 (Figure 19).

The PCR with a mix of three primers for the amplification of three Staphylococcal species (359 bp for *S. aureus*, 430 bp for *S. intermedius* and 926 for *S. pseudintermedius*) was performed to compare the results of QIIME2 taxonomy assignment. Twelve samples amplified for *S. pseudintermedius*, and ten for *S. intermedius* as showed in Figure 20. Not all sample amplified for this primers mix, meaning that other *Staphylococcus* species are present.

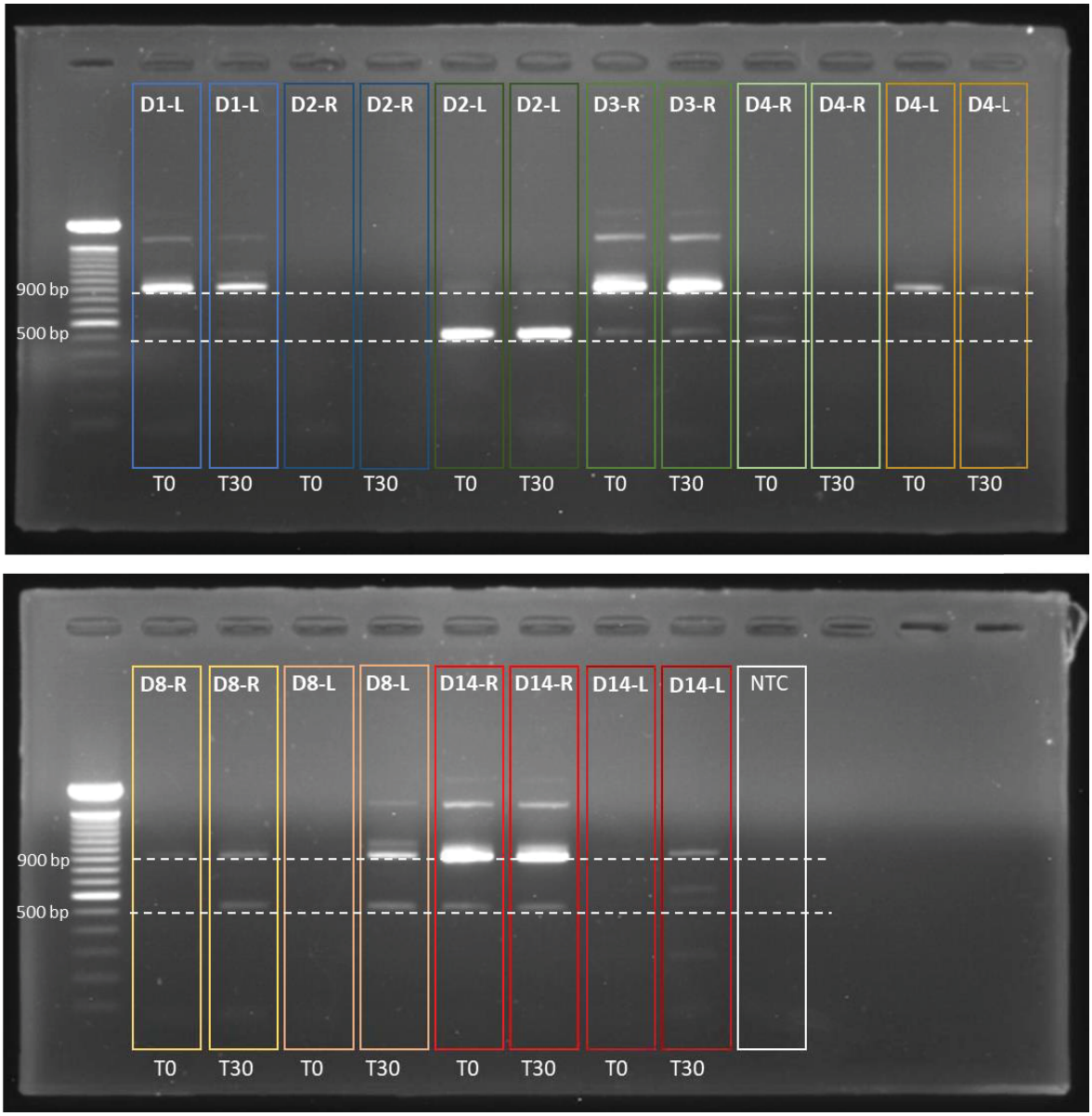

Figure 20. Agarose gel for *Staphylococcus* species in samples from the skin of dogs affected by otitis. The white lines showed samples that amplified for *S. pseudintermedius* (900 bp approximately) and *S. intermedius* (430 approximately).

3.4) Long Amplicons PCR approach: taxonomy skills of a rapid detection in microbiological fungal cultures and in complex otitis samples

We aimed to develop a long-amplicon PCR approach and nanopore sequencing to detect fungal microbiota present on complex microenvironments, and to apply it to clinical samples (canine otitis). As positive sample controls, we used microbiological fungal cultures and fungal strains from a mock community. Petri dishes of each culture were kindly provided from LETI laboratories.

All samples amplified for both amplicons size, 3.5 Kb and 6 Kb. In 3.5 Kb, we included those domains that better help in taxonomic classification of fungi as it is showed in Figure 21.

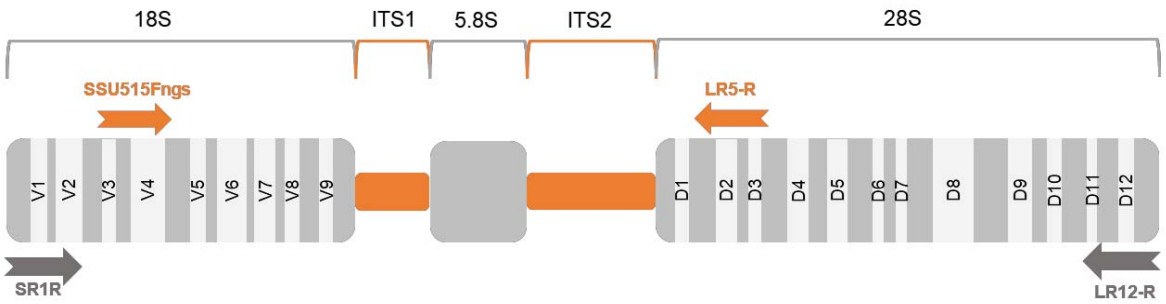

Figure 21. Primers set used for the amplification of the ITS region (3.5 Kb) are shown in orange in the upper part of the operon, and the ones for amplification of the full operon (6 Kb) are shown in grey in the lower part (Vilgalys lab 1992; Tedersoo et al. 2015).

Both amplicons correctly detected and identified the ZymoBIOMICS™ mock community fungal strains (*Saccharomyces cerevisiae*, *Cryptococcus neoformans)*, and *Malassezia pachydermatis* and *Microsporum canis* from microbiological cultures. Looking in detail, *Saccharomyces cerevisiae*, *Cryptococcus neoformans* and *M. pachydermatis* were detected up to 100% by both 3.5 Kb and 6 Kb long fragments, while 6 Kb amplicon better detected *Microsporum canis* (Figure 22). Both fragments identified *Aspergillus* genus as the main one found in the culture, but looking at species level, *A. fumigatus* was the most abundant one. *Alternaria* spp. and *Candida* spp. showed different results of what we expected (Figure 23).

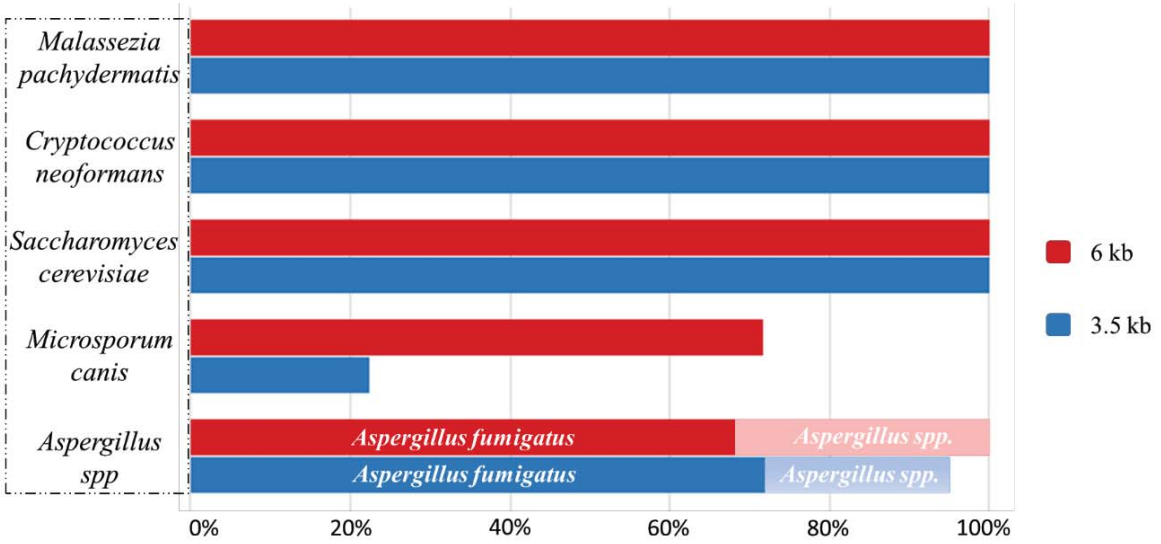

Figure 22. ZymoBIOMICS mock community (*S. cerevisiae* and *C. neoformans*) and microbiological cultures of fungi after taxonomical classification of the 3.5 Kb and 6 Kb ribosomal amplicons.

***Alternaria* spp.** Both amplicons size detected *Metarhizium brunneum* as the main fungus found in these cultures, while *Alternaria alternata* was the species found in really low relative abundance (0.2% for 3.5 Kb and 15.6% for 6 Kb). No similarities between these fungi were found: they belong to different order, Pleosporales and Hypocreales. *Alternaria* is a ubiquitous filamentous fungi found in soil air and human/animal skin (Pastor and Guarro 2008); *Metarhizium* is commonly found as parasite of insects and symbiont of plant (Samish et al. 2014; Tiago and Oliveira 2014).

Looking at the nomenclature of this fungus, *Metarhizium brunneum* belonged to *Metarhizium anisopliae* strain (Tiago and Oliveira 2014; Yousef et al. 2018) but no correlation with *Alternaria alternata* was found.

***Candida* spp.** In Petri dish the colonies of this fungus were red/orange, and sequences revealed the presence of *Rhodotorula graminis* and only few reads were associated to *Candida* spp. (Figure 23). *Rhodotorula* is a carotenoid biosynthetic yeast, part of the Basidiomycota phylum, easily identifiable by distinctive yellow, orange or red colonies (Yadav et al. 2014). This yeast is commonly associated with

plants and it produces three major carotenoids: b-carotene, torulene and torularhodin (http://www.antimicrobe.org/f16.asp#t1).

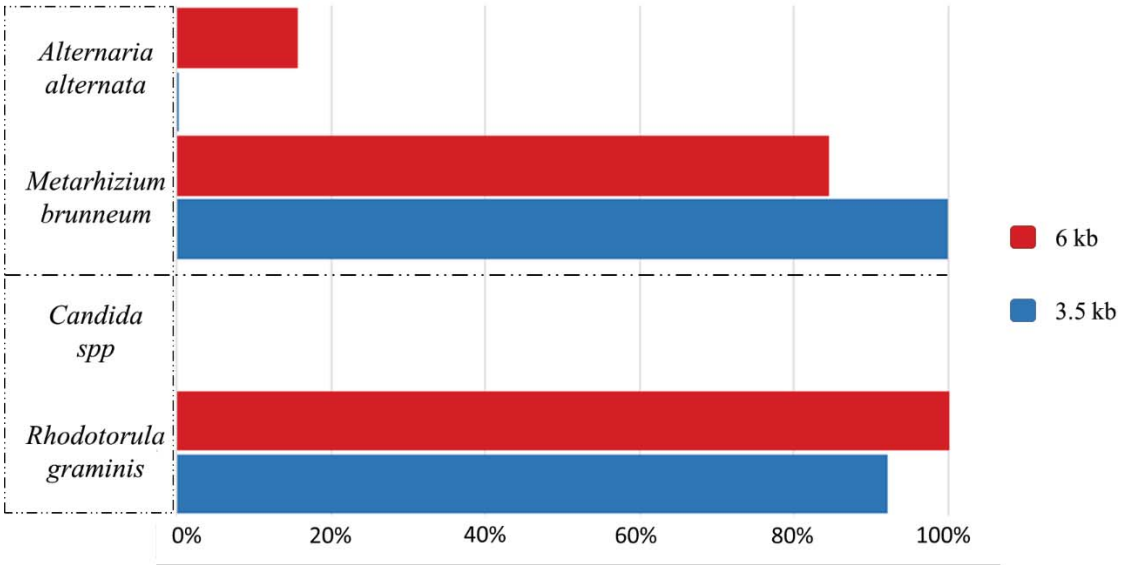

Figure 23. Fungal microbiological cultures showed unexpected results in the taxonomical classification after sequencing. Few reads from the Alternaria culture belonged to *Alternaria* spp., and it was classified at species level as *A. alternata*, but the most abundant fungus found was *Metarhizium brunneum*. No reads from the Candida culture were classified as *Candida* spp. because of the presence of *Rhodotorula graminis*.

Canine otitis. Conscious that no differences were found in *M. pachydermatis* analysis using both fragments sizes (Figure 22), we sequenced microbiological cultures of *M. pachydermatis* as positive controls and four complex samples with 3.5 Kb amplicon size. We run WIMP for fungal communities' detection: all positive controls were identified as *M. pachydermatis*, while the complex samples showed other *Malassezia* species (Table 4).

Two of the samples correspond to the same dog, one from a healthy ear (S02) and the other one (S03) with clinical signs compatible with otitis externa, and *M. pachydermatis* is the main fungal species detected in both ears. The other two samples (S01 and S04) came from the ear with otitis externa of two dogs. In that case, other *Malassezia* species were detected together with *M. pachydermatis*, such as *M. globosa* and *M. sympodialis.*

Table 4. Relative abundance of *Malassezia* species found in *M. pachydermatis* microbiological culture, and in four complex samples belonged to three different dogs affected by otitis. Samples S02 and S03 belong to the same dog, while S04 and S01 belong to two different dogs.

	M. pachydermatis	*M. globosa*	*M. sympodialis*	*M. Unidentified*	*Others*
M01	98.9%	0.2%	0.2%	0.2%	0.5%
M02	99.0%	0.1%	0.2%	0.2%	0.5%
M03	98.9%	0.2%	0.3%	0.2%	0.4%
S02_healthy	98.2%	0.1%	0.2%	0.2%	1.3%
S03_affected	95.1%	1.1%	1.7%	0.4%	1.7%
S04_affected	78.8%	4.0%	5.0%	3.9%	8.3%
S01_affected	73.7%	4.7%	5.1%	5.3%	11.2%

3.5) Assembly and annotation of *Malassezia pachydermatis* genome

The analysis of a clinical isolate of *M. pachydermatis* from a dog affected by otitis externa was performed using the Oxford Nanopore Technology: sequence of long reads was performed with the MinION device.

De novo assembly resulted in a genome size of 8.24 Mbp and 10 contigs: the longest one obtained was 1.8 Mb and the assembly quality score (N50) was 1.42 Mb (Figure 24). The completeness obtained with BUSCO analysis was 76.6%. The genome mode was the basidiomycota_odb10 (2019-11-20, number of species: 133, number of BUSCOs:1764).

Bandage was used for contig visualization, and the results obtained were compared with the two only available genome assemblies for *M. pachydermatis* from the collection strain CBS 1879 and with the Illumina short-reads: GCF_001278385.1 (Triana et al. 2015) and GCA_001264975.1 (Wu et al. 2015) (Table 5).

Table 5. Comparison among the *M. pachydermatis* contigs found with Oxford Nanopore Technology and the Illumina short reads reported from Triana et al. 2015 and Wu et al. 2015. Available at www.ncbi.nlm.nih.gov.

Isolate origin	Size	Scaffolds	Contigs	N50	Reference
Clinical sample	8.24 Mb		10	1.42 Mb	Nanopore
CBS 1879	8.15 Mb	91	118	0.64 Mb	GCF_001278385.1
CBS 1879	8.16 Mb	61	80	0.64 Mb	GCA_001264975.1

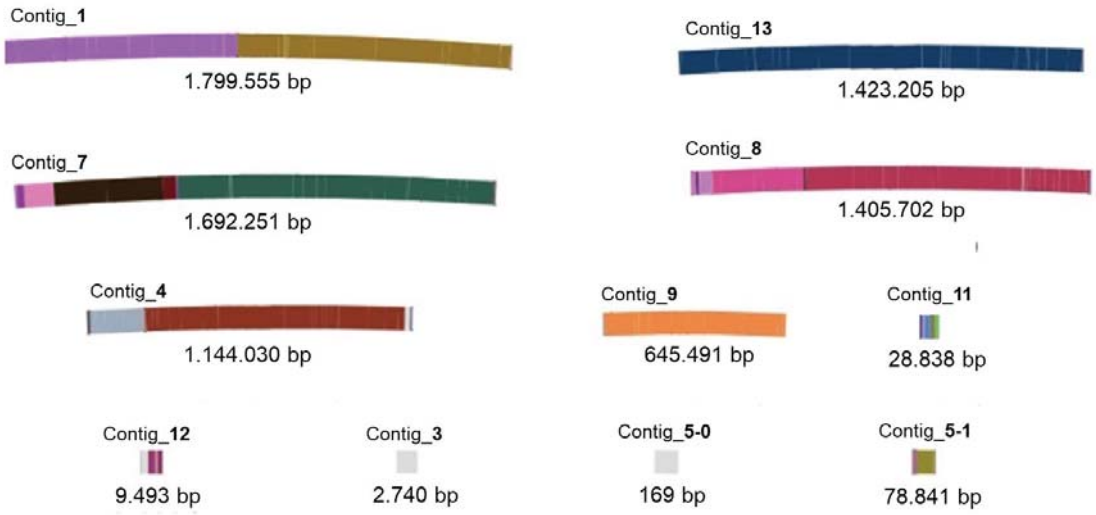

Figure 24. Contigs visualization by Bandage. Six contigs (contig_1, contig_13, contig_7, contig_8, contig_4 and contig_9) might correspond to the 6 already reported chromosomes of *M. pachydermatis*.

4. DISCUSSION

This thesis aimed to investigate the fungal communities in dogs' skin, focusing on dogs with clinical otitis. That included the choice of the hypervariable regions from the fungal ribosomal operon, sequencing by high-throughput technologies (Ion Torrent and Nanopore), and analyses using different bioinformatics tools and pipelines.

4.1) On the skin: the bacterial and fungal communities

The first goal of this study was to investigate the bacterial and fungal microbiota in the ear canal of dogs with ceruminous, but not purulent, otitis externa, and to compare the efficacy of two different topical treatments over time. Treatment A was prednisolone plus an otic cleanser with pomegranate extract. Treatment B was prednisolone plus antimicrobial therapy and a saline otic cleanser *(Effect of an anti-inflammatory pomegranate otic treatment on clinical evolution and microbiota profile of dogs with otitis externa; Authors: Anna Puigdemont, Sara D'Andreano, Laura Ramió-Lluch, Anna Cuscó, Olga Francino and Pilar Brazis; Veterinary Dermatology, 2020: in press).*

The worldwide spread of antibiotic-resistant pathogens such as *Staphylococcus aureus* has led to the search of antimicrobial compounds from natural sources, including the plants (Al-Zoreky 2009). Among all constituents of the plants, the polyphenols, tannins, and flavonoids have received a great deal of attention for their antimicrobial activity. Natural compounds such as pomegranate extract (rich in tannins) helped to develop new possibilities against the growth of some pathogenic bacteria/fungi strains (Orak et al. 2011). There are proves that the polyphenols extracted from the fruit rind are active against phytopathogenic fungi (Foss et al. 2014) and are also used as topical antifungal agent in treatment of *Candida albicans* or *C. krusei* (Orak et al. 2011; Foss et al. 2014). About the fungal genus of *Malassezia*, studies on the species *M. furfur* checked the effect of the pomegranate used as an herbal formulation to combat skin infections (Gopalakrishnan 2016). Against bacteria, the pomegranate showed inhibition effects on *S. aureus* and *S. epidermidis*, *Propionibacterium acnes* and *P. granulosum* (Betanzos-cabrera et al. 2015; Henning et al. 2019).

On the other hand, microbial culture-based tests have proven ability to identify the most common bacterial and fungal populations present in ear canals of healthy and atopic dogs. Despite a strong correlation with microbiology culture-based results, microbiome studies with next-generation sequencing can evidence the presence of a multitude of bacterial and fungal families, most of which are difficult or impossible to identify through a standard microbiological culture.

The microbiota observed in different sites of dog' skin, showed significant differences in bacterial composition; looking at the auditory canal skin, the microbiome profile has recently been characterized in healthy and atopic dogs. A dysbiosis characterized by increase of *Staphylococcus* spp and *Ralstonia* spp was observed in atopic dogs (Ngo et al. 2018).
Chermprapai et al. (2019) tested an antimicrobial shampoo in three atopic and six healthy dogs, showing a non-significant increase in bacterial diversity while the opposite effect was described in fungal diversity. Moreover, they found no significant differences between atopic and healthy dogs, both before and after treatment. The authors observed that the changes in microbiota composition were more dependent on individuality than skin site, health status, or treatment effect. The strong effect of the individual with its environment and its influence on the composition and variability of microbiome has been reported in previous works on healthy dogs (Cuscó et al. 2017b; Cuscó et al. 2017a).

In the present study, the clinical status, mycobiota, and microbiota from the ear of dogs suffering otitis externa were compared before and after two topical treatments, one of them based on natural pomegranate extract and the other based on antifungals and antibiotics. Dogs were assigned into two groups and samples were taken from the inner part of the ear at the time of enrolment (T_0), 15 days after either treatment A or treatment B (T_{15}), 15 days after the receiving only the otic cleanser (pomegranate or saline) (T_{30}) and after no treatment (T_{45} and T_{60}). Bacterial (16S rRNA gene) and fungal (ITS2) communities were analysed and compared with clinical scores. We chose the V4 region of the bacterial 16S rRNA gene and the ITS2 of the fungal ribosomal operon as the most suitable for taxonomic assignments. After sequencing with Ion Torrent platform, short reads were obtained and combined with bioinformatics tools such as QIIME2 software and DADA2 pipeline, to obtain the taxonomy of the microbes presented in all samples.

The sequencing results agreed with the microbiological cultures at the time of enrolment. *Malassezia*, *Staphylococcus*, and *Corynebacterium* were assessed as the causal agents of infection through classical microbiological culture and were confirmed by 16S rRNA gene or ITS2 taxonomic analysis. The three genera have

also been detected with next-generation sequencing techniques in dogs' skin, and in both healthy ears and otitis (De Martino et al. 2016; Ngo et al. 2018).

The mycobiome analyses detected that the genus *Malassezia* was the most abundant fungus in otitis samples defining a common profile among them. Only two of the otitis samples with a negative microbiological culture for both bacteria and fungi presented other fungal species rather than *Malassezia*, such as Pleosporaceae and Cladosporiaceae families. This pattern was only partially detected using microbiological cultures, confirming the superior sensitivity of sequencing techniques. Cladosporiaceae (*Cladosporium* genus) and Pleosporaceae (*Alternaria* and *Curvularia* genera), which are commonly found in household dust, have been previously investigated as being putatively responsible from allergies in atopic dogs (Meason-Smith et al. 2015). Also, both fungi were reported as generally found on skin of dogs and cats with atopic dermatitis (Bond et al. 2020).

In microbiota analyses, we detected Staphylococcaceae along with Corynebacteriaceae, Propionibacteriaceae, Micrococcaceae, and Streptococcaceae as the most abundant bacterial families. The first two bacterial families may produce a variety of infections, including external otitis (Oliveira et al. 2008). Other genera found in taxonomy such as *Streptococcus, Micrococcus,* and *Moraxella*, that we do not find in microbiological cultures, were previously reported as bacteria that live in the healthy ear canal (Ngo et al. 2018).

Longitudinal results after the treatment (T_{15}) and the follow-up (T_{30}) suggest control of the bacterial and fungal overgrowth and changes in the fungal communities, also reducing the clinical signs when pomegranate treatment was applied. After 15 days of treatment, the reduction in clinical signs was statistically significant in both groups of treatment (prednisolone + pomegranate otic cleanser and prednisolone + gentamycin sulphate and ketoconazole+ saline otic cleanser).

This positive clinical evolution correlates to the decrease in *Malassezia* spp. relative abundance and the subsequent increase in fungal diversity between the initial and middle time points. The mycobiota composition was strongly modified in both treatments with a significant increase in fungal richness, linked to the decrease of *Malassezia* species and the appearance of new fungal diversity. An increase in

fungal diversity was the most significant change after both treatments. In both treatments, the profiles mainly characterized by *Malassezia* spp. at T_0 were replaced by more diverse communities, including new families, such as Cladosporiaceae, Pleosporaceae, and Aspergillaceae.

M. pachydermatis has been reported to be the causal agent of otitis in several studies (Gaitanis et al. 2012; Harada et al. 2015; Theelen et al. 2018; Guillot and Bond 2020), and *Malassezia* spp. was investigated in our samples at the species level in each treatment. At the onset, *M. pachydermatis* species was the most prevalent species, while *M. globosa* and *M. restricta* were detected after both treatments with improved clinical signs. We also detected a group of *Malassezia* spp., which were not identified at the species level in the taxonomic analysis.

On the other hand, no significant differences were observed in microbiota among the different time points within the two treatments, even though one of the treatments included antibiotics. That could be explained by the high individuality of dog skin microbiota and the likely environmental influences, as has already been reported (Cuscó et al. 2017a). The dogs included in the study form a heterogeneous cohort of pets that come from different places and environments. In fact, the bacterial composition of otitis ears was more variable when compared to fungal one. For mycobiota studies, this cohort seems appropriate to detect differences; however, a larger cohort size would be beneficial for studying the microbiota.

A topical corticosteroid treatment accompanied by a natural antimicrobial agent (pomegranate extract) was able to control bacterial and fungal overgrowth and dramatically changed the fungal profile, replacing *M. pachydermatis* with other fungal communities, and reducing the clinical signs. Next-generation sequencing analyses allowed a better understanding of the dynamics of canine otitis, linking the improvement to the decrease in *Malassezia* species and an increase in fungal diversity. More studies should be addressed to limiting or even avoiding antibiotic treatments and thereby helping to limit bacterial resistance. Natural compounds like pomegranate would be good antimicrobial alternatives in mild human and animal infections.

Staphylococcaceae and Malasseziaceae are common families found on the skin of dogs, both in health and disease, and they were the most abundant bacterial and fungal genera in our samples, so we performed a more in-depth analysis to reach the species level.

The Staphylococcal ability to develop resistance against antimicrobial treatments has been concerning both in veterinary and human medicine for years, especially the resistance to methicillin (Findik et al. 2018). When Staphylococcal infection occurs, they are associated with S. aureus in humans or S. pseudintermedius in dogs and cats since they are commensal bacteria on the skin and mucosae and also opportunistic pathogens (Findik et al. 2018). The presence of *S. pseudintermedius* has been reported in dogs affected by otitis externa, sepsis, and pyoderma, but this species may also be misidentified with *S. intermedius* (Wedley et al. 2014; Verstappen et al. 2017).

For the Staphylococcaceae family, the taxonomy confirmed the genus *Staphylococcus*, but no species were assigned in our study. Staphylococcal species are challenging to discriminate only with phenotypic differences (Sasaki et al. 2010). The PCR is a suitable method that helps in recognizing different species from a bacterial genus. In the case of *Staphylococcus*, specific primers sets to amplify the nuc gene, which shows moderate diversity among the *Staphylococcus* genus (Sasaki et al. 2010), allow the identification of *Staphylococcus* species (Verstappen et al. 2017). However, the specific-primers require a priori knowledge of the species we expect to detect.

In our case, we used primers specific for *S. aureus*, *S. intermedius*, and *S. Pseudintermedius* to confirm *Staphylococcus* genus at the species level. We analysed 20 samples corresponding to samples at T_0 and T_{30} from six dogs (four dogs with two affected ears, and two dogs with one affected ear). *S. pseudintermedius* was detected in three dogs at T_0 and T_{30}, in one dog only at T_0, and in two other dogs only at T_{30}. *S. intermedius* was detected in one of the dogs at T_0 and T_{30}.

Not all samples amplified for the species we were looking for, suggesting the presence of other species, such as *S. epidermidis* or *S. schleferii*.

4.2) Fungi and the analysis of the ITS regions compared with the full ribosomal operon

The second objective aimed to improve the taxonomy assignment for fungal communities. We evaluated the third-generation sequencing, trying to solve the problems of the short-reads with the introduction of long fragments, and the validation of bioinformatics tools for taxonomy assignment and assembly (D'Andreano et al. 2020). The problem of the technologies like PacBio and Nanopore is related to the error rates and the lower accuracy of the reads sequenced, because it may affect the correct assignation of taxonomy. As for the short reads, there are many algorithms designed to exploit long-reads data. The long reads, despite the high error rate, may improve the classification accuracy, especially for some taxa, and can help in *de novo* assemblies for metagenomic studies (Pearman et al. 2019).

The main question about the analysis of fungal communities regarded the correct size of the amplified operon is: Is the ITS region long enough to reach the species level? Or would it be better to sequence the whole ribosomal operon?
Primers used to amplify domains of the fungal ribosomal operon should be chosen depending on the fungus, but no standard markers are defined yet. The longest amplicons should be considered to describe the communities at lower taxonomy classification, due to differences in length of the fungal ribosomal operon (Tedersoo et al. 2018; Wurzbacher et al. 2018b). With the MinION device from Oxford Nanopore Technologies, it was possible to sequence longer fragments and improve the taxonomy assignment in real-time to compare the skills of the ITS region versus the whole operon.
We aimed to understand if long amplicons are suitable markers for the analysis of mycobiome in dog skin, and which size could be the best for fungal communities (3.5 Kb or 6 Kb). Samples from microbiological cultures and a standard mock community (ZymoBIOMICS^{TM}) were chosen together with four samples from dogs affected by otitis externa. The microbiological cultures and ZymoBIOMICS^{TM} mock community standards were essential for the study as positive controls because their genome sequences validated the correct detection of fungi in WIMP.

Saccharomyces cerevisiae, *Cryptococcus neoformans*, *Malassezia* spp., *Microsporum canis* and *Aspergillus* spp. were correctly detected and identified from the microbiological cultures and the mock community of ZymoBIOMICS™. We found that some fungi from the genus *Malassezia*, *Saccharomyces*, or *Cryptococcus*, showed no difference in taxonomy assignment at the species level with either the ITS regions (3.5 Kb amplicon) or the full ribosomal operon (6 Kb amplicon). On the other side, in fungi such as *Microsporum canis*, the full operon showed higher taxonomical skill respect of the ITS region.

Furthermore, we were able to identify *M. pachydermatis* as the main species in the microbiological culture, while for the *Aspergillus* genus, the most abundant species was *A. fumigatus* followed by other species with low percentages.

Besides, nanopore sequencing also underlined the unexpected taxonomy of fungi such as *Candida* spp. or *Alternaria* spp. The morphology of the colonies and the colour from their cultures indicated the possibility of finding other fungi, as so it was. The results showed unexpected fungi found in our cultures.

The microbiological cultures corresponding to *Alternaria* spp. and *Candida* spp. were misidentified as per classical microbiology, and other fungi were detected. It is noteworthy that the samples plated came from dog skin, which is prone to environmental contamination, as has been previously described in skin microbiome of healthy dogs (Cuscó et al. 2017a; Cuscó et al. 2019).

Few of the reads from the *Alternaria* culture were classified as *A. alternata* with the 6 Kb amplicon, while most classified as *Metarhizium brunneum*. Discovered in Spain and used as an herbicide against fly *Bactrocera oleae* (Yousef et al. 2018), this fungus belongs to the same phylum of Ascomycota, but it differs at lower taxonomy levels. The *Candida* microbiological culture was misclassified, even when showing an orange colour, caused by *Rhodotorula graminis*.

Finally, we investigate the possibility of reaching species level in complex samples from the skin of dogs affected by otitis, finding that *Malassezia* was the most abundant genus, and the classification at species level was performed to investigate possible changes between health status and diseased one.

M. pachydermatis is the most abundant species in the ear canal of healthy dogs (Korbelik et al. 2018). WIMP correctly identified all the *Malassezia* samples, and we

were able to identify *Malassezia* at the species level from four complex canine otitis samples. Two of the samples corresponded to the same dog, one from a healthy ear (S02) and one with clinical signs (S03) that were compatible with otitis externa. *M. pachydermatis* is the main fungal species detected (98% of the reads for the healthy ear – S02 – and 95% for the sample with clinical signs compatible with otitis externa – S03). The other two samples (S01 and S04) came from the ear with otitis externa of two other dogs. In those cases, other *Malassezia* species were detected together with *M. pachydermatis*, such as *M. globosa* and *M. sympodialis*.

That was the first approach to assess nanopore sequencing and the long-read amplicon for the analysis of fungi in complex samples. We assess the taxonomical power of two different amplicons targeting 3.5 Kb and 6 Kb of the ribosomal operon, respectively, with the longest one providing a better taxonomy assignation. We used positive controls from the ZymoBIOMICS™ mock community and from microbiological cultures that cannot always be considered pure cultures. We demonstrate this approach's suitability to characterize the fungal community of complex samples, either healthy samples or samples with clinical signs of infection, such as otitis. The next steps will involve the analysis of complex samples from different origins to detect the causal agent of the disease in a clinical metagenomics approach.

4.3) *Malassezia pachydermatis* and its genome

As Oxford Nanopore showed its suitable for the analysis of fungi and bacteria using long amplicons, the third and final objective was to sequence the whole genome of *M. pachydermatis* from a microbiological culture to improve the completeness of the genome reference (Triana et al. 2015).

Malassezia is one of the most-studied fungi of the skin in humans and animals. It includes a variety of species, 18 currently accepted, that are found typically in humans, mammals, and even in birds. The last three species found were from parrots and bats, suggesting that, if investigated on wild animals, new other *Malassezia* species may be discovered (Lorch et al. 2018). The genome of *Malassezia* lacks the genes coding for fatty acid synthase (Wu et al. 2015), so they need a supply of lipids to grow. Otherwise, the specific gain of lipid hydrolases could be related to its adaptation to life on the skin. As it is part of the healthy microbiota of the skin, *Malassezia* can find the lipids necessary for their development, and most of the species are considered lipid-dependent (Wu et al. 2015; Puig et al. 2017; Cabañes 2019). Among all *Malassezia* species, *M. pachydermatis* is the common one found in healthy individuals, and its interaction during skin diseases is not entirely understood yet (Gaitanis et al. 2012; Theelen et al. 2018).

Concerning the genome of *M. pachydermatis*, two groups reported genomes of the collection strain 1879 sequenced with Illumina HiSeq 2000 platform (Triana et al. 2015; Wu et al. 2015). The assembly of a whole genome can be challenging to complete with short reads to when there are repeated sequences in the chromosome because they cause different structures in the assembly graph. The use of Bandage (Wick et al. 2015) helps in the identification of these sequences. Bandage is also useful when a single query (for example, a gene) splits in multiple nodes. Contigs can also be investigated with a BLAST integration when they are not connected, or the user needs to research on the possible matches with other reported sequences from the same organism.

We compared the *M. pachydermatis* genome assembly obtained with nanopore sequencing with a built database of the reference genome for the collection strain CBS 1879. The final number of bases was similar for both technologies (≈8.2 Mb) as the genome completeness (78,6% with Illumina HiSeq and 76,6% with

Nanopore-only), while the number of contigs found in the reference genomes was higher (118 and 80 for the reported CBS 1879; 11 for Nanopore-only data). The N50 statistic value showed a better assembly quality in terms of contiguity with Nanopore data, suggesting an improvement of the genome assembly (0.64 Mbp for the CBS 1879 and 1.42 Mbp for Nanopore data). The whole reference genome matches with nine out of the eleven contigs assembled with nanopore-only corrected reads. The remaining 2 contigs represent less than 0.03% of the genome (2,909 bp) and are likely to be due to contamination.

Long nanopore corrected reads resulted in a consensus sequence suitable to de novo assemble the genome of our clinical isolate of *M. pachydermatis*, improving the reference scaffolding genome (*Genome assembly of Malassezia pachydermatis isolated from a canine otitis clinical sample; Authors: D'Andreano Sara, Viñes Pujol Joaquim, Francino Olga; DOI 10.5281/zenodo.3874381; Microbiology Resource Announcements; under revision)*. Further investigations will include a comprehensive analysis of the new assembly of *M. pachydermatis* for in-depth characterization at the gene content and functional levels.

In conclusion, when we read the word "microbiome," we usually think about bacteria communities that inhabit different niches in a host or the environment and about their capacity to interact, contribute or adapt to the host immunity in health and disease status. Most research and interest have focused on bacterial microbiota, but anywhere there is a bacterial community, there is a fungal and viral and archaea community too. The methods and approaches to detect, identify, and analyse a fungal community have significantly evolved during the years, and the fungal databases began to expand even if they still lag those for bacteria.

5. CONCLUSION

In this thesis, the investigation of fungal communities with second and third-generation sequencing permitted to highlight:

1. The region ITS2 of the fungal ribosomal operon showed better taxonomic skills respect the ITS1 for the study of fungal communities with short-read sequencing.

2. There was a strong correlation between microbiology culture-based results and next-generation sequencing ones regarding the study of otitis externa in dogs.

3. Topical treatment with corticosteroid plus a natural antimicrobial agent (pomegranate extract) was able to control the bacterial and fungal overgrowth during otitis externa in dogs. In particular, there was a drastic change in the fungal profile regarding *Malassezia pachydermatis, which was* replaced by other species and other fungal communities.

4. Next-generation sequencing analyses allowed a better understanding of the dynamics of canine otitis, linking the clinical improvement to the decrease in *Malassezia* species and the increase in fungal diversity. Natural compounds like pomegranate would be good antimicrobial alternatives for mild infection in humans and animals.

5. The comparison of nanopore long reads between the full ITS amplicon (3.5 Kb) and the full ribosomal operon (6 Kb) showed that, depending on the fungal genus, the longest amplicon provided better taxonomy assignation.

6. Nanopore long reads from the fungal ribosomal operon correctly identified *Saccharomyces cerevisiae*, *Cryptococcus neoformans*, *Malassezia* spp., *Microsporum canis,* and *Aspergillus* spp., as well as the misclassified

Candida and *Alternaria* colonies. This technology is suitable for the characterization of fungal communities in complex samples, either healthy or with clinical signs of infection, such as otitis in dogs.

7. Consensus sequence and *de novo* assembly of *Malassezia pachydermatis* with nanopore long reads resulted in a genome of 8.24 Mbp in 10 contigs (longest 1.8 Mbp, N50 1.42 Mbp), with similar completeness to the reference genomes, and improving their scaffolding up to the chromosomal level.

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