Description of the Otic Bacterial and Fungal Microbiota in Dogs with Otitis Externa Compared to Healthy Individuals

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ABSTRACT

DESCRIPTION OF THE OTIC BACTERIAL AND FUNGAL MICROBIOTA IN DOGS WITH OTITIS EXTERNA COMPARED TO HEALTH INDIVIDUALS

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Otitis externa is a common multifactorial disease with an incidence in dogs as high as 10-20%. The diversity of the cutaneous microbiota in dogs appears to decrease in diseased states. However, little is known about the microbiota of the canine ear and how it is altered by disease. The objective of this study was to compare the otic bacterial and fungal microbiota in dogs with otitis externa versus controls. Samples were collected from 30 dogs with clinical and cytological evidence of otitis externa and ten clinically normal dogs. DNA from each sample was isolated and Illumina sequencing of V4 hypervariable region of the 16S rRNA gene and the ITS region amplicons was performed. Sequences were processed using the bioinformatics software MOTHUR. The otic microbiota is much more complex than has been identified with previous culture-based studies, and otitis externa is accompanied by broad and complex differences in the microbiota.

DECLARATION OF WORK PERFORMED

All work contained in this manuscript was performed by Juraj Korbelik, with the exception of:

- Approximately 30% of sample processing (DNA extraction, PCR amplification and attachment of Illumina primers) was performed by a lab technician, Joyce Rousseau.
- Illumina Next-generation sequencing was conducted by the staff at the Advanced Analysis Centre – Genomics Facility at the University of Guelph.

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CHAPTER ONE: LITERATURE REVIEW

1.1. Introduction

The microbiota is the vast microbial population that colonizes the body. It outnumbers the host's cells by a factor of approximately 10.[1] It is estimated that approximately 1 billion bacteria inhabit a typical square centimetre of skin.[2] By virtue of the size of this microbial population, and increasing evidence of its close interaction with the host, some have referred to the microbiota as our 'second genome'.[3] The microbiota and the sum of its genetic materials (the microbiome) interact with the host through a complex and often poorly understood mechanism, playing a critical role in health and disease.[4] The microbiota has a profound influence on physiology and nutrition, and is crucial for the life of the host.[4] Beneficial microorganisms aid in digestion, prevent establishment of pathogens, interact with the immune system and produce anti-inflammatory factors, pain-relieving compounds, antioxidants and vitamins.[3] Harmful bacteria can produce toxins, initiate inflammation, produce mutagens and have negative local and systemic effects. In humans, the microbiota has been implicated as playing a role in a wide range of diseases, including inflammatory bowel disease (IBD), psoriasis, atopic dermatitis (AD), various immune-mediated disorders, obesity, metabolic disorders, neurocognitive disorders and neoplasia.[5-11] While study has been more limited in dogs, the microbiota has been associated with AD, IBD, neoplasia and obesity, among others.[12-15]

1.2. Assessment of the microbiota

Until recently, many of the bacterial and fungal species on the skin could not be analyzed using culture-based methods, limiting our existing knowledge.[1,16] The development of molecular techniques to identify and quantify microbial organisms has revolutionized our view of the microbial world.

There are two main genomic approaches to microbiota assessment, amplicon-based and shotgun. Amplicon based assessment involves amplification of a consequence gene and sequencing of the PCR products. This typically relies on sequencing and analysis of 16S ribosomal RNA (rRNA) genes. These genes are present in all bacteria and Archaea but not in eukaryotes. The 16S rRNA gene contains variable regions, which allows for taxonomic classification.[16] The sequencing of these genes means an organism does not need to be cultured to be identified[16] and has revealed highly diverse microbiota that previously has not been demonstrated by conventional methods.[17-19] A critical step of 16S rRNA gene analysis is the choice of primers.[20] Using suboptimal primers can lead to under-representation or even exclusion of some species or groups. Consequently, this can lead to questionable conclusions on community membership.[20] The most commonly used primer pair for Bacteria is S-D-Bact-0564-a-S-15/S-D-Bact-0785-b-A-18.[20] This primer pair has high overall coverage (89.0%) and reasonable domain specificity. It only fails to detect four rare bacterial phyla (Chlorofexi, Elusimicrobia, BHI80-139 and Candidate division OP11).[20]

Ribosomal RNA gene based sequencing can detect the predominant members in a community but may not be sensitive enough to detect the rare members of a community.[21] Primer bias and low depth of sampling, which could account for some of these limitations, can be improved using shotgun sequencing of the whole genome, if adequate depth is used.[21] This strategy is not limited by sequence conservation or bindings site variability within a specific gene target.[21] Whole genome shotgun sequencing allow for identification of many different microbial genes which encode many different metabolic and biochemical functions, allowing for the assessment of similar biological functions or community phenotypes.[21] A limitation of this technique is the potential for contamination of the host's genetic material and high numbers of genes with unknown function or genes which lack annotation.[21] Additionally, this method requires a relatively large quantity of starting DNA and is ill suited for studies with low microbial burden, where obtaining a large amount of material may be difficult.[21] Further, greater sequence depth is required, resulting in added cost and complexity.

Evaluation of the fungal microbiota is more challenging. For fungi, two phylogenetic markers within the rRNA region are widely used for genomic characterization: 18S rRNA gene and Intervening Internal Transcribed Spacer (ITS) regions.[22,23] These act as conserved regions and as binding sites for polymerase chain reaction (PCR) primers yet, like 16S rRNA genes, have interspersed hypervariable regions that allow for differentiation (see Figure 1.1).[24] Early investigations of the mycobiota targeted the 18S rRNA gene, which is analogous to the 16s rRNA gene in prokaryotes.[24] However, this gene is less discriminatory for fungi when compared to the bacterial 16s rRNA gene and

does not allow accurate classification of fungi to the genus or species level.[24] The two ITS regions (ITS1 and ITS2) flank the 5.8s rRNA gene and are post-transcriptionally removed. Since they are dispensable for ribosomal function, they experience less evolutionary pressure. This leads to higher sequence variability allowing for discrimination between closely related taxa.[24]

Another key step when using molecular techniques is efficient and unbiased DNA extraction.[25] Kennedy *et al*. (2014) highlighted the importance of ensuring that all samples being analyzed are prepared using the same DNA extraction method as different kits can have significantly different DNA yields.[25] Compositional analysis between two commercially available kits used to extract DNA from stool samples showed different proportions of certain bacterial genera between the kits.[25] DNA extraction methods which rely on enzymatic treatment without physical disruption tend to give reduced recovery of Gram-positive organisms and artificially elevate numbers of Gram-negative organisms.[26] This is presumably because Gram-negative organisms are more easily lysed.[26] Since almost all metagenomic studies require extraction of DNA as a first stop, difference at this point will influence downstream results. Therefore, caution is needed when comparing studies using different extraction methods.[25] Kennedy *et al*. suggests that studies, which have used different extraction kits, should not be considered cross-comparable.[25] This highlights the need for standardization of metagenomic analyses, so that results between studies are comparable.

1.3. Characterization of the cutaneous bacterial microbiota in humans

The human skin is colonized by a wide array of bacteria, many of which are beneficial and symbiotic.[2] It is generally accepted that the cutaneous microbiota is split into two groups: resident and transient microbes. Resident microbes are a relatively fixed group of microorganisms that are routinely found on the skin and re-establish themselves if disrupted.[16] They are typically considered to be commensal and may provide some benefit to the host.[16] Transient microbes do not establish themselves permanently on the skin surface, but can be present for hours to days.[16] Resident and transient microbes are not pathogenic under normal conditions.[16] However, if the normal resident microbiota, skin barrier function or immune response is disrupted, resident and transient bacterial populations can colonize and proliferate, resulting in disease.[16] Which microbes are transient and which are resident is difficult to determine as the skin is constantly exposed to the environment.[16] Additionally, next generation sequencing cannot distinguish between dead and living organisms, thus molecular studies may technically be assessing species which have historically contributed to the microbiota.[16] The human skin is an ecosystem harbouring bacterial species from more than 19 distinct phyla.[17,27] The four most common phyla are Actinobacteria, Firmicutes, Proteobacteria and Bacteroidetes.[17,27] Three distinct microenvironment types have been described: (i) sebaceous, such as on the manubrium, (ii) moist, such as the axillary vault and (iii) dry, such as on the volar forearm.[16,27] Each of these microenvironments harbours a different bacterial microbiota, with *Propionibacterium* spp. (Phylum Actinobacteria) and *Staphylococcus* spp. (Firmicutes) predominating the sebaceous sites, *Corynebacterium* spp. (Actinobacteria) predominating the moist sites and a mixed

population with the greatest prevalence of Betaproteobacteria (Proteobacteria) and Flavobacteriales (Bacteroidetes) dominating in the dry sites (see Figure 1.2).[27] In contrast to adults, infants are colonized predominantly by Firmicutes, followed in relative abundance by Actinobacteria, Proteobacteria, and Bacteriodetes. The difference in the colonization of adults versus infant skin is most likely due to the state of structural development and composition of skin in infants.[18] Many other factors can influence the skin microbiota. For example, a study by Leung *et al*. (2015) showed that skin microbiota varies between individuals of different racial groups.[28] Both community membership and structure contributed to differences in microbial communities between racial groups.[28] Additionally, it was found that individuals living in the same household had similar skin microbiota when compared to individuals living in different households.[28] However, it is not clear from this study whether the differences in microbiota observed are due to race or due to differences in environment exposure, diet and lifestyle, which are also known to influence cutaneous microbiota.[28]

Very little study has been performed specifically on the otic bacterial microbiota in humans. When the external auditory canal is sampled in humans, it is typically grouped under the sebaceous microenvironment.[17,27] As such, it is predominantly colonized by propionibacteria and staphylococci.[17,27] Taxa likely derived from earwax, such as Carnobacteriaceae and Bifidobacteriales, were also noted.[17] Chan *et al*. (2017) sampled the external auditory canal and middle ear of children with otitis media with effusion.[29] Next generation sequencing of these samples revealed that *Alloiococcus* (58.0%), *Staphylococcus* (20.8%), *Pseudomonas* (3.2%), *Corynebacterium* (2.7%) and

Enterobacteria (1.4%) were the most common genera found in the externa auditory canal (41). *Alloiococcus* (37.5%), *Haemophilus* (14.4%), *Moraxella* (10.0%), *Staphylococcus* (8.2%), *Streptococcus* (3.8%), *Corynebacterium* (3.1%) and *Pseudomonas* (3.0%) were the most common genera found in the middle ear effusion.[29] It was found that the external auditory canal (along with the nasopharynx) could serve as a reservoir for microbiota of the middle ear.[29]

1.4. Characterization of the cutaneous fungal microbiota in humans

To date, very few studies have characterized the fungal microbiota (mycobiota) of human skin using next generation sequencing.[30] In contrast to bacterial diversity, which is more dependent on site physiology (eg. sebaceous, dry, or moist), fungal community structure more strongly correlates with the site location.[30] Significantly greater diversity was observed on the feet in both number of genera observed and variation between individuals.[30] Fungal community structure was stable over time at the core and arm body sites, but not on the feet.[30]

In three studies, *Malassezia* spp. predominated at the majority of sites sampled.[30-32] All three studies found that *M. restricta* and *M. globosa* were the two predominant species with variation between body sites.[30-32] In one study, 11 of the 14 known *Malassezia* spp. were identified amongst body sites.[30] *M. restricta* predominated in the external auditory canal, retroauricular crease and glabella, while *M. globosa* was more common on the back, occiput and inguinal crease.[30] Additionally, in two studies, the percentage of *Malassezia* carriage was higher in healthy individuals than those with atopic dermatitis[31]

or psoriasis.[32] Additionally, Oh *et al*. (2013) found elevated fungal diversity and increased representation of opportunistic fungi, such as *Candida* and *Aspergillus* spp. in patients with primary immunodeficiencies.[33] A single study characterized the mycobiota of human the scalp.[12] Basidiomycota (*Filobasidium* spp.) was the most common phylum associated with individuals afflicted with dandruff while Ascomycota (*Acremonium* spp.) was common in individuals with healthy scalps.[12]

Several studies suggest that the skin may act as a reservoir for the recurrent fungal infections observed in immunocompromised individuals.[31,33] However, this is yet to be proven. Several studies have found that fungal diversity on the skin increases in disease states.[31-33] This differs from findings in bacterial microbiota in which a decreased level of diversity is noted in individuals with AD.[6,35]

1.5. Characterization of the cutaneous bacterial microbiota in dogs

As in humans, very few studies have examined the bacterial microbiota of canine skin. Recent studies that analyzed species diversity using molecular methods have demonstrated that the skin microbiota of dogs is much richer and more diverse than previously reported by culture-based methods.[13,35] One study identified bacteria from 17 different phyla on the skin of healthy dogs.[13] The most abundant phyla from all sites sampled were Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes and Cyanobacteria (see Figure 1.3). While Proteobacteria were the most abundant at all sites sampled, the relative order of the other predominant phyla changed according to the site tested.[13] At the class level, more diversity was noted between sites. Family

Oxalobacteriaceae (Proteobacteria) was the most abundant group sampled. *Ralstonia* spp. was the most abundant genus identified, ranging from 5 to 35% of all taxa identified.[13] *Bacillus*, *Staphylococcus*, *Alicyclobacillus* and *Streptococcus* were some of the most predominant genera sampled but composition varied greatly between sites.[13] In another study, the predominant bacteria sampled from the skin were *Porphyromonas*, *Staphylococcus*, *Streptococcus*, *Propionobacterium* and *Corynebacterium* spp.[35] In previous culture-based studies, the most common bacteria were found to be *Micrococcus* spp., coagulase-negative staphylococci, alpha-hemolytic streptococci, *Clostridium* spp., *Propiobacterium acnes, Acinetobacter* spp. and Gram-negative aerobes.[36] Another study found *Micrococcus* spp., Gram-negative aerobes, *Bacillus* spp. and *Staphylococcus pseudintermedius* to be the most common bacteria isolated.[37]

High variability was discovered between dogs and between sites on the same individual.[13] In this study, it was not stated whether variability was greater between individuals or between sites tested. In humans, there is greater variability between individuals then between sites sampled.[27] Higher numbers of bacterial species were found on haired skin than non-haired skin.[13] Bacterial richness and diversity varied greatly between body sites, with the nostril and conjunctiva harbouring the fewest and the dorsal nose the greatest number of bacteria taxa.[13] It has been shown in humans that the microbiota of neonates differs to that of adults.[18] In a preliminary study in dogs, results suggest that the microbial community is not influenced by individual factors (e.g. breed, sex, age, environmental, ectoparasites);[13] however, the youngest patient

sampled in this study was 8 months old.[13] Human studies also indicate that environmental and individual factors do affect cutaneous microbiota.[28]

1.6. Characterization of cutaneous fungal microbiota in dogs

Until recently, only culture-based studies had been performed to identify fungal organisms on the skin and ears of dogs.[38-42] One study only focused on the distribution of *Malassezia* spp. at different body sites[38] while the other identified the presence of additional fungi including *Alternaria*, *Cladosporium* and *Aspergillus* spp.[39] Three studies strictly sampled the ears of dogs that were either healthy or had otitis externa.[40-42] In these studies, the only fungal organisms cultured in affected ears were *Malassezia*, *Candida* and *Aspergillus* spp.[40,41] The only fungal organism cultured from the ear of healthy dogs in one study was *M. pachydermatitis*.[40] However, a more recent study identified *Penicillium* spp. in 64% of normal ears cultured and *Aspergillus* spp. in 19% of ears cultured.[42] The remaining 17% comprised of various other saprophytic fungal organisms.[42]

To date, there has been very limited study describing the cutaneous mycobiota of dogs using next generation sequencing. One study analyzed the mycobiota from 10 sites in healthy dogs and 6 sites from dogs diagnosed with allergic disease.[43] As with bacterial microbiota, the number of fungal species found was much higher than with previous culture-based techniques.[43] The most plentiful fungi genera present on canine skin, on all body sites, were *Alternaria* and *Cladosporium*, with *Malassezia* and *Epicoccum* being the third and fourth most plentiful fungi present in the ear canal (see Figure 1.4).[43]

Unlike the bacterial microbiota, the mycobiota does not appear to be influenced by body site in healthy skin.[43] The exception was the mucosal sites, which had significantly reduced species richness.[43] This is similar to the findings of Rodrigues Hoffmann *et al*. (2014), which also found decreased bacterial microbiota richness and diversity at mucosal sites.[13] The mycobiota from body sites in healthy dogs tended to be similar within a dog but a high degree of inter-dog variability existed.[43] Human skin also exhibits a high degree of interpersonal variability; however, the human cutaneous mycobiota was more dependent on body site.[30] The authors of this canine study postulated that this could be due to several factors, such as the lack of distinct microenvironments in canine skin, differences in skin pH and lipid content between human and canine skin, and increased environmental exposure and decreased bathing in dogs.[43]

1.7. The effect of disease states on cutaneous and otic microbiota

Otitis externa is a common multifactorial disease affecting 1-2.5% of people a year[44], while the prevalence in dogs has been reported as high as 10% to 20%.[45,46] Additionally, allergic dermatitis and otitis externa were the top two reasons for claims in dogs for 2016 at VPI Pet Insurance, one of the biggest pet insurance companies in the U.S.A.[47] Primary causes of otitis externa include hypersensitivity and autoimmune disorders, endocrine diseases, otic parasites, foreign bodies and conformational defects.[48] The otic microbiota can act as secondary opportunists and complicate otitis externa.[48] In both species, a common underlying cause of otitis externa is AD.[44,49] AD is a common inflammatory condition of the skin that affects 10% of children[6] and 10% of

dogs.[50] Different mechanisms have been proposed to be the cause of alteration in cutaneous microbiota of humans with AD and psoriasis. These include reduced levels of antimicrobial peptides, increased expression of extracellular matrix proteins, alterations in epidermal barrier function and Toll-like receptor 2 mutations.[51] Mutations in genes encoding filaggrin, a protein that contributes to epidermal barrier function, may also contribute to AD.[52] Recent studies in an Adam17-deficit mouse model suggest that *S. aureus* drives lesion formation.[53] Toxins produced by *S. aureus* are hypothesized to trigger or exacerbate AD.[54] A recent study showed that delta-toxins produced by *S. aureus* induce mast cell degranulation and promote inflammation of the skin.[55] Studies in humans with AD have shown a predominance of *S. aureus* during active flares, strengthening the theory that *S. aureus* and alterations in skin microbiota play a role in inflammation.[6] Infections with *S. aureus* correlates with clinical severity of atopic dermatitis flares in humans.[56] Allergic flares in dogs with AD are associated with superficial infection by *S. pseudintermedius* and *S. schleiferi*.[57,58]

In many cutaneous diseases, it is unclear if the condition is caused by an alteration in the cutaneous microbiota or if the alterations are the result of the disease itself.[59] Capone *et al.* (2011) showed that samples from children with AD had a lower microbial diversity during flares of AD when compared to baseline samples.[18] Similarly, skin samples from dogs with AD showed lower diversity and species richness when compared to samples from healthy dogs.[13] Diversity, a measure that takes into account the number of taxa present and their abundance in the community, was also significantly lower in dogs with AD.[35] Although similar taxa were observed in samples

from healthy and allergic dogs[13,35], taxa that represented less than 1% of the skin microbiota in healthy dogs were often absent in allergic dogs[13]. Significant differences between allergic and healthy dogs were identified for a few taxa. The proportions of the betaproteobacterium *Ralstonia* spp. were significantly lower in samples from allergic dogs when compared to healthy dogs and accounted for less than 0.02% of the total taxa identified.[35] *Ralstonia* spp. made up 5 to 35% of total taxa in healthy dogs.[13]

Results from studies using culture-based methods showed that the skin and nasal mucous membranes of humans with AD are more often colonized with *Staphylococcus aureus* than healthy individuals. [6,50] Similarly, the skin of dogs with AD is more likely to be colonized with *S. pseudintermedius*, when compared to controls.[57,58] These findings were similar to data from more recent studies using next generation sequencing indicating that *S. aureus* dominates the affected skin regions in children with AD.[6] Baseline and post-flare samples from children with AD also had a higher relative abundance of *S. aureus* compared to the skin of healthy children.[6] The findings are similar in dogs: one study found that dogs with AD had significantly increased relative abundance of *Staphylococcus* spp. when compared to control across all sampled sites.[35] This differs from the results of another study which did not show a relative increase in abundance of *Staphylococcus* spp. between dogs with AD and controls.[13] Bradley *et al.* (2016) also found that there was a decreased abundance of *Porphyromonas* spp. in samples taken from the pinna and axilla of dogs with AD when compared with normal dogs.[35] In the groin there was a significant median increase in the relative abundance of *Corynebacterium* spp. in dogs with AD vs controls.[35]

As with bacterial microbiota, the mycobiota of dogs with AD was significantly less rich than that of healthy skin.[43] This differs from one previous study that found increased fungal diversity in lesional skin of humans with AD.[33] The genera *Blumeria*, *Wallemia*, *Candida*, *Schizophyllum* and *Exserhilum* were increased in the dogs with healthy skin while *Sporobolomyces*, *Hydnum*, *Irpex*, *Periconia*, *Cochliobolus* and *Microascales* were increased in dogs with allergic skin.[43] Although *Malassezia* has been implicated as an allergen and a trigger in both human and canine AD,[31,60,61] no significant difference was detected in the relative abundance of these fungi between healthy and allergic dogs.[43] However, 50% of samples from ears of allergic dogs were predominated by the genus *Malassezia* or *Sporobolomyces.*[43]

As 'normal' (and its variations) are starting to be defined and changes that occur in disease states are being described, there is increasing interest in factors that modify the cutaneous microbiota. The impact of antimicrobials on the cutaneous microbiota has received very limited study. Kong *et al.* (2012) found that children receiving intermittent treatment with topical medication (antibiotics, corticosteroids or calcineurin inhibitors) in the previous 7 days or oral antibiotics in the previous 4 weeks had markedly increased bacterial diversity when compared with patients receiving no treatment during flares of AD.[6] These findings were similar in dogs with AD, with a recent study showing that treatment with oral antibiotics during atopic flares restored microbial diversity. No statistical difference was found in the diversity of cutaneous microbiota between controls and atopic dogs after 4-6 weeks of treatment with systemic antibiotics.[33]

Interestingly, significant differences re-emerged 4-6 weeks post treatment suggesting that antimicrobial therapy restores diversity of the skin microbiota in atopic dogs but that the effects dissipates once treatment is stopped.[35]

Mitigation of dysbiosis with antimicrobials clearly ameliorates disease severity; however, methicillin- and multi-drug resistance are now commonplace in both veterinary and human medicine.[35] Although resistance to antifungals is still relatively uncommon, there is evidence that it too is emerging in both humans and animals.[62-64] Understanding the impact of disease states on the otic microbiota is important for several reasons: (i) to understand how the microbiota responds to insults, (ii) to understand the pathophysiology of diseases affecting the skin, (iii) to identify specific changes that might be markers of microbiota disruption as aids for diagnosis or for selective therapeutic repopulation. In the future, these findings may provide information on developing non-antimicrobial approaches to controlling otitis externa in skin disease.

The aim of this study was to describe the otic bacterial microbiota and mycobiota of healthy dogs and compare it to that of dogs with otitis externa. Our hypothesis was that the alpha diversity of the otic microbiota and mycobiota would be reduced in dogs with otitis externa when compared to healthy controls. Additionally, we hypothesized that beta diversity would also be altered in dogs with otitis externa when compared to healthy dogs.

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1.9. Figures

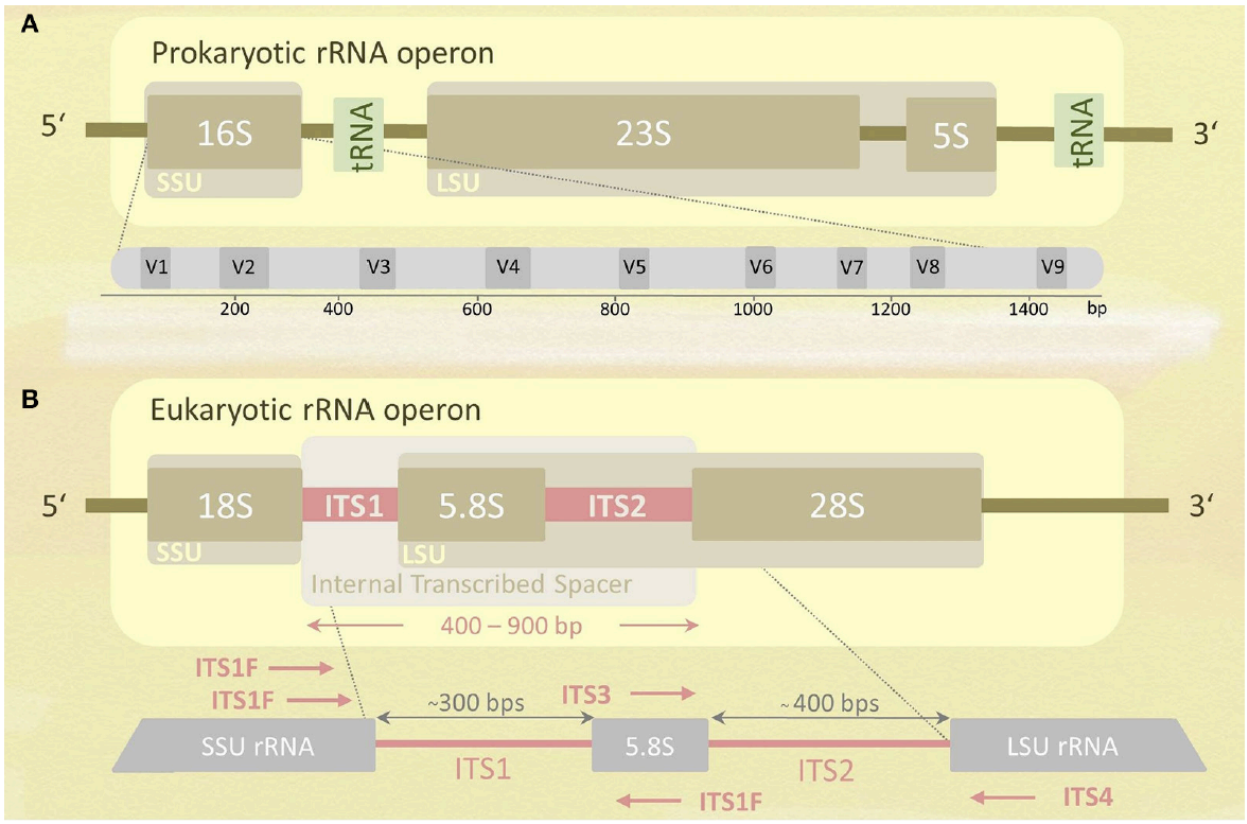

Figure 1.1. Schematic representations of rRNA operons. (A) Prokaryotic and (B) eukaryotic rRNA operons. Position and orientation of oligonucleotide primers used for ITS amplification are schematically indicated. SSU, small subunit; LSU, large subunit; tRNA, transfer RNA; V1-V9, variable regions; ITS, internal transcribed spacer; bps, base-pairs. Figure from Halwachs *et al*. (2017).[24]

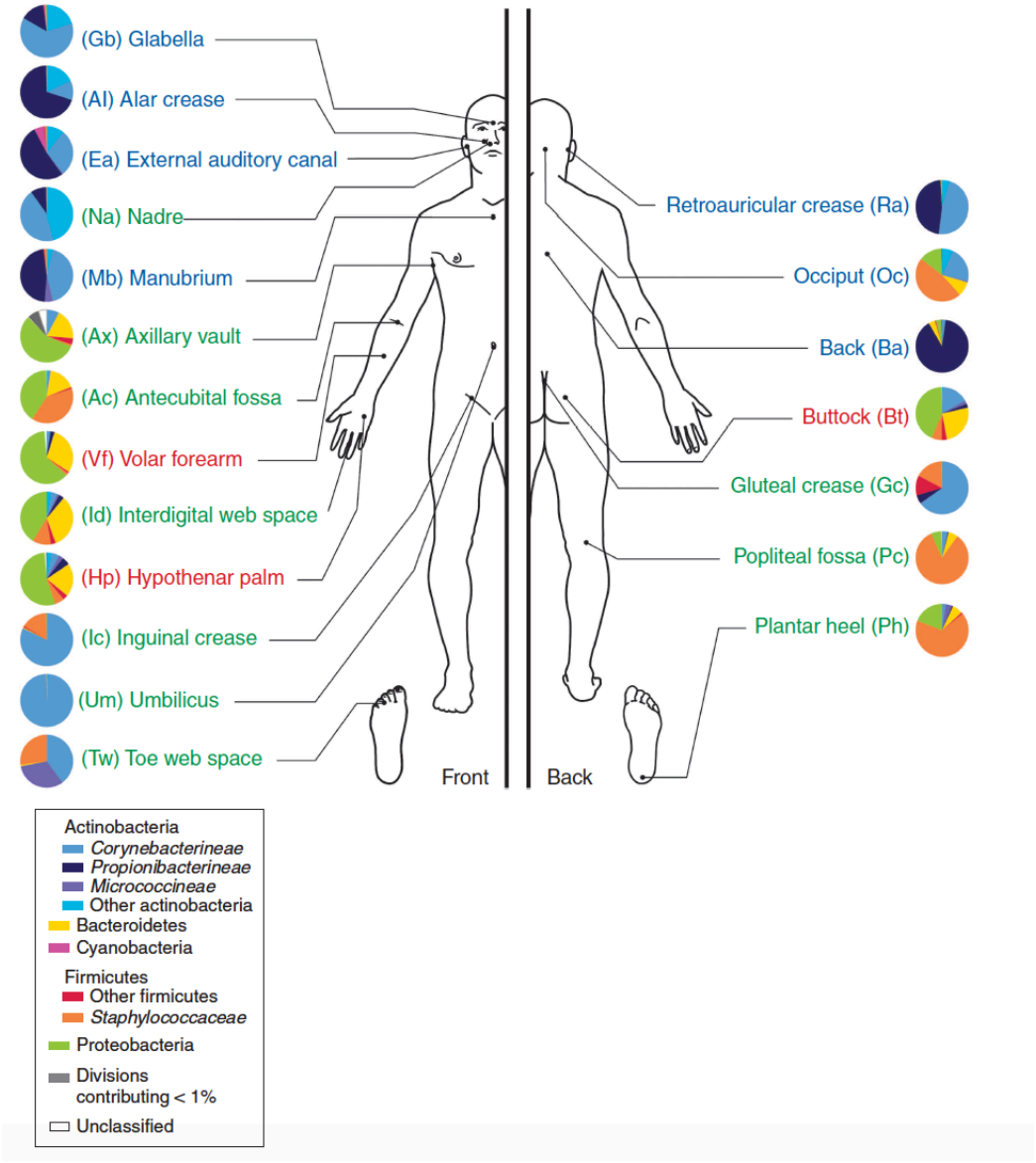

Figure 1.2. Topical distribution of bacteria on the skin: Sebaceous sites are labelled in blue, moist sites are labelled in green and dry sites are labelled in red. Figure from Kong *et al*. (2011)[16], data from Grice *et al*. (2009).[27]

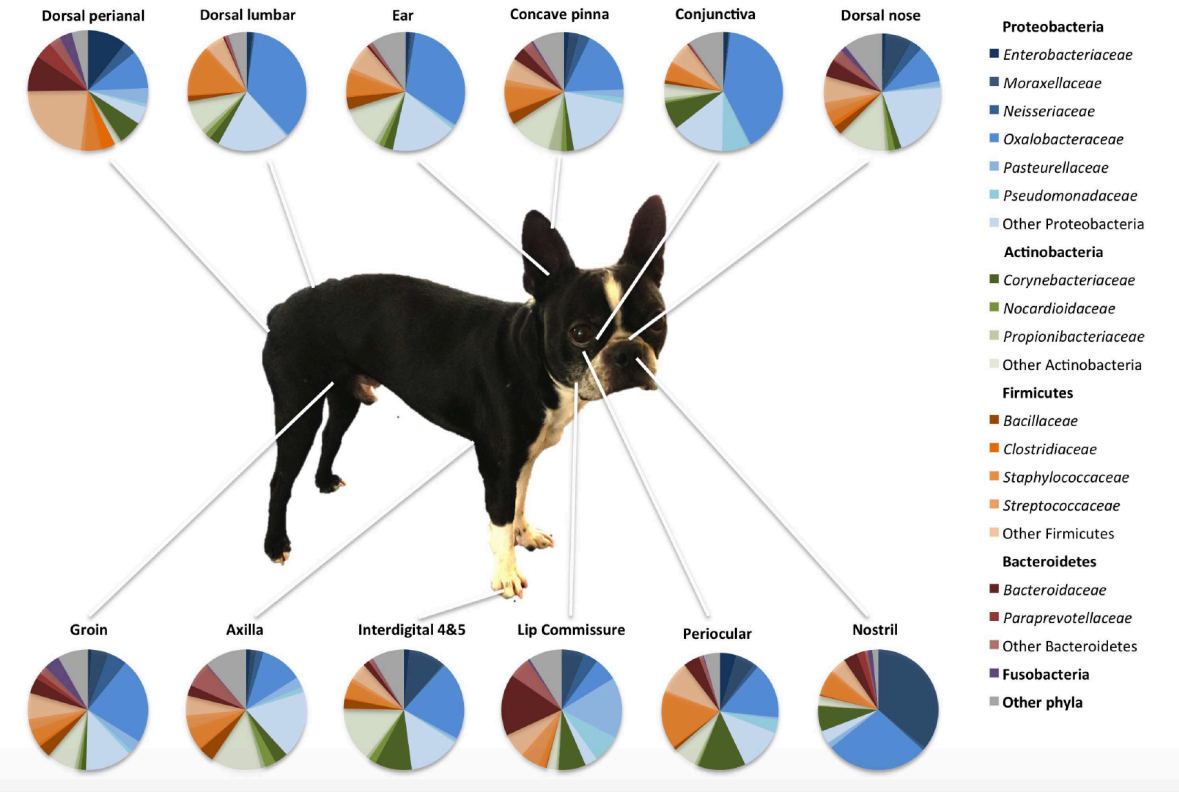

Figure 1.3. Bacterial phyla and families in healthy dogs. Average of most common bacterial phyla and families identified in different sites in the skin of healthy dogs. Figure from Rodrigues Hoffman *et al*. (2014).[13]

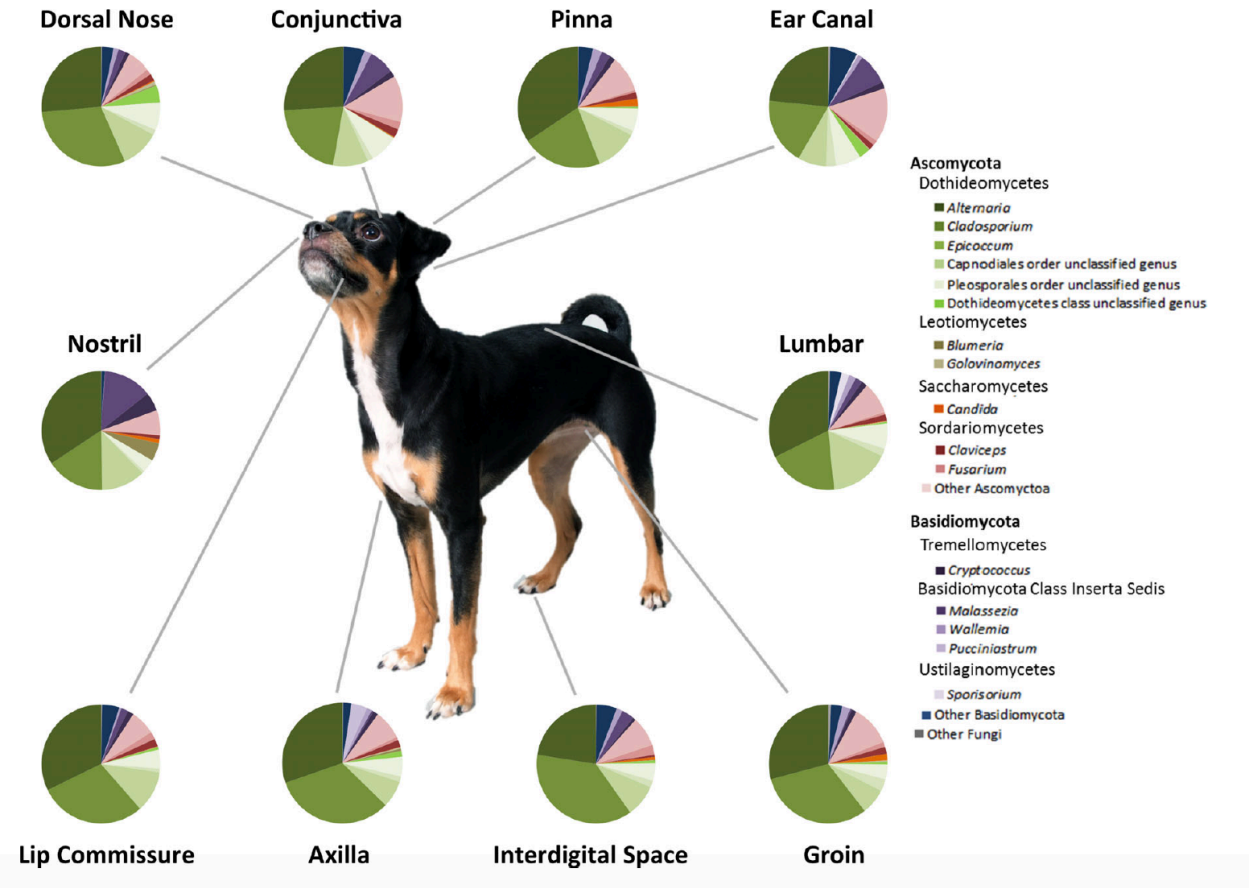

Figure 1.4. Average relative abundance of fungal taxa by body site in healthy dogs. Figure from Meason-Smith *et al*. (2015).[43]

CHAPTER TWO: CHARACTERIZATION OF THE OTIC BACTERIAL MICROBIOTA IN DOGS WITH OTITIS EXTERNA COMPARED TO HEALTHY INDIVIDUALS

2.1. Introduction

Otitis externa is a common multifactorial disease with a prevalence as high as 20% in dogs. [1,2] Primary causes of otitis externa include hypersensitivity and autoimmune disorders, endocrine diseases, otic parasites, foreign bodies and conformational defects.[3] The otic microbiota can act as secondary opportunists and complicate otitis externa.[3] In dogs, a common underlying cause of otitis externa is AD.[4,5] AD is a common inflammatory condition of the skin that affects 10% of dogs.[6] Different mechanisms have been proposed to be the cause of alteration in cutaneous microbiota of humans with AD and psoriasis. These include reduced levels of antimicrobial peptides, increased expression of extracellular matrix proteins, alterations in epidermal barrier function and Toll-like receptor 2 mutations.[6] In many cutaneous diseases, it is unclear if the condition is caused by an alteration in the cutaneous microbiota or if the alterations are the result of the disease itself.[7]

Until recently, many of the bacterial species on the skin and in the ears could not be analyzed using culture-based methods, limiting our existing knowledge.[8,9] The recent development of molecular techniques to identify and quantify microbial organisms has revolutionized our view of the microbial world. Recent studies, which analyzed species diversity using molecular methods, have demonstrated that the skin microbiota of dogs is much richer and more diverse than previously reported by culture-based methods.[10,11]

Results from studies using culture-based methods showed that the skin of dogs with AD is more likely to be colonized with *S. pseudintermedius*, when compared to controls.[12,13] These findings were similar to data from more recent studies using next generation sequencing indicating that dogs with AD had significantly increased relative abundance of *Staphylococcus* spp. when compared to controls across all sampled sites.[11] Another study found that there was a decreased abundance of *Porphyromonas* spp. in samples taken from the pinna and axilla of dogs with AD when compared with normal dogs.[11] In the groin there was a significant median increase in the relative abundance of *Corynebacterium* spp. in dogs with AD versus controls.[11]

To date, very few studies have assessed the otic microbiota in dogs. The aim of this study was to investigate how the otic microbiota of dogs with otitis externa is altered compared to clinically normal dogs. Methicillin- and multi-drug resistance are now commonplace in both veterinary and human medicine.[11] Understanding the impact of disease states on the otic microbiota is important to identify specific changes that might be markers of microbiota disruption as aids for diagnosis or for selective therapeutic repopulation. In the future, these findings may provide information on developing non-antimicrobial approaches to controlling otitis externa.

2.2. Materials and Methods

2.2.1. Study population

All animals for this study were recruited through the Guelph Veterinary Specialty Hospital following a protocol approved by the University of Guelph's Animal Care

Committee. Thirty dogs that presented to the Dermatology Department with clinical and cytological evidence consistent with otitis externa were enrolled in the study. Clinical signs consistent with otitis externa included at least one ear with evidence of erythema and discharge from the external ear canal. In order to be included in the study, at least one ear must have had cytological evidence of infectious otitis externa. This was described as having greater than or equal to 2+ bacteria or yeast on a semi-quantitative assessment scale.[14] All dogs were examined by the same dermatology resident. Dogs were excluded if they had received any topical ear treatments or cleaners within the two weeks previous to sampling or systemic antifungals or antibiotics within the three months previous to sampling.

For the control group, ten dogs that were presented to the Guelph Veterinary Specialty Hospital Surgery Department for elective orthopedic procedures were enrolled. In order to be enrolled in the study, the dogs must not have received any systemic antimicrobials within the last three months and had not received any topical ear treatments or cleaners in the previous 2 weeks. Dogs with any prior history of ear or skin disease were excluded from the study. Each dog was examined by a dermatology resident for evidence of otitis externa or signs of skin disease. This included erythema, discharge from the ears, alopecia, excoriations, hyperpigmentation of the skin or papular or pustular eruptions. Dogs with any signs of skin or ear disease were excluded from the study. Otic cytology was performed from both ears. Any evidence of yeast or bacteria (1+ or greater) on cytology would lead to exclusion from the study.

2.2.2. Sample collection and DNA extraction

Samples were collected from affected and control dogs using a regular tipped culture swab (ESwab Regular Collection Kit, BD Diagnostics™, Franklin Lakes, NJ, USA). Each swab was inserted into external ear canal up to the junction between the vertical and horizontal canals. The swab was then rotated 360 degrees before being withdrawn. Both ears were sampled separately in all dogs. Each sample was labeled and stored at 4°C for no longer than 7 days before being transferred to a -80°C freezer until analysis.

DNA was extracted from each sample using the MoBio Power Soil DNA Extraction kit, following the manufacturer's protocol (Qaigan Inc., Toronto, ON, Canada). DNA quantity and quality was assessed by spectrophotometry (NanoDrop, Roche, Mississauga, ON, Canada).

2.2.3. Amplification and sequencing

In order to assess the bacterial microbiota, PCR amplification of the V4 hypervariable region of the 16S rRNA gene using the forward primer S-D-Bact-0564-a-S-15 and reverse primer S-D-Bact-0785-b-A-18 was performed as per Klindworth *et al*.[15] The forward and reverse primers were designed to contain an overlapping region of the forward and reverse Illumina sequencing primers in order to anneal them to primers containing the Illumina adaptors plus the 8 base pairs (bp) identifier indices.

After amplification, the PCR products were evaluated by electrophoresis in 2% agarose gel and purified with the Agencourt AMPure XP (Beckman Coulter Inc, Mississauga, ON, Canada) by mixing 20µL of amplicon with 40µL of AMPure on a 96 well plate. After

incubating for 5 minutes at room temperature, the beads were separated and washed twice with 80% ethanol and then eluted in 32µL of 10mM Tris pH 8.5 buffer solution.

A second PCR was performed to attach dual indices and Illumina® sequencing adapters using the Nextera XT Index kit (Illumina®, San Diego, CA, USA). After purification of these amplicons, the samples were quantified by spectrophotometry using the NanoDrop® (Roche, Mississauga, ON, Canada) and normalized to a final concentration of 2nM. Normalization and sequencing of the library pool was performed at the University of Guelph's Advanced Analysis Centre using an Illumina® MiSeq (San Diego, CA, USA) and 2x250 kit.

2.2.4. Data analysis

The MOTHUR package of algorithms (v1.39.5) was used for analysis.[16] Paired end reads were aligned. Sequences >242 or less than 239 bp were removed, as well as those containing ambiguous base calls, long runs of holopolymers (>8 bp) or those that did not align with the correct region. Chimeras were detected using UCHIME and removed.[17] Sequences from Chloroplasts, Mitochondria, Archaea and Eukaryotes were removed. Sequences were binned into operational taxon units (OTUs) at a 3% (0.03) dissimilarity level using the nearest neighbour approach, and taxonomy assigned to both individual sequences and OTUs using the RDP database[18]. Subsampling was performed to normalize sequence number across samples for assessment of alpha and beta diversity to a depth of 22,979 sequences. In order to maximize sequence depth, samples that yield relatively low sequence numbers (<20000 sequences) were repeated. One ear sample was selected at random from each dog for comparison

between affected and control ears.

Richness (Chao1), evenness (Shannon's evenness) and alpha diversity (inverse Simpson's) indices were calculated. Comparison of the relative abundances of taxa at different levels (phylum through species) was performed using Wilcoxon test in JMP® (v13.2.0, SAS Institute Inc., 2016). A $P<0.05$ was considered significant for all comparisons. Adjustment for false discovery rate was performed using the Benjamini Hochberg technique[19] in R (v3.3.3, "Another Canoe", RStudio Inc., 2017). Beta diversity was assessed through the creation of dendrograms using both the Yue & Clayton measure of dissimilarity (a measure of community structure, which considers shared OTUs and their relative abundances) and traditional Jaccard coefficient (a measure of community membership, which considers the number of shared OTUs, not their abundance). Unweighted UniFrac tests were applied to evaluate the impact of otitis externa on microbial population structure.[20] Principal coordinate analysis (PCoA) was performed based on both Jaccard coefficient and Yue & Clayton measure. Linear discriminant analysis effect size (LEfSe) was used to identify OTUs that were enriched or depleted.[21]

2.3. Results

From the total of 60 affected ears sampled, 31 were removed from analysis of bacterial microbiota due to poor DNA yield or repeated inability to obtain adequate sequence numbers. Of the 20 control ears sampled, 5 were removed from analysis of bacterial microbiota due to repeatedly low number of sequences or DNA yield. This left 29

samples from 18 affected dogs and 15 samples from 8 control dogs for the analysis (Table 2.1). Dogs enrolled into the affected group (those with otitis externa) ranged from one to 10.5 years of age (median=6.25 years), and consisted of six female spayed, nine male castrated and three intact female dogs. Twelve pure bred and six mixed breed dogs were enrolled into the affected group. Four spayed females and four castrated males ranging from1 to 9 years of age (median= 5.75 years) were enrolled into the control group. This group consisted of five pure bred and three mixed breed dogs.

After quality processing, the total number of sequences from the ears of healthy dogs was 805,196 with a median of 55,852 sequences per sample (range=27,113-80,634). The total number of sequences found in affected ears was 1,706,195 with a median of 47,364 sequences per sample (range=19,451-141,643).

Bacteria from 27 different phyla were identified. In healthy ears, the predominant bacterial organisms were from the phyla Firmicutes (relative abundance 38.4-86.0%, median=71.4%), Proteobacteria (7.64-19.9%, median=11.9%) and Bacteroidetes (3.05-7.38%, median=4.97%). Within the phylum Firmicutes, the three most common genera in healthy ears were *Romboutsia* (1.17-39.3%, median=26.9%), *Megamonas* (0.545-13.6%, median=9.12%) and *Holdemanella* (1.33-8.03%, median=6.05%). Within the phylum Proteobacteria, the three most common genera were *Succinivibrio* (0.527-2.79%, median=2.08%), *Haemophilus* (0.530-2.95%, median=1.63%) and *Anaerobiospirillum* (0.027-1.58%, median=1.07%). *Prevotella* (0.088-3.23%, median=2.62%), *Bacteroides* (0.727-3.37%, median=1.77%) and *Porphyromonas*

(0.041-0.972%, median=0.142%) were the three most common genera within the phylum Bacteroidetes (Figure 2.1).

In affected ears, the phyla Firmicutes (0.531-99.7%, median=30.7%), was the most abundant, followed by Actinobacteria (0.013-79.9%, median=28.8%) and Proteobacteria (0.036-99.2%, median=4.16%). Within the phylum Firmicutes, the three most common genera were *Staphylococcus* (0-99.4%, median=3.09%), *Streptococcus* (0.008-28.6%, median=0.374%) and *Blautia* (0-0.240%, median=0.054%). The three most common genera in the phylum Actinobacteria were *Corynebacterium* (0.005-74.3%, median=10.9%), *Bifidobacterium* (0-1.76%, median=0.194%) and *Collinsella* (0-0.155%, median=0.018%). The most common genera in the phylum Proteobacteria was *Pseudomonas* (0-95.3%, median=0.674*%), Escherichia/Shigella* (0-6.56%, median=0.186%) and *Acinetobacter* (0-0.943%, median=0.009%) (Figure 2.1).

After controlling for false discovery rate, the relative abundance of 24 taxa was increased in control samples when compared to affected samples, including the phyla Firmicutes (P=0.008), Bacteroidetes (P< 0.001) and the genera *Rombutsia* (P<0.001), *Fusobacterium* (P=0.021) and *Megamonas* (P<0.001), among others (Table 2.2).

LEfSe analysis identified 164 OTUs that were differentially abundant (P<0.05). One hundred and fifty-three OTUs were over-represented in healthy ears, including the genus *Rombutsia, Anaerobiospirillum, Corynebacterium, Megamonas* and *Faecalibacterium.* Eleven OTUs were over-represented in the affected ears, including

those from the genera *Staphylococcus, Pseudomonas, Parvimonas, Bacteroides* and *Actinomyces* (Figure 2.2). Of the 153 OTUs over-represented in healthy ears, 85 OTUs (55.6%) were from the phylum Firmicutes. Of these 85 OTUs, 58 (68.2%) were from the order Clostridiales.

Observed richness (P=0.004), estimated richness (P<0.001), evenness (P=0.004) and alpha diversity (P=0.002) were significantly lower in affected ears (Figure 2.3). Unweighted Unifrac analysis indicated that there were significant differences in both bacterial community structure (P<0.001) and membership (P<0.001) between affected and control ears. These differences were depicted using PCoA plot and dendrogram that demonstrate clearly separated clusters of samples from healthy dogs and those with otitis externa (Figure 2.4).

Principal coordinate analyses were performed based on both Jaccard and Yue & Clayton coefficients to compare the bacterial community and membership structure from samples within the same dog and samples between dogs. In dogs with bilateral otitis externa, the majority of samples from the same dog were clustered close together. This depicted that the samples from the same dog were more similar to each other, than to samples from the ears of other affected dogs (Figure 2.5). In contrast to the inter-dog similarity in affected ears, ears from the same healthy dog appeared no more similar to each other than those of samples from other control dogs (Figure 2.6).

2.4. Discussion

This study identified a much richer bacterial community than had previously been identified using culture-based methods,[22,23] as was expected based on the advantages of sequence-based methods. Comparison of the microbiota of healthy dogs versus those with otitis externa identified various interesting differences. Bacterial alpha diversity was reduced in samples from ears affected by otitis externa when compared to samples from health ears. Beta diversity was also altered in affected ears when compared to health controls. This is consistent with previous studies in dogs and humans, which have shown decreased cutaneous bacterial diversity in samples from atopic individuals when compared to healthy controls.[10,11,24] This differs with studies of canine gastrointestinal microbiota which found no significant difference in diversity measure in dogs with intestinal lymphoma and inflammatory bowel disease when compared to healthy individuals.[25] These changes indicate that there were significant differences not only in the overall membership of the population, but also the distribution of those members within the microbiota. Thus, the changes present go well beyond simple changes in one or a few individual components of the microbiota.

Bacteria from 27 different phyla were identified in the ears of normal dogs. This is higher than a previous study that used next generation sequencing and identified only 17 distinct phyla from the skin and ears of dogs.[10] In this study, the most common phyla identified in normal ears were Proteobacteria (median=49.4%), Firmicutes (16.7%), Actinobacteria (16.4%) and Bacteroidetes (1.00%).[10] While our study also identified these same four phyla as the most common, the order was altered, with the phylum

Firmicutes being the most common (median=71.4%), followed by Proteobacteria (11.9%), Bacteroidetes (4.97%) and then Actinobacteria (3.00%). Although the preliminary study found that the microbial community did not appear to be influenced by individual factors and environment in dogs,[10] several human studies indicate that these factors do affect cutaneous microbiota.[26,27] This may explain the difference in findings between our two studies. Additionally, this study employed primers which encoding the V1-V3 region of the 16s rRNA gene, used 454-pyrosequencing and assigned taxonomy using a different database than our study.[10] These dissimilarities in methodology could also account for differences between our studies.

The predominance of Firmicutes, and most notably Clostridiales, as over-represented taxa in normal ears in LEfSe analysis suggests that these may either be important determinants of aural health or taxa that are most readily disrupted during otitis externa. Unfortunately, due to the cross-sectional nature of this study, the cause versus effect cannot be discerned.

A comparison of relative abundance between affected and control ears identified that bacteria from the genus *Porphyromonas* were increased in samples from normal ears when compared to those affected with otitis externa. A recent study also found that the genus *Porphyromonas* was preferentially decreased on the pinna and axillae of dogs with AD.[11] Bacteria from the genus *Porphyromonas* are anaerobic, gram-negative bacilli.[28] Some species are commensals of the bovine rumen while others have been implicated in respiratory, mucosal, dental and skin infections in both humans and

animals.[28] The significance of this genus' presence in the healthy canine ears, and it's absence from those affected with otitis externa, is unknown at this point. The genus *Porphyromonas* may be important indicators for the presence of a bacterial dysbiosis due to a disease state.

Bacterial from the genera *Staphylococcus* and *Pseudomonas* are commonly associated with otitis externa in dogs.[22,23] These two genera were identified by LEfSe analysis to be over-represented in affected ears. As a cross-sectional study, we could not discern whether proliferation of these genera, or the depletion of normal bacterial flora and subsequent replacement with these genera, was the driving force for the development of otitis externa. Bacteria from these two genera are important causes of otitis externa for separate reasons. Infections with *Staphylococcus spp.* in humans promote inflammation of the skin.[29] Delta-toxins produced by *S. aureus* induce mast cell degranulation and promote inflammation of the skin.[29] Studies in humans with AD have shown a predominance of *S. aureus* during active flares, strengthening the theory that *S. aureus* and alterations in skin microbiota play a role in inflammation.[30] Infections with *S. aureus* correlates with clinical severity of atopic dermatitis flares in humans.[31] Allergic flares in dogs with AD are associated with superficial infection by *S. pseudintermedius* and *S. schleiferi*.[32,33] A recent study also found that the genus *Staphylococcus* was preferentially increased in all sites sampled for dogs with AD.[11] Otitis externa caused by *Pseudomonas aeruginosa* has been reported to have an incidence of 11-13%.[34] It has been reported to be the most resistant microbe to cause otitis externa and is resistant to many commonly used antibiotics.[34]

Interestingly, the genera *Parvimonas* and *Fusobacterium* were increased in dogs with otitis externa. *Parvimonas micra*, the only species in this genus[35], is able to form biofilms and has been implicated in causing chronic periodontitis in both humans and dogs.[36,37] Biofilm formation is an important virulence factor that helps facilitate resistant bacterial infections within the ear canal and hamper the elimination of infections by both the immune system and antimicrobials.[38] *P. micra* acts as a catalyst for increased biofilm production.[36] In conjunction with other microorganisms (including *Fusobacterium spp*.), *P. micra* allows for the production of biofilms with proteolytic activity, which causes progressive tissue destruction and inflammation in canine periodontal disease.[36] It is possible that *P. micra* has the same effects in chronic otitis externa and as a strict anaerobe has been overlooked in culture-based studies, although further study would be needed to confirm this hypothesis.

The role of otitis externa caused by *Corynebacterium spp*. has been disputed due to its presence as part of the normal microbiota on the external ear canal and because the genus has always been reported in combination with other micro-orgasms in cases of otitis externa.[39] This study found that the genus *Corynebacterium* was increased in healthy ears. Analysis of relative abundance did not find that this genus was significantly increased in dogs with otitis externa. This would support the hypothesis that this bacterium is not pathogenic and requires the presence of other bacteria in order to proliferate.[39]

This study found that bacterial species diversity, richness and evenness were significantly reduced in samples from affected ears when compared to healthy ears. This is similar to findings of a previous study, which also found decreased bacterial richness and alpha diversity on the skin of dogs affected by AD.[10,11] This is supported by several studies in humans which also found a decrease in bacterial alpha diversity and richness in patients with AD.[30,40] In addition to the decrease in alpha diversity, there was a clear decrease in bacterial beta diversity in dogs with otitis externa. Significant alterations in both community membership and structure were present within the microbiota of healthy dogs and those with otitis externa. Therefore, the changes noted go beyond simple alterations in individual components of the bacterial microbiota but affected its entire composition.

In general, inter-individual variability is less than intra-individual variability when assessing various body sites in different species. PCoA plots to compare the bacterial community and membership structure in dogs with bilateral otitis externa indicated that samples from within the same dog were more similar to each other, than to samples from the ears of other affected dogs. Surprisingly, the same was not true with PCoA plots of healthy ears. A decrease in diversity and proliferation of a few bacterial genera in affected ears could cause samples from the same dog become more uniform and thus cluster closer together.

A limitation of this study is that several of the dogs enrolled in the affected group were receiving immunomodulatory therapy at the time of sampling. A recent study determined

that treatment with ciclosporin and corticosteroids does not significantly impact the cutaneous microbiota.[41] It is unknown whether treatment with oclacitinib, immunotherapy or antihistamines could alter the cutaneous microbiota. Additionally, we had difficulty obtaining adequate DNA and sequence yields from samples resulting in a large proportion of samples being removed from analysis. Since a larger proportion of samples were removed from the affected group (51.7%) than the control group (25%), we hypothesize that secretions in affected ears may produce a large number of PCR inhibitors, which interfere with DNA extraction. Low bacterial DNA burdens in control samples may be responsible for poor DNA and sequence yields. This lead to the analysis of samples from only a subset of the enrolled population and it is unclear whether this could have introduced bias into this study. Additional cycles during DNA amplification may have improved DNA yield but this was avoided as it could have also introduced bias resulting in less meaningful results. Additionally, a control was not performed to ensure there was no bacterial contamination of reagents. However, contaminants would be expected to be evenly distributed amongst samples, and since the focus was comparing diseased and healthy ears, there would be little potential impact.

Next generation sequencing has revealed that the bacterial microbiota is much more complex than previously identified using culture-based studies. Reduced bacterial richness and diversity is present in ears affected with otitis externa indicating that a dysbiosis is present. This study did not discriminate between different underlying causes of otitis externa and further study is needed to investigate whether alterations in

the microbiota are present in otitis externa caused by different primary disease states.

2.5. References

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2.6. Tables

Table 2.1. Signalment, cytology results and concurrent medications of the affected (n=18) and healthy dogs (n=8) in which samples were sequenced

Patient #	Health Status	Sex	Breed	Age (years)	Ear	Cytology	Concurrent Medication
A1	Affected	FI	Miniature Pinscher	9	left	4+ rods & cocci	none
A2	Affected	MN	Jack Russell	10.5	left	4+ yeast	oclacitinib
A3	Affected	FS	Fox Terrier	8	right	4+ rods & cocci	none
A4	Affected	MN	Mix breed	6	right	4+ cocci & rods	none
A5	Affected	MN	German shepherd	1	left	4+ rods & cocci	oclacitinib
A6	Affected	FS	Maltese	6.5	right	4+ cocci	oclacitinib
A7	Affected	FS	Chihuahua	7.5	left	4+ yeast	none
A8	Affected	MN	French Bulldog	1.5	right	2+ yeast	none
A9	Affected	FS	Boxer	1	right	3+ yeast	oclacitinib
A10	Affected	FI	Mix breed	1	left	4+ yeast	none
A11	Affected	MN	Spino Italiano	2	right	4+ rods, 3+ cocci, 1+ yeast	prednisone
A12	Affected	MN	Mix	9	left	4+ rods & cocci	prednisone
A13	Affected	FS	Mix	9	right	4+ rods, 2+ cocci	prednisone
A14	Affected	MN	Mix	5	left	4+ rods & cocci	meloxicam
A15	Affected	FI	Mix	2.5	right	4+ yeast	none
A16	Affected	MN	German shepherd	6	left	3+ cocci, 4+ yeast	none
A17	Affected	MN	Mastiff	7.5	right	4+ cocci	prednisolone/ trimeprazine tartate
A18	Affected	FS	Cocker Spaniel	7	right	4+ rods & cocci	none
C1	Healthy	FS	Mix	8	left	NSF	none
C2	Healthy	FS	Dachshund	9	right	NSF	none
C3	Healthy	MN	Burnese Mountain dog	5.5	left	NSF	meloxicam
C4	Healthy	MN	Mix	6	left	NSF	meloxicam
C5	Healthy	FS	Mix	4	right	NSF	meloxicam
C6	Healthy	MN	Pug	6	right	NSF	none
C7	Healthy	FS	Cane Corso	2.5	left	NSF	none
C8	Healthy	MN	Havanese	4	right	NSF	none

Signalment: MN male neutered, FI female intact, FS female spayed, Mix mixed breed.
Cytology results: NSF no significant findings.

Table 2.2. Relative abundance of taxa that were significantly different in the aural microbiota of dogs with otitis externa ('affected', n=18) or healthy controls ('control', n=8)

Taxonic Level	Taxon	Control median	Affected median	P-Value	Adjusted P-Value
Phylum	Firmicutes	71.4	30.7	0.008	0.023
Phylum	Bacteroidetes	4.97	0.12	<0.001	0.001
Genus	*Romboutsia*	26.9	0.018	<0.001	0.001
Genus	*Fusobacterium*	0.75	0.026	0.021	0.049
Genus	*Megamonas*	9.12	0.006	<0.001	0.001
Genus	*Faecalibacterium*	5.98	0.026	<0.001	0.001
Genus	*Catenibacterium*	5.7	0.004	<0.001	0.001
Genus	*Holdemanella*	6.05	0.035	<0.001	0.001
Genus	*Parvimonas*	0.353	0	<0.001	0.001
Genus	*Lactobacillus*	2.4	0.047	<0.001	0.001
Genus	*Bacteroides*	1.77	0.026	0.001	0.003
Genus	*Bifidobacterium*	1.41	0.194	0.005	0.015
Genus	*Parasutterella*	0.189	0	<0.001	0.001
Genus	*Clostridium sensu stricto*	1.64	0.031	<0.001	0.001
Genus	*Prevotella*	2.62	0.01	<0.001	0.001
Genus	*Haemophilus*	1.63	0.003	<0.001	0.001
Genus	*Succinivibrio*	2.08	0.008	<0.001	0.001
Genus	*Turicibacter*	1.6	0.004	<0.001	0.001
Genus	*Porphyromonas*	0.142	0.005	0.003	0.009
Genus	*Neisseria*	0.102	0	0.012	0.03
Genus	*Anaerobiospirillum*	1.07	0.006	<0.001	0.001
Genus	*Phascolarctobacterium*	0.301	0.021	0.009	0.026
Genus	*Blautia*	0.68	0.054	<0.001	0.001
Genus	*Actinomyces*	0.177	0.008	0.008	0.023

Wilcoxon tests were used to compare groups. *P* values were adjusted using the Benjamini Hochberg technique.

2.7. Figures

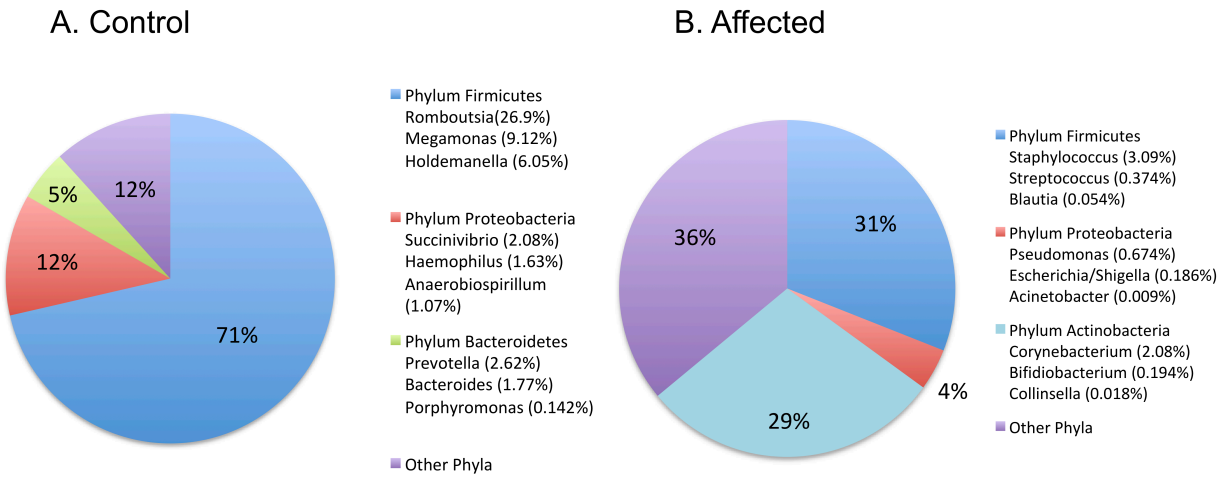

Figure 2.1. Median relative abundance of the most common taxa isolated from healthy dogs (A, n=8) and those affected with otitis externa (B, n = 18).

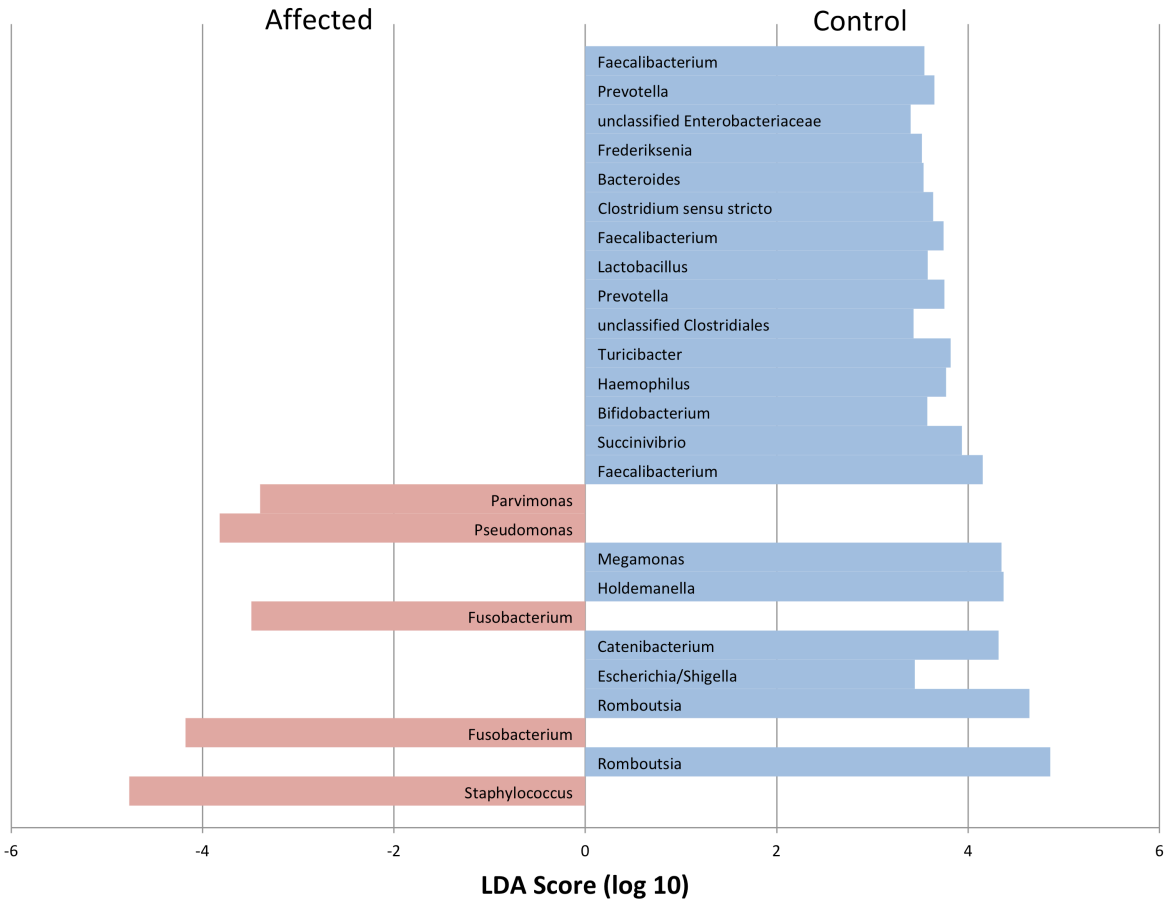

Figure 2.2. Operational taxonomic units (OTUs) identified by LEfSe analysis as differentially abundant (*P*<0.05) in the aural microbiota of dogs affected with otitis externa (n=18) and healthy control dogs (n=8). Only OTUs with a linear discriminant analysis (LDA) >3.4 are depicted.

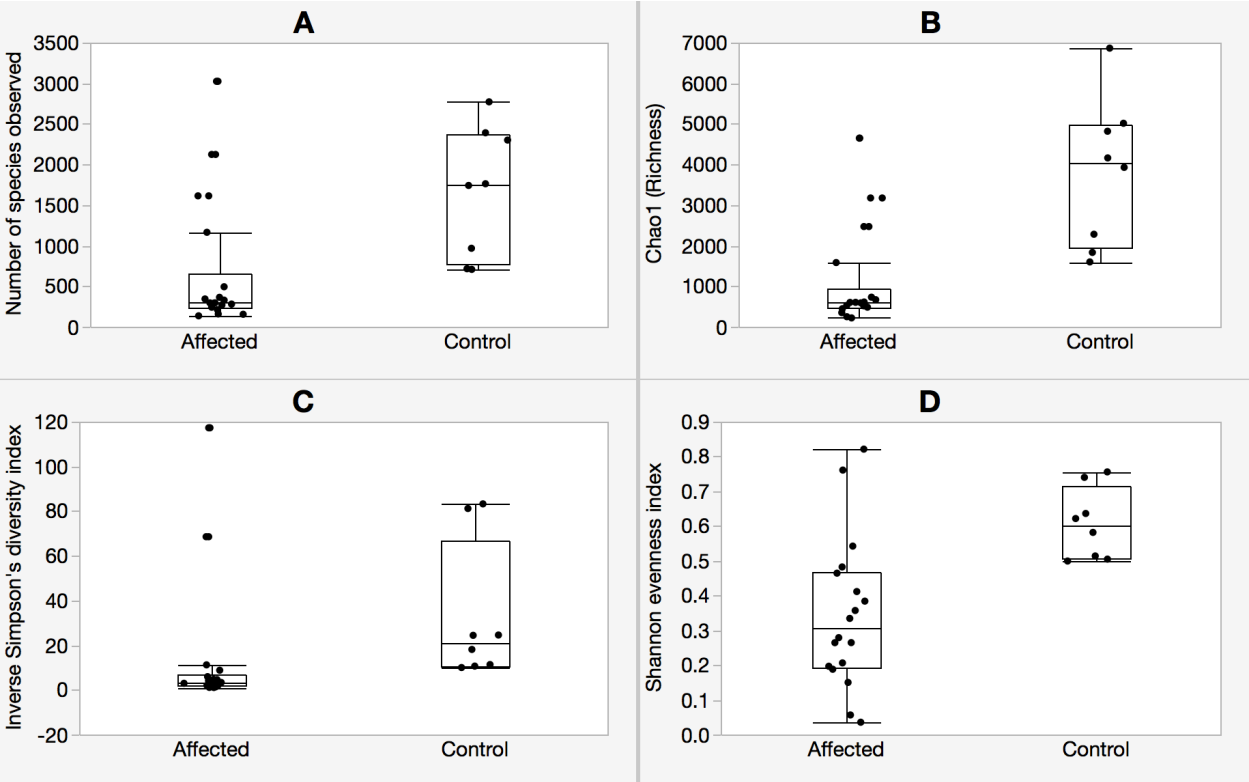

Figure 2.3. Alpha diversity measurements in dogs with otitis externa (affected, n=18) and healthy dogs (control, n=8). (A) Number of species observed (B) Estimated richness (Chao1) (C) Estimated diversity (inverse Simpson's diversity index) (D) Estimated evenness (Shannon's evenness index).

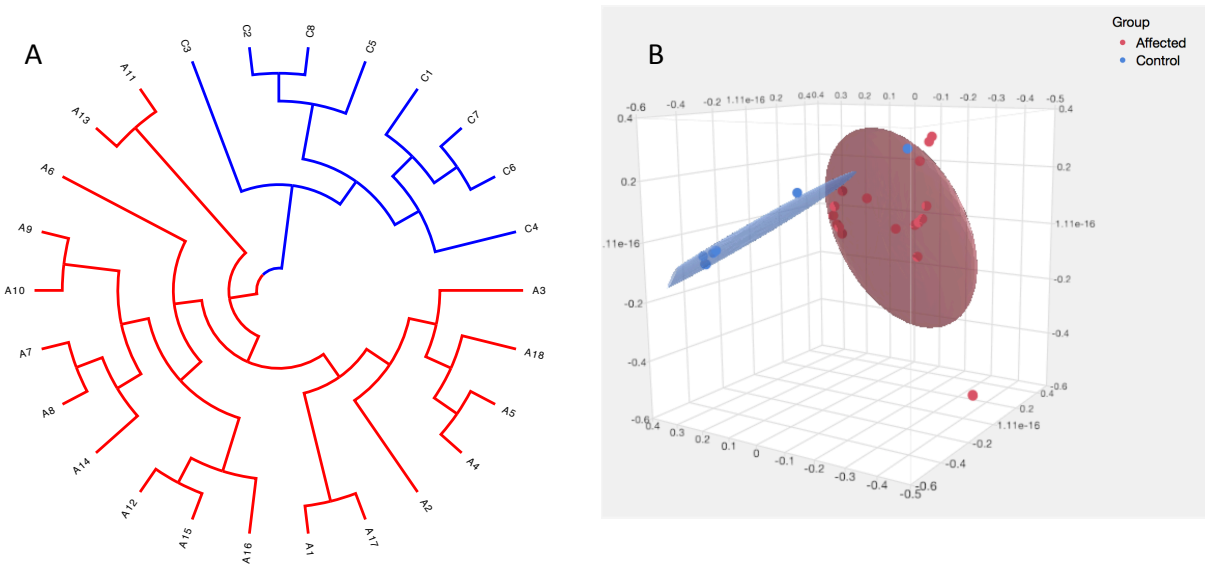

Figure 2.4. (A) Dendrogram depicting Yue & Clayton measure of dissimilarity in the aural microbiota of dogs with otitis externa (red) and healthy controls (blue). (B) PCoA plot was generated by JMP® using distance calculation performed in MOTHUR using the Jaccard coefficient metric. Clear coverage differences are depicted between affected samples (red) and control samples (blue). Ellipsoid coverage is 50%.

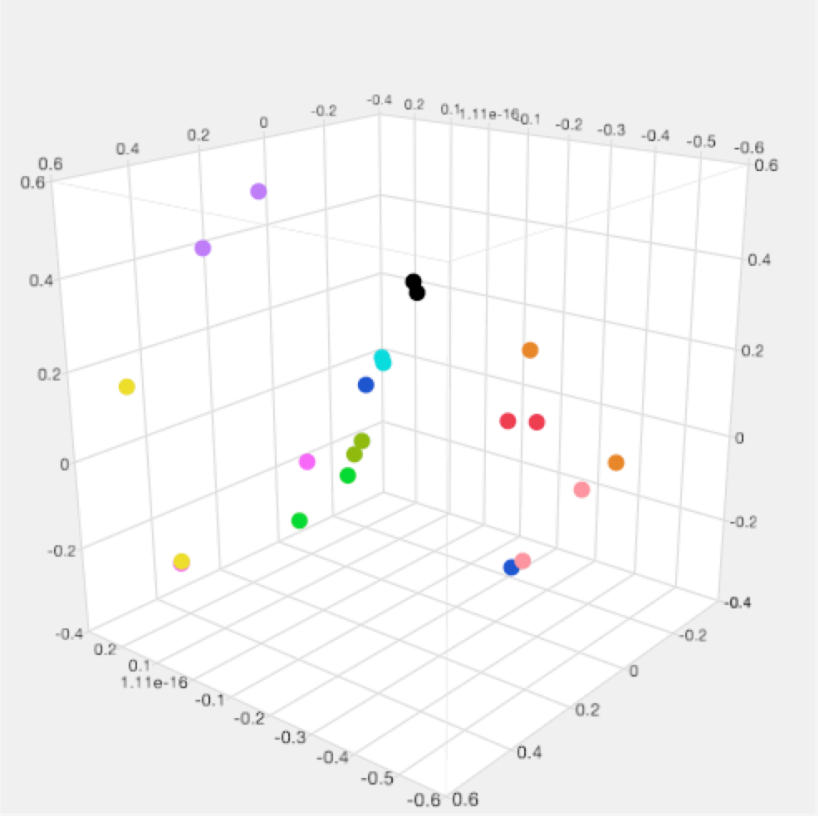

Figure 2.5. PCoA plot of samples from affected ears (n=11) generated by JMP® using distance calculation performed in MOTHUR using the Jaccard coefficient metric. Samples from the same dog are indicated using the same colour.

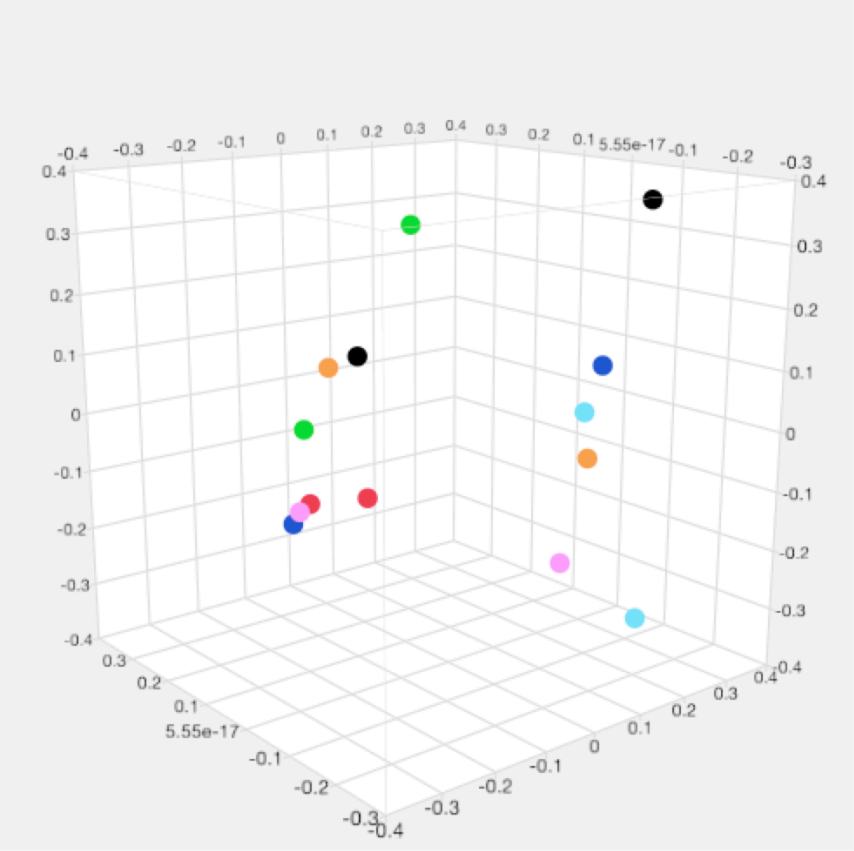

Figure 2.6. PCoA plot of samples from control ears (n=7) generated by JMP® using distance calculation performed in MOTHUR using the Jaccard coefficient metric. Samples from the same dog are indicated using the same color.

CHAPTER THREE: ANALYSIS OF THE OTIC MYCOBIOTA IN DOGS WITH OTITIS EXTERNA COMPARED TO HEALTHY INDIVIDUALS

3.1. Introduction

Otitis externa is a common multifactorial disease with a prevalence in dogs that has been reported as high as 10 to 20%.[1,2] Allergic dermatitis and otitis externa were the top two reasons for insurance claims in American dogs for 2016.[3] Primary causes of otitis externa include hypersensitivity and autoimmune disorders, endocrine diseases, otic parasites, foreign bodies and conformational defects, but clinical manifestation of disease is predominantly the result of secondary bacterial or fungal infection, most commonly from the dog's commensal bacterial microbiota and fungal microbiota (mycobiota).[4]

Until recently, many of the bacterial and fungal species on the skin and in the ear canal could not be analyzed using culture-based methods, leading to a rather superficial understanding of these complex microbiota populations.[5,6] The development of culture-independent molecular techniques has revolutionized our view of the microbial world. Genomic characterization of fungal microbiota (mycobiota) usually relies on the sequencing and analysis of two phylogenetic markers within the rRNA gene region: 18S rRNA gene and intervening Internal Transcribed Spacer (ITS) region.[7,8] These act as conserved regions and as binding sites for PCR primers and sequence analysis of PCR amplicons generated from within these regions can be used to identify the mycobiota in a non-culture-dependent manner.[6] The use of culture-independent methods has

revealed highly diverse microbiotas that previously have not been demonstrated by conventional methods.[9-12]

While study of the bacterial microbiota is now commonplace, there are comparatively few studies of mycobiotas, particularly in animals. In contrast with the bacterial skin microbiota, the human skin mycobiota appears to more strongly correlate with the site location rather than site physiology (eg. sebaceous, dry, or moist).[13] In three studies, *Malassezia* spp. predominated at the majority of sites sampled, with *M. restricta* and *M. globosa* being the two predominant species.[13-15] Some degree of body site specificity was noted, with *M. restricta* predominating in the external auditory canal.[13] Additionally, in two studies, the percentage of *Malassezia* carriage was higher in healthy individuals than those with atopic dermatitis (AD)[14] or psoriasis,[15] suggesting that fungal diversity increases with disease states in humans.[13-15] This is further supported by a study that found elevated fungal diversity and increased representation of opportunistic fungi, such as *Candida* and *Aspergillus* spp., in patients with primary immunodeficiencies.[16] Several studies suggested that the skin may act as a reservoir for the recurrent fungal infections observed in these patients.[11,16] This differs from findings in bacterial microbiota, where decreased diversity is noted in individuals with AD.[17,18]

Culture-based studies of the canine skin and aural mycobiota have indicated limited fungal richness.[19-23] Three studies strictly sampled the ears of dogs that were either healthy or had otitis externa.[21-22] In these studies, the only fungal organisms cultured in affected ears were *Malassezia*, *Candida* and *Aspergillus* spp.[21,22] The only fungal

organism cultured from the ear of healthy dogs in one study was *M. pachydermatitis*.[21] However, a more recent study identified *Penicillium* spp. in 64% and *Aspergillus* spp. in 19% of normal ears.[23] The remaining 17% comprised of various other saprophytic fungal organisms.[23]

Culture-independent study of the cutaneous mycobiota of dogs has been very limited. One study analyzed the mycobiota from 10 sites in healthy dogs and six sites from dogs diagnosed with allergic disease, including the ear.[24] As with humans, the number of fungal species found was much higher than with previous culture-based techniques.[24] The most plentiful fungal genera present on canine skin, at all body sites, were *Alternaria* and *Cladosporium*.[24] Additionally, *Malassezia* and *Epicoccum* were the third and fourth most plentiful fungi present in the ear canal.[24] Unlike the bacterial microbiota, the mycobiota does not appear to be influenced by body site in healthy skin.[24] The exception was mucosal sites, which had significantly reduced species richness.[24] This is similar to the findings of Rodrigues Hoffmann *et al*., which also found decreased bacterial richness at mucosal sites.[12] The mycobiota from body sites in healthy dogs tended to be similar within a dog but a high degree of inter-dog variability existed.[24] The mycobiota of dogs with AD was found to be significantly less rich than that of healthy skin.[24] This differs from one previous study that found increased fungal diversity in lesional skin of humans with primary immunodeficiencies.[16] Although *Malassezia* has been implicated as an allergen and a trigger in both human and canine AD[14,25,26], no significant difference was detected in the relative abundance of these fungi between

healthy and allergic dogs.[24] However, 50% of samples from ears of allergic dogs were predominated by the genus *Malassezia* or *Sporobolomyces.*[24]

Understanding the impact of disease states on the otic mycobiota is important for several reasons: (i) to understand how the mycobiota responds to insults, (ii) to understand the pathophysiology of diseases affecting the skin, (iii) to identify specific changes that might be markers of microbiota disruption to aid diagnosis or for selective therapeutic repopulation. The aim of this study was to investigate the mycobiota of dogs with fungal otitis externa and clinically normal controls.

3.2. Materials and Methods

3.2.1. Study population

All animals for this study were recruited through the Guelph Veterinary Specialty Hospital following a protocol approved by the University of Guelph's Animal Care Committee. Thirty dogs that presented to the Dermatology Department with clinical and cytological evidence consistent with fungal otitis externa were enrolled in the study. Clinical signs consistent with otitis externa included erythema and discharge from the external ear canal. In order to be included in the study, at least one ear must have had cytological evidence of fungal otitis externa. This was described as having greater than or equal to 2+ yeast on a semi-quantitative assessment scale.[28] All dogs were examined by the same dermatology resident. Dogs were excluded if they had received any topical ear treatments or cleaners within the two weeks previous to sampling or

systemic antifungals or antibiotics within the three months previous to sampling. Treatment with other concurrent medication was permitted. Patients treated with oclacitinib (Apoquel®; Zoetis Inc, Kalamazoo, MI, USA), ciclosporin (Atopica®; Elanco Animal Health, Greenfield, IN, USA), allergen specific immunotherapy, corticosteroids, antihistamines, and non-steroidal anti-inflammatories were not excluded from the study.

For the control group, ten dogs that were presented to the Guelph Veterinary Specialty Hospital Surgery Department for elective orthopaedic procedures were enrolled. In order to be enrolled in the study, the dogs must not have received any systemic antifungals or antibiotics for at least the last three months and had not received any topical ear treatments or cleaners in the previous two weeks. Three of the five control dogs were being treated with meloxicam (Metacam®; Boehringer Ingelheim Vetmedica Inc., St. Joseph, MO, USA) at the time of sampling and were included. Dogs with prior history of ear or skin disease were excluded. Each dog was examined by a dermatology resident for evidence of otitis externa or signs of skin disease. Dogs with any signs of skin or ear disease were excluded from the study. Otic cytology was performed from both ears and dogs were excluded if there was any evidence of yeast or bacteria (1+ or greater) on cytology.

3.2.2. Sample collection and DNA extraction

Samples were collected from affected and control dogs using a regular tipped culture swab (ESwab Regular Collection Kit, BD Diagnostics™, Franklin Lakes, NJ, USA). Each swab was inserted into the external ear canal up to the junction between the vertical

and horizontal canals. The swab was then rotated 360 degrees before being withdrawn. Both ears were sampled separately in all dogs. Each samples was labelled and stored at 4°C for no longer than 7 days before being transferred to a -80°C freezer until analysis.

DNA was extracted from each sample using the MoBio Power Soil DNA Extraction kit, following the manufacturer's protocol (Qaigan Inc., Toronto, ON, Canada). DNA quantity and quality was accessed by spectrophotometry (NanoDrop, Roche, Mississauga, ON, Canada).

3.2.3. Amplification and sequencing

PCR amplification of the ITS hypervariable region was performed using the primers ITS1F (5-CTTGGTCATTTAGA GGAAGTAA-3) and ITS2R (5-GCTGCGTTCTTCATCGATGC-3) as per Findley *et al*.[13] The forward and reverse primers were designed to contain an overlapping region of the forward and reverse Illumina sequencing primers in order to anneal them to primers containing the Illumina adaptors plus the 8 base pairs (bp) identifier indices.

After amplification, the PCR products were evaluated by electrophoresis in 1.5% agarose gel and purified with the Agencourt AMPure XP (Beckman Coulter Inc, Mississauga, ON, Canada) by mixing 20µL of amplicon with 40µL of AMPure on a 96 well plate. After incubating for 5 minutes at room temperature, the plate was placed on a magnetic stand for 2 minutes then the supernatant was removed and the beads were

washed twice with 80% ethanol and subsequently eluted in 32µL of 10mM Tris pH 8.5 buffer solution.

A second PCR was performed to attach dual indices and Illumina® sequencing adapters using the Nextera XT Index kit (Illumina®, San Diego, CA, USA). After purification of these amplicons, the samples were quantified by spectrophotometry using the NanoDrop® (Roche, Mississauga, ON, Canada) and normalized to a final concentration of 2nM. Normalization and sequencing of the library pool was performed at the University of Guelph's Advanced Analysis Centre using an Illumina® MiSeq (San Diego, CA, USA) and 2x300 kit.

3.2.4. Data analysis

The MOTHUR package of algorithms (v1.39.5) was used for analysis.[28] Paired end reads were aligned. Sequences less than 200 bp were removed, as well as those containing ambiguous base calls or long runs of holopolymers (>20 bp). Chimeras were detected using VSEARCH and removed.[29] Sequences from plants and protists were removed. Comparison of the relative abundances of taxa at different levels (phylum through species) was performed using Wilcoxon test in JMP® (v13.2.0, SAS Institute Inc., 2016). To control for false discovery, *P* values were adjusted using the Benjamini Hochberg technique[30] in R (v3.3.3, "Another Canoe", RStudio Inc., 2017). After classification using the UNITE (v7.2) fungal ITS database[31], sequences were binned into operational taxonomic units (OTUs) using the Phylotype command in MOTHUR, a closed OTU picking approach.[28]

Subsampling was performed to normalize sequence number across samples for assessment of alpha and beta diversity to a depth of 81087 sequences. Richness (Chao1), evenness (Shannon's evenness) and alpha diversity (inverse Simpson's) indices were calculated. As data were not normally distributed, Wilcoxon tests were used to compare groups. A $P<0.05$ was considered significant for all comparisons. Beta diversity was assessed through the creation of dendrograms using both the Yue & Clayton measure of dissimilarity (a measure of community structure, which considers shared OTUs and their relative abundances) and traditional Jaccard coefficient (a measure of community membership, which considers the number of shared OTUs, not their abundance). Analysis of similarity (ANOSIM) tests were applied to evaluate the impact of otitis externa on fungal population structure.[32] Principal coordinate analysis (PCoA) was performed based on both Jaccard coefficient and Yue and Clayton measure. Linear discriminant analysis effect size (LEfSe) was used to identify OTUs that were enriched or depleted.[33]

3.3. Results

Dogs enrolled into the affected group (those with otitis externa) ranged from one to 10.5 years of age (median=6 years), and consisted of 14 female spayed, 12 male castrated and four intact female dogs. Twenty-three pure bred and seven mixed breed dogs were enrolled into the affected group. Six spayed females and four castrated males ranging from 1 to 9 years of age (median= 5.75 years) were enrolled into the control group. This group consisted of seven pure bred and three mixed breed dogs.

Despite repeated efforts, obtaining adequate DNA yield and sequence data was a challenge. Ultimately, data were obtained from five control samples and six affected dogs (Table 3.1). The affected samples were from ears with 3+ or more yeast on cytology. After quality processing, the total number of sequences from the ears of healthy dogs was 707,068 with a median of 143,297 sequences per sample. The total number of sequences found in affected ears was 1,438,339 with a median of 204,557 sequences per sample.

Fungi from ten different phyla were identified (Figure 3.1). In healthy ears, the predominant fungal organisms were from the phyla Ascomycota (relative abundance 31.6-98.8%, median=94.2%) and Basidiomycota (1.11-64.0%, median=5.78%). Within the phylum Ascomycota, the three most common genera in healthy ears were *Aureobasidium* (6.35-24.4%, median=21.8%), *Alternaria* (1.21-44.5%, median=9.74%) and *Mycosphaerella* (0.043-6.61%, median=0.003%). Within phylum Basidiomycota, the three most common genera were *Vishniacozyma* (0.043-1.34%, median=0.190%), *Rhodotorula* (0.025-0.509%, median=0.130%) and *Filobasidium* (0.006-2.79%, median=0.098%).

In affected ears, the phylum Basidiomycota (64.0-98.4%, median=96.6%) was the most abundant in all samples, followed Ascomycota (0-31.6%, median=0.036%). Within the phylum Basidiomycota, the three most common genera ears were *Malassezia* (55.7-98.4%, median=96.8%), *Filobasidium* (0-0.269%, median=0.001%) and *Holtermanniella* (0-0.262%, median=0.001%). The three most common genera in the phylum

Ascomycota were *Mycosphaerella* (0-1.70%, median=0.002%), *Cladosporium* (0-0.394%, median=0.001%) and *Candida* (0-0.058%, median=0.001%). Unsurprisingly, the most common species from affected ears was *M. pachydermatis* (55.7-98.4%, median=96.8%). The identity of *M. pachydermatis* was confirmed using BLASTN (v2.7.1) based on 100% identification, and only 84% similarity to the next closest species (*M. japonicas*).[34] Relative abundance of the genus *Malassezia* was increased in sampled from dogs affected with otitis externa (*P*=0.016). The relative abundance of ten other genera was increased in control samples when compared to affected samples, including *Cladosporium* (*P*=0.016), *Alternaria* (*P*= 0.016) and *Mycosphaerella* (*P*= 0.04), among others (Table 3.2).

LEfSe analysis identified 42 OTUs that were differentially abundant (*P*<0.05). Thirty-nine OTUs were over-represented in healthy ears, including several species from the genus *Alternaria, Cladosporium* and *Epicoccum.* With the exception of five OTUs from the phylum Basidiomycota, the majority of OTUs over-represented in healthy ears were from the phylum Ascomycota. Three OTUs were over-represented in the affected ears, including *M. pachydermatis* and two OTUs that could not be classified at the phylum level (Figure 3.2). One of these unclassified OTUs was identified as *M. pachydermatis* using BLASTN with 94% identity.[34]

Observed richness (*P*=0.008), estimated richness (*P*=0.008), evenness (*P*=0.008) and alpha diversity (*P*=0.008) were significantly lower in affected ears (Figure 3.3). ANOSIM analysis indicated that there were significant differences in both fungal

community structure (R=1, P=0.003) and membership (R=0.853, P=0.002) between affected and control ears. These differences were depicted using PCoA plot and dendrogram that demonstrate clearly separated clusters of samples from healthy dogs and those with otitis externa (Figure 3.4).

3.4. Discussion

Although otic fungal richness and diversity were low when compared to that of bacteria,[12] this study identified a much richer fungal community within the canine external ear canal than had previously been identified using culture-based methods.[21-23] Similar to the findings of Meason-Smith *et al*., this study also found that fungi from the phyla Ascomycota and Basidiomycota predominated.[24] While we also found the genus *Alternaria* to be one of the most common in healthy ears, our study found the genera *Aureobasidium* and *Vishniacozyma* rather than *Cladosporium* and *Epicoccum* to be more abundant.[24] The different findings between our two studies may be related to two factors. First is geographic location, as the dogs sampled in the study performed by Meason-Smith *et al*. were presumably recruited from within the state of Texas, USA.[24] while the dogs in our study were recruited from the Ontario, Canada. A recent study found that the bacterial microbiota of dogs was influenced by their environment and lifestyle.[35] Therefore, it is likely that climate and other geographic factors could influence the composition of the otic mycobiota. Time of year, with differences in temperature and humidity, could also impact an external surface such as the ear. Moreover, individual variation accounts for 45% of differences in cutaneous bacterial microbiota between dogs.[36] The same is likely true for changes in the otic mycobiota. Additionally, Meason-

Smith *et al*. used the GenBank ITS-1 database for the taxonomic classification of OTUs[24] while we used the UNITE database.[31] Although database difference had the potential to influence findings between our two studies, this does not appear be the cause. While there were some minor differences in organization of OTUs, classification of our data using the GenBank ITS-1 database did not meaningfully alter these findings (data not presented).

This study found that various aspects of the otic fungal microbiota are different in dogs with otitis externa. Fungal species diversity, richness and evenness were all significantly reduced in samples from affected ears when compared to healthy ears. This is similar to findings of a previous study, which also found decreased fungal richness and diversity in ears of dogs affected by AD.[24] This differs from findings in cats, where a significant decrease in fungal alpha diversity was not identified in healthy vs. allergic cats.[37] This species may be more refractory to alterations in their cutaneous mycobiota and thus less likely to develop secondary infections.[38]

Interestingly, of those OTUs that were preferentially increased in samples from healthy individuals, the majority (34/39) were from the phylum Ascomycota. This suggests that species from this phylum may comprise the majority of the normal mycobiota of the canine ear, and is replaced by species from other phyla (particularly Basidiomycota) when fungal otitis externa is present. Alternatively, it is possible that proliferation of certain members of Basidiomycota is a driving force both for development of disease and alteration of the mycobiota. As a cross-sectional study, direction of relationships

(i.e. cause vs. effect) cannot be discerned.

The majority of the genera isolated from the canine ear were comprised of fungi that are generally considered non-pathogenic, ubiquitous, environmental organisms. Additionally, fungi from the genera *Alternaria*, *Cladosporium* and *Epicoccum* were over-represented in healthy ears. Fungi from these genera are known allergens of both dogs[39] and humans.[40] Meason-Smith *et al*., postulated that the difference in carriage of these fungi between individual dogs could play a role in hypersensitivity to these fungi.[24] A recent study found that early-life environment influences the bacterial microbiota in dogs and may play a rule in the development of AD in dogs. It is possible that increased carriage of these fungi in healthy individuals, especially at a young age, may lead to a more tolerant immune response and reduce the chance of these individuals developing a hypersensitivity reaction to these fungi.

In addition to differences in relative abundances and alpha diversity, there were clear differences in beta diversity. There were significant differences in both the overall membership of the population, as well as the distribution of those members within the mycobiota (structure). Thus, while it is interesting to note certain taxa that were significantly different, the changes that were present go well beyond simple changes in one or a few individual components of the mycobiota. Previous studies have also found a reduction in cutaneous fungal alpha and beta diversity in dogs with atopic dermatitis.[24] Similarly, cutaneous bacterial diversity is also reduced in dogs with atopic dermatitis.[12,41] However, results from this study are different from several human studies

that reported increased fungal diversity in lesional skin of people with during diseased states.[13-16] These differences could be attributed to the fact that normal human skin is predominately inhabited by the genus *Malassezia*.[13-15] Thus, a disturbance of the mycobiota could lead to a decrease of the predominant fungal resident and allow for invasion of other organisms, leading to an increase in overall fungal diversity.[24]

Our results indicated that species from the genus *Malassezia* (phylum Basidiomycota), specifically *M. pachydermatis*, were predominant in all samples collected from dogs with otitis externa and was present at lower levels in healthy ears. This genus accounted for a median of 96.8% of all sequences from affected ears. This is similar to the findings from Meason-Smith *et al*. in which 50% of ears sampled of allergic dogs were predominated by one species, 2/3 of which were from the genus *Malassezia*.[24] However, that study did not find an increase in relative abundance of this genus between healthy and allergic individuals when all cutaneous sites were taken into account.[23] Interestingly, several culture-based studies also found *M. pachydermatis* to be the most common fungus isolated from infected ears.[21,22] LEfSe analysis in our study confirmed that the genus *Malassezia* was over-represented in samples collected from dogs with otitis externa. As our approach analyzed relative, rather than absolute abundance, it is difficult to determine whether the changes in relative abundance occurred because of suppression of other (non-*Malassezia*) taxa or increases in *Malassezia*. It is also possible that both events are occurring. Several studies have implicated *Malassezia* as an allergen and a trigger in both humans and dogs with AD.[14,25,26] Although it is not clear from this study whether this organism triggers otitis

externa or is a perpetuating factor, our findings suggest that an increased relative abundance of Malassezia, specifically *M. pachydermatis*, plays a significant role in otitis externa in dogs.

A limitation of this study was the difficulty of obtaining adequate DNA and sequence yields from samples. This could be because of low fungal DNA burdens or the presence of inhibitors in samples.[42] Halwachs *et al*. also found that bead-beating leads to significant decrease in fungal DNA yield.[42] This may have lead to difficulty extracting adequate DNA from our samples. This lead to the analysis of samples from only a subset of the enrolled population (five healthy and six affected dogs), and it is unclear whether this could have introduced bias into this study. These results should perhaps be taken as a comparison of dogs with severe fungal otitis externa versus controls, since higher fungal burdens are likely present with more advanced disease and the samples from dogs with otitis externa that could be analyzed all had large numbers of *Malassezia* yeast on cytology. Additional cycles during DNA amplification may have improved DNA yield but this was avoided as it could have also introduced bias resulting in less meaningful results. Two dogs in the affected group were receiving immunomodulatory therapy at the time of sampling; one with oclacitinib and one with ciclosporin and corticosteroids. A recent study determined that treatment with ciclosporin and corticosteroids does not significantly impact the cutaneous microbiota (bacterial and *Malassezia*).[43] It is unknown whether treatment with oclacitinib has an impact on cutaneous microbiota.

Next generation sequencing has revealed that the fungal mycobiota is much more complex than previously identified using culture-based studies. Reduced fungal richness and diversity is present in ears affected with otitis externa indicating that a dysbiosis is present. This study did not discriminate between different underlying causes of otitis externa and further study is needed to investigate whether alterations in the mycobiota are present in otitis externa caused by different primary disease states.

3.5. References

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3.6. Tables

Table 3.1. Signalment, ear lesions, cytology results and concurrent medications of the affected (n=6) and healthy dogs (n=5) in which sampled were sequenced

Patient #	Health status	Sex	Breed	Age (years)	Clinical Lesions	Cytology Results	Concurrent medication
A1	Affected	MN	Jack Russell	10.5	thickening of pinna, hyperpigmentation, ceruminous discharge	4+ yeast	oclacitinib
A2	Affected	FI	Mix	2.5	erythema, brown discharge	4+ yeast	none
A3	Affected	MN	German Shepherd	6	erythema, stenosis, yellow discharge	4+ yeast, 3+ cocci	none
A4	Affected	MN	Mix	9	erythema, brown discharge	4+ yeast	ciclosporin, prednisone
A5	Affected	FS	Mix	7	erythema, brown discharge	3+ yeast	none
A6	Affected	FS	Havanese	1	erythema, brown discharge	4+ yeast	none
C1	Healthy	FS	Havanese	4	None	NSF	none
C2	Healthy	FS	Mix	8	None	NSF	none
C3	Healthy	FS	Dachshund	9	None	NSF	none
C4	Healthy	MN	Bernese Mountain dog	5.5	None	NSF	meloxicam
C5	Healthy	MN	Mix	6	None	NSF	meloxicam

Signalment: MN male neutered, FI female intact, FS female spayed, Mix mixed breed.
Cytology results: NSF no significant findings.

Table 3.2. Relative abundance of taxa that were significantly different in the aural mycobiota of dogs with otitis externa ('affected', n=6) or healthy controls ('control', n=5)

Taxonic Level	Taxon	Affected Median	Control Median	P-Value	Adjusted P-Value
Phylum	Ascomycota	0.032	94.2	0.004	0.015
Phylum	Basidiomycota	96.8	5.78	0.004	0.015
Family	Nectriaceae	0	0.005	0.005	0.016
Class	Dothideomycetes	0	8.29	0.004	0.016
Genus	Malassezia	96.8	0.084	0.004	0.016
Genus	Cladosporium	0.001	1.25	0.007	0.016
Genus	Gibberella	0	0.197	0.005	0.016
Genus	Neoascochyta	0.001	0.082	0.008	0.016
Genus	Aureobasidium	0	21.8	0.008	0.016
Genus	Mycosphaerella	0.002	5.21	0.022	0.04
Genus	Alternaria	0	9.74	0.007	0.016
Genus	Debaryomyces	0	0.036	0.005	0.016
Genus	Rhodotorula	0	0.13	0.005	0.016

Wilcoxon tests were used to compare groups. *P* values were adjusted using the Benjamini Hochberg technique.

3.7. Figures

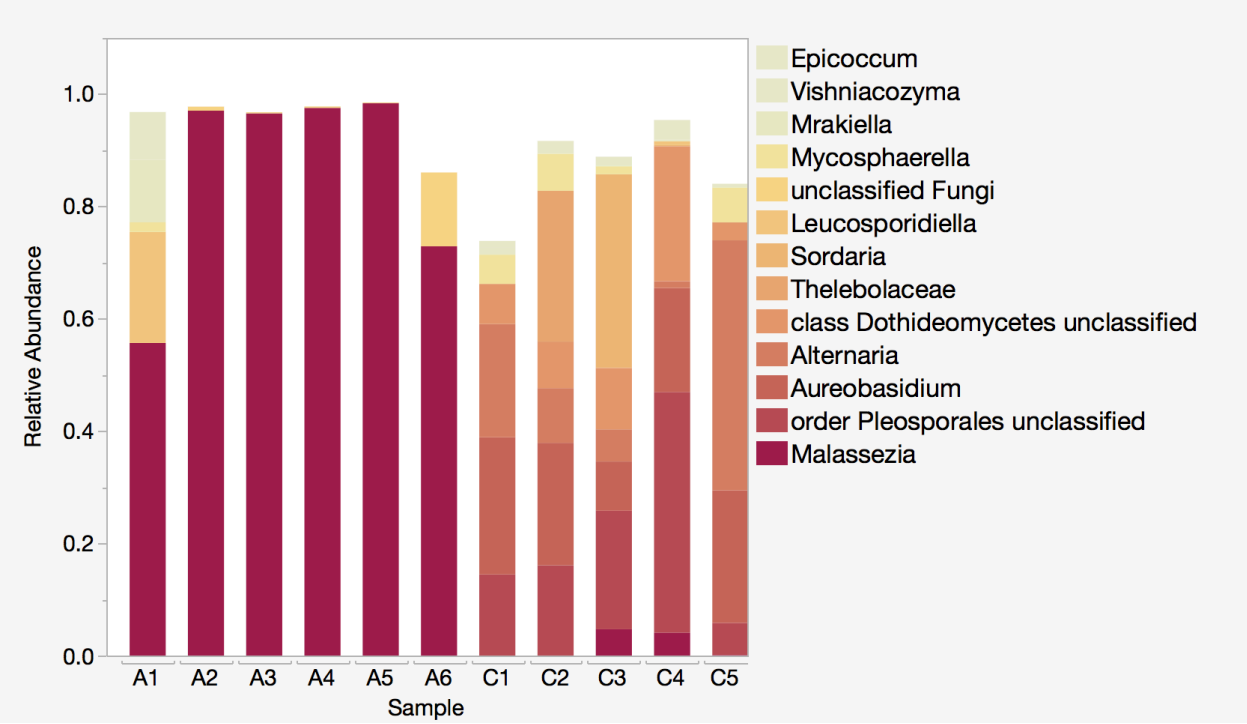

Figure 3.1. Relative abundance of the predominant taxa in the aural mycobiota of dogs with otitis externa (A, n=6) and healthy dogs (C, n=5).

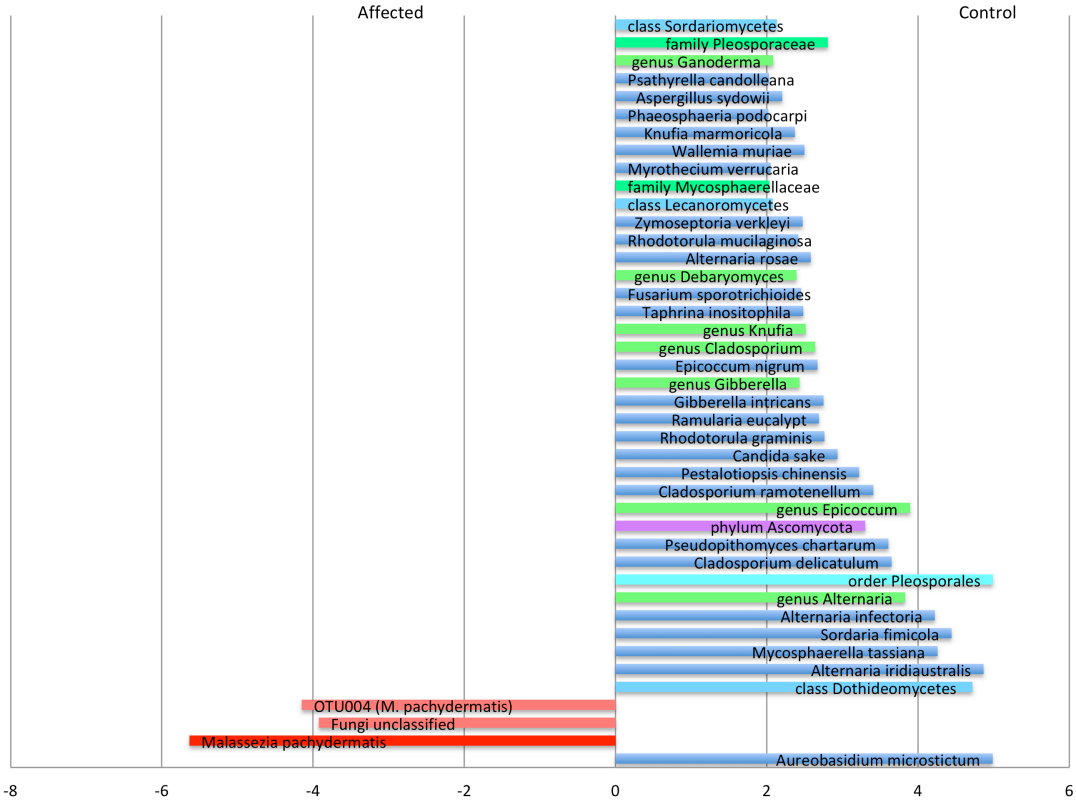

Figure 3.2. Operational taxonomic units (OTUs) identified by LEfSe analysis as differentially abundant (P<0.05) in the aural mycobiota of dogs affected with otitis externa (n=6) and healthy control dogs (n=5). Those OTUs not listed to species level could not be classified further. LDA: linear discriminant analysis.

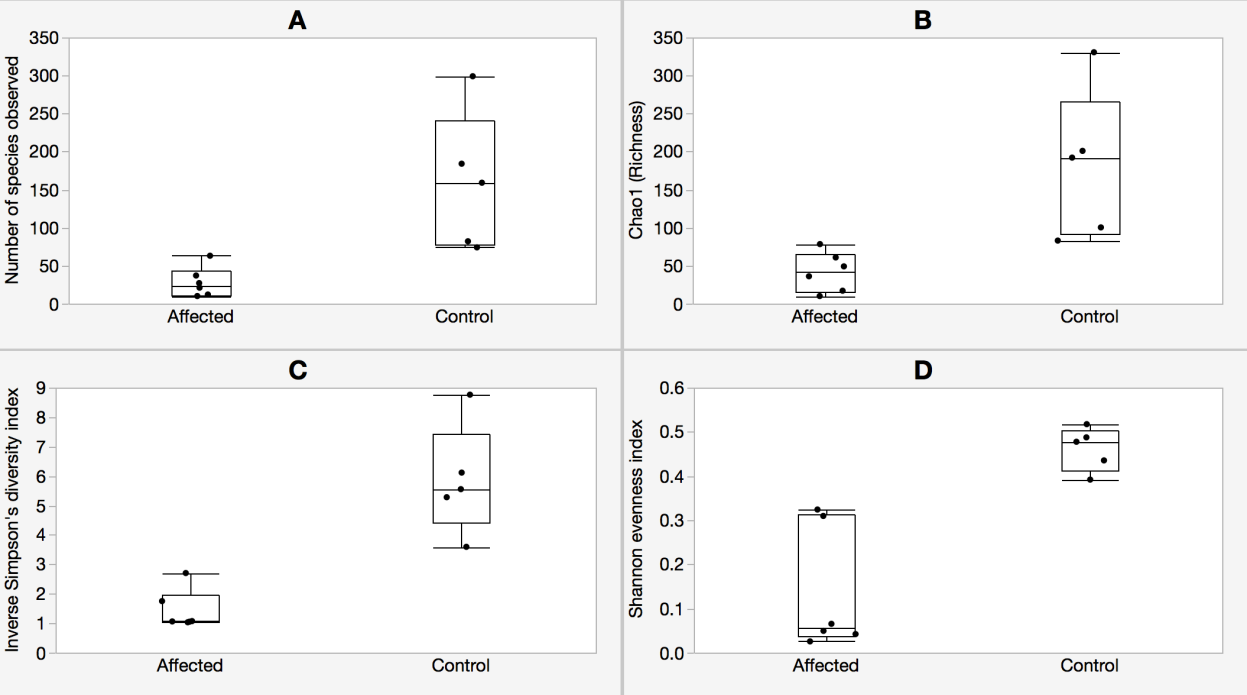

Figure 3.3. Alpha diversity measurements in dogs with otitis externa (affected, n=6) and healthy dogs (control, n=5). (A) Number of species observed (B) Estimated richness (Chao1) (C) Estimated diversity (inverse Simpson's diversity index) (D) Estimated evenness (Shannon's evenness index).

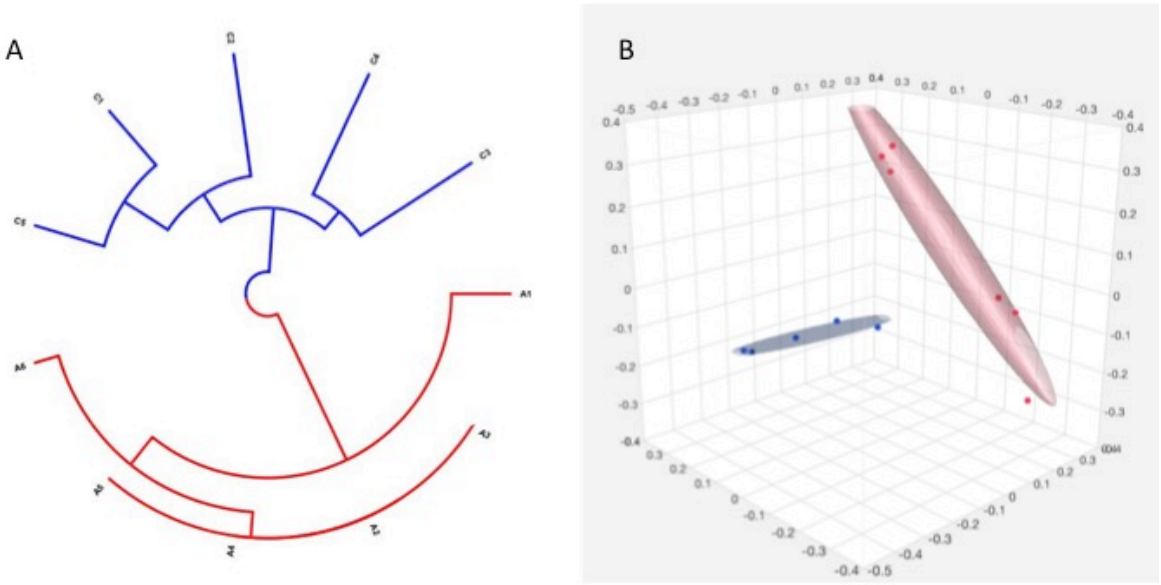

Figure 3.4. (A) Dendrogram depicting Yue & Clayton measure of dissimilarity in the aural mycobiota of dogs with otitis externa (red) and healthy controls (blue). (B) PCoA plot was generated by JMP® using distance calculation performed in MOTHUR using the Jaccard coefficient metric. Blue points indicate samples from healthy ears; red points indicate samples from dogs with otitis externa. Clear coverage differences are depicted between affected samples (red) and control samples (blue). Ellipsoid coverage is 50%.

CHAPTER FOUR: SUMMARY AND CONCLUSIONS

4.1. Summary

Otitis externa is one of the most common diseases to affect domestic dogs.[1,2] Primary causes of otitis externa can be difficult to identify and address leading to relapse in many patients.[3] Additionally, secondary causes, along with perpetuating and predisposing factors, can make this condition difficult to resolve.[3] Chronic otitis can lead to permanent changes in the ear canal, which can make the condition intractable to medical treatment.[3] Otitis externa is a painful condition that can lead to permanent hearing loss and affect the human and animal bond,[3] and it accounts for abundant antimicrobial use in an era where antimicrobial stewardship is of increasing concern.

In many cutaneous diseases, it is unclear if the condition is caused by an alteration in the cutaneous microbiota or if the alterations are the result of the disease itself.[4] Until recently, many of the bacterial species on the skin and in the ears could not be analyzed using culture-based methods, limiting our existing knowledge.[5,6] Recent studies, which analyzed species diversity using molecular methods, have demonstrated that the skin microbiota of dogs is much richer and more diverse than previously reported by culture-based methods.[7,8] However, few studies have assessed the otic microbiota.

The first objective of this study was to describe the otic bacterial and fungal microbiota. It was found that the otic bacterial and fungal microbiota were much more diverse than

previously found through culture-based methods. Bacteria from 27 and fungi from ten different phyla were identified in healthy ears. This, itself, was not surprising as the superiority of this approach to culture has been well established. Yet, previous studies using next generation sequencing, which focus mainly on the cutaneous microbiota, identified only 17 distinct bacterial phyla[7] and three fungal phyla[9]. Firmicutes was the most abundant bacterial phylum present in the ear, followed by Actinobacteria and Proteobacteria. Although previous studies identified the same phyla as most abundant, their prevalence was altered in our study. As with previous studies, the predominant fungi in the otic mycobiota were from the phyla Ascomycota and Basidiomycota. Our study shows that the bacterial and fungal microbiota of the ear is much more complex and diverse than previously thought. Conditions within the ear canal are moist, warm and sheltered, making it ideal for microbial growth. While cerumen produced within the ear canal contains immunoglobulin and other peptides, which can inhibit the growth of organisms,[3] it can also act as a source of nutrients for properly adapted organisms. This microenvironment allows for an even more diverse microbiota and mycobiota than is present on exposed skin. The ecosystem within the ear canal can be likened to a rainforest, which is one of the most bio-diverse ecosystems on the planet. This diverse micro and myco-biota play a protective role. When the normal environmental conditions are altered, as in disease states, the normal microbiota is disrupted allowing for the proliferation of pathogenic species.

The second objective of this study was to describe the effect of otitis externa on the otic microbiota. It was hypothesized that there would be dramatic differences between

healthy and affected ears. This study found that both bacterial and fungal species alpha diversity, richness and evenness were significantly reduced in samples from affected ears when compared to healthy ears. A decrease in richness and diversity are a common finding in studies of various diseases, such as IBD[10] and AD[11]. It appears that many disease states either result in, or are due to, a decrease in diversity of the myco and micro-biota. Species diversity is important as many of the organisms within this ecosystem rely on each other. If the balance is disrupted, species diversity rapidly declines either due to de-population or the over-growth of certain members of the population. Additionally, there were clear differences in both bacterial and fungal beta diversity. Significant differences were present in both the overall membership of the population, as well as the distribution of those members within the microbiota. Thus, the changes that were present go well beyond simple changes in one or a few individual components of the bacterial and fungal microbiota. This indicates that otitis externa results in (or from) a major disturbance in the microbiota. The microbiota plays a protective role, its disruption allows for the proliferation of pathogenic species, thus exacerbating and perpetuating the original primary cause. Unfortunately, as with many disease states, it is not clear from our study whether the cause of otitis externa is due to proliferation of certain pathogenic taxa or whether these taxa take advantage of the disruption in the normal environmental. This author favours the theory that a disruption in the otic microenvironment results in a decrease in microbial diversity and allows for certain members within the micro and myco-biota to multiply and become pathogenic for several reasons: (i) Multiple primary conditions can cause otitis externa, (ii) most of the

organisms which cause otitis externa can be found at low numbers in health ears and (iii) healthy dogs do not develop otitis externa.

Bacteria from the genera *Staphylococcus* and *Pseudomonas,* which are commonly associated with otitis externa,[12,13] were identified by LEfSe analysis to be over-represented in affected ears. As a cross-sectional study, we could not discern whether proliferation of these genera, or the depletion of normal bacterial flora and subsequent replacement with these genera, was the driving force for the development of otitis externa. Recent studies suggest that toxins produced by *Staphylococcus aureus* trigger or exacerbate AD, strengthening the theory that this species and alterations in skin microbiota play a role in inflammation.[14,15]

Our study identified that the genus *Porphyromonas* was preferentially abundant in healthy ears, while the genus *Staphylococcus* was preferentially increased in ears affected with otitis externa. A previous study, which used next generation sequencing to assess changes to the cutaneous microbiota in dogs with AD, also found that these genera were preferentially decreased or increased, respectively, in a diseased state.[8] These two genera may be important indicators for the presence of a bacterial dysbiosis due to a disease state. To this date, there is no definitive test to confirm AD in dogs. In the future, there is potential that these indicator organisms could be used to help definitively diagnose this and other conditions. Additionally, bacteria from the genus *Porphyromonas* could potentially be used for selective re-population of the otic microbtiota and help resolve the dysbiosis caused by otitis externa. Similar treatments

are already being used in the form of fecal transplantation in patients with IBD. As resistant infection become more commonplace, these types of treatments become more necessary to reduce the need for, and potentially replace, antimicrobial-based therapy.

The role of *Corynebacterium spp*. in otitis externa has long been disputed.[14] This study found that the genus *Corynebacterium* was increased in healthy ears. This would support the hypothesis that this bacterium is not pathogenic;[16] however, inability to speciate is an inherent limitation of this method and the potential role of virulent species in a background of commensal *Corynbacterium* spp. cannot be ruled out.

Unsurprisingly, the genus *Malassezia*, and in particular *M. pachydermatis*, predominated in samples from affected ears. However, LEfSe analysis identified 39 fungal OTUs that were preferentially increased in samples from healthy individuals, the majority (34/39) were from the phylum Ascomycota. This suggests that species from this phylum comprise the majority of the normal otic mycobiota, and that species from this phylum are replaced by species from other phyla (particularly Basidiomycota) when fungal otitis externa is present. Alternatively, it is possible that proliferation of certain members of Basidiomycota is the driving force both for development of disease and alteration of the mycobiota. Again, cause versus effect could not be discerned.

Despite the contributions to our knowledge of the otic microbiota this study provided, it did have some limitations. Several of the dogs enrolled in the affected group were receiving immunomodulatory therapy at the time of sampling. Additionally, several dogs

in the control group were receiving treatment with non-steroidal anti-inflammatories (NSAIDs) at the time of sampling. It is possible that the concurrent use of these medications could have affected the microbiota. It is difficult find patients with chronic conditions that have been withdrawn from treatment for extended periods of time and it would be unethical to withdraw patients from these treatment for the purposes of this study. However, a recent study determined that treatment with ciclosporin and corticosteroids does not significantly impact the cutaneous microbiota.[17] It is unknown whether treatment with oclacitinib, immunotherapy, NSAIDs or antihistamines could alter the otic microbiota. Additionally, we had difficulty obtaining adequate DNA and sequence yields from a large proportion of samples, especially in the mycobiota portion of this study. This can occur as a result of various factors, including poor sampling, low DNA abundance on swabs, inefficient DNA extraction and the presence of PCR inhibitors. Since a larger proportion of samples were removed from the affected group than the control group, we hypothesize that secretions in affected ears may produce a large number of PCR inhibitors, which interfere with DNA extraction. Further methodological studies to evaluate inhibitors and mitigate their effects are warranted for future otic microbiota studies. Low bacterial or fungal DNA burdens in control samples may be responsible for poor DNA and sequence yields. This lead to the analysis of samples from only a subset of the enrolled population and it is unclear whether this could have introduced bias into this study, as it is possible that the study population was biased towards animals with greater overall bacterial and fungal populations in their ears. Additional cycles during DNA amplification may have improved DNA yield but this was avoided as it could have also introduced bias resulting in less meaningful results. A

control was not performed to ensure there was no bacterial contamination of reagents. However, contaminants would be expected to be evenly distributed amongst samples, and since the focus was comparing diseased and healthy ears, there would be little potential impact. The number of samples that did not yield adequate DNA or sequence numbers further reduces the likelihood that contaminants were a major concern. Regardless, study of the 'kitome' is increasingly being performed and would be useful to include in future studies, especially those of healthy ears where the presumed low bacterial burden leads to a greater influence of contaminants. Furthermore, while the presence of a dysbiosis was identified in dogs with otitis externa, as a cross sectional study, we could not discern the cause versus effect of the dysbiosis present. Further study is needed to identify whether the initiation of the dysbiosis is due to proliferation of particular taxa or due to the decreased diversity in normal flora and replacement with these taxa. Finally, by only sequencing the V4 hypervariable region of the 16s rRNA gene, the characterization of bacterial sequences below the genus level was not possible. This made it impossible to accurately distinguish between different species of the same genus, limiting our knowledge. Additionally, the use of this technique does not distinguish between viable and dead organisms. Since several studies have shown that the microbiota changes temporally[6], this is research would be more accurately portrayed as the historical microbiota of canine ear.

If this study were to be repeated, I would include control samples to ensure that contamination of the reagents with environmental bacteria or fungi did not alter the results. This would allow us to increase the number of cycles during DNA amplification

and improve DNA yield, as concern for introducing bias would be mitigated by using a control. Furthermore, I would remove bead-beating of samples during the DNA extraction process for the mycobiota portion of this study, as it was subsequently shown to significantly decrease fungal DNA yield.[18] Unfortunately, this study was published after our experiment was performed. Collecting serial samples from affected patients before, during and post-treatment would give us valuable longitudinal data. This would allow us to determine whether changes in otic microbiota were due to environmental changes, which resulted in decreased diversity or whether proliferation of certain organisms is a driving force both for development of disease and alteration of the microbiota.

4.2. Conclusions

This research was able to provide several important insights on how the otic microbiota is comprised and how it is altered with disease. It was also able to identify several key taxa that may be indicators for the presence of dysbiosis. Understanding the impact of disease states on the otic microbiota is important for several reasons: (i) to understand how the microbiota responds to insults, (ii) to understand the pathophysiology of diseases affecting the skin, (iii) to identify specific changes that might be markers of microbiota disruption to aid diagnosis or for selective therapeutic repopulation.

Several opportunities for further study have arisen from the results of this research. *M. pachydermatis* is thought to have a symbiotic relationship with commensal staphylococci on the skin of dogs.[19] It would be interesting to compare the results of the

bacterial and fungal portions of this study to confirm whether this relationship is also found in the ear and identify any similar relationships between bacterial and fungal species that may be present. Additionally, as this study did not differentiate between different primary causes of otitis externa, further investigation is needed into whether alterations in the microbiota are present in otitis externa caused by different primary disease states.

4.3. References

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