ON THE PATH FROM DESCRIPTION TO PATHOGENESIS: INVESTIGATION OF THE CUTANEOUS FUNGAL MICROBIOTA IN HEALTHY AND ALLERGIC COMPANION ANIMALS

A Dissertation

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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May 2019

Major Subject: Veterinary Pathobiology

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ABSTRACT

Next-generation sequencing (NGS) of host-associated microbes has revolutionized our understanding of commensal microbiota diversity and their interaction with the host to influence health and disease. The purpose of this work was at first descriptive, to undertake the founding studies using NGS to describe the cutaneous fungal microbiota (mycobiota) of dogs and cats, and to investigate alterations in allergic animals. The final study investigated the species level distribution and temporality of one fungal commensal, *Malassezia*, owing to its role in secondary yeast dermatitis of allergic dogs. A greater diversity of fungi was sequenced from skin swabs than was previously described using culture-dependent methods. The cutaneous mycobiota was predominated by environmental fungi and was more diverse on haired body sites than mucosal sites. The skin of allergic dogs harbored fewer types of fungi compared to healthy controls and a fungal dysbiosis was identified in allergic dogs and cats. Further analysis revealed a species level dysbiosis of *Malassezia* with significantly more *M. restricta* and *M. globosa* on the healthy canine skin, and *M. pachydermatis* on allergic skin. *M. pachydermatis* was 8-fold more abundant on the skin of laboratory atopic dogs prior to allergen exposure. These findings raised new questions regarding the cause of fungal dysbiosis. Some proposed explanations include immunologic dysfunction in the allergic individual or alterations to the skin barrier functions including hydration and nutrient availability, especially triglycerides and ceramides for *Malassezia*. Future studies with greater numbers of animals, as well as simultaneous investigations of immune function, skin morphology, and skin lipidomics might provide insights into the pathogenesis of fungal dysbiosis and secondary yeast infections in allergic animals.

DEDICATION

This dissertation is first dedicated to my parents Robert Meason and Linda Oberrender for their unconditional support and love. Without them I would not have accomplished this and so many other achievements in my young life. Their shining examples of strength, determination, and compassion have shaped me into the woman, wife, mother and scientist that I am proud to be today. Secondly this dissertation is dedicated to my daughter Cassidy Jean Smith. Cassidy in all her brilliance and independence has taught me patience and a love so deep I had never imagined possible. She is truly the light of my world and my everlasting inspiration. Last but surely not least, this is dedicated to my husband Jeremy Smith for his never ending support, patience and love throughout this challenging journey.

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Aline Rodrigues Hoffmann, and my committee members, Dr. Jan Suchodolski, Dr. Sara Lawhon, Dr. Adam P. Patterson, and Dr. Vince Gresham for their guidance and support throughout the course of this research. I would like to thank two collaborators, Dr. Alison Diesel and Dr. Thierry Olivry, who also provided guidance and support. I would like to thank Dr. Laura Bryan for serving as a substitute committee member and providing valuable feedback and guidance.

Thanks also go to my friends and colleagues and the department faculty and staff for making my time at Texas A&M University a great experience- especially Ms. Caitlin Older, who was a co-author on many of my studies.

Our sequencing studies would not have been possible without the expertise of the sequencing centers including MRDNA, Dr. Scot E. Dowd, and the University of Minnesota sequencing center.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a dissertation committee consisting of Professor Aline Rodrigues Hoffmann, advisor, Professor Sara D. Lawhon of the Department of Veterinary Pathobiology, and Professor(s) Jan S. Sucholdolski, co-advisor, and Professor Adam P. Patterson of the Department of Small Animal Clinical Sciences. All work for the dissertation was completed independently by the student.

Funding Sources

Chapter II was made possible in part by the American Kennel Club Canine Health Foundation under Grant Number [ACORN No. 02111]. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the American Kennel Club Canine Health Foundation. Chapter III was made possible in part of by the Winn Feline Foundation under Grant ID MT14-018. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the Winn Feline Foundation.

NOMENCLATURE

NGS	Next-generation sequencing
DNA	Deoxyribonucleic acid
ITS	Internal Transcribed Spacer
OTUs	Operational Taxonomic Units
CFU	Colony Forming Units
qPCR	Real-time Quantitative Polymerase Chain Reaction
AD	Atopic dermatitis
cAD	Canine atopic dermatitis

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

A brief history on the microbiome revolution

Microbiome is defined as the collection of microbes and their gene products that colonize a discrete habitat. *Microbiota* is a term that refers only to the microbial organisms within a microbiome, formerly known as microflora.[1] The mammalian microbiome can be further divided into microbial communities inhabiting anatomically and physiologically distinct body surfaces including the oral cavity, respiratory tract, gastrointestinal tract, urogenital tract and skin. The main categories of microbes within the mammalian microbiome in decreasing order of abundance are bacteria, fungi, viruses and parasites.[2]

One of the major propelling forces of the 'microbiome revolution'[3] was the establishment of the Human Microbiome Project (HMP) in 2007.[4] This international consortium, partially funded by the NIH roadmap initiative, set forth the overarching goals of understanding how the human microbiome impacts health, and developing novel therapeutic strategies to control the microbiome. The HMP promised to tackle challenges facing the field of microbiome research that would enable participation by the broader scientific community. Although the HMP was critical in the advancement of microbiome research, they surely were not the first to investigate effects of the microbiota on human health.[5] Previous researchers though were limited in their ability to cultivate host-associated microbes in the laboratory. It has been estimated that 20-60% of microbes within the microbiome are not readily cultured.[4] This does not come as a surprise to the field of microbiology as the 'great plate count anomaly' was revealed 35 years ago based on

the observation that fewer colonies grew on a plate than the number of bacterial organisms seen under a microscope.[6]

Technological advancements were an integral part of this revolution, as was the collaboration of scientific disciplines. The advent of massively parallelized DNA sequencing, or next-generation sequencing (NGS), in the early 2000's enabled simultaneous molecular characterization of entire microbial communities.[7] This was a vast improvement from Sanger chain terminated sequencing that required pure culture for sufficient sequence quality to identify single microbes in a sample. Microbial phylogenetic markers developed in the 1990s, such as the 16S ribosomal RNA (16S rRNA) coding region of bacteria and the Internal Transcribed Spacer (ITS) region of fungi, were harnessed for targeted amplicon sequencing of bacterial and fungal communities. The release of bioinformatics platforms such as QIIME[8] and mothur[9] provided researchers with standardized protocols and software packages to analyze the massive amounts of data generated from microbial NGS. One of the early concerns of the HMP was whether ecological analyses formerly applied to macro-scale environments would still be accurate for micro-scale environments such as the microbiome.[10] Ecologists and statisticians were involved in the seminal microbiome studies to apply and develop appropriate diversity and community distance analyses such as UniFrac.[11]

The anticipated outcome of foundational microbiome studies was a need to correlate functional impacts of the microbiome with human health. We quickly saw the birth of new fields of 'omics' and investigations into the metabolome, proteome and lipidome.[12] The ultimate goal of microbiome research was the formulation of prebiotics and probiotics that could alter the microbiome to remedy disease or promote health in an individual.[13] A short decade after the HMP was formed, we have already seen the development and testing of various pre- and

probiotics.[14] The success of microbiome studies in human medicine prompted our investigation into the microbiome of our veterinary patients, and the aims presented within this thesis.

The human skin microbiome in health

The healthy human skin microbiome is shaped by a number of biological and environmental factors. Pioneers of skin microbiome research began by describing the microenvironment of human skin across the entire body, thereby laying a framework for interpretation of the presence and distribution of skin microbiota.[15] The skin microenvironment is largely determined by the anatomy and physiology of skin.

Briefly, the skin is comprised of discrete layers with microbes generally colonizing the outermost layer, the epidermis, although new research documents the presence of rare bacteria in the deeper layer, the dermis.[16] The epidermis contains multiple layers of cells at differing stages of development with the outermost layer, stratum corneum, being likened to brick and mortar. The bricks represent anucleate skin cells, corneocytes, solidified within a mortar of lipid lamellae containing ceramides, cholesterol and fatty acids. Other important features of the stratum corneum that contribute to the barrier function of skin are keratohyaline granules containing the protein Filaggrin, and the envelope surrounding corneocytes made of proteins such as involucrin, loricrin, and keratolinin. Scattered throughout the epidermis are appendages including hair follicles and glands that extend down into the dermis. Sweat glands are named based on their differing secretions and function. Eccrine sweat glands secrete a liquid substance concentrated with sodium chloride and function in thermoregulation. Apocrine sweat glands secrete a substance rich in steroids that are thought to play a role in pheromone production. Sebaceous glands are associated with hair follicles and secrete an oily substance, sebum, containing lipids

that function in retaining moisture.[15,17] *Grice et al.* concluded that human skin has a varied topography that includes dry, moist and oily microenvironments or niches.[15]

The basic tenets of microbiology require that microbes have a metabolism adapted to the nutrient availability of their surroundings and possess adequate defense mechanisms to thrive in the face of natural competitors. The skin is much like a desert compared the gastrointestinal tract. The surface of the skin is overall acidic, dry and replete of carbohydrate sources.[15,17] Some examples of major skin commensals adapted to specific skin microenvironments include *Staphylococcus* and *Malassezia*. *Staphylococcus* spp. secrete proteases utilizing urea from sweat glands as a nitrogen source, and are halotorerant, able to withstand the high concentrations of salt in sweat.[18] *Malassezia* spp, that are not able to synthesize their own lipids, secrete lipases utilizing triglycerides in sebum as an energy source,[19] and phospholipases that allow for invasion of host tissue; phospholipases have been shown to vary in virulence dependent upon strain of *Malassezia*.[20] Initial studies using NGS to investigate bacterial microbiota of healthy human skin demonstrated that certain bacterial taxa were more abundant at specific body sites and that there was low inter-individual variability. For example, *Propionibacterium* that are lipid metabolizing, were more abundant in oily or sebaceous areas such as the face and chest, and *Staphylococcus* and *Corynebacterium* were more abundant on moist skin such as the elbow crease or axillae.[21]

Temporality of the skin microbiome in healthy individuals has also been investigated including post-natal colonization, then extending throughout all life stages, and lastly focusing on stability within a life stage. The route of initial colonization of the skin in neonates was shown to influence the composition of the gut microbiota for up to 7 years of age.[22] The skin of babies delivered by vaginal route was initially colonized by vaginal microbes such as *Lactobacillus* or *Prevotella*, and the skin of babies delivered by C-section were colonized by skin microbes such

as *Staphylococcus* and *Corynebacterium*.[23] During the post-natal period commensal microbiota educate the immune system in a process called tolerance so that following maturation of the immune system, commensals will not induce inflammation, and the body will know how to respond to invading pathogens.[24] Specifically microbiota can influence the immune system through the production of antimicrobial peptides, increasing expression of complement, control of the expression of Interleukin 1, and tuning of local T cells.[25] It is not yet clear how differences in early colonization mechanistically influence the immune system but some studies have shown the prevalence of celiac disease, asthma, type 1 diabetes and obesity is greater in children that were delivered by C-section compared to the vaginal route.[26]

The skin microbiome during puberty has been extensively studied due to the prevalence of acne in pubescent individuals. Studies have shown that pre-pubescent individuals have a greater diversity of bacterial microbiota whereas adolescent and post-adolescent individuals have a predominance of *Propionibacterium* and *Corynebacterium*.[18] These alterations have been attributed to increasing sex hormones that in turn stimulate an increased production of sebum, number of apocrine glands, and density of hair follicles. The result of these physiologic changes is increased epidermal lipids thus providing a niche for lipid loving bacteria like *Propionibacterium* and *Corynebacterium*. However, once reaching adulthood, the skin microbiome has been shown to be stable over a two-year span with most of the stability apparent at bacterial species and strain levels, and sebaceous areas demonstrating the greatest stability.[27]

A recent review on the geographic variation of the healthy human microbiome emphasizes the major challenge in differentiating between genetic, ethnic, environmental and cultural factors such as hygiene and diet.[28] The authors concluded that mode of subsistence (hunter gatherer, rural agriculturalist, western urban industrialized) overshadows ethnic or

geographic influences. They found that overall there was a core microbiome and microbial diversity was reduced as populations succeeded through stages of subsistence; corresponding with decreasing exposure to the environment (soil and natural bodies of water) and changing diets. While these effects are more pronounced in the GI microbiota, some studies have found the skin to be similarly affected. *Hospodsky et al 2014* found increased abundances of Propionibacteriaceae, Staphylococcaceae, and Streptococcaceae on the hands of women from the US compared to women from Tanzania who had increased abundances of soil associated Rhodobacteraceae.[29] Even within a focal geographic location, *Ting et al 2015* found that urban Chinese women had higher abundances of *Propionibacterium* on the glabella, while rural Chinese women had higher abundances of *Corynebacterium*.[30] A factor that is likely more pronounced in western civilization is the effect of co-habitation with pets. Cohabitation has been shown to be a strong influencing factor in the degree of shared skin microbiota between cohabiting individuals.[31,32] Cohabitation with dogs was shown to increase the level of shared microbiota in cohabiting individuals, with the greatest sharing occurring between the persons' palms and the dogs' forehead and paws.[31] The impact of sharing microbiota between pets and cohabiting people is not fully understood. However, studies demonstrating carriage of multi-drug resistant bacteria on the skin of healthy pets[33-37] reminds us that the effects of cohabitation with pets are indeed important to human health.

The human skin microbiome in disease

The perception of microbial pathogenesis has even evolved from a pathogen-centered focus, to the damage response framework factoring equal contributions from the host and pathogen, and now the effects of entire microbial communities on infectious disease. Koch postulated that an individual microorganism could be proven as the etiologic agent of a specific

disease. In the early 2000s the damage response framework presented a new way of thinking about infectious disease where pathogen virulence is a function of host damage ranging from a weakened to hyper-reactive immune system.[38] This framework came in a time when the prevalence of opportunistic infections in immunosuppressed individuals was on the rise and microbes, previously not considered pathogenic, were causing disease in these individuals. Colonization resistance was coined in the 1960s to explain how a single bacterium could protect from invasion by pathogenic bacteria.[39] The microbiome revolution brought into context how the entire microbiome collectively acting together could protect against colonization of pathogenic bacteria through direct antagonism and enhancement of mucosal immunity.[40] Most recently *Bird and Segre* published a letter proposing an adaption to Koch's postulates that infectious disease causation should be interpreted in a systems biology approach including the microbiome.[41] We now believe that due to the constant interaction between the host and its microbiota, the microbiome is involved with many infectious diseases by either preventing or predisposing to disease.

Dysbiosis, alterations to commensal microbiota, has recently been associated with numerous disease processes. The question remains for many of these diseases: which comes first? Is the dysbiosis the cause of disease, or does the disease result in dysbiosis? On almost every body surface there is now a correlation of disease with dysbiosis including but not limited to: bacterial vaginosis,[42] ulcerative colitis,[43] periodontal disease,[44] and cystic fibrosis.[45] Even systemic and metabolic disorders such as obesity,[46] diabetes mellitus[47] and neoplasia[48] have now been associated with dysbiosis. Diseases affecting skin include acne, chronic wound healing, diabetic ulcers, psoriasis, and atopic dermatitis.[18] For many of these affecting skin, commensal

bacteria become pathogenic, leading one to consider these in a damage response framework with contributions from both the host and the entire microbiome.

Acne vulgaris is a pustular skin disease affecting pubescent individuals. The bacterium isolated from pustules is *Propionibacterium acnes*. Interestingly *P. acnes* is found on both healthy and diseased skin and is one of the most abundant bacterial microbiota on human skin. *P. acnes* has been shown to induce inflammation within the follicle as well as systemically in diseased individuals.[49] Disease severity has been associated with increasing sebum activity,[18] but importantly specific strains of *P. acnes* have been isolated more frequently from diseased individuals.[50-53] Further work has shown that these specific strains of *P. acnes* from diseased individuals have differing effects on sebocytes and keratinocytes.[54,55] These findings taken together suggest that there is strain specific microbial pathogenesis as well as host factors that contribute to the disease acne vulgaris.

Inflammatory disease has long been suspected of being influenced by microbiota, and accumulation of reports that microbiota and the host immune system are highly interactive provides further indication. Two inflammatory skin diseases under ongoing investigation are psoriasis and atopic dermatitis (AD). Psoriasis is characterized by plaque formation subsequent to an initial inflammatory insult. Streptococcal throat infections have been linked to the onset of one type of psoriasis, although *Streptococcus* is a commensal and alone does not induce psoriatic lesions in healthy skin.[49] Recent microbiome studies have not identified a consistent dysbiosis signature associated with Psoriasis, but they have demonstrated an increased diversity of microbiota in lesional skin compared to non-lesional skin. Host genetic mutations affecting CD8T cells has been associated with up to 60% of psoriatic patients indicating an immune dysregulation is likely involved. Several studies have demonstrated shifts in microbiota

following treatment of psoriasis[56] leaving researchers to find the link between inflammation and dysbiosis in psoriasis.[57]

Dysbiosis has been consistently demonstrated as a significant factor in atopic dermatitis. This disease has a prevalence of 15-20% in children worldwide and is characterized by erythema and pruritus that progresses to plaque formation.[18,49] A portion of these patients then succumbs to what is known as the "atopic march," a progressive series of allergic diseases such as asthma and food allergies. This disease is multifactorial with contributions from skin barrier disruption, immune dysregulation and dysbiosis. In a subset of patients, a mutation in the gene encoding for the stratum corneum protein Filaggrin results in an impaired skin barrier which is thought to provide increased exposure to environment.[58] The immune dysregulation involves a Th2 predominated immune response and recruitment of mast cells and allergen specific IgE to the skin. *Staphylococcus aureus* carriage is reported at 30-100% in atopic individuals.[58] Founding NGS studies of AD demonstrated a correlation between atopic flares and increasing relative abundance of *S. aureus*.[18] This bacterium has been shown to induce further inflammation and damage to the skin barrier thereby exacerbating lesion severity.[49] Recently *Nakatsuji et al.* has shown that atopic skin is lacking colonization of coagulase negative *Staphylococci* (CoNS), such as *S. epidermidis* and *S. hominis*. CoNS have been shown to produce antimicrobials that keep *S. aureus* abundances in check. Lack of CoNS on infant skin has also been shown to increase the risk of developing AD later in life. Researchers conclude that exposure to CoNS may be preventative for the development of AD and future therapeutics may be developed targeting the balance of CoNS on skin.[58]

The fungal microbiome and its unique challenges

The fungal microbiome and its effects on human health have been studied far less than the bacterial microbiome. This is in part due to the presence of fewer fungal genomes in the human microbiome thus posing a challenge for adequate sequencing depth. A recent review documents that up to 10% of samples in a fungal microbiome study can be expected to be lost due to low number of sequences.[59] Although the genomic copy number of fungi is lower than that of bacteria, the size of fungal organisms tend to be 100 times greater than bacteria.[60,61] Theoretically the surface area covered by fungi is greater than bacteria, and the overall microbial biomass should be greater. This leads one to question whether the impact of fungal metabolites on the host could be greater too. The few number of fungal genomes is further compounded by the fact that skin has a lower overall microbial biomass compared to other human microbiomes such as the gastrointestinal tract.

Aside from genomic copy number, other unique challenges of studying the fungal microbiome are nuances of the ITS region, incomplete databases, and the lack of standardized protocols. Large-scale bioinformatics studies have shown that the ITS region is ideal for barcoding due to its high level of sequence variability between genera.[62] However, this sequence variability prevents it from being used to calculate a kingdom wide phylogenetic reference tree.[59] Many of the OTU picking algorithms and diversity analyses, such as UniFrac,[11] designed for the study of bacteria cannot be used for fungi due to the lack of a consensus tree. This has likely contributed to the lack of standardized bioinformatics protocols available. Additionally, fungi tend to be fastidious growers in the laboratory leaving our fungal databases incomplete. Many fungal microbiome studies are left with numerous sequences that cannot be classified, potentially missing biologically important associations between uncultivated taxa and health or disease.

Another complication of many available databases is the inclusion of both anamorph and teleomorph names for the same organism, which can lead to inaccurate interpretations of biological significance and over or under representation of specific taxa.

Skin was the first part of the human mycobiome to be described.[63] In contrast to the bacterial microbiome of skin, the mycobiome was predominated by only one genus, *Malassezia*, except at the feet that have a higher diversity, comprised of environmental fungi. These findings correlated well with previous cultured-dependent studies of skin. Temporal studies of the skin mycobiome found that core body sites remained stable and represented a greater portion of the metagenome than did mycobiota of the feet that were low in abundance and exhibited high variability.[27] Although *Malassezia* is the predominant fungal commensal in adults, children possess a much greater diversity of mycobiota with increased abundances of Euritiomycetes (which includes the fungi responsible for causing dermatophyte infections).[64] This finding was interesting given the prevalence of pediatric dermatophytosis. It is thought that just as increasing epidermal lipids during puberty promote the growth of *Propionibacterium*, so it does for *Malassezia*. Just recently in 2017 *Petrosino et al.* described the gastrointestinal mycobiome of the HMP cohort and found *Malassezia*, *Candida* and *Saccharomyces* to be the most abundant commenals.[65] This group also found there was a high degree of intra and inter-individual variability. In the last couple of years high quality reviews have been published on the methodology of mycobiome studies and so we hope more studies investigating its role in health and disease will be conducted in the near future.[59,61,66]

Similar to bacteria, fungi have recently been shown to modulate the immune system.[67] Fungi interact with the immune system through mannans present in their cell walls with toll-like receptors and c-type lectins. On the skin, *Malassezia* secrete metabolites such as indole that bind

to aryl hydrocarbon receptors and function in epithelial repair, melanogenesis, and barrier homeostasis. Additionally, *Malassezia* metabolize triglycerides in sebum into short chain fatty acids that have been shown to have a number of immunomodulatory effects. Interactions of fungi with the immune system continue to be an area of investigation especially with increasing evidence of the association of fungal dysbiosis with disease.

To date fungal dysbiosis has been associated with disorders of the immune system, delayed epithelial repair and inflammatory skin disease, although these studies are present in low numbers and caution should be taken in deriving strong conclusions. Patients with primary immune deficiencies possess varying genetic mutations resulting in impaired immunity but all experience atopic-like eczema. These patients were found to have increased fungal diversity comprised of fungi that commonly cause chronic opportunistic infections in these patients including *Candida* and *Aspergillus*.[68] Delayed wound healing has been associated with increased abundances of pathogenic or allergenic fungi in the mycobiome of diabetic foot ulcers.[64,69] This study identified the mycobiota as a prevalent component of the DFU metagenome, and that fungal diversity increased following antibiotic administration. The authors concluded that antibiotic administration could be contributing to the prolongation of healing by increased fungal colonization of wounds. One study has been performed investigating the mycobiota in Psoriasis.[70] This studied identified an increased fungal diversity on the skin of psoriatic patients compared to healthy controls with an overall reduction in *Malassezia*. Similar findings have been reported for atopic dermatitis, along with changes to the species distribution of the genus *Malassezia*. *M. slooffieae* and *M. dermatis* were found to be increased on atopic skin compared to healthy controls.[71] Interestingly in all four diseases discussed here, increased fungal diversity

and decreased abundances of the main fungal commensal *Malassezia,* have been associated with various disease states.

A note on terminology regarding inflammatory skin disease in companion animals

The terms ‘allergy’ and ‘allergic’ will be used throughout this thesis to describe inflammatory skin disease and the animals diagnosed with these conditions. “Atopic dermatitis’ is a term in veterinary medicine that is reserved for dogs who have a hypersensitivity to specifically environmental allergens and thus some of the disease factors are similar to AD in people. However, dogs can also have hypersensitivities to proteins in their food, most commonly to beef and chicken, or to flea saliva. Regardless of the inciting allergen these dogs, collectively referred to as ‘allergic,’ are at increased risk of developing secondary infections including *Staphylococcus* pyoderma and *Malassezia* dermatitis. Due to this reason, the assumption has been made that underlying pathologies that are likely similar, and thus we have included them within the same study group. Another reason is that allergic dogs will sometimes have hypersensitivity to more than one allergen (food, flea and environment), preventing stratification of these patients into separate study groups. Future studies may investigate disease factors and the microbiota separately based on inciting allergen, but was not the purpose of our work.

Cats are not known to possess the same similarities of their hypersensitivity dermatitis with atopic people. However, they can be hypersensitive to the same inciting allergens as dogs including food, fleas and the environment. As such the terminology reserved for their hypersensitivity dermatitidies are again different and include: flea-bite, food-induced, and non-flea non-food induced hypersensitivity dermatitis. Collectively these cats will be referred to as ‘allergic’.

Inflammatory skin disease in companion animals

Much of what we know about the role of microbiota in inflammatory skin disease comes from studies of AD in people. Contextual interpretation of the microbiota in inflammatory skin disease of animals must consider important differences in skin anatomy, physiology and disease presentation. Dogs and cats are covered with pelage of differing thickness, hair quality, and length which can affect the temperature and moisture of the skin surface.[17] This tends to vary most significantly by breed of dog, however in-breeding has likely also resulted in the accumulation of additional genetic mutations that could contribute to the skin microenvironment. The potential role of breed heritable effects on the skin microenvironment has not been investigated in depth. Gland distribution is also different in dogs and cats. Eccrine sweat glands tend be concentrated on the foot pads, whereas apocrine glands are more evenly distributed across the body.[17] Aside from anatomical differences, the composition of gland secretions and lipid content of lamellae in animals is not fully understood. Although general functions of glands are likely to be similar, differences in proportions of proteins and lipids could have a profound effect on the microbiota. Further investigation is warranted to appreciate the distribution of skin microbiota in animals.

Inflammation in the skin of animals and people causes similar clinical signs such as erythema and pruritus, however, subsequent lesion development differs. Instead of dry eczematous plaques such as is observed in atopic people, allergic dogs develop lesions associated with secondary skin infections- most commonly *Staphylococcus* pyoderma, and *Malassezia* dermatitis and otitis.[17] *Staphylococcus* pyoderma is characterized by superficial dermatitis or a pustular folliculitis, and *Malassezia* dermatitis is characterized by grease, scale, crust and occasional pustules. These infections have been shown to contribute to pruritus and commonly

are recurrent throughout an atopic dog's lifetime. Chronic *Malassezia* dermatitis causes lichenification and hyperpigmentation of skin, although chronic inflammation of the skin may also present in an identical manner. In addition to classical atopic dermatitis, dogs may also present with similar skin lesions in response to food allergies, known as cutaneous adverse food reaction. The distribution of these lesions in dogs, regardless of inciting allergen, occurs predominantly on the face, ears, feet, folds such as the axilla and inguinal region, and flexure surfaces. Importantly, this lesion distribution is identical to that of atopic people making dogs an appropriate model to study AD.

Allergic skin disease in cats is characterized by markedly different lesion presentation with four main patterns: cervicofacial dermatitis, eosinophillic skin disease complex, milliary dermatitis and self-induced alopecia.[72] The eosinophillic skin disease complex is a rather unique disease presentation in cats and can include the presence of indolent ulcers of the upper lip, eosinophilic plaques of the abdomen and medial thighs, and eosinophillic granulomas of the oral cavity, chin or caudal thighs. Unlike allergic dogs that are highly predisposed to secondary infections these are relatively uncommon in cats, but when they do occur are predominantly *Staphylococcus* pyoderma, and *Malassezia* dermatitis or otitis.

The underlying pathogenesis of atopy in dogs shares both similarities and differences with the same condition in people.[73] A subset of dogs was identified as having a mutation in filaggrin shown to contribute to skin barrier impairment in canine atopic dermatitis. Also similar to people, studies have shown that atopic dogs have transepidermal water loss, ceramide abnormalities, and increased levels of IgE in the skin against environmental allergens. Members of the taskforce on canine atopic dermatitis caution that cAD is a clinical syndrome and not all patients will exhibit this exact combination of abnormalities.[74] Onset in companion animals tends

to occur early in life around 1 to 3 years old.[75] An interesting thought that has yet been unexplored, possibly due to the lack of comparative mammalian studies, is that atopic dermatitis in people is generally a disease of children, whereas atopic dermatitis in companion animals is a lifelong condition. People undergo significant changes in their skin lipid content during puberty causing a shift in their skin microbiota. To our knowledge, this phenomenon does not occur in most of our companion animals, possibly because they are spayed and neutered at a young age. Future studies comparing the prevalence of atopy or composition of epidermal lipids in intact animals compared to spayed or neutered animals may help answer this question.

The microbiome in companion animals

Exponentially less is known about the skin microbiota of animals and whether dysbiosis is associated with disease in dogs and cats. The first canine microbiome to be described was in the gastrointestinal tract. Major bacterial phyla in the canine gastrointestinal tract are similar to those in people. Shifts in the gastrointestinal microbiota were documented in both acute and chronic diarrhea in dogs, most notably a loss of commensal bacteria and increased abundances of *Clostridium perfringes*.[76,77] Dysbiosis in canine inflammatory bowel disease differs with increased abundances of Proteobacteria such as Enterobacteriaceae and decreased abundances of Firmicutes such as *Clostridia*.[78] Based on these findings, the administration of probiotics has become more common in the treatment of diarrhea and inflammatory bowel disease in veterinary medicine. While some studies have shown beneficial outcomes of probiotic administration in diarrhea and IBD, a recent exhaustive review cautions these effects may be strain dependent and the results are not consistent.[79]

Prior to the initiation of this thesis work, the skin microbiota of dogs and cats had not been investigated using NGS with the exception of one publication in 2014 describing the

bacterial microbiota of healthy and allergic dogs.[80] This study looked at a variety of factors including breed, age, sex, presence of fleas, housing habitat, and indoor and outdoor environments. Compared to human skin, the skin of dogs had more Proteobacteria and it was hypothesized this could be due to increased environmental exposure. In support of this hypothesis, a recent study of the skin microbiome across all mammals found that the human skin microbiome was distinct from all other mammals.[81] Non-human mammals in this study had greater abundances of soil-associated bacteria. *Ross et al.* concluded that hygiene and environmental exposure were likely influencing the differences observed in humans and non-human mammals.

More recently several studies have investigated breed, environmental factors, and temporality affecting the healthy skin microbiota of dogs.[82,83] Sampling of the skin microbiota in cohabiting dogs over time demonstrated a significant influence of cohabitation.[82] This study also concluded that season was an influencing factor, however, for this to be true, the authors should have sampled the dogs over multiple years. The study design that only sampled dogs over the course of one year, only allows for correlation of microbiota with temporality and not season. Another study controlled for breed and environment influences by only sampling dogs from a related background that shared the same environment.[83] This group found that the individual followed by body site were the main driving factors. These studies taken together suggest that breed, environment, cohabitation, and seasonality may all be important variables to control for in the study design of skin microbiome studies of animals.

Following the undertaking of this thesis work, the skin of allergic dogs has been more extensively studied. As of now the skin microbiome in allergic dogs has been evaluated in longitudinal studies of atopic flares, and after the administration of immunomodulatory drug or

antimicrobials. *Bradley et al.* sampled atopic dogs at lesion flare, 4 weeks after starting antibiotic administration, and 4-6 weeks following cessation of antibiotics.[84] They found *Staphylococcus pseudintermedius* predominated atopic dogs during flares, and abundance of *S. pseudintermedius* correlated with lesions severity. Abundance of *S. pseudintermedius* and lesion severity was significantly reduced following antibiotic administration. However, the authors caution that with the rise of antibiotic resistance alternative approaches for controlling *S. pseudintermedius* in dogs should be investigated. A similar study by *Pierezan et al.* investigating the skin microbiota in an inducible canine model of atopic dermatitis demonstrated increased abundances of *Staphylococcus* following exposure to allergen using both NGS and real-time quantitative PCR.[85]

Immunomodulatory drugs such as glucocorticoids and cyclosporine are commonly prescribed treatments for atopic dermatitis in dogs. *Widmer et al.* tested the effects of these drugs on the skin microbiota of atopic dogs who were not experiencing flares or infections.[86] They concluded that these drugs did not have any effect on the bacterial diversity or community structure. Despite their conclusions, some trends indicated the opposite, and the small sample size possibly influenced lack of significance in this study, since only 6 dogs were included. Another group tested the effect of topical antimicrobial shampoo with both antibacterial and antifungal spectrum on healthy and atopic dogs.[87] This study demonstrated significant changes to the bacterial and fungal community composition following treatment. The overall bacterial diversity increased and fungal diversity decreased with treatment. One limitation to this study was a small sample size of only 9 dogs, and future larger studies are warranted to determine the effects of topical antimicrobials used to treat atopy in dogs. It remains difficult to interpret the impact of changes to microbiome structure following treatment, but could serve as tool to monitor treatment success or prevention of future flares.

Thesis Aims

Prior to the this thesis work the skin mycobiome of companion animals had not been described using NGS, and the role of the mycobiome in canine atopic dermatitis and feline hypersensitivity dermatitis was unknown. Atopic dogs are at a higher risk of developing secondary *Malassezia* dermatitis and otitis compared to healthy dogs, and recent works have demonstrated inter-microbial interactions that could influence host health. This lead us to the aims of this thesis project which were to describe the skin mycobiota of dogs and cats, and then to investigate how the skin mycobiota may be altered in allergic animals. Due to *Malassezia pachydermatis* being a common opportunistic pathogen in allergic dogs, we also aimed to derive species level abundances of *Malassezia* in healthy and allergic dogs. Lastly we aimed to determine whether shifts in the abundance of *Malassezia* species occur as a result of allergen exposure in a longitudinal study of inducible canine atopic dermatitis.

CHAPTER II [1]

CANINE CUTANEOUS MYCOBIOTA

Introduction

Skin diseases are often characterized by multifaceted etiology with potential contributions coming from the host's genetics, skin barrier integrity, immune system, and inflammatory components, which can be exacerbated by environmental exposure and hygiene practices.[88] The cutaneous microbiota associated with skin diseases have only recently been investigated in humans. Through this work, dysbiosis (an alteration to the normal microbiota) of cutaneous microbiota has been associated with a variety of human skin diseases including psoriasis (PS),[70,89] acne vulgaris,[90] and atopic dermatitis (AD).[68,91] Fewer studies have focused on how the microbiota influences skin health of other host species such as dogs.[80] In addition to improving animal health, these studies are needed to evaluate how animal behavior, anatomy, or environmental exposure affects cutaneous microbiota, and ultimately health status of the host. Interest in the microbial communities of companion animals is growing as we begin to recognize how their microbiota can influence our own[31] and possibly affect human health for people cohabiting with pets.[92]

Atopic dermatitis (AD) is a pruritic condition characterized by a skin barrier dysfunction and hyper-sensitization to environmental allergens.[93,94] In both people and dogs, there are increased levels of IgE to environmental allergens, an initial Type I hypersensitivity reaction characterized by increased numbers of T-helper type-2 (Th2) cells in lesional skin, and later Type IV hypersensitivity in chronic cases, supporting a similar pathogenesis of AD in these two

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[1] Meason-Smith C, Diesel A, Patterson AP, et al. What is living on your dog's skin? Characterization of the canine cutaneous mycobiota and fungal dysbiosis in canine allergic dermatitis. *FEMS Microbiol Ecol* 2015;91.

host species.[94] Fungi are generally thought to be less influential in the pathogenesis of AD, however *Malassezia* hypersensitivity has been implicated in both human and canine AD through patch testing, IgE studies, and responsiveness to antifungal therapy.[95-100] Recently, next generation sequencing (NGS) studies have identified bacterial dysbiosis associated with affected human skin in AD[91] and PS,[89] and with non-affected skin in dogs with allergic skin disease, which includes AD.[80] Bacterial dysbiosis associated with AD and PS is characterized by a reduction of bacterial diversity in affected skin, and shifts in relative abundance of particular bacterial species.[89,91] Fungal dysbiosis has also been reported for these diseases in humans, but instead of reduced diversity as found for the bacterial microbiota, there is an increase in fungal diversity at the site of lesions,[68,70,101] and clustering by health status in principle coordinates analysis.[68,70,101]

Prior to investigating how a disease process has altered the host microbiota, or if the microbiota might play a role in disease pathogenesis, there must be the initial studies of healthy skin microbiota and determination of the factors influencing their ecological distribution and function. A few studies have characterized the fungal microbiota (mycobiota) of human skin using NGS.[63,68,70,101-103] One study revealed that healthy human skin is predominantly colonized by the genus *Malassezia* with body site differences seen only for the different species of *Malassezia*.[63] Fungal diversity was dependent upon body site, and the greatest diversity was found in samples from feet. Retesting of individuals over time demonstrated a stable fungal community structure at the core and arm body sites, but not at the feet. Overall fungal community structure was strongly correlated with the site location (head, torso, arms, feet),[63] in contrast to bacterial communities that are more dependent on site physiology (dry, moist, sebaceous).[21]

We are still in the early stages of describing the skin microbiota in companion animals using NGS. To date there has been one NGS study published by our group characterizing the bacterial microbiota of canine skin.[80] Similar to human skin, bacterial community composition is significantly different between body sites. The predominant phylum across all body sites in dogs is *Proteobacteria*, unlike human skin that is predominantly colonized by *Actinobacteria* and *Firmicutes*.[21] Richness and diversity of bacterial taxa varies across the canine body sites with the nostril and conjunctiva harboring the fewest, and the dorsal nose the greatest.[80]

Descriptions of the mycobiota in dogs using NGS has been limited to the fecal mycobiota in healthy dogs and those with diarrhea.[104] The only studies aimed at characterizing fungi on the skin of dogs have been culture-based.[105-111] One study sampled strictly the ears of dogs (n=194), that were either healthy, atopic or had otitis.[110] In this study, the most abundant fungal organism cultured was *Penicillium* spp., and the second was *Malassezia pachydermatis*. Another study from France cultured fungi from the conjunctiva and adjacent skin on the nose of dogs and identified the presence of additional fungi including *Alternaria*, *Cladosporium*, and *Aspergillus*.[111] While these studies are all valuable, it is well documented that molecular-based studies provide a more comprehensive picture of the microbial landscape due to the non-cultivable nature of some microbes or "selective" culture of others.[112]

The goal of this study was to characterize the canine cutaneous mycobiota using NGS, and determine whether body sites influenced the distribution of fungal organisms. Ten distinct body sites, consisting of haired skin, mucosal surfaces and one mucocutaneous junction were sampled in ten healthy dogs. We expected to find a greater diversity of fungal commensals than what has been detected by fungal culture alone. Similar to human skin, we expected to see a dependence of fungal communities on site location. To additionally investigate the role of the

mycobiota in canine allergic skin disease, we also collected skin swabs from eight dogs with clinical allergic skin disease at six body sites that are commonly affected by cutaneous manifestation of allergies. We expected to find changes in the diversity, membership and structure of fungal communities, as well as increased abundances of *Malassezia pachydermatis* owing to its implication in canine AD.

Materials and Methods

Subject Recruitment

All samples for this study were collected following a protocol approved by the Texas A&M University Institutional Animal Care and Use Committee. Ten dogs (D1-D10) with no history of skin disease were recruited for collection of healthy skin samples (Table 1). These dogs ranged from 1.5 to 11 years old, and included five castrated males and five spayed females. There were four mixed breed dogs, two Jack Russell Terriers, one Beagle, one Pitbull, one Boston Terrier, and one German Shepherd. A board certified veterinary dermatologist clinically evaluated the ten healthy dogs, and also evaluated eight additional dogs (D11-D18) for inclusion in the allergic group (Table 1). Six dogs were diagnosed with AD using standard diagnostic methods including fulfillment of Favrot's criteria and exclusion of other pruritic dermatoses.[113] One dog was diagnosed with chronic pododermatitis and cutaneous adverse food reactions (CAFR), and one dog was diagnosed with only CAFR. The allergic dogs ranged from 2 to 10 years old, included four castrated males, and four spayed females. The breeds of allergic dogs were two Boston Terriers, two Cavalier King Charles Spaniels, one Shetland Sheepdog, one Australian Shepherd, one Labrador retriever, and one mixed breed dog. To be included in the study, dogs could not have displayed overt clinical signs of bacterial or fungal skin infections at the time of sample collection. Five out of the eight allergic dogs were receiving medication for

their AD: oral immunotherapy (2), oclacitinib (Apoquel®, Zoetis) (2), and oral cyclosporine (Atopica, Novartis) (1). All healthy study participants did not receive systemic antibiotics or antifungals six months prior to collection of samples, and allergic study participants one month prior. Additionally, no dog was allowed to be bathed one week prior to the beginning of the study. Healthy dogs had not received steroids previously, and all but one of the allergic dogs had not received steroids within the last month prior to the study. Allergic dogs were allowed to have their allergic disease managed with either long-term medication and/or immunotherapy without the need for withdrawal for study inclusion purposes. None of the allergic dogs were experiencing lesions or flares at the time of sample collection.

Table 1. Medical histories and environmental exposures of dogs enrolled in this study. Allergy pruritus, ear problems and fleas were part of the clinical history and not clinically present at the time of sample collection.

Dog	Health Status	Breed	Age	Sex	Allergy Pruritus	Ear Problems	Fleas	Time Indoors	Outdoor Environment	Indoor Environment	Allergy Treatments	Steroids	Previous Antibiotic Usage
D1	Healthy	Jac	9	M	N	N	N	80	TGW	CTFB	N/A	N	Y
D2	Healthy	Mix	1.5	M	N	N	N	90	GW	CTFB	N/A	N	N
D3	Healthy	Mix	2	M	N	N	N	80	TGW	CTFB	N/A	N	N
D4	Healthy	Mix	3.5	M	N	N	N	90	TGW	CTFB	N/A	N	N
D5	Healthy	Bea	2	M	N	N	N	90	GW	CTFB	N/A	N	N
D6	Healthy	Mix	1.5	F	N	N	N	70	TGW	CTFB	N/A	N	N
D7	Healthy	Pit	9	F	N	N	Y	90	TGW	TF	N/A	N	N
D8	Healthy	Bos	3	F	N	N	Y	70	TGW	TF	N/A	N	N
D9	Healthy	Jac	11	F	N	N	Y	90	TGW	CTFB	N/A	N	N
D10	Healthy	Ger	7	F	N	N	N	50	TGW	CTFB	N/A	N	N
D11	Allergic	Bos	2	M	Y	Y	N	98	W	CTFB	Oral Immunotherapy	DepoMedrol	N
D12	Allergic	Bos	7	M	Y	Y	N	98	W	CTFB	Oral Immunotherapy	DepoMedrol	N
D13	Allergic	Mix	6	M	Y	Y	N	70	TGW	TFB	Apoquel	Prednisone	Y
D14	Allergic	Cav	2	F	Y	N	Y	99	TGW	CTFB		Y	Y

Table 1. Continued

Dog	Health Status	Breed	Age	Sex	Allergy Pruritis	Ear Problems	Fleas	Time Indoors	Outdoor Environment	Indoor Environment	Allergy Treatments	Steroids	Previous Antibiotic Usage
D15	Allergic	She	5	F	N	Y	N	95	TGW	CTFB	Cyclosporine	N	N
D16	Allergic	Aus	3	M	N	N	N	50	TGW	CTFB		Prednisone	N
D17	Allergic	Lab	10	F	Y	N	Y	95	TGW	T	Fluconazole	Prednisone	N
D18	Allergic	Cav	10	F	Y	Y	Y	90	TGW	TF	Apoquel	N	N

Jac: Jack Russell Terrier, Mix: Mixed breed, Bea: Beagle, Pit: Pitbull Terrier, Bos: Boston Terrier, Ger: German Shephard, Cav: Cavalier King Charles Spaniel, She: Shetland Sheepdog, Aus: Aussie, Lab: Labrador, T: Trees, G: Grass, W: Weeds, C: Carpet, T: Tile Floors, F: Furniture, B: Bedding. Allergy treatments were concurrent, and D16 was the only dog currently taking steroids, all others with a Y had not received steroids in the last month (Meason-Smith, 2015).

Sample Collection and DNA Extraction

Ten body sites on healthy dogs were swabbed including the axilla, conjunctiva, dorsal nose, ear canal, groin, interdigital space, lip commissure, lumbar, nostril, and pinna. Six body sites on allergic dogs that are commonly affected by cutaneous manifestation of allergies were swabbed including the axilla, ear canal, groin, interdigital space, lumbar, and nostril. Samples were only collected from the right side of all dogs. Gloves were changed between dogs and the exam table was wiped down with DNA away (Molecular BioProducts, Inc., San Diego, CA) after each dog was sampled. Three superficial skin swabs (Isohelix,Cell Projects Ltd. UK) were used for each body site, with swabs being rubbed ten times on each side of the swab within an area of approximately one square inch. Two swabs were immediately stored in lysis buffer from the MoBio Power Soil DNA Extraction kit (MoBio Laboratories, Inc., CA) to be extracted and sent for sequencing, and the third was retained in a sterile tube for archiving purposes. All swabs were stored at 4°C for no more than one week before extraction and final storage at -80°C. DNA was isolated using the MoBio Power Soil DNA Extraction kit, following the manufacturer's protocol. Negative controls were also included in sequencing: including sterile swabs that were processed following the extraction protocol, and the reagents only, with no swab included.

For comparison of sequences between our extraction protocol and the protocol followed by Findley *et. al.* 2013, we collected four swabs from the right ear of five healthy dogs taking the same precautions as above. Two swabs were used following the above DNA extraction protocol and two followed the Findley protocol.

ITS Sequencing and Sequence Analysis

Extracted DNA was submitted to MR DNA Laboratory (Shallowater, TX) for Illumina sequencing on a MiSeq Instrument using ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and

ITS4R (5'-TCCTCCGCTTATTGATATGC-3') primers. Resultant sequences from the forward reads were processed in Mothur,[9] an open-source bioinformatics software. First, sequences were trimmed for quality, sequences less than 200 bases were culled out and the remaining were chopped at 250 bases. Next, chimeras were removed with Uchime,[114] and OTUs were binned by taxonomic classification (phylotype) with the ITS-1 Findley *et. al.* 2013 database following their recommended parameters. Alpha diversities including inverse Simpson, non-parametric Shannon, Chao1, and observed species were calculated with a rarefaction depth of 1900 sequences. To assess both the membership and structure of fungal communities, distance matrices for healthy samples, allergic samples, and shared sites were generated using Bray-Curtis (membership), Jaccard (membership) and Yue-Clayton theta coefficient metrics (structure), with a rarefaction to 1900 sequences. These distance matrices were formatted for use within QIIME to generate principle coordinates of analysis (PCoA) plots.

Statistical Analysis

Alpha diversity estimators and relative abundances were first confirmed non-normal with the Shapiro-Wilk test in the statistical software JMP Pro 11 (SAS Institute, Inc.). A significance value of $P<0.05$ was selected for all statistical tests. A Kruskal-Wallis test was performed to determine if the alpha diversity of at least one body site or dog was significantly different from the others. When significance was identified, a Steel-Dwass All Pairs test was performed to identify the body sites or dogs that were significantly increased or decreased (JMP). A Mann-Whitney test was performed for each shared body site (a body site that was sampled in both healthy and allergic dogs; n=6), to determine whether the samples for one health status were significantly different from the other (JMP). Analysis of similarities (ANOSIM) function in the statistical software package PRIMER 6 (PRIMER-E Ltd., Luton, UK) was performed on

Mothur-generated distance matrices (Bray-Curtis, Jaccard, and Yue-Clayton theta coefficient) to determine the influence of various factors (body site, individual dog, health status) on the dissimilarity between mycobiota of the groups being examined. The relative abundance tables generated in Mothur for each taxonomic level were combined and filtered to only include taxa that were present in at least 20 samples at greater than or equal to 0.1%. To identify taxa whose relative abundance was significantly different between body sites, individual dogs or health statuses, the filtered relative abundance table was imported into JMP and Kruskal-Wallis tests were performed. The filtered relative abundance table was also formatted for linear discriminant analysis (LDA) effect size (LEfSe)[115] to identify significant differences in taxa between health statuses. All p-values were corrected for multiple comparisons using the Benjamini & Hochberg False discovery rate.[116]

Raw sequences were uploaded to the NCBI Sequence Read Archive under the project number: PRJNA293511.

Results

From the 148 canine body sites sampled, four were removed from analysis due to low number of sequences. The total number of fungal sequences amplified from the remaining 144 samples totaled 4 477 229 after quality processing and chimera removal; the median number of sequences per sample was 30 354.

Fungal Diversity Analyses of Healthy Canine Skin

Two factors were considered in the diversity analyses of healthy dogs: the influence of body site and of the dog. To test the effect of body sites, the same sites from all dogs were analyzed as a group. Conversely, to test the effect of the dog, all body sites from the same dog were analyzed as a group. Next, the diversity estimators for each group were compared. If there

were significant differences between the groups, we concluded that factor had an influence on the diversity of the cutaneous mycobiota. We found an overall significant effect of body site on the richness (observed species, $P=0.0002$; Supplementary Table S1) and diversity (Shannon, $P=0.028$; Supplementary Table S1) of the cutaneous mycobiota. The mucosal surfaces, nostril and conjunctiva, accounted for most of this difference and had a significantly reduced number of observed species compared to all other sites ($P<0.05$; Fig.1a). However, when taking into account evenness with the Shannon metric, we found that only the nostril was significantly less diverse ($P<0.05$; Fig. 1b) than all other sites. We also found a significant effect of the individual dog on the richness (observed species $P<0.0001$; Supplementary Table S1) and diversity (Shannon $P=0.0003$; Supplementary Table S1) of mycobiota. The mycobiota of dog number 10 was more rich ($P<0.05$; Fig. 1c) and diverse ($P<0.05$; Fig. 1d) than that of all other dogs. Median values of alpha diversity for each body site and dog are reported in Supplementary tables S2 and S3.

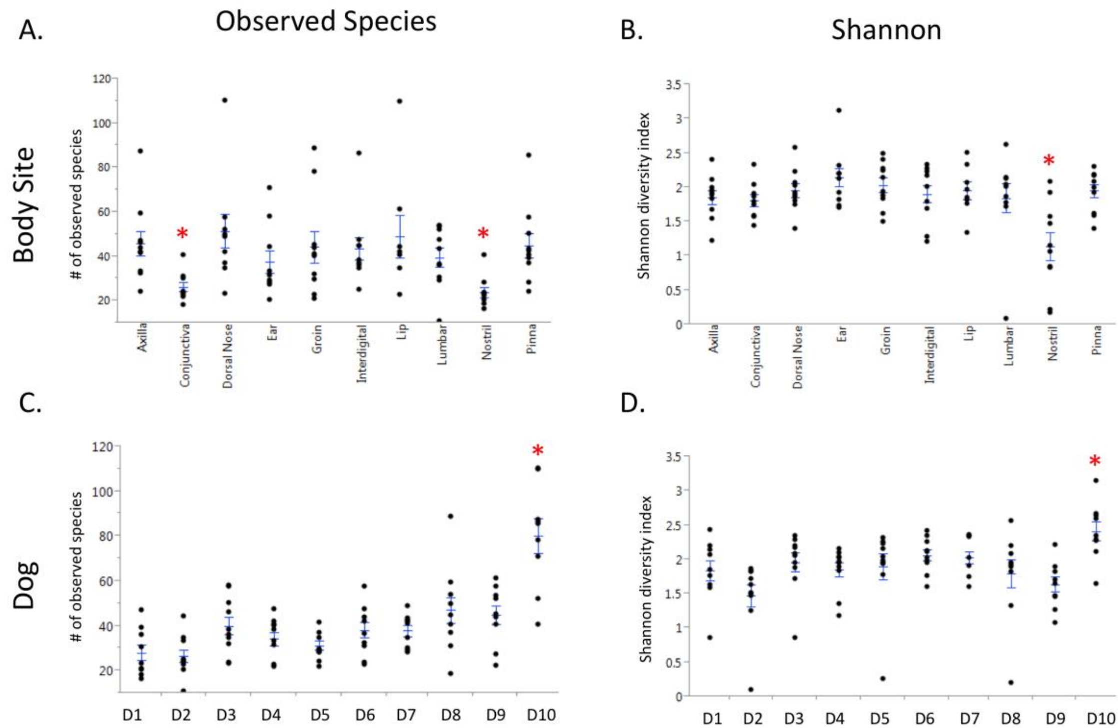

Figure 1. Alpha diversity of healthy dogs. Asterisks denote body sites or dogs that are significantly different from all other sites or other dogs (Kruskal-Wallis, multiple comparisons test, P<0.05). (a) Species richness estimator was calculated with observed species and samples were grouped by body site. (b) Diversity estimator was calculated with Shannon, and samples were grouped by body site. (c) Species richness estimator was calculated with observed species and samples were grouped by dog. (d) Diversity estimator was calculated with Shannon, and samples were grouped by dog. Means and mean error bars plotted in blue (Meason-Smith, 2015).

The same two factors were examined to determine their influence on the membership and structure of fungal communities (beta-diversity) residing on healthy canine skin. To answer this question, ANOSIM was performed on rarefied distance matrices (membership: Bray Curtis and Jaccard metrics; structure: Yue-Clayton coefficient). To determine the effect of one factor on the membership or structure, pairwise comparisons were made between all body sites or between all dogs. An R value and p-value were produced for each comparison, and an R value closer to zero indicated similarity between the pair, whereas an R value closer to one indicated dissimilarity

between the pair. When examining a factor, higher R values indicated that factor has an influence on the beta-diversity.

We found that the individual dog factor had a greater influence (median R values=0.338, 0.535, 0.381 respectively; Supplementary Table S4) on the beta-diversity of cutaneous mycobiota than did the body site factor, which was not a significant influencing factor for any of the 45 pairwise comparisons made between healthy body sites (Fig. 2a). From the 45 comparisons between healthy dogs, 38 pairs were significantly dissimilar ($P<0.05$; Supplementary Table S4). These findings were visualized with principal coordinates of analysis (PCoA) plots generated from rarefied Bray-Curtis distance matrices, which showed no sample clustering by skin microenvironment (Fig. 2b), or body site (Fig. 2c), but showed clustering of all body sites for several dogs (Fig. 2d). However, not every dog demonstrated tight clustering of body sites.

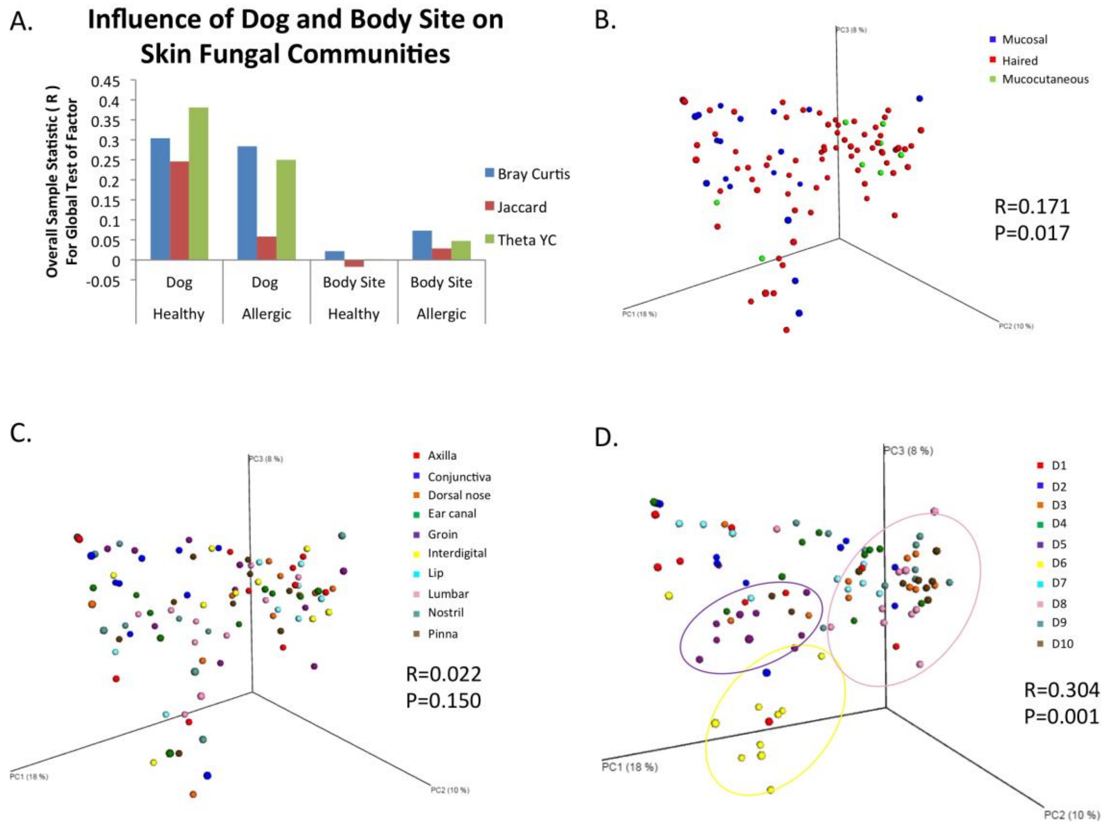

Figure 2. The influence of skin microenvironment, body site, and dog on fungal community membership. (a) The overall sample statistic (R) from the global test for the two factors 'Body Site' and 'Dog' were calculated in Primer6 with ANOSIM on the distance matrix containing only healthy or only allergic dogs. Pairwise distance calculations were performed in Mothur using the following metrics: Bray Curtis (blue), Jaccard (red), and the Yue-Clayton theta coefficient (green). The R-values for each test are plotted as bars and grouped by factor tested (top), and health status of samples included (bottom). (b) PCoA plot was generated in QIIME with samples colored by skin microenvironment, and pairwise distance calculations were performed in Mothur using the Bray-Curtis metric. (c) PCoA plot was generated in QIIME with samples colored by body site, and pairwise distance calculations were performed in Mothur using the Bray-Curtis metric. (d) PCoA plot was generated in QIIME with samples colored by dog, and pairwise distance calculations were performed in Mothur using the Bray-Curtis metric (Meason-Smith, 2015).

Fungal Community Composition of Healthy Canine Skin

In addition to diversity analyses, the taxonomic composition of the mycobiota was also determined. The predominant phylum of fungal organisms sequenced from healthy canine skin was Ascomycota followed by Basidiomycota. The major class of Ascomycetes was

Dothideomycetes and the most abundant genera within this class included *Alternaria*, *Cladosporium*, and *Epicoccum*. The most abundant Basidiomycete genera included *Cryptococcus* and *Malassezia*. There were also other Ascomycete taxa that were abundant but unable to be classified to the genus level based on available fungal databases (Fig. 3).

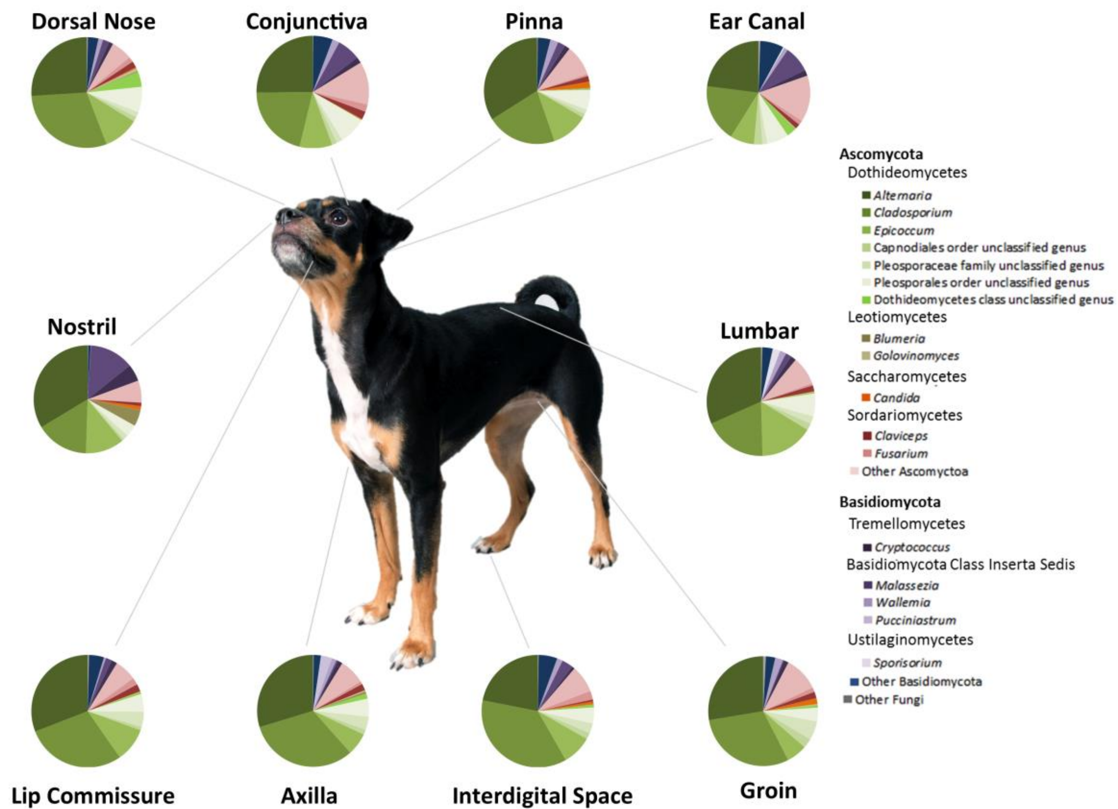

Figure 3. Average relative abundance of fungal taxa by body site in healthy dogs. The average relative abundance of predominant taxa was calculated for each body site and represented by pie charts. The averages were taken from D1-10 (Meason-Smith, 2015).

To determine whether the relative abundance of specific fungal taxa differed between body sites or individual dogs, statistical analysis was performed on the relative abundance tables generated from Mothur. The relative abundance table, including all taxonomic levels, was

filtered to only include taxa that were present in at least 20 samples at greater than 0.1%. Using this filtered table that included 193 taxa, we identified four fungal taxa that were significantly different between body sites in healthy dogs, and 153 that were significantly different between dogs (P<0.05; Supplementary Table S5). The LEfSe analysis did not reveal any taxa that were significantly different between body sites. These findings are further visualized by stacked bar plots of the relative abundances of fungal taxa for each sample, which showed a high degree of variation between dogs (columns) for the same body site (rows) (Figs. 4a and 4b). Similar to the diversity analysis, the individual dog factor had a greater influence on community composition than did the body site factor, indicating that specific taxa were found across all body sites in one dog, but not present on other dogs.

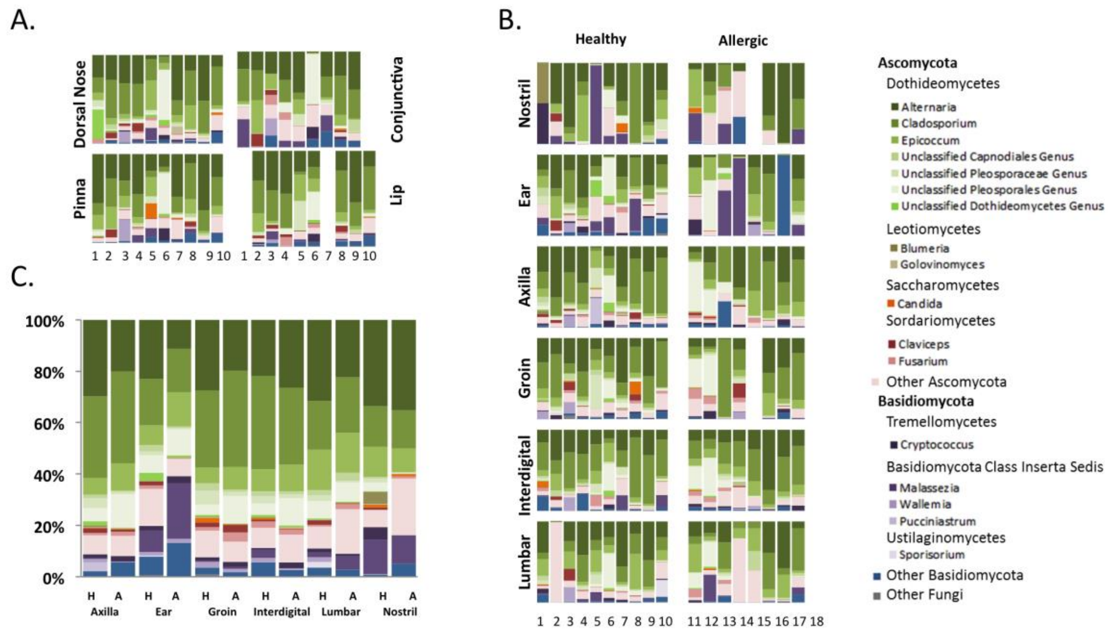

Figure 4. Fungal taxa summary plots for healthy and allergic canine skin. Stacked bar plots represent the predominant fungal taxa present within a sample. (a) Body Sites are arranged in rows with each column representing the body site of one dog (numbered at the bottom). (b) Shared sites between healthy and allergic dogs are arranged with healthy on the left and allergic on the right in a similar orientation as (a). (c) The relative abundance of predominant fungal taxa was averaged across all dogs in each health status group for each body site. H represents healthy dogs and A represents allergic (Meason-Smith, 2015).

To test if the extraction protocol used in this study had an effect on the ability to extract *Malassezia* DNA, we compared this extraction protocol to that of Findley *et. al.* 2013. Comparison of the two extraction protocols yielded no significant differences in the relative abundance of *Malassezia* detected in the ears of five dogs (Supplementary Fig. S1a), and both protocols yielded similar most abundant taxa: *Cladosporium* and *Epiccocum (*Supplementary Fig. S1b). However, there did exist an influence of protocol on the data, as relative proportions of *Cladosporium* and *Epiccocum* varied between the two protocols, samples clustered separately by protocol on PCoA, and overall community membership and structure were significantly different (ANOSIM R=0.3880, P=0.0370; Supplementary Fig. S1c).

Fungal Diversity Analyses of Baseline Allergic Canine Skin

Similar to the approach in healthy dogs, we were also interested in how body site or dog influenced the mycobiota of allergic skin in dogs. There was an overall significant effect of body site on richness (observed species, P<0.0001) and diversity (Shannon, P=0.030) of cutaneous mycobiota (Supplementary Table S1). Specifically the nostril was less rich (observed species, P<0.01) than the axilla, groin, interdigital, and lumbar sites, and less diverse (Shannon, P<0.05) than the axilla, groin, and interdigital sites. In addition, the ear in allergic dogs was both less rich (observed species, P<0.05) than the axilla, groin and lumbar, as well as less diverse (Shannon, P<0.05) than the interdigital space. In contrast to the findings in healthy dogs, there was no influence of the individual dog on the richness or diversity of the skin mycobiota. Exact values of alpha diversity for each body site and dog are reported in Supplementary tables S6 and S7.

Although there were no significant differences in beta diversity of cutaneous mycobiota between body sites in healthy dogs, differences between body sites in allergic dogs were identified. Fungal community membership (Bray Curtis) was significantly different in the nostril

compared to the axilla (R=0.331; P=0.015; Supplementary table S8), and interdigital space (R=0.441; P=0.015; Supplementary table S8). Fungal community structure (Yue-Clayton theta coefficient) was different between the nostril and axilla (R=0.294; P=0.030; Supplementary table S8), groin (R=0.213; P=0.030; Supplementary table S8) and interdigital space (R=0.300; P=0.045; Supplementary table S8). Similar to healthy dogs, the beta diversity was more dependent on the individual dog than body site (Fig. 2a) with 11 out of 28 comparisons between allergic dogs being significantly different with a median R-value of 0.306 for membership (Bray Curtis, median P<0.05; Supplementary table S4) and 0.297 for structure (Yue-Clayton theta coefficient, median P<0.05; Supplementary table S4).

Shifts in Attributes of Cutaneous Mycobiota between Healthy and Allergic Dogs

For the comparison of mycobiota between healthy and allergic canine skin, only the 'health status' was considered. First, differences in alpha diversity between the two groups were evaluated. The mycobiota of the nostril and ear from allergic dogs were less rich than their counterpart in healthy dogs (observed species, P<0.05 and, P<0.01; Fig. 5a). The mycobiota of the ear was the only allergic body site that was less diverse (Shannon, P=0.003; Fig. 5b) than the same body site in healthy dogs. Overall allergic canine skin was significantly less rich in fungal species compared to healthy canine skin (observed species, P<0.001; Fig. 5c), but not significantly different for the evenness measurements (Fig. 5d).

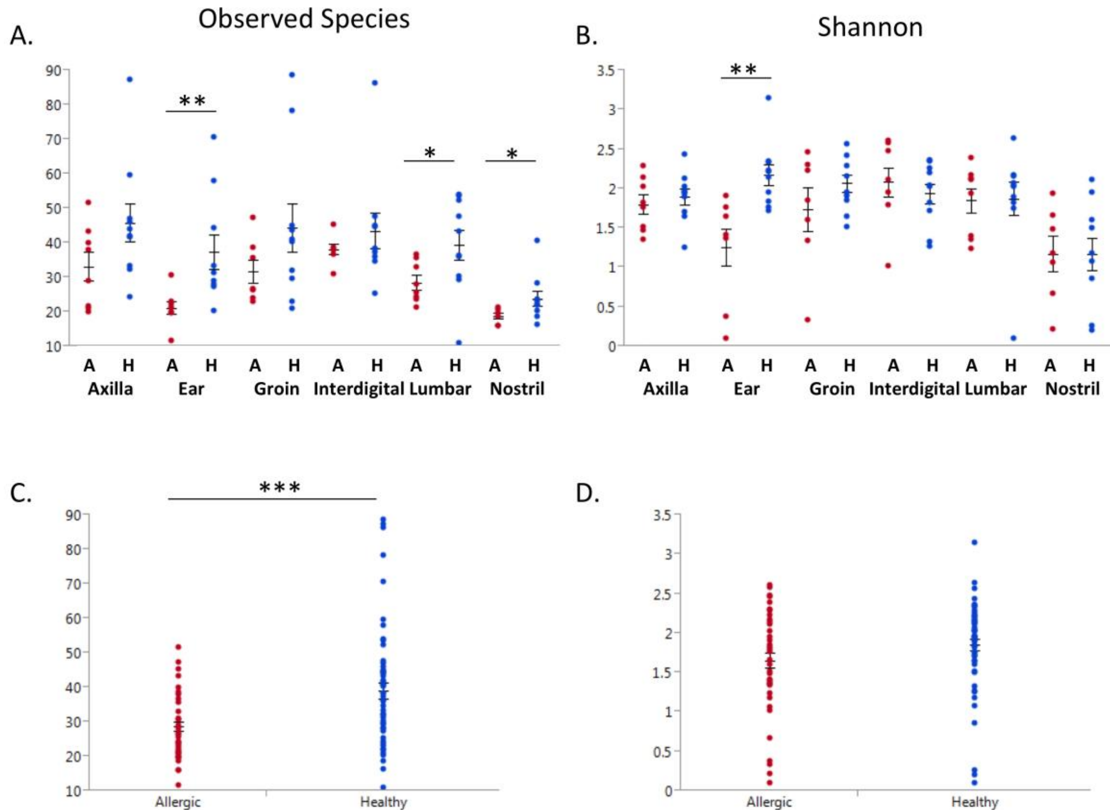

Figure 5. Comparison of alpha diversity and richness between healthy and allergic dogs. Means are marked with straight lines and mean error bars plotted using JMP. Significant differences between health statuses are denoted by asterisks (*P<0.05, **P<0.01, ***P<0.001). (a) The fungal richness of each body site was calculated with observed species, and are grouped by body site and health status. (b) The fungal diversity of each body site was calculated with Shannon, and are grouped by body site and health status. (c) The fungal richness of body sites was calculated with observed species, and are grouped by health status. (d) The fungal diversity of body sites was calculated with Shannon, and are grouped by health status (Meason-Smith, 2015).

We also wanted to know whether health status influenced beta-diversity. To accomplish this, ANOSIM was performed on the Bray-Curtis, Jaccard, and Yue-Clayton theta coefficient distance matrices. An overall significant effect of the factor 'health status' was identified with only the Jaccard distance matrix (Fig. 6a), which was mainly due to differences in cutaneous mycobiota between health status groups at three body sites: ear canal (R=0.249, P=0.026; Supplementary table S8), groin (R=0.264, P=0.024; Supplementary table S8), and interdigital

space (R=0.402, P=0.012; Supplementary table S8). These differences were visualized with PCoA plots that demonstrated clear clustering of allergic dog samples separate from healthy dog samples for individual body sites (Figs. 6b 6c and 6d). These findings were further supported by testing of individual taxa between the two health status groups done in JMP using the filtered relative abundance table (Supplementary Table S5), and through LDA Effect Size (LEfSe) analysis (Fig. 7).

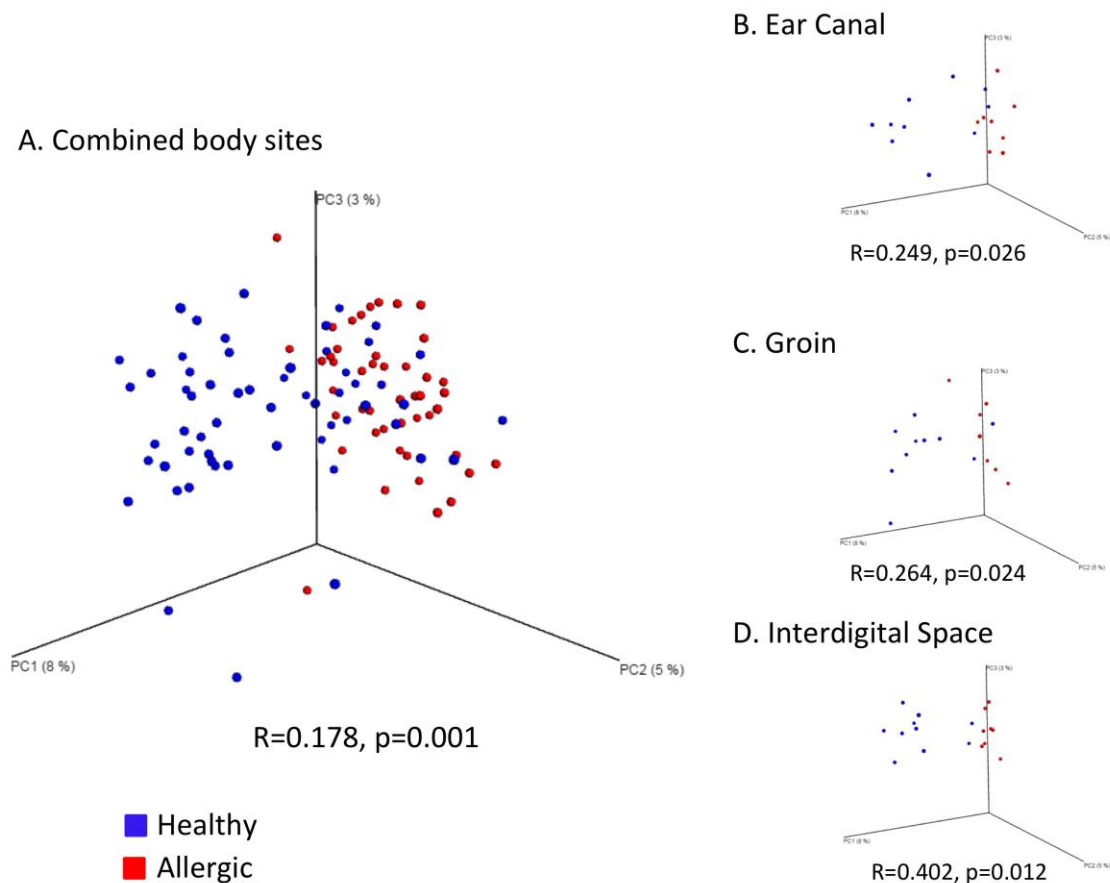

Figure 6. Dissimilarity between healthy and allergic skin fungal communities. (a) PCoA plot of all samples coming from the shared sites in healthy and allergic dogs. Dissimilarity in fungal community membership was estimated with the Jaccard metric in the Mothur package, and 3-D PCoA plots were generated in QIIME. Each dot represents a body site from one dog, with all healthy dogs colored in blue and all allergic dogs colored in red. (b) PCoA plot of only the ear samples for healthy and allergic dogs. (c) PCoA plot of only the groin samples for healthy and allergic dogs. (d) PCoA plot of only the interdigital space samples for healthy and allergic dogs (Meason-Smith, 2015).

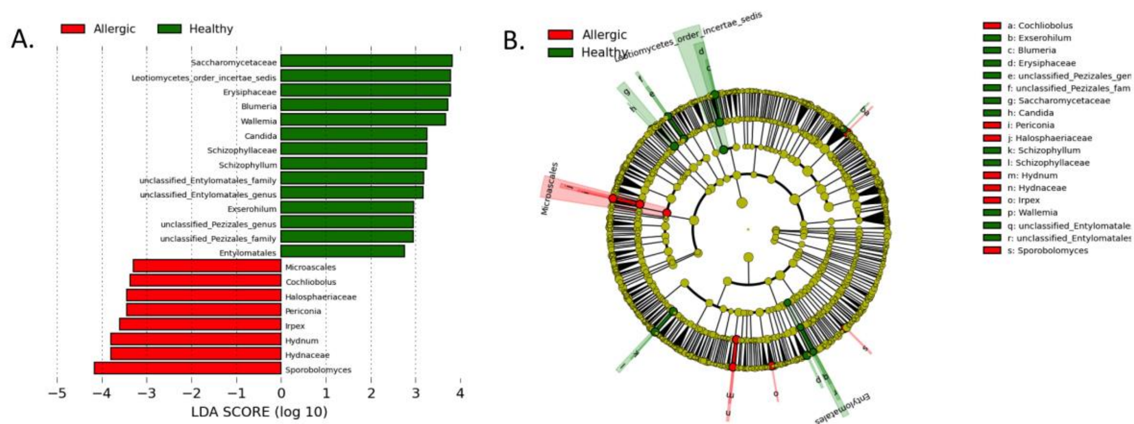

Figure 7. Differential abundances of fungal taxa between healthy and allergic canine skin. (a) LDA Effect Size (LEfSe) analysis revealed 14 fungal taxa significantly more abundant in healthy skin, and 8 taxa more abundant in allergic skin. (b) Cladogram plotted from LEfSe analysis showing the taxonomic levels represented by rings with phyla in the innermost ring and genera in the outermost ring, and each circle is a member within that level. Those taxa in each level are colored by health status for which it is more abundant (P<0.05; LDA Score 2.5) (Meason-Smith, 2015).

In addition to alpha and beta-diversity, differences in relative abundance of specific taxa were also identified between healthy and allergic dogs. Overall, there were 85 taxa found to be significantly increased or decreased in allergic skin when compared to healthy skin by the Kruskal-Wallis test performed in JMP (P<0.05; Supplementary table S5). Stacked bar plots were used to visualize changes in presence and abundance of fungal taxa between healthy and allergic skin at six body sites (Fig. 4c). LEfSe analysis identified twelve taxa that were more abundant in healthy dogs, and seven taxa that were more abundant in allergic dogs (Fig. 7). The genera that were increased in healthy skin included *Blumeria*, *Wallemia*, *Candida*, *Schizophyllum* and *Exserhilum*. The genera increased in allergic skin include *Sporobolomyces*, *Hydnum*, *Irpex*, *Periconia*, *Cochliobolus*, and *Microascales*. Furthermore, 50% of the allergic ears were predominated by one genus: 58% of the mycobiota in the ear of D13 was *Malassezia*, 94% of

D14 was *Malassezia*, and 99% of D17 was *Sporobolomyces* (Basidiomycete, Sporidiobolales family *incerta sedis*) (Fig. 4b).

Discussion

Consistently throughout the diversity analyses, and comparison of relative abundances of fungal taxa, cutaneous mycobiota of the ten dogs sampled in this study were more dependent on the individual dog than the body site. Although the mycobiota associated with body sites were very similar within the dog, there existed a high degree of inter-dog variability. Human skin also exhibits a high degree of interpersonal variability,[63] but unlike canine skin, as demonstrated by the current study, human cutaneous mycobiota were dependent on body site. Although body site was not a major influencing factor of cutaneous mycobiota in healthy dogs, we did find reduced fungal diversity at the mucosal sites. The mucosal sites in dogs, including the nostril and conjunctiva, are bathed in fluid and are also more protected from the environment, which could explain the reduction in richness, a finding that also exists for the bacterial microbiota of canine skin.[80]

Several physiological differences exist between canine and human skin which may account for some of the differences noted between the mycobiota of the two species. Human skin has a varied topography and morphology, producing distinct dry, sebaceous or moist skin microenvironments,[15] whereas canine skin is more uniform across areas of haired skin containing both sebaceous and apocrine glands.[117] Additionally, canine skin is more acidic than human skin.[118,119] There also exists differences in lipid content of the skin that could influence the colonization of cutaneous mycobiota.[117]

Other than skin morphology and topography, additional factors may be responsible for the differences in distribution of mycobiota observed between canine and human skin. These

include differences between human and animal behavior, hygiene habits, and amount of environmental exposure. It is generally well accepted that dogs are more exposed to outdoor elements than humans due to closer proximity to the ground, and behaviors such as rolling in the grass and laying on the floor inside the home where shoes track in environmental contaminants. In addition to greater environmental exposure, dogs are bathed less frequently than humans, which could enable colonization of more diverse fungi.

The types of environmental exposure could affect beta-diversity of cutaneous mycobiota, which may explain the high degree of inter-dog variability. For example, dogs from different homes (and backyards) may have variable exposures to different types of trees, plants, grasses, and bodies of water, such as ponds, swimming pools or bayous. Contact with the floor inside a home is another type of environmental exposure that likely influences diversity. It is interesting, and possibly correlated, that the only areas of human skin possessing high fungal diversity are the feet,[63] which are often in contact with the floor. Similarly, dogs spend most of their time laying their entire body on the floor, and a diverse mycobiota, as identified in this study, would be expected to colonize different regions of their bodies. Specifically, there was an abundance of *Epicoccum* on the feet of humans,[63] and the current study found this particular taxa was also abundant on the skin of dogs. Additional indoor exposures, including cohabitation with other people or animals, can also influence the cutaneous microbiota.[31] Further studies are required to find a true correlation between cohabitation and sharing of cutaneous mycobiota amongst human and animal members of the same household.

We also found that health status had a significant effect on the cutaneous mycobiota of dogs. Comparing the ten healthy dogs in this study to the eight dogs diagnosed with allergic skin disease, we found the skin of allergic dogs had reduced fungal richness. Additionally, allergic

ears, a site commonly infected in allergic dogs, had reduced fungal diversity as well. Interestingly, we identified differences in cutaneous mycobiota between body sites in allergic dogs, but not in healthy dogs. Also, the cutaneous mycobiota was more similar within a dog for healthy dogs than for allergic dogs. These findings taken together suggest that the stability of cutaneous mycobiota within a dog is disturbed by allergic skin disease, leading to changes at distinct body sites affected by this disease, and thus more pronounced body site differences in the allergic dogs. A similar phenomenon was observed for human primary immunodeficiency (PID) patients where the dependence of bacterial communities on body site in healthy individuals was diminished in the affected individuals.[68]

The significant changes identified for allergic skin in dogs who were not experiencing any observable clinical lesions at the time of sample collection suggests an association of fungal dysbiosis to the underlying mechanisms of allergic skin disease. In people, loss of function mutations to the filaggrin gene and resultant skin barrier dysfunction have been proposed as one of the most important factors in development of AD.[120] Altered filaggrin expression has been identified for atopic dogs,[121] along with transepidermal water loss, and decreased ceramide concentrations.[122] It is possible that the reduced diversity we see in both bacteria and fungi living on non-lesional allergic canine skin could be attributed to skin barrier impairment in allergic dogs, or to changes in nutrient (water and lipid) availability in the skin caused by the allergic skin disease. Likewise, the chronic use of steroids, antibiotics, antifungals, fatty acids, and topical treatments in allergic dogs could alter the skin microbiota.

A previous study reported an increase in fungal diversity in lesional skin of atopic people.[68] Although we did not find the same trend in our study, this may be attributed to the fact that the mycobiome of healthy human skin is naturally less diverse than healthy canine skin, and

predominated by one genus, *Malassezia*. The healthy human vaginal microbiota lacks bacterial diversity and is predominated by *Lactobacillus.*[123,124] An increased diversity of anaerobic bacteria, coupled with a decrease in *Lactobacillus*, has been identified in bacterial vaginosis (BV) through NGS.[123,124] In both of these cases of increased microbial diversity associated with diseased skin and mucosa (fungal microbiota in AD and bacterial microbiota in BV), the baseline or healthy microbiota is predominated by one genus, and a disturbance to the microbiota lead to a decrease of the major microbial resident allowing for invasion of other microbes, thus an increase in overall diversity. On the other hand, a disruption to an already diverse microbiota could allow for one or several microbes to increase in relative abundances, and predominate in lesional skin. Another possibility is that decreased microbial diversity was present in non-lesional canine skin, but had lesional skin been sampled, an increase in diversity might have been observed. We plan to evaluate the differences between lesional and non-lesional canine allergic skin in future studies.

Although *Malassezia* has been implicated in both human and canine AD as an allergen and trigger of disease symptoms,[95-98,100] we were unable to detect any significant differences in the relative abundance of *Malassezia* between healthy and allergic groups. Three ears were predominated by *Malassezia* (greater than 50% relative abundance), one from a healthy dog and two from allergic dogs. All dogs were examined by veterinarians and there were no reported ear infections at the time of sample collection; thus these would either represent asymptomatic ear infections or the invasion and predominance of one genus. Perhaps if more dogs had been sampled we would have seen a true significant increase in *Malassezia* for the allergic group. The relatively low abundance of *Malassezia* across all body sites was an unexpected finding since culture-based studies have reported *Malassezia* as being one of the most cultured fungi from

canine skin.[110,125-127] This genus absolutely predominated in human skin in two NGS studies targeting the ITS[63] and large rRNA subunit (LSU) regions.[101] We ruled out the possibility of the extraction protocol influencing the amount of *Malassezia* detected through comparison of our extraction protocol to that of the Findley *et al.* 2013 protocol, and found no significant differences in the amount of *Malassezia*.

Our results demonstrate a very rich mycobiome compared to human skin with a predominance of Dothideomycetes such as *Alternaria*, *Cladosporium* and *Epicoccum*. These three genera are responsible for environmental allergies in two groups of people: 20-30% of atopic people, and 6% of the general population (non-atopic people).[128] In addition to serving as human allergens to hypersensitive people, these fungi are also known allergens for atopic dogs,[129] and have been identified in house dust.[130] Interestingly, the relative abundances of *Alternaria* and *Cladosporium* were significantly different between dogs (Supplementary Fig. S3) and future studies may help to elucidate why the skin of some dogs harbor more of these allergenic fungi than do others, and whether carriage of these fungi on dogs could impact humans or dogs who are hypersensitive to these fungi. Furthermore, it is possible that cohabitation with dogs, whose skin is inhabited by these allergenic fungi, at an early age could de-sensitize children to fungal allergens and possibly protect against the development of allergies to these fungi.[131] Another possible mechanism for desensitization to the allergenic fungi present on the coat of dogs is through fetal exposure in a pregnant mother who cohabits with dogs.[132,133]

In summary, next generation sequencing of canine skin has revealed a much more diverse cutaneous mycobiota than what was previously described with culture-based techniques. The cutaneous mycobiota appear to be influenced by various factors including environmental exposure, cohabitation with other pets and skin health status. Since the majority of the dogs in

our study came from separate households and were different breeds, the high degree of inter-dog variability could be explained by differences in environmental exposure, genetic differences between breeds, or pelage characteristics. Our study only included 18 dogs and so the influence of these factors should be confirmed with future studies having increased numbers of animals and evaluating each factor separately. The host-microbiome interaction in allergic dogs also warrants investigation through immunologic and metagenomic studies, as we have now seen both the bacterial and fungal microbiota in non-lesional canine allergic skin disease are disrupted, with increased abundances of particular taxa present in the allergic skin, and an overall reduction in microbial diversity. The predominant fungal taxa inhabiting the skin of dogs suggest human cohabitation with dogs could have an effect on sensitization to fungi, and other microbes, however this relationship and mechanism remain unclear.

CHAPTER III [2]

FELINE CUTANEOUS MYCOBIOTA

Introduction

In the midst of the microbiome revolution, next-generation sequencing (NGS) technologies have provided a methodology to more comprehensively characterize host-associated microbial communities (microbiota), and have revealed a much more diverse microbiota than what was previously thought to exist (Human Microbiome Consortium).[88] In humans, NGS studies have shown that skin-associated bacterial microbiota are distributed according to physiological niches,[15] such as dry, moist, and sebaceous skin microenvironments,[21] whereas the distribution of the fungal microbiota (mycobiota) is more dependent upon body site location such as core body versus feet.[63] In contrast to what is observed in people, the bacterial microbiota on canine skin do not prefer physiological niches, but are instead influenced by body site.[80] The mycobiota are more likely to be distributed evenly across body sites within a dog, and significant differences in mycobiota are observed between dogs.[134]

The specific bacterial and fungal taxa present on canine skin differs from those present on human skin. Canine skin is predominated by bacteria in the phylum Proteobacteria, Firmicutes and Actinobacteria,[80] and environmental fungi such as *Alternaria* and *Cladosporium*,[134] whereas human skin is more abundantly colonized by bacteria in the phyla Actinobacteria and Firmicutes,[21] and the fungal genus *Malassezia*.[63] Hygiene practices and environmental exposures are thought to contribute to the differences in diversity and taxa between host species,[134] although studies are still required to better investigate their influence on the microbiome. To

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[2] Meason-Smith C, Diesel A, Patterson AP, et al. Characterization of the cutaneous mycobiota in healthy and allergic cats using next generation sequencing. *Vet Dermatol* 2017;28:71-e17.

date, the microbial communities present on feline skin have only been investigated using culture-dependent methods.[108,135-143] The results of these studies are variable, and fungal genera commonly isolated include *Penicillium*, *Cladosporium*, *Aspergillus*, *Alternaria* and *Malassezia.*

Inclusion of study participants diagnosed with atopic dermatitis (AD), or more broadly an allergic dermatitis, into these NGS studies enables the comparison of skin-associated microbiota between a healthy skin environment, and one affected by skin barrier impairment, immunologic dysfunction, altered epidermal kinetics or epidermopoiesis (migration of nucleated cells from the stratum basale to anuclear cornified cells in the stratum corneum), and inflammation as seen in these disease processes. Bacterial and fungal dysbiosis (alteration to the normal microbiota) has now been identified in both human AD,[68,91] and canine allergic dermatitis.[80,134] The lesional skin of human atopic patients possesses a reduced bacterial diversity, with proportionate increases in *Staphylococcus* species,[91] and increased fungal diversity.[68] On the contrary, non-lesional skin of allergic dogs possessed reduced diversity of both bacterial and fungal microbiota.[80,134]

Cats also suffer from an allergic dermatitis sometimes resembling human and canine AD, referred to as nonflea nonfood induced hypersensitivity dermatitis (NFNFIHD), suggesting triggers of the environment as the cause of allergic skin disease.[144] However, the pathogenesis of NFNFIHD is incompletely understood and to our current knowledge does not share some of the defining characteristics of human and canine AD.[145,146] These include the lack of proven genetic predisposition for any subgroup of NFNFIHD, except for three littermates,[147] clinical presentation,[148] and uncertainty as to whether the skin barrier is impaired in NFNFIHD. Furthermore, there has been variable reports on the role of IgE to environmental allergens in NFNFIHD.[149,150] To date the skin microbiota of NFNFIHD cats has yet to be investigated with either NGS or culture-dependent methods. Only a single study using cytologic examination of

tape strips demonstrated an overgrowth of *Malassezia* in allergic cats compared to control cats.[140]

The goals of this study were to characterize the mycobiota of feline skin using NGS, and to determine whether alterations to the mycobiota exist in allergic feline skin. Similar to previous studies, the influence on the mycobiota of skin physiology, body site, and individual was assessed for healthy and allergic cats. The overall fungal diversity and relative abundances (the amount of a fungal taxon sequenced in a sample relative to the total amount of fungal DNA sequenced for that sample) of select taxa were compared between healthy and allergic feline skin. It was hypothesized that the mycobiota of feline skin would be similar to canine skin, and that fungal dysbiosis would also exist in feline allergic dermatitis.

Materials and methods

Subject recruitment

All samples for this study were collected following a protocol approved by the Texas A&M University Institutional Animal Care and Use Committee. Eleven cats (C1-C11) were enrolled in this study on the basis of no current or prior dermatological conditions, as determined by a board certified veterinary dermatologist, and were assigned to the healthy group (Table 2). These cats ranged from 2 to 17 years of age, five were castrated males and six spayed females, and there were six domestic shorthairs, two domestic mediumhairs, and three domestic longhairs.

Table 2. Signalment and medical histories of twenty cats enrolled in this study.

Cat	Health status	Breed	Age	Sex	Fleas	Time Indoors	Indoor Environment	Outdoor Environment	Previous antibiotics usage
C1	Healthy	DLH	5	MC	Y	100	n/a	n/a	N
C2	Healthy	DSH	2	FS	N	100	TFB	n/a	N
C3	Healthy	DSH	13	MC	N	100	CTFB	n/a	N
C4	Healthy	DSH	7	MC	N	70	TFB	TGW	N
C5	Healthy	DMH	4.5	FS	N	99	CTFB	TGW	N
C6	Healthy	DSH	7	FS	N	100	TFB	n/a	N
C7	Healthy	DSH	9.5	FS	N	50	B	TGW	N
C8	Healthy	DLH	13	FS	N	100	CTFB	n/a	N
C9	Healthy	DLH	15	FS	Y	0	n/a	TGW	N
C10	Healthy	DMH	6	MC	N	100	CTFB	n/a	N
C11	Healthy	DSH	17	MC	N	100	CTF	n/a	N
C12	Allergic	DSH	9	MC	N	100	TFB	n/a	N
C13	Allergic	Sia	8	MC	N	100	TFB	n/a	N
C14	Allergic	DSH	11	MC	Y	95	CFB	TGW	N
C15	Allergic	Sia	9	FS	N	100	TFB	n/a	N
C16	Allergic	DSH	5	FS	N	60	CTFB	TGW	Y

Table. 2 Continued

Cat	Health status	Breed	Age	Sex	Fleas	Time Indoors	Indoor Environment	Outdoor Environment	Previous antibiotics usage
C17	Allergic	DSH	9	FS	N	100	CTFB	n/a	N
C18	Allergic	Per	4	MC	Y	100	CTB	n/a	Y
C19	Allergic	DSH	7	FS	N	100	CTFB	n/a	N
C20	Allergic	DSH	8	FS	Y	95	TFB	TGW	N

Fleas and ear problems were part of the medical history and not present at the time of sample collection.

Abbreviations. Signalment: DLH-Domestic long hair, DMH-Domestic medium hair, DSH-Domestic short hair, Per-Persian, Sia-Siamese, MC-Castrated male, FS-Spayed female. Indoor environment: C-Carpet, T-Tile, F-Furniture, B-Bedding. Outdoor environment: T-Trees, G-Grass, W-Weeds. Y-Yes, N-No (Meason-Smith, 2017).

An additional nine cats (C12-C20) were also evaluated by a board certified veterinary dermatologist and were included in the allergic cat group (Tables 2 and 3). The allergic cats ranged from 4 to 11 years of age, and included four castrated males and five spayed females. The breeds of allergic cats included six domestic shorthairs, two Siamese, and one Persian. Cats were included in this group based on the diagnosis of a hypersensitive dermatitis (HD), and exclusion of other pruritic dermatoses caused by ectoparasites and bacterial or fungal infections.
The classification of HD for each cat is presented in Table 3: flea bite hypersensitivity (FBH, n=8), food induced hypersensitivity (FIHD, n=1), and nonflea nonfood induced hypersensitivity (NFNFIHD, n=4). Four cats were diagnosed with more than one type of HD, and one cat had been diagnosed with FBH whose pruritus was not completely resolved with appropriate adulticidal flea prevention, but had not completed a food trial and thus was classified as also having nonflea bite hypersensitivity (NFBH). The age of onset of lesions ranged from 3 to 6 years of age, with two cats that experienced a gradual progression of clinical signs with an unknown exact age of onset. Most cats (7/10) did not experience seasonal exacerbation of signs except for one that had flares during the summer only, and two that experienced flares during the spring and summer. The most common clinical signs included pruritus and self-induced alopecia. There was a wide range of lesion distribution (Table 3). Six cats had documented steroids usage, but only two (C14 and C15) were receiving steroids at the time of sample collection. Additionally, three cats were receiving therapies including oral cyclosporine (C15 and C16) (Atopica®, Novartis), sublingual immunotherapy (C17), and oral antihistamines (C16).

Table 3. Hypersensitivity classification, age of onset, seasonality, clinical signs and distribution, and treatments for nine allergic cats.

Cat	Breed	Age	Type of HD	Age of onset	Seasonality	Clinical signs	Lesion distribution	Ear problems	Allergy treatments	Steroids
C12	DSH	9	FBH	6	N	Pruritus, self-induced alopecia	Limbs	N	N	N
C13	Sia	8	FBH	6	N	Pruritus, self-induced alopecia	Dorsum	N	N	N
C14	DSH	11	FBH	G	Summer	Pruritus, self-induced alopecia crusting	Rump, tail, ears, ventral abdomen	Y	N	Y
C15	Sia	9	FBH, FIHD, NFNFIHD	3	Spring, summer	Pruritus, cervicofacial, self-induced alopecia	Face, neck, ears	Y	Cyclosporine	Y
C16	DSH	5	FBH, NFNFIHD	4	N	Pruritus, self-induced alopecia, excoriations	Chest, ventral abdomen, dorsum, tail, limbs	Y	Cyclosporine, antihistamines	Y
C17	DSH	9	FBH, NFNFIHD	6	N	Pruritus, cervicofacial dermatitis, self-induced alopecia, eosinophilic plaques	Face, ventral abdomen, limbs	N	Sublingual immunotherapy	Y
C18	Per	4	FBH, NFBH	3	N	Pruritus, self-induced alopecia	Ears, ventral abdomen, rump, tail, limbs	Y	N	N
C19	DSH	7	NFNFIHD	6	N	Pruritus, cervicofacial	Face, ears	N	N	Y
C20	DSH	8	FBH	G	N	Pruritus, self-induced alopecia	Ventral abdomen, limbs	N	N	N

Table 3. Continued

Allergy treatments were concurrent. All cats with a Y in the steroids column had previously received steroids, except for C14 and C15 that were receiving steroids at the time of sampling. C18 was diagnosed with a ringworm infection, treated with lime sulfur dips, and lesions resolved three months prior to sample collection.

DSH-Domestic short hair, Per-Persian, Sia-Siamese, FBH-Flea bite hypersensitivity, FIHD-Food induced hypersensitivity, NFNFIHD- Non-flea non-food induced hypersensitivity, NFBH- non-flea bite hypersensitivity, G-gradual, Y-Yes, N-No (Meason-Smith, 2017).

All healthy cats had not received systemic antibiotics or antifungals in the preceding six months, allergic cats in the preceding one month, and none of the cats had been bathed in the week prior to sample collection. Furthermore, no cats sampled showed any signs of secondary bacterial or fungal infections at the time of collection.

Sample collection and DNA extraction

Twelve body sites were sampled on healthy cats including axilla, chin, conjunctiva, dorsal nose, dorsum, ear canal, groin, interdigital space, nostril, oral cavity, preaural space, and reproductive tracts. Only six sites, commonly affected by allergic signs, were sampled on allergic cats and included axilla, ear canal, dorsum, groin, interdigital space, and nostril. Samples were collected by rubbing sterile skin swabs against skin, DNA was extracted and stored as previously described.[134]

ITS Sequencing and sequence analysis

Illumina sequencing of all samples was performed on a MiSeq instrument at the XXXX, using the ITS1F (5'- CTTGGTCATTTAGAGGAAGTAA-3') and ITS2R (5'-GCTGCGTTCTTCATCGATGC-3') primers that amplify the internal transcribed spacer (ITS-1) region, a non-coding segment of genome found within the ribosomal genes of all eukaryotes. Sequences from only the forward reads were then processed in the open-source bioinformatics software Quantitative Insights into Microbial Ecology, QIIME.[8] Quality filtering was performed and operational taxonomic units (OTUs; group of similar sequences that represents a taxonomic unit of a fungal species or genus) generated using the open reference picking command, and the Findley *et al*. 2013 ITS sequence database.[63] Taxonomic assignments were made with a formatted version of the Findley et. al. 2013 ITS taxonomy file. OTU tables were rarefied at 3100 sequences for healthy only samples, 5000 for allergic only samples, and 3300 for the table

including only the six sites sampled in both healthy and allergic cats. Alpha diversity was measured using Chao1, observed OTUs, and Shannon metrics. Beta diversity was measured using weighted Jaccard, Bray Curtis, and Pearson metrics.

Statistical Analysis

All statistical analyses were completed as previously described,[134] except that distance matrices and relative abundance tables were generated in QIIME. The relative abundance tables were combined for all taxonomic levels (Phylum, Class, Order, Family and Genus) and filtered to include taxa present at greater than 1% in at least 3 samples for allergic cats, or 5 samples for healthy cats. Data was tested for normality, and Kruskal-Wallis tests were performed to determine whether the mean value (relative abundance or alpha diversity) of at least one cat or body site was significantly different from all others ($P < 0.05$). When significant, a Steel-Dwass all pairs test was performed to identify the cat(s) or body site(s) with significant differences (JMP). A Mann-Whitney test was performed to determine significant differences between health statuses (JMP). To determine whether the beta diversity of samples was significantly influenced by cat, body site, skin physiology, steroids, or healthy status, the analysis of similarities (ANOSIM) function in the statistical software PRIMER 6 (PRIMER-E Ltd, Luton, UK) was performed on the distance matrices generated in QIIME using the Jaccard, Bray Curtis, and Pearson metrics. The combined and filtered relative abundance tables were also used in linear discriminant analysis (LDA) effect size (LEfSe)[115] to determine significant differences between cats, body sites, or health statuses. All P-values were corrected for multiple comparisons using the Benjamini and Hochberg false discovery rate.[116]

Raw seqeunces were uploaded to the NCBI Sequence Read Archive under project number: PRJNA315159.

Results

One hundred thirty two samples were collected from healthy cats, and 54 from allergic cats. Due to low number of sequences (less than 3000), 24 samples from healthy cats and 15 from allergic cats were removed from downstream analyses. Following quality processing, the total number of sequences from the remaining healthy samples was 7,249,611, with a median of 42,742 sequences per sample. The total number of sequences from allergic samples was 2,521,229, with a median of 49,684 sequences per sample.

Skin Fungal Diversity Analyses of Healthy Cats

The alpha diversity (diversity within a sample) of fungi sampled from feline skin was estimated using three different alpha diversity metrics. The observed OTUs estimator measures the number of OTUs per sample, which is thought to be a close representation of the number of fungal species present (i.e. fungal richness), the Chao1 estimator is a richness estimator that accounts for sequencing depth (likelihood OTUs were not identified in acquired sequencing data), and the Shannon Index is a diversity measure that accounts for OTU abundance and evenness. To determine whether fungal richness and diversity of skin microbiota was different between cats, body sites or skin physiologies, the alpha diversity measures for each metric were analyzed across all body sites within a cat ('Cat'), across all cats at one body site ('Body Site'), or for all body sites within a skin physiology category ('Skin Physiology') (Supplementary table 9).

For healthy cats, there was a significant difference in fungal richness and diversity between cats (Observed OTUs, $P<0.001$; Shannon, $P=0.022$), and body sites (Observed OTUs, $P=0.044$; Shannon, $P<0.0001$). Specifically, the skin of C9 harbored a more rich and diverse mycobiota than other cats (Figure 8). The conjunctiva and reproductive tracts of healthy cats

were the least diverse body sites, while the preaural space was the most rich and diverse (Figure 8). Fungal diversity was also significantly different between skin physiologies (Shannon, P<0.0001), with the mucosal sites (including conjunctiva, nostril and reproductive tracts) being significantly less diverse than oral, sebaceous (chin), and haired sites (Figure 8).

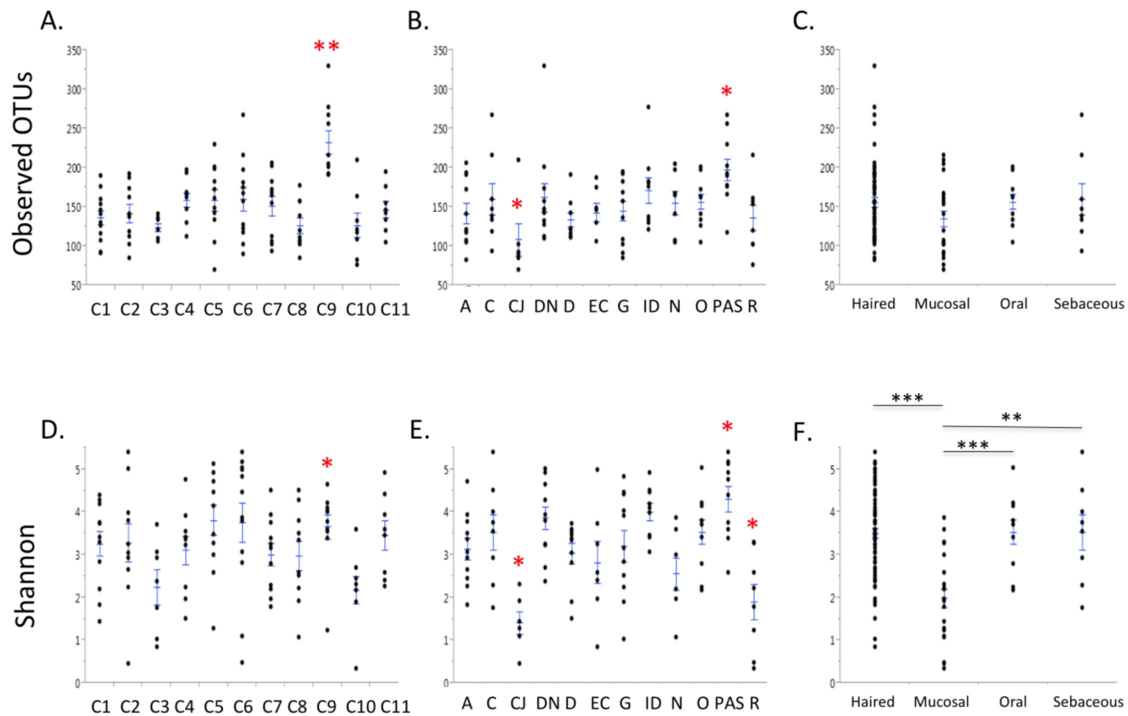

Figure 8. Alpha diversity of healthy cats. a-c: Alpha diversity estimated with observed OTU's and samples grouped by (a) cat, (b) body site, and (c) skin physiology. d-f: Alpha diversity estimated with Shannon diversity metric, and samples grouped by (d) cat, (e) body site, and (f) skin physiology. Means and mean error bars are plotted in blue for each group. Groups with a mean significantly different from other means are denoted by asterisks, with associated P-values (Steel-dwas multiple comparisons test, of *<0.05, **<0.01, ***<0.001. A-axilla, C-chin, CJ-conjunctiva, DN-dorsal nose, D-dorsum, EC-ear canal, G-groin, ID-interdigital space, N-nostril, O-oral, PAS-preaural space, R-reproductive tract (Meason-Smith, 2017).

The beta diversity (diversity between samples) of feline skin mycobiota was estimated using three different non phylogenetic based metrics. The Jaccard estimator is calculated by comparing the presence of shared fungal taxa between samples, while the Bray Curtis and Pearson estimators further account for differences in amounts of fungal taxa between samples. These calculations are performed for each possible pair of samples, and the distance matrix generated is then used to create the 3-dimensional PCoA plots. ANOSIM was performed on the distance matrices to determine statistical significance of a factor (cat, body site, skin physiology) on the dissimilarity between samples.

The results of performing ANOSIM on the distance matrices generated by all three metrics produced comparable results as demonstrated in Figure 9. The R statistic indicates the effect a variable has on the dissimilarity between samples. This value ranges from zero to one, with an R value of one indicating complete dissimilarity between two groups within a factor (e.g. axilla and groin are the groups, body site is the factor). An R value of one would also indicate that factor has a very strong influence on the presence and/or abundance of mycobiota. R values are calculated for each pairwise comparison between groups (significant comparisons summarized in supplementary table 10), and a global R statistic is calculated for the factor under study (cat, body site, skin physiology).

Some clustering of healthy cat samples (n=108) by cat can be observed in the PCoA plot of the Bray Curtis pairwise distances between healthy cats, indicating similarity of fungal communities in the sites that cluster together (Figure 9; ANOSIM, R=0.324, P=0.001). Nineteen of the pairwise comparisons between cats were significantly different, with an average R value of 0.215 and P-values ranging from 0.003 to 0.038 (Supplementary table S10). Clustering was

less apparent by skin physiology (Figure 9; ANOSIM global R=0.208; P=0.002), and absent by body site (Figure 9; ANOSIM global R=0.083; P=0.001).

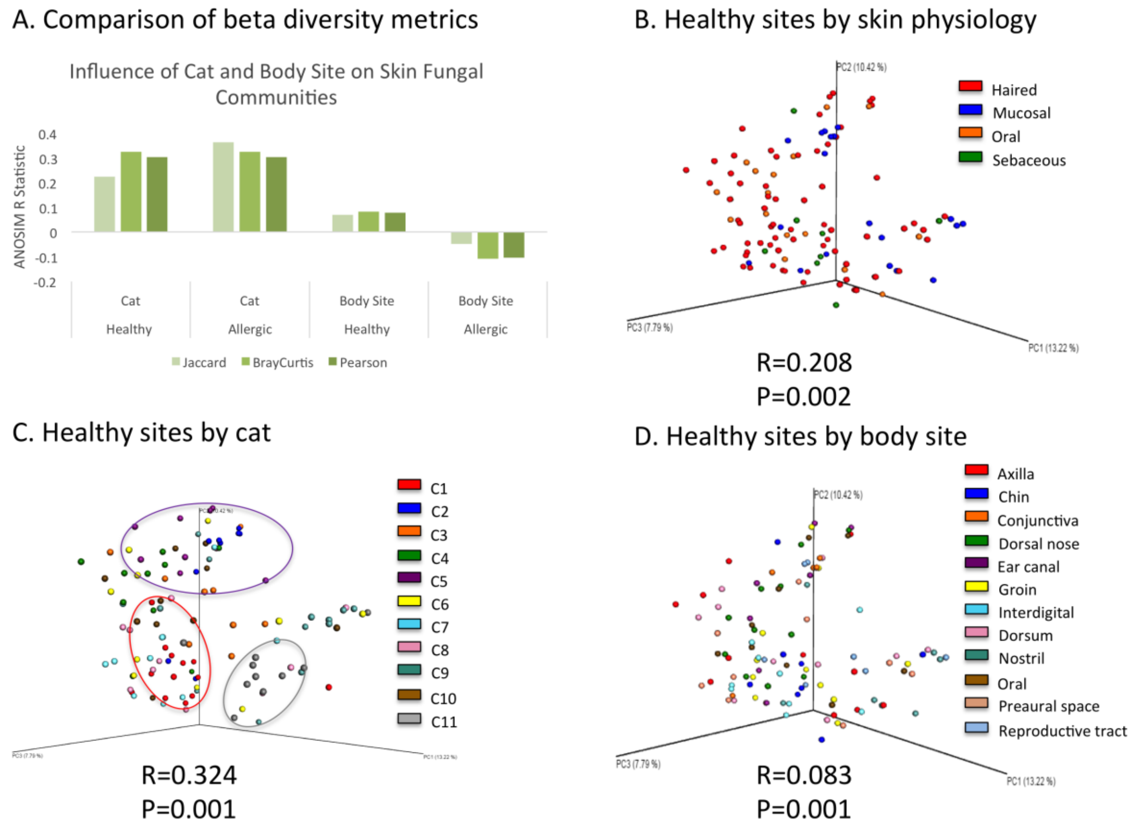

Figure 9. Beta diversity of healthy cats. (a) Comparison of ANOSIM global R statistic between three metrics, Jaccard, Bray Curtis and Pearson for the factors of cat and body site in both health status groups. (b-d) PCoA plot of Bray Curtis pairwise distances for healthy cat samples,with associated ANOSIM global R statistic, and P-value; colored by (b) skin physiology, (c) cat, and (d) body site (Meason-Smith, 2017).

Skin Fungal Taxonomic Composition of Healthy Cats

The most abundant fungal phylum identified was Ascomycota accounting for 79% of fungal sequences from healthy cats, and the most abundant class within this phylum was Dothideomycetes accounting for 48% of healthy sequences. The three most abundant genera

within this class were *Cladosporium*, *Alternaria*, and *Epicoccum* (Figure 10). There was also a remarkable amount of fungal sequences (21%) that were classified within the Ascomycota phylum, but could not be classified further (Figures 10 and 11; Other Ascomycota). The most abundant genus within the Basidiomycota phylum was *Cryptococcus*. Although these were the most abundant taxa across healthy sites sampled, a high degree of variability between samples was noted, as presented in the taxa plots of Figure 11. *Malassezia* was sequenced from 30% of healthy cat samples (n=35), but was present at greater than 1% relative abundance in only 5% of samples (n=6) (Supplementary figure S2). The median relative abundance of unassigned sequences was 6%, however there were several samples that had greater than 50% of unassigned sequences. Due to the fact fungal databases are still undergoing curation, these sequences may be assigned to fungal taxa in future studies.

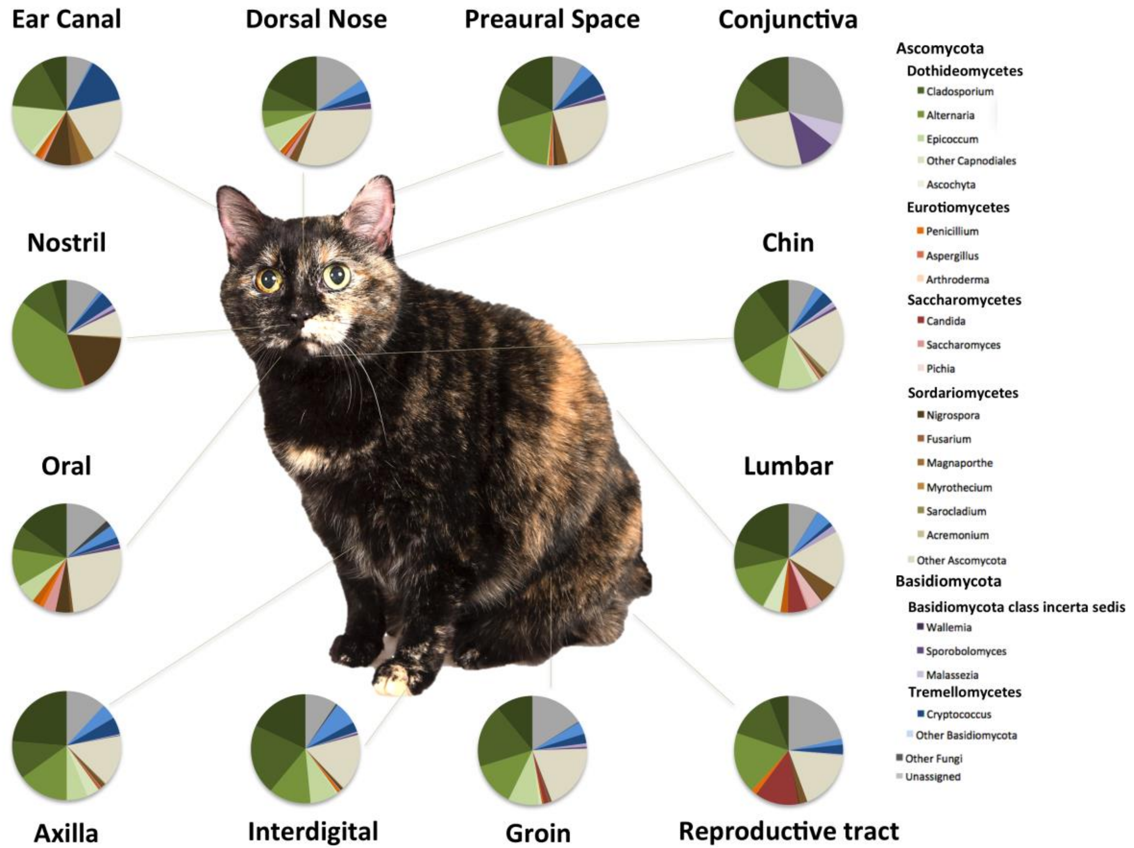

Figure 10. Fungal taxonomic composition of healthy cat body sites. The relative abundances of the most common taxa were averaged by body site and are represented by pie charts (Meason-Smith, 2017).

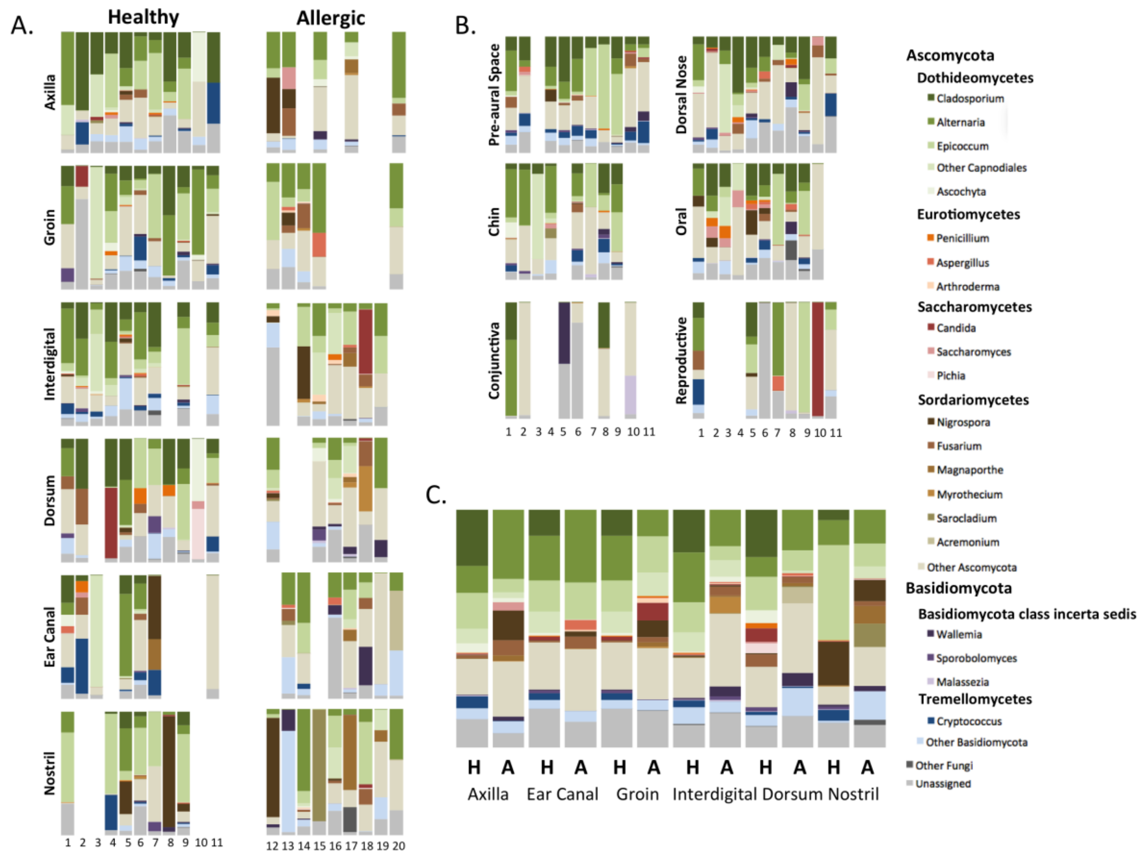

Figure 11. Fungal taxonomic composition of healthy and allergic feline skin. (a-b) Relative abundance of fungal taxa are presented for each sample, and colored by fungal genus. (c) Comparison of most abundant fungal genera between healthy and allergic skin, averaged for each of six sites (Meason-Smith, 2017).

Two types of statistical testing, Kruskal-Wallis and LEfSe, were performed to determine whether specific taxa (phylum, class, order, family, or genus levels) were differentially abundant between cats or body sites. Kruskal-Wallis testing performed in JMP revealed that the relative abundance of 53 taxa were significantly different between cats (Supplementary table S11; FDR adjusted $P<0.05$), only two taxa were significantly different between body sites, and eight taxa were different between skin physiologies. The relative abundance of the three most abundant fungal genera on the skin of healthy cats, *Cladosporium*, *Alternaria*, and *Epicoccum*, were

significantly different between cats (Supplementary table S11). LEfSe analysis did not identify any significant differences in fungal taxa between healthy cats or body site.

Skin Fungal Diversity Analyses of Allergic Cats

Alpha diversity was estimated for allergic samples with the Chao1, Observed OTUs (fungal richness), and Shannon (fungal diversity) metrics and all median values are found in Supplementary table 12. No significant differences in fungal richness or diversity between allergic cats, nor between allergic body sites (Supplementary figure S3) were identified with Kruskal-Wallis tests. Similar to healthy cats, allergic cats possessed reduced fungal diversity at the mucosal sites (conjunctiva, nostril and reproductive) (Supplementary figure S3; Kruskal-Wallis, $P<0.05$). No differences in fungal richness or diversity were identified between allergic cats that had received, or were currently receiving steroids, and allergic cats that had never received steroids (Supplementary figure S3).

The beta diversity of allergic cat samples (n=43) were calculated using the weighted Jaccard, Bray Curtis and Pearson metrics to determine if there were any differences between cats, body sites, skin physiologies and steroid usage. PCoA plots revealed some clustering of sites by cat (Supplementary figure S4; ANOSIM, R=0.324, P=0.001), but no clustering by body site. Although the ANOSIM R statistic was low for steroid usage (R=0.100, P=0.020), sample clustering is visually apparent in the PCoA plot of Bray Curtis pairwise distances between allergic cat samples. Skin physiology did not have a major effect on differences in beta diversity between allergic samples (Supplementary figure S4; ANOSIM, R=0.208, P=0.047). ANOSIM performed on the Bray Curtis distance matrix for allergic cat samples revealed that the beta diversities of six pairs of cats were significantly different with an average R value of 0.370 and

FDR adjusted *P*-values of 0.041 (Supplementary table S10). No pairwise comparisons of allergic body sites were significantly different for any beta diversity metric.

Skin Fungal Taxonomic Composition of Allergic Cats

The most abundant fungal phylum sequenced from the skin of allergic cats was Ascomycota accounting for 77% of all sequences, and the most abundant class within this phylum was Dothideomycetes, accounting for 34% of sequences (Figures 9 and 10). The three most abundant Ascomycete genera were *Cladosporium, Alternaria and Nigrospora.* The most abundant Basidiomycete genus was *Cryptococcus. Malassezia* was sequenced from 21% of allergic cat samples (n=8), but was present at greater than 1% relative abundance in only one sample (Supplementary figure S2).

Kruskal-Wallis tests were performed on taxa present in at least three samples with a relative abundance of greater than 1% to determine whether they were differentially abundant between allergic cats, or between body sites. Six taxa were differentially abundant between allergic cats, but no taxa were identified as significantly different between body sites (Supplementary table S13). Two of the genera that were significantly different between cats were *Arthroderma* (sexual stage of *Microsporum,* causative agent for dermatophytosis) and *Fusarium* (Supplementary figure S5). *Arthroderma* and *Fusarium* were more abundant on C18 compared to other cats. These results were further corroborated in LEfSe analysis that revealed *Fusarium* as a taxon significantly more abundant on C18 compared to all other cats (Supplementary figure S6; LDA score of 5). LEfSe analysis also showed that an unclassified *Tremellales* genus, phylum basidiomycete, was more abundant on the dorsum of allergic cats compared to other body sites on allergic cats (Supplementary figure S6; LDA score of 4.5).

Comparison of Skin-associated Fungi between Healthy and Allergic Cats

For the comparison of fungi colonizing the skin of healthy cats to that of allergic cats, only the six shared sites (axilla, dorsum, ear canal, groin, interdigital space and nostril) were included in the following analyses. For these sites, the estimated alpha diversities were not significantly different between the two groups (Supplementary figure S7 and supplementary table S14), and neither were the estimated beta diversities influenced by health status overall (Supplementary table S15). However, the Jaccard pairwise comparisons at two sites were significantly affected by health status: axilla (ANOSIM, R=0.378, FDR adjusted P=0.03), and interdigital space (ANOSIM, R=0.255, FDR adjusted P=0.036). Clustering by health status can be observed for most samples at these two sites in PCoA plots of the Jaccard pairwise distances (Figure 12).

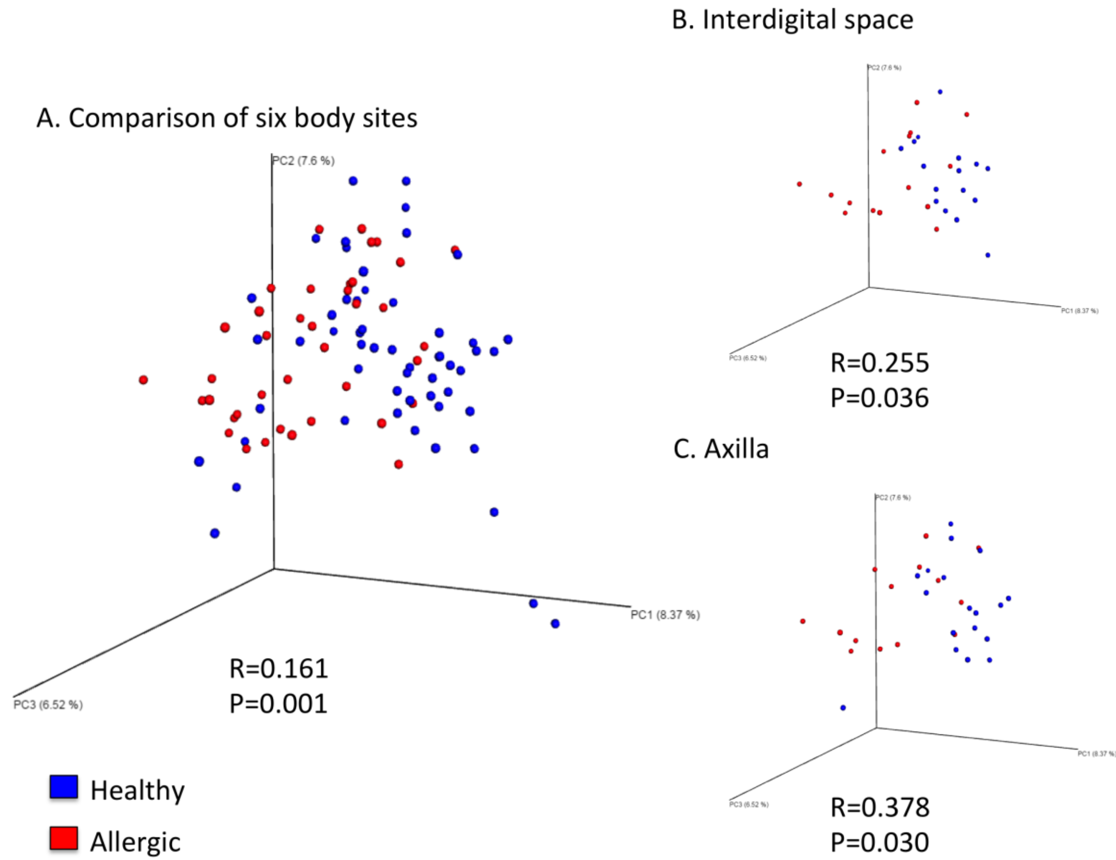

Figure 12. Comparison of beta diversity between healthy and allergic skin. PCoA plots of Jaccard pairwise distances for healthy and allergic feline skin samples, with associated ANOSIM global R statistic and associated P-value for (a) six sites, (b) only the interdigital spaces, (c) only the axillas. Colored by health status (Meason-Smith, 2017).

Only fungal taxa that were present in at least three samples with a relative abundance of greater than 1% were analyzed by Kruskal-Wallis tests and LEfSe to determine if any taxa were differentially abundant between health statuses. The Kruskal-Wallis tests revealed that nine taxa were significantly different between health statuses including the genera *Epicoccum* and non-classified Capnodiales order (Supplementary table S16), which were also identified as significantly more abundant in the healthy group by LEfSe analysis (Figure 13; LDA score of 4 to 5). The classes Agaricomycetes and Sordariomycetes were also identified as significantly different between health statuses (Supplementary table S16), and LEfSe analysis found these

classes to be significantly more abundant in the allergic group (Figure 13; LDA score of -3 to -4). Figure 10 visually demonstrates differences in averages of fungal taxa between healthy and allergic groups at each of the six sites included in this comparison of skin health groups.

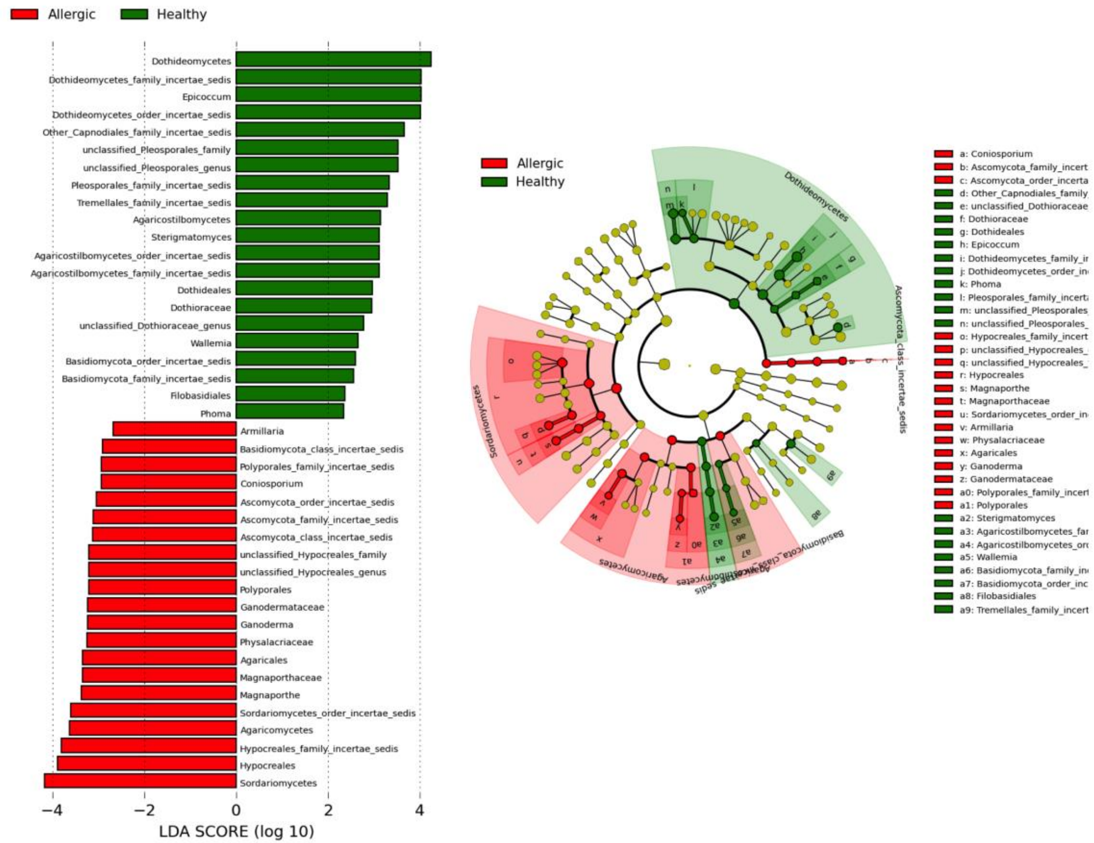

Figure 13. LDA effect size (LEfSe) analysis of healthy and allergic cats. Fungal taxa that are significantly increased or decreased in healthy or allergic skin are presented in two forms- as bar blots showing the LDA score, and as a cladogram demonstrated the phylogenetic relationships. Taxa are colored according to the health status group in which they are increased in abundance (Meason-Smith, 2017).

Discussion

Similar to what has been previously reported in dogs,[134] this study demonstrated that fungi colonizing the skin of cats tended to be similar across the entire body of the cat, with

differences observed between cats. It is possible that the grooming habits of cats may influence the dissemination of mycobiota across the entire body of the cat. This study also identified reduced diversity at the mucosal sites, and a predominance of Dothideomycetes (*Cladosporium, Alternaria*, and *Epiccocum*), similar to what was found for canine skin. While it is not possible to quantitatively compare the results of two NGS studies, qualitatively the diversity of fungi sequenced from feline skin appears to be comparable to that of canine skin,[134] and much more diverse than what has been found on the human body, except at the pedal sites.[63] A previous study suggested that outdoor exposures might explain the predominance of environmental fungi sequenced from the skin of dogs,[134] however, the same taxa of fungi were also abundant on cats, many of which, in this study, were strictly indoor (13/20). Further studies are warranted to evaluate how outdoor exposures might influence the colonization of fungi on the skin of companion animals.

Aside from the influences on diversity of fungi inhabiting the skin of people and animals, there remain many questions regarding the temporal stability of these fungi on animal skin. One of the cats in this study was diagnosed with dermatophytosis a few months prior to collection of samples for this study. The skin lesions in this cat resolved with application of lime sulfur dips and no clinical signs were observed at the time of sample collection. Statistical analysis of the relative abundances of fungi sequenced from the skin of this cat compared to the skin of other cats revealed significantly higher amounts of the fungus *Arthroderma*, which is the sexual stage of *Microsporum*, one of the causative agents of dermatophytosis. Although this finding was isolated to one cat, combined with its clinical history, this indicates the possibility of dermatophytosis causing long term effects to the skin mycobiota across the entire cat. This finding also raises continued concern regarding a potential carrier state for dermatophytosis in

cats,[151] and demonstrates the ability of NGS to detect this state in the absence of clinical signs. Additional studies including increased numbers of animals would certainly be required in order to confirm long term alterations to the fungal skin microbiota and a carrier state following resolution of lesions. This finding also offers evidence in support of the sampling and sequencing techniques used in this study, and others like it, suggesting representative sampling of the skin microbiota rather than transient environmental contamination. Interestingly, this cat (C18) also had a significant increase in *Fusarium* DNA across all of its body sites, compared to other cats. The relationship between colonization of *Fusarium* and *Arthroderma*, may also be of interest for future studies.

Malassezia has been implicated as a significant allergen in human[96,97] and canine AD,[100] however has yet to be associated with feline HD. Several studies have cultured *Malassezia* spp. from the skin of healthy cats[137,152] and cats with otitis.[108,136,138,141] In one of these studies *Malassezia* was cultured from approximately 40% of healthy cats.[136] In the present study, *Malassezia* DNA was sequenced from around 30% of healthy cat samples, but at a low abundance relative to all fungi sequenced. There has also been documented breed differences in the type and amount of *Malassezia* colonization of feline skin; in a study including 73 cats, *Malassezia* was isolated from 90% of Devon Rex cats, 39% of Cornish Rex cats, and 50% of domestic short hair cats.[152] Another study identified an overgrowth of *Malassezia* spp. from the skin of allergic cats using cytological examination of tape strips.[140] We were not able to replicate these findings in the current study; *Malassezia* was sequenced from 21% of allergic cats, and no significant difference in abundance of *Malassezia* was identified between healthy and allergic samples. A previous NGS study of healthy and allergic canine skin also reported an unexpectedly low abundance of *Malassezia*.[134] Future studies including additional methodologies

may be required to confirm the relative abundances of *Malassezia* spp. on the skin of companion animals and whether there exists any increased relative abundances of *Malassezia* on the skin of allergic animals.

The allergic cats enrolled in this study were diagnosed with a range of HD including flea bite hypersensitivity (FBH), nonflea nonfood induced HD (NFNFIHD), and combinations of FBH and NFNFIHD in the same cat. The lesion distribution was also varied amongst study participants, but in accordance with typical cutaneous reaction patterns associated with the type of HD experienced by that cat. Although the allergic cats in this study were affected by varying etiological triggers of HD with varying lesion types/distributions, there were still some significant changes to the mycobiota of their skin as a group, namely the increase or decrease of particular fungal taxa. Fungal dysbiosis has also been identified in both canine and human AD,[68,134] and fungal richness and diversity has differed between species, with previous studies showing increased fungal diversity in human atopic patients, and reduced fungal richness in allergic dogs. Unlike in dogs with allergic dermatitis,[134] there was not an overall reduction in fungal diversity in the allergic cats. Some factors that might explain this discrepancy include the differences in distribution and phenotypic presentation of lesions between canine AD and NFNFIHD in cats,[153] or differences in immune regulation of the skin in these two species. Another possible explanation could be related to lack of skin barrier impairment in allergic cats, which is often described in atopic dogs[154] and people.[120] There has yet to be any studies to provide evidence for or against the impairment of the skin barrier in allergic cats, nor has there been any studies comparing the transepidermal water loss between healthy and allergic cats.

A complex dialog between skin microbiota and host immune system is known to occur.[25,155] For instance, the host commensal microbiota is capable of inducing expression of

antimicrobial peptides,[156] which can then alter or modulate the presence and abundance of certain skin microbes. There is still debate as to whether microbial dysbiosis observed in inflammatory skin disorders is the cause of it, or rather an effect of, immune dysfunction. Regardless, microbial dysbiosis identified in canine allergic dermatitis and the results of this study in allergic cats, suggest that there is some alteration to this dialog between host and commensal microbiota in allergic dermatitis of companion animals.

In summary, NGS performed on skin swab samples of healthy and allergic feline skin identified a diverse mycobiota with predominances of environmental fungi such as *Cladosporium,* and *Alternaria*. These findings correlate well with what has been shown through culture dependent studies of feline skin,[135,136,139,142,143] and NGS studies of canine skin.[134] The mucosal sites, including conjunctiva, nostril and reproductive sites, harbor fewer fungal species, while the preaural space harbored the most. Fungal dysbiosis was identified for allergic feline skin, which was colonized by increased abundances of the fungal classes Agaricomycetes and Sordariomycetes, and decreased amounts of the fungal genus *Epicoccum*, one of the most abundant fungi on the skin of healthy cats. Fungal and bacterial dysbiosis has also been reported for allergic canine skin, with reduced numbers of microbial species present on the skin of allergic dogs compared to healthy dogs.[80,134] One of the most interesting and unexpected findings from this study was the widespread distribution of dermatophyte DNA across the entire body of one cat that had a history of dermatophytosis, but no clinical signs at the time of sample collection. Further studies with larger numbers of animals are needed to confirm these findings, and to evaluate the role of the environment on the skin microbiota. For the skin of cats, NGS has enabled confirmation of culture dependent studies, while also providing novel and exciting data.

Investigation into the immune regulation of feline skin, and pathogenesis of feline NFNFIHD might help to explain the differences identified in this study compared to that of allergic dogs.

CHAPTER IV

MALASSEZIA DYSBIOSIS IN CANINE ALLERGIC DERMATITIS

Introduction

Next-generation sequencing (NGS) technologies have revolutionized the study of skin microbiota. Perturbations to the commensal microbiota (dysbiosis) are associated with numerous cutaneous diseases.[18] Inflammatory conditions such as human and canine atopy have been of particular interest owing to secondary infections affecting diseased individuals.[157] Recent studies have shown that for both human and canine atopy flares of inflammation coincide with increasing abundances of *Staphylococcus* and progression of lesions.[85,91,158] Looking beyond the bacterial microbiota, dysbiosis of fungal microbiota (mycobiota) has now been documented in human atopy,[71] psoriasis,[70] chronic wounds,[69] seborrheic dermatitis[159] and canine allergic dermatitis.[134] These cutaneous diseases are often multifactorial and are thought to involve an interplay between the host, environment, and microbiota. However, much is still unknown regarding the exact mechanism of disease pathogenesis.[57,58,74,160]

While many parallels exist between human and canine atopy including transepidermal water loss (TEWL), ceramide alterations, and microbial dysbiosis,[74,157] there are some clinical aspects that are unique to canine atopy. For example, many atopic dogs will develop secondary *Malassezia* dermatitis or otitis as a chronic and recurrent feature of their disease.[74] Over time a recurrent otitis externa will lead to hyperproliferation of the ear canal ultimately resulting in mineralization and the need for removal of the ear canal in a surgical procedure known as a TECABO, total ear canal ablation and bulla osteotomy. *Malassezia pachydermatis* is the etiologic agent of secondary yeast infections in allergic dogs, and previous work has demonstrated *M. pachydermatis* is more abundant on the skin of dogs with allergies by both

culture,[110] and cytology.[161] One proposed explanation for the prevalence of *Malassezia* infections in atopic dogs is simply that these dogs harbor more *M. pachydermatis* on their skin. Other research has focused on pathogen virulence traits and found an association between strains that produce specific phospholipases and severity of disease.[162-165]

Our lab conducted the first fungal NGS study on healthy and allergic dogs, and did not find an increased relative abundance of *Malassezia* on the skin of non-lesional allergic dogs.[134] The discrepancy between our results and other culture-dependent studies demonstrating increased *Malassezia* could be due to differences in methodology. However, an alternative explanation is that dysbiosis is present at the species level in allergic dogs. Findley *et al.* found that even though *Malassezia* was the predominant commensal across all body sites, excluding the feet, species level predilections existed between different body sites.[63] It has been thought that *M. pachydermatis* is the predominant *Malassezia* commensal on canine skin. We wanted to investigate whether other species of *Malassezia* might be present on canine skin using NGS, and if species level dysbiosis might be occurring on allergic canine skin. Previous culture-dependent studies have isolated lipid dependent *Malassezia* from canine and feline skin,[136,138,166-168] supporting our suspicion that other species may be present.

Another yet unexplored facet of canine atopy is how the mycobiota, especially *Malassezia*, are affected by flares of inflammation. Interaction between the mycobiota and host immune system has recently been documented, prompting more investigations into the role of mycobiota in inflammatory conditions.[25,60,67,169,170] Both atopic people and dogs have been shown to be hypersensitive to *Malassezia* by patch testing,[98,171] providing more evidence that specifically *Malassezia* is interacting with the host immune system in inflammatory skin conditions. Multiple longitudinal studies have shown that *Staphylococcus* increases during atopic

flares in dogs,[85,158] but our previous fungal NGS study only looked at the skin of allergic dogs who were not experiencing flares of inflammation and did not have any skin lesions.

To address these questions we performed phylogenetic analysis on the *Malassezia* sequences from the aforementioned NGS study of healthy and non-lesional allergic dogs to derive species level classification of sequences. To confirm the results, we additionally performed realtime quantitative PCR (qPCR) targeting specific species of *Malassezia*. Secondarily we performed the same analyses on skin swabs from a laboratory colony of inducible atopic dogs in a longitudinal study of allergen induced atopic lesional flares.

Materials and Methods

Study subjects

Client owned dogs were enrolled into the healthy (n=10) and non-lesional allergic (n=8) groups.[134] The healthy dogs ranged in age from 1.5 to 11 years old, and included 5 spayed females and 5 castrated males. The allergic dogs ranged in age from 2 to 10 years old, and included 4 spayed females and 4 castrated males. The cause of allergies varied in these dogs including cutaneous adverse food reactions, flea allergy dermatitis, environmental atopy, and combinations. Inclusion criteria included a documented history of allergies diagnosed by a board certified dermatologist. Exclusion criteria included receiving antibiotics in the last month, and having been bathed in the last week. Body sites sampled in both the healthy and allergic groups included axilla, ear canal, groin, interdigital space, lumbar and nostril.

Purpose bred maltese-beagle dogs were included in the lesional atopic group (n=8).[85] These dogs had been previously exposed to and challenged with Dermatophagoides farinae house dust mite (HDM). They ranged in age from 2 to 14 years old and included four intact females, two spayed females, and two intact males. Exclusion criteria included receiving

antimicrobials, anti-inflammatory or immunosuppressive drugs in the last three months. Dogs were exposed to HDM daily for three days and skin swabs were taken prior to HDM application (1), at lesion development (2), and after lesion resolution (3). These dogs were under the care of and scored by a board certified veterinary dermatologist.

Sample collection and DNA extraction

All skin swabs included in this study had previously been collected and DNA extracted as described.[85,134] Briefly, sterile swabs (Isohelix,Cell Projects Ltd. UK) were rubbed on the skin for a total of 20 times and placed in a sterile microcentrifuge tube that was stored at 4C until extractions performed. Genomic DNA was extracted from skin swabs using a commercially available kit (MoBio Power Soil DNA Isolation kit, Carlsbad, CA). Negative controls included swab only and reagent only samples. Samples were stored for short term at -20C and long term at -80C.

NGS and bioinformatics

Fungal DNA from client owned healthy and allergic dogs was sequenced as previously described.[134] Fungal DNA from atopic dogs was presently amplified using ITS1 and ITS2 primers, with 40 cycles and sequenced on an Illumina MiSeq instrument (2x 300) at the University of Minnesota Genomics Center. Mothur[9] and the *Findley et al.* 2013 database were used to classify sequences to the genus level according to their recommended guidelines.[63] Briefly, sequences were quality filtered based on quality score threshold of 25, chimeric sequences removed, and k-nearest neighbor algorithm used to classify sequences. Next, *Malassezia* sequences were extracted in mothur, aligned to a *Malassezia* reference tree in pplacer[172] using a reference package kindly supplied by the Segre lab, and species level taxonomic strings exported using the guppy command. New taxonomic files were imported back

into mothur for further downstream analysis including making taxa plots by body site, time point and group.

Realtime quantitative PCR

M. globosa (MYA-4612) and *M. restricta* (MYA-4611) ATCC strains and a clinical isolate of *M. pachydermatis* from a dog were used for standard curves. All strains were grown on modified Dixons agar for 2-7days at 32°C. Plates were scraped and Power soil kit used to extract genomic DNA. Concentrations of DNA were measured in duplicate using Qubit high sensitivity kit on an CFX Connect Real-Time System (Bio-Rad Laboratories Inc., Hercules, CA) instrument. ITS-1 PCR and conventional sequencing was performed to confirm identification of all standards (data not included). Primers were previously published[173] and included a common forward primer (ITS-ANA-F), and three reverse primers specific to each species targeted (GlobR, RestrR, PachyR). These primers were chosen because they target the ITS region which enables a consistent comparison with the NGS data, and were previously shown to be species specific.[173] Standard curves consisted of five to six serial dilutions depending on the limit of detection for each primer pair. All standards, non-template controls, and samples were run in duplicate on a CFX Connect Real-Time System (Bio-Rad Laboratories Inc., Hercules, CA) instrument. Reactions consisted of 5ul Super mix, 0.5ul forward primer, 0.5ul reverse primer, 2ul water, and 2ul standard or sample for a total volume of 10ul. Cycle parameters were identical for all three qPCRs: initial heating step at 98°C for 3 min, and 40 cycles of 95.0°C for 30 second and 60°C for 30 seconds. Genome equivalents were calculated from the nanogram:Cq curve using published genome sizes of *M. globosa* (8.9Mb), *M. restricta* (7.2Mb) and *M. pachydermatis* (8.2Mb).[19]

Statistical analysis

NGS relative abundances and qPCR genome equivalents were tested for normality using JMP Pro 14 software (SAS Institute, Inc.) and the Shapiro-Wilk test. All data was confirmed to be non-normal and Kruskal-Wallis, a non-parametric test, was chosen for subsequent analysis. A significance value of $p<0.05$ was selected for all statistical tests. Relative abundances and genome equivalents were tested for significant differences between individual dogs, body sites, time points (inducible atopic dogs- time points 1, 2 and 3), and groups (healthy, non-lesional allergic, inducible atopic). When testing for differences between the client-owned dogs and the inducible atopic dogs, only the groin samples of healthy and allergic dogs were included for a more accurate comparison. A Steel-Dwas All Pairs, non-parametric, test was used for determining significance between pairs of individuals, body sites, time points, or groups.

Results

Malassezia populations of client-owned healthy and non-lesional allergic dogs

NGS

Extraction of *Malassezia* sequences from the NGS dataset including 108 samples from six body sites on 10 healthy dogs and 8 non-lesional allergic dogs yielded 151,793 sequences with an average of 1,405 sequences per sample. The main three *Malassezia* species represented in order of decreasing relative abundance were *M. restricta* (range: 0-99.2, mean=43.6), *M. pachydermatis* (range: 0-99.9, mean=40.9), and *M. globosa* (0-99.1, mean=7.7). Kruskal-wallis testing demonstrated that *M. pachydermatis* was significantly increased on allergic skin ($p=0.0005$), while *M. restricta* ($p=0.0369$) and *M. globosa* ($p<0.0001$) were significantly increased on healthy skin (Figure 14). Body site was not an influencing factor for either healthy or allergic dogs.

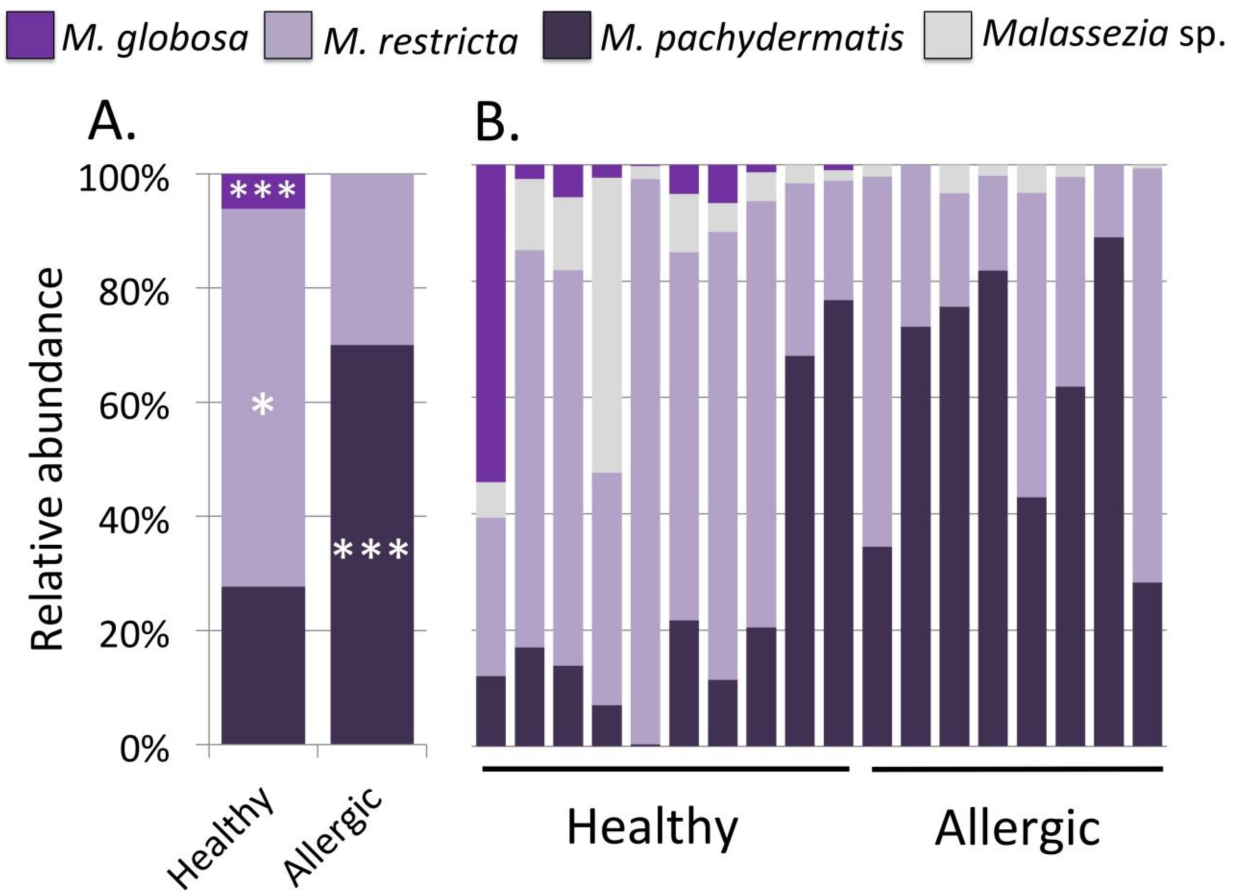

Figure 14. *Malassezia* species level relative abundances in client owned healthy and non-lesional allergic dogs. Significant differences between health statuses are denoted by asterisks (*p<0.05, **p<0.01, ***p<0.001). a) Mean relative abundances of *Malassezia* species were calculated for client owned study groups including samples from all body sites and individuals. b) Mean relative abundances across all body sites were calculated for each individual dog. Relative abundances in this plot are a function of only *Malassezia* sequences, not all fungal sequences.

qPCR

Species specific quantitative PCRs were performed for *M. pachydermatis*, *M. restricta* and *M. globosa* on all 108 healthy and non-lesional allergic dog samples. Genome equivalents were determined by a nanograms:Cq ratio, calculated using DNA concentration of the culture-derived standards and the genome size of these three species. Overall *M. restricta* (range: 0-3876, mean=75) was the most abundant by this method, followed by *M.pachydermatis* (range: 0-

244, mean=5) and lastly *M. globosa* (range: 0-213, mean=3). Kruskal-wallis testing demonstrated that *M. globosa* was significantly increased on healthy skin (p<0.0001, Figure 15). Genome equivalents of *M. restricta* was increased on healthy skin but this finding was non-significant, and genome equivalents of *M. pachydermatis* did not differ between groups.

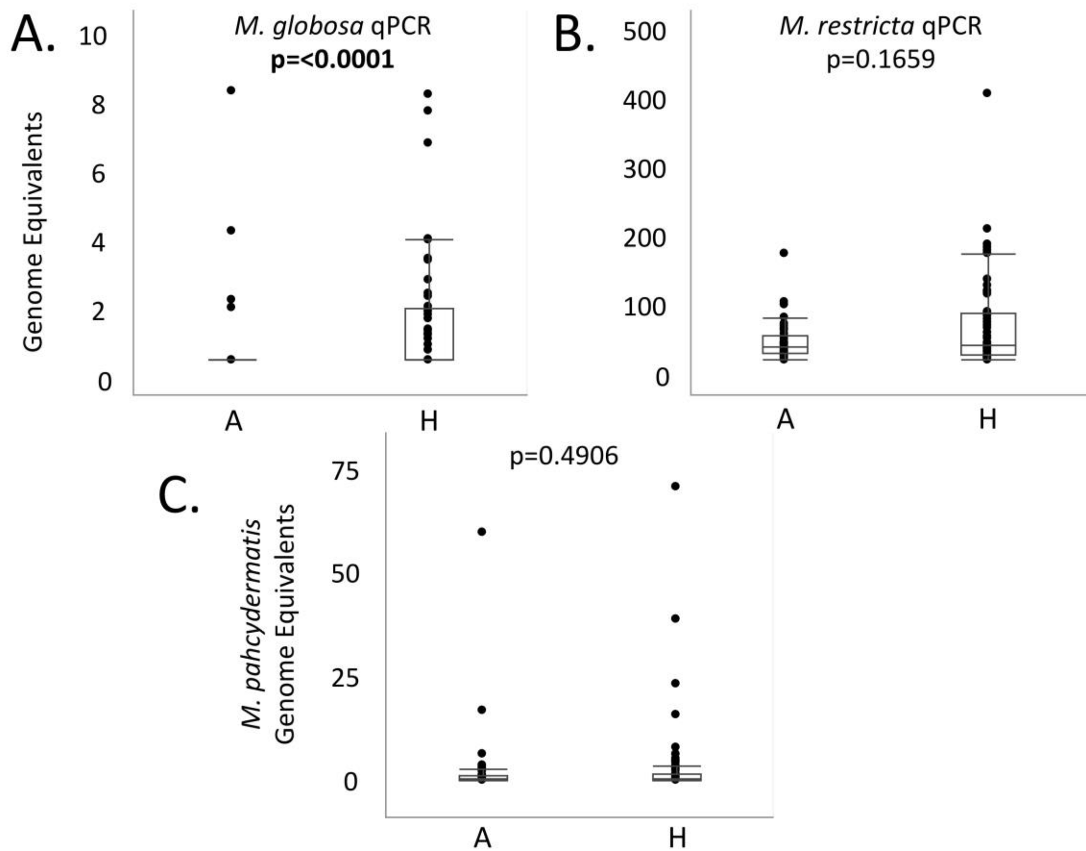

Figure 15. Genome equivalents of *M. globosa*, *M. restricta*, and *M. pachydermatis* by health status. Genome equivalents of a) *M. globosa*, b) *M. restricta*, and c) *M. pachydermatis* were compared between client-owned healthy and allergic dogs. Significant differences between health statuses are denoted by asterisks (*p<0.05, **p<0.01, ***p<0.001).

In addition to health status, *M. restricta* was found to significantly vary by body site in both healthy and allergic dogs (Figure 16). Body site was not an influencing factor on genome equivalents of *M. pachydermatis* or *M. globosa*.

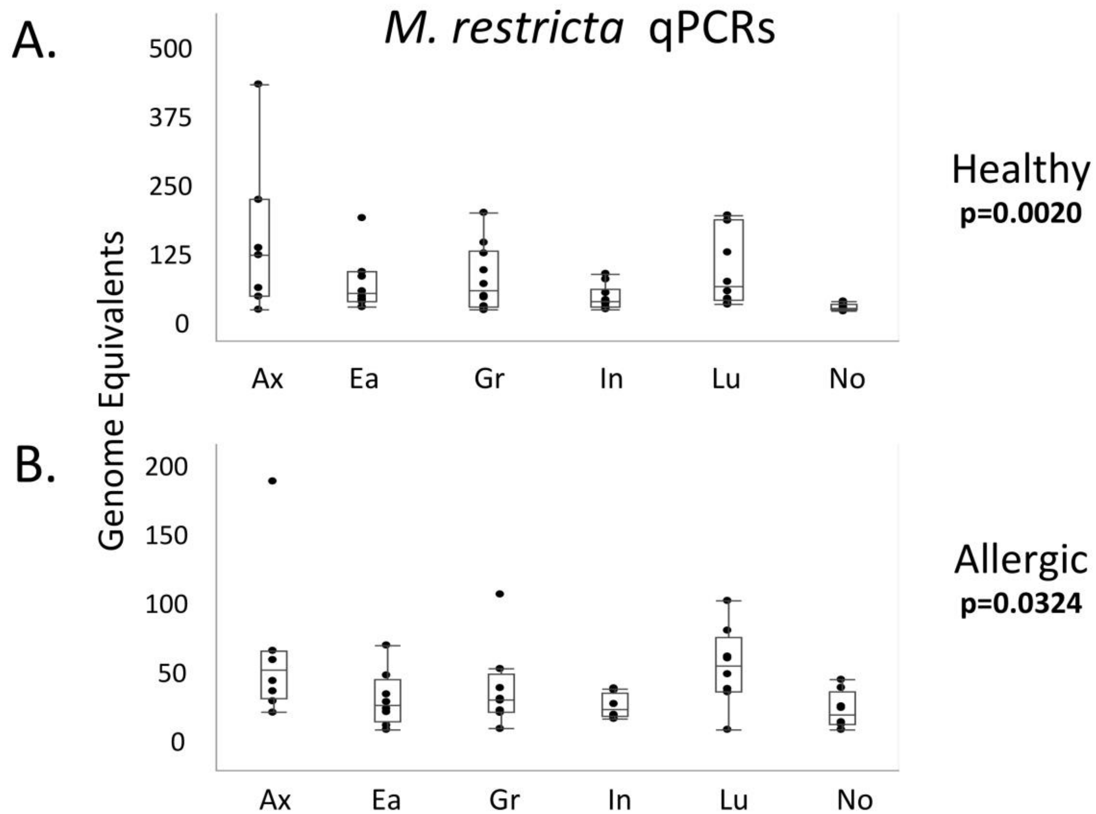

Figure 16. Genome equivalents of *M. restricta* by qPCR varies by body site in both a) healthy and b) allergic dogs.

The individual dog was also identified as an influencing factor on genome equivalents of *M. restricta* and *M. pachydermatis* (but not *M. globosa*) for both healthy and allergic dogs (Table1). By Kruskal-Wallis testing, the individual was identified as the strongest influencing factor, followed by group, and lastly by body site. Correlation between NGS and qPCR existed for 9/15 comparisons presented in Table 4.

Table 4. Correlation of client-owned healthy and allergic dog findings between two methods- NGS and qPCR. Significant differences between groups (health status, body site, individual) are denoted by asterisks ($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$).

Kruskal-wallis P values	*M. restricta* NGS	*M. restricta* qPCR	*M. globosa* NGS	*M. globosa* qPCR	*M. pachydermatis* NGS	*M. pachydermatis* qPCR
Healthy v Allergic	*	-	***	***	***	-
Healthy body sites	-	**	-	-	-	-
Allergy body sites	-	*	-	-	-	-
Healthy individual	**	*	**	-	***	**
Allergic individual	-	-	-	-	-	***

Malassezia populations during atopic lesion flares of inducible laboratory dogs

NGS

Fungal NGS was performed on a colony of laboratory dogs for the first time and 24 samples yielded 2,108,463 sequences. Extraction of *Malassezia* sequences from the NGS dataset including 24 samples of 8 laboratory dogs over three time points yielded 845,038 sequences with an average of 31,971 sequences per sample (Figure 17).

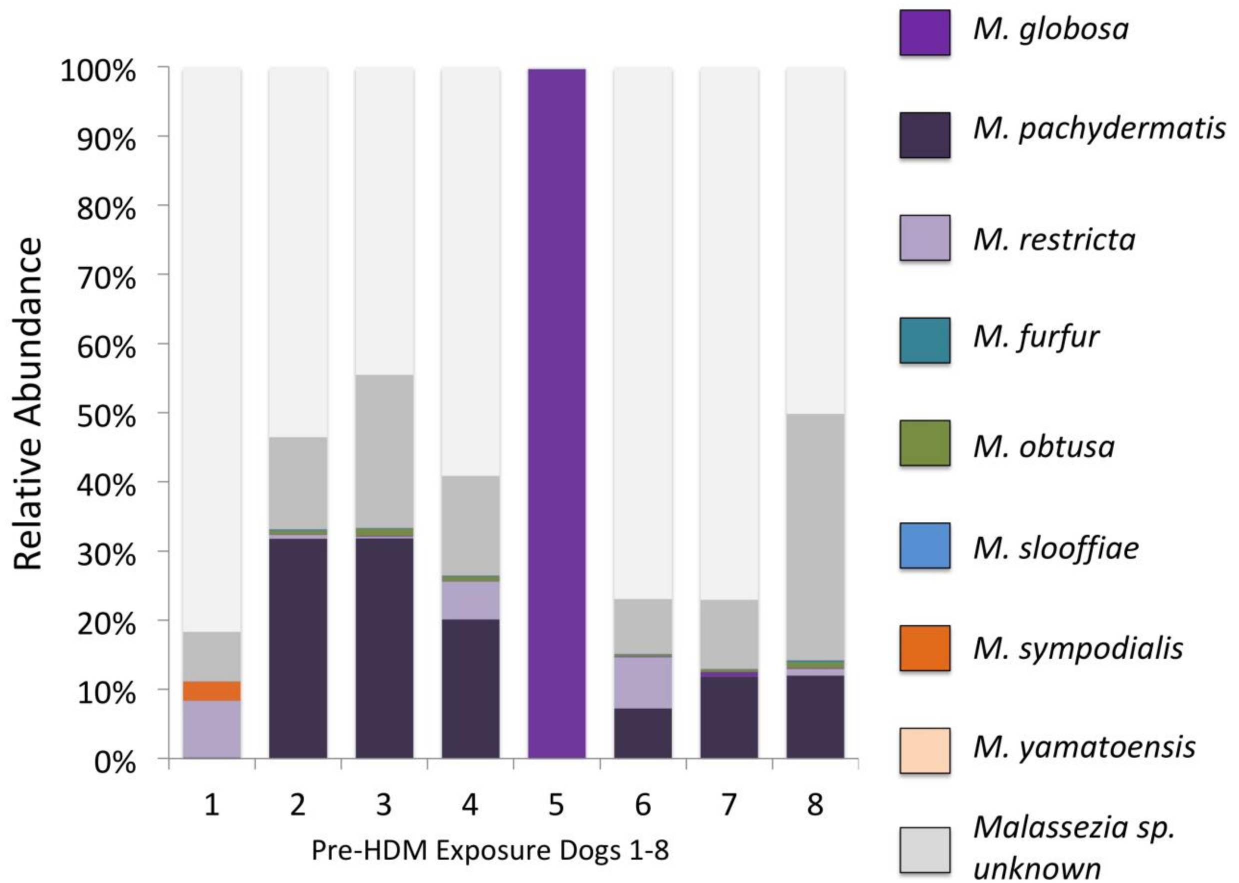

Figure 17. Relative abundance of fungal genera in eight inducible atopic laboratory dogs. The mean relative abundance of *Malassezia* species for each dog prior to HDM exposure are presented in a taxa plot. All other fungal genera sequenced from each sample are not portrayed to simplify the figure. Relative abundance in this plot is a function of all fungal sequences.

Kruskal-wallis testing demonstrated that *Malassezia* genus relative abundances (range: 9.5-99.7, mean=40.8) were significantly different between individual dogs (p=0.0267, Figure 18), but not between time points (p=0.8332, Figure 18).

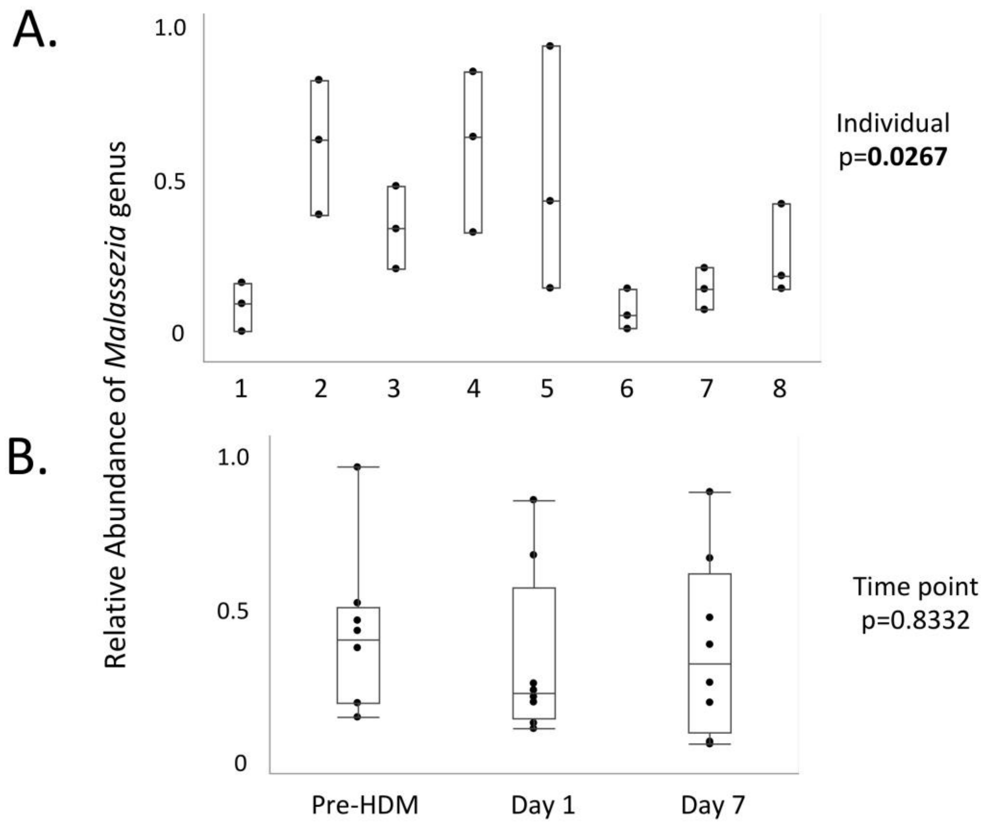

Figure 18. *Malassezia* genus relative abundances are shaped more by a) individual than b) time point.

The *Malassezia* species represented in order of decreasing relative abundance were *M. pachydermatis*, *M. restricta*, *M. obtusa, M. furfur,* M. *globosa, M. slooffiae* and *M. sympodialis* (Figure 19).

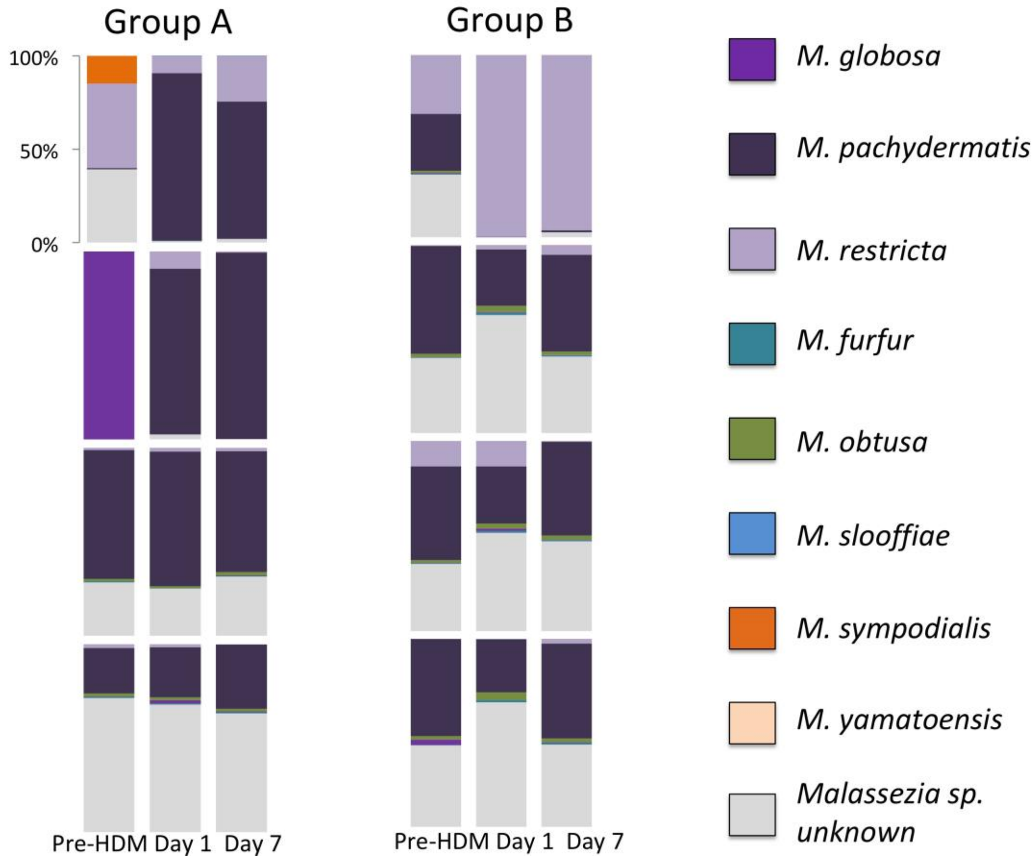

Figure 19. *Malassezia* species relative abundances in eight inducible atopic dogs. All dogs are grouped based on the change in *M. pachydermatis* elicited by HDM exposure- increasing (group A), or decreasing (group B), relative abundance. Each row within a group corresponds to one dog, and the three bars represent each time point sampled. Relative abundance in this plot is a function of only *Malassezia* sequences, not including other fungal genera.

M. pachydermatis and *M. restricta* were considered the most abundant species, while the mean relative abundance of all other species was less than 5%. Kruskal-wallis testing demonstrated that species relative abundance significantly varied by individual but not time point (Table 5, Supplementary table S17).

Table 5. Correlation of inducible atopic dog findings between two methods- NGS and qPCR. Significant differences between groups (individual and time point) are denoted by asterisks (*p<0.05, **p<0.01, ***p<0.001).

Kruskal-wallis P values	*Malassezia*	*M. pachydermatis*		*M. restricta*	
	NGS	NGS	qPCR	NGS	qPCR
Individual	*	-	**	*	-
Time	-	-	-	-	-

Looking only at *M. pachydermatis*, there were two overall trends of increasing (Group A) or decreasing (Group B) relative abundance following HDM exposure. Kruskal- wallis testing demonstrated that *M. pachydermatis* significantly decreased in Group B (p=0.0304, Figure 20).

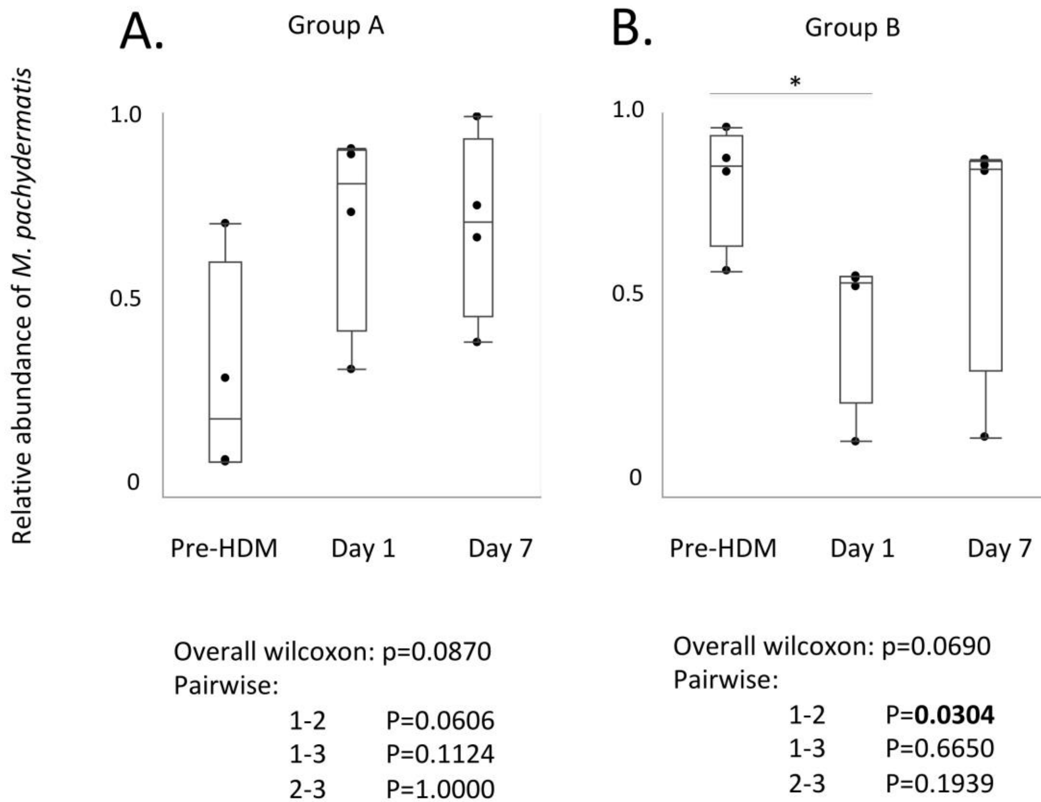

Figure 20. Two trends following exposure to HDM- increasing or decreasing relative abundance of *M. pachydermatis*

qPCR

Species specific quantitative PCRs were performed for *M. restricta* (range 6-160, mean=55) and *M. pachydermatis* (range: 0-252, mean=39) on all 24 laboratory dog samples. Neither species were found to vary significantly between time points by this method. Individual was a significant influencing factor on genome equivalents of *M. pachydermatis* (Table 5, Supplementary table S18).

Comparison of genome equivalents between all three study groups (client-owned healthy groin, client-owned allergic groin, pre-HDM exposure groin) demonstrated that *M.*

pachydermatis was significantly increased by 8-fold on laboratory dogs (Figure 21). *M. restricta* was not significantly different between the three study groups.

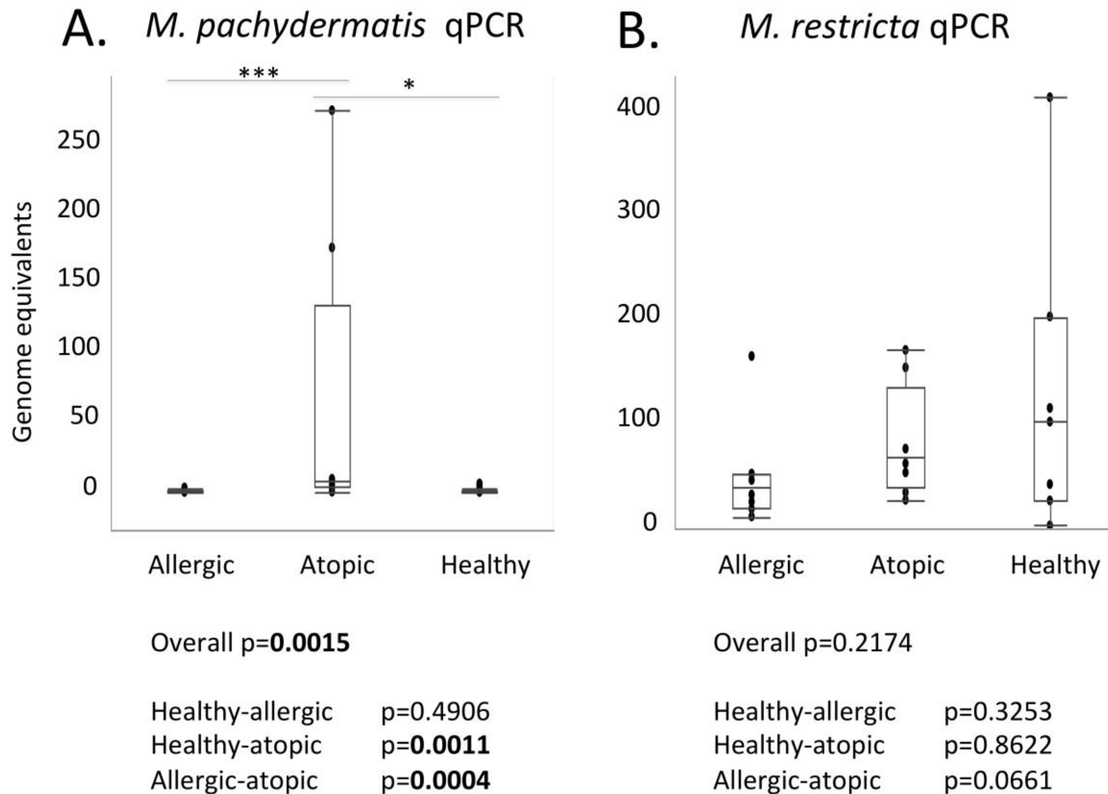

Figure 21. Comparison of *M. pachydermatis* and *M. restric*ta genome equivalents across three study groups- healthy, non-lesional allergic, and pre-lesional inducible atopic dogs.

Discussion

For the first time we have examined cutaneous *Malassezia* species populations in dogs using NGS and qPCR. One of the most significant findings from this study is that healthy canine skin is predominated by lipid-dependent yeast *M. restricta* and *M. globosa*. To our knowledge these yeast have not been previously isolated from dogs, but studies using NGS identified them as the predominant yeast on healthy human skin. Possible explanations for why this yeast has not

previously been isolated from canine skin are that *M. globosa* is a rather fastidious organism to culture in the laboratory, not assimilating any of the tween reagents in contrast to other species in this genus,[174] and is not routinely looked for in dogs. *Cafarchia et al.* found that lipid dependent yeast were more commonly isolated from healthy canine skin compared to allergic skin, but the species identification was not carried out.[167] Realtime quantitative PCR corroborated our phylogenetic analysis on NGS data showing that *M. globosa* was significantly more abundant on healthy canine skin and nearly absent on the skin of dogs with diagnosed allergies.

Increased abundances of *M. globosa* on healthy canine skin is of particular interest because recently researchers have concluded this yeast may play a beneficial role to the host.[175] These authors found that *M. globosa* secretes an aspartyl protease with antibiofilm activities against *Staphylococcus aureus*, a known opportunistic pathogen in human atopic dermatitis flares. It is not known whether *M. globosa* secretes a protease that has similar action on *S. psuedintermedius*, the opportunistic pathogen in canine atopic dermatitis, but it is tempting to speculate how *M. globosa* may also play a protective role in healthy canine skin. Furthermore, its absence on atopic canine skin might contribute to secondary *Staphylococcus* infections that are frequently observed clinically in these patients.

Skin lipid content is known to be altered in canine atopic dermatitis[122,176] and might explain why *M. globosa*, with very strict lipid dependency, thrives on healthy skin but not atopic skin. This particular dysbiosis might provide a niche for *M. pachydermatis* with a more versatile ability to metabolize a broader range of lipids[177] and contribute to its overgrowth on atopic canine skin. A recent lipidomics assay demonstrated that triglyceride content is significantly more abundant on the forehead of healthy individuals where *Malassezia* predominates as a fungal commensal compared to the feet where the diversity of fungal commensals is much

greater.[178] Future studies performing simultaneous analysis of skin lipidomics and fungal microbiota in healthy and allergic dogs may offer more evidence for this idea that skin lipid content influences which yeast are the abundant commensals.

We previously identified *Malassezia* as being on average 6% of the skin mycobiota in healthy and allergic dogs, but presently found that laboratory inducible atopic dogs have an 8 fold increased relative abundance of *Malassezia* on their skin. A variety of factors may contribute to this phenomenon. This colony of dogs is highly genetically related, and recent work has shown that the breed or genetic makeup of an animal has an influence on their skin microbiota.[152,179] Additionally, these dogs are kept mostly indoors with increased humidity and greater hygiene compared to client-owned animals. It has been proposed that hygiene could play a significant factor in why people harbor more *Malassezia* on their skin compared to dogs and cats that have greater exposure to the environment and decreased hygiene practices.[81] Lastly, as part of this colony's animal welfare regimen, these dogs are fed a diet supplemented with fatty acids that have been shown to have anti-inflammatory effects and enhance the skin barrier in atopic dogs.

The sequencing of *M. obtusa* only from the laboratory dogs is also interesting and one other potential cause for differences between these two cohorts is that the client-owned animals were all from Texas, USA while the laboratory dogs are housed in North Carolina, USA. Geographic restriction may possibly exist in this genus given that *M. japonica* and *M. yamatoensis* are commonly isolated in SE Asia but not elsewhere.[20] Geographic influence on the skin and gut microbiome was recently reviewed by *Gupta et al.* and found to be a significant factor in shaping the presence of commensals.[28] Future comparisons of skin *Malassezia* populations should consider the location of individuals sampled when drawing conclusions about

the presence and distribution of these species. The three main species across all study groups regardless of location were *M. restricta*, *M. pachydermatis*, and *M. globosa* indicating a potential role of commensalism for these three species on canine skin.

Although we were mainly interested in studying populations of species within a genus, and have discussed a lot of potential inter-microbial and host-microbial interactions, the individual pathogen virulence must also be considered. The common etiologic agent of secondary *Malassezia* infections in allergic dogs is specifically *M. pachydermatis*. Phospholipase activity has been associated with increased virulence of *M. pachydermatis* strains causing otitis externa compared with strains from healthy animals. Furthermore, strains from dogs and cats differ in their abilities to form biofilms. [180] These strain-associated virulence factors should be further investigated in the context of the entire mycobiota of affected animals, as well as in conjunction with lipid content of the skin. It remains unclear why *M. pachydermatis*, generally the most virulent of all species in this genus, colonizes the skin of dogs but not people.

Canine atopic dermatitis can have variable presentations which has been attributed to its multifactorial pathogenesis.[74] The prevalence of *Staphylococcus* pyoderma or *Malassezia* dermatitis in allergic dogs is not currently known. For some dogs these will occur simultaneously, but in other dogs their disease will be predominated by one or the other. There were clear alterations to the abundance of *Malassezia* species in client owned allergic dogs which may indicate a widespread predisposition to *Malassezia* dermatitis in allergic dogs. However, changes to *Malassezia* during flares of inflammation as assessed by the longitudinal study of laboratory inducible dogs was less clear. We identified two trends following exposure to allergen- increasing or decreasing abundances of *M. pachydermatis*. These trends could explain why some allergic dogs are more prone to *Malassezia* dermatitis than others. However, there

were very few dogs sampled in this study and future studies with larger numbers of animals would be needed to clarify this phenomenon. We also found that the individual was a strong influencing factor on the *Malassezia* populations which could have contributed to the variability in changes observed following allergen exposure. Importantly, the laboratory dogs did indeed harbor more *M. pachydermatis* compared to the client-owned healthy dogs which would support the conclusions that increased abundances of *M. pachydermatis* are present on allergic canine skin and predisposes them to *Malassezia* dermatitis. Future longitudinal studies of *Malassezia* populations during flares of inflammation might benefit from simultaneous investigation of the bacterial microbiota, skin lipid content, and other host factors such as TEWL and history of *Malassezia* dermatitis.

Interestingly, individual was a strong influencing factor for all three study groups. This was evident in that 1) some dogs would harbor more *Malassezia* across all body sites compared to other dogs 2) *Malassezia* abundances on laboratory dogs demonstrated similarity across longitudinal time points for individual dogs. The latter finding indicates there may be stability of *Malassezia* abundances over time, however these time points were over the course of 7-10 days and future studies with longer intervals would be needed to clarify this. Other temporal studies of the skin microbiome have found that there is stability at the species and strain levels.[18] Body site was only an influencing factor for *M. restricta* abundances, however, this was the most abundant species across all study groups. Previous culture-independent studies also found that *Malassezia* populations varied between body sites.[167] This might be explained by differences in lipid content of body sites, however this has not been described for canine skin.

Advantages and limitations exist for both culture-dependent and -independent techniques. Studies are underway to determine the exact lipid preferences of each *Malassezia* species and

may lead to development of more robust culture methodologies. Currently with such vast differences in the ability of *Malassezia* spp. to grow in culture, quantitatively comparing the relative abundances of *Malassezia* species between samples is nearly impossible. Molecular methods offer an advantage for mycobiota studies, however, we still do not know the exact biases introduced by copy number variation in the ITS region, primer efficiencies or contaminating fungal DNA.[59] Upon publishing complete *Malassezia* genomes, Dawson and colleagues were able to show that some reports of *Malassezia* in metagenomic datasets was likely inaccurate.[19] Future studies using metagenomics might better confirm the results presented in this study, however, corroboration between NGS and qPCR supports our conclusions.

The results in our study clearly show *Malassezia* spp. dysbiosis occurs in allergic dogs that are more predisposed to develop *Malassezia* infections and redefine our existing knowledge of healthy *Malassezia* populations. Harnessing this canine model of atopy, we have an animal model to further investigate interactions between skin microbiota, such as between *Malassezia* and *Staphylococcus*. *Malassezia* act as an opportunistic pathogen in a variety of human conditions including Pityriasis versicolor, dandruff and seborrheic dermatitis. Sheynius and colleagues recently showed that *Malassezia* interacts with the host via nanovesicles and may be contributing to inflammation in the host.[181] It is unknown whether this mechanism may also occur in allergic dogs but the identification of *M. globosa* on healthy canine skin indicates that dogs would be an appropriate animal model for future studies. Finally, this canine model could serve as an opportunity to study how different therapeutic interventions affect the mycobiota in correlation with clinical outcomes.

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

The work presented in this thesis represents the seminal studies of skin mycobiota in veterinary medicine. We applied NGS for the first time to describe the healthy skin mycobiota of dogs and cats and identified a greater diversity of fungi than was previously shown with culture. Environmental fungi were predominant on both canine and feline skin, unlike human skin that is predominated by one genus, *Malassezia*. We identified dysbiosis of the skin mycobiota associated with inflammatory skin disease of dogs and cats which was characterized by an overall decreased diversity. The final chapter is moving us from description to pathogenesis through identification of dysbiosis at the species level that can be explained by altered lipid content in allergic skin. These works lay the foundation for future investigation into the mechanisms underlying the overgrowth of *M. pachydermatis* on allergic skin and animal models to test novel pre-biotics.

These foundational studies raised more questions regarding the temporality of fungi sequenced from skin swabs of animals. It remains unclear whether the environmental fungi sequenced were transient mycobiota or stably colonized on the skin surface. The hair coat of dogs and cats has been likened to a mop that collects fungal spores from the environment it contacts. Even though spores may only be transiently present on the hair coats of animals they may still be interacting with the host immune system in a significant way. Future studies sampling both shaved and unshaved animal skin over time may provide insight into the existence of a stable core microbiome versus transient colonizers. Decreased hygiene practices may also contribute to the presence of transient mycobiota that are less likely to be sampled on human skin

that is washed frequently. Two recent large scale studies concluded that hygiene and environmental exposure are strongly influencing factors on the skin microbiome. Mode of subsistence was identified as a geographic stratifier, where people with greater exposure to the environment (hunter-gatherers) had more diverse skin microbiota compared to western civilizations.[28] Non-human mammals also were shown to have a more diverse skin microbiota compared to people with obvious increased hygiene practices.[81] Future studies involving hair clipping or bathing frequency can also have clinical implications in veterinary dermatology for management guidelines on treating recurrent skin infections.

The presence of lipid dependent *Malassezia* as dominant commensals on healthy canine skin is demonstrated for the first time using NGS and confirmed by secondary primers and quantitative PCR. These results could have important implications for both understanding disease pathogenesis and pursuing novel therapeutics such as a lipid pre-biotics that promote the growth of lipid dependent healthy commensals. NGS was a superior method to culture due to the striking differences in ability to culture these *Malassezia* species precluding the use of culture to quantitatively compare *Malassezia* populations between samples. That being said, culture will be a valuable next step for studying lipid preferences of *M. restricta* and *M. globosa* in vitro. Additionally, competition assays between *Malassezia* species and studying the effects of these species on *Staphylococcus pseudintermedius* biofilms will also be important in elucidating more details regarding the pathogenesis of secondary infections in allergic dogs.

While NGS has proven to be a valuable technique, we are still unsure of all the biases introduced by NGS of the ITS region in mycobiome studies. The ITS region has been shown to vary in copy number between fungal genera[182,183] which could introduce bias into relative abundances within one sample, but should not affect the comparison of populations between

study groups or over time following treatment. To date there is not a clear consensus on whether ITS-1 or ITS-2 is the preferred target due to primer biases[66,184] and future studies employing metagenomic sequencing would help to confirm findings from this thesis work. Other gene targets such as Beta-tubulin or Chitin synthase-2 may prove to be more accurate in studying animal skin mycobiota or specifically *Malassezia* species populations. Metagenomics would also be valuable in studying inter-microbial interactions as a result of treatment or prevention regimens used for allergic dermatitis in veterinary medicine. Metagenomics studies of human skin has have also been harnessed to study the overall function of the skin microbiome regardless of taxonomic classifications.[185,186]

Treatment effect on the skin microbiota has only recently been investigated in human medicine. Grice and colleagues looked at the effect of topical antimicrobials and antiseptics. They found that antimicrobial use resulted in a shift in resident bacterial communities that are thought to be protective against *Staphylococcus aureus* colonization, whereas antiseptics only resulted in minor shifts. This can obviously have very important clinical implications in the treatment of dermatologic conditions. They also concluded there is a stable and resilient skin microbiome with certain taxas such as *Propionibacteria* being most resilient. Segre and colleagues have recently tested the effects of emollients in prevention of atopic dermatitis in infants and found that emollient use promoted the growth of *Streptococcus salivarius*, a known beneficial commensal with anti-inflammatory effects. The increasing prevalence of antibiotic and antifungal resistance in both human and veterinary medicine is an important motivating factor for pursuing alternative approaches to preventing and treating skin infections. Future studies in veterinary medicine should include testing antiseptics and emollients on the skin of dogs and cats.

Canine and feline allergic dermatitis are multifactorial diseases which include host factors such as skin barrier impairment and immune dysregulation. More studies are needed to understand the genetic contributions to these phenomena. In-breeding of animals has shown to introduce strong hereditary contributions to other diseases.[187,188] Strong breed predilections exist for canine atopic dermatitis[74] and more GWAS studies could help to identify genetic mutations contributing to disease pathogenesis. These genetic contributions to skin barrier and immune dysregulation could also have effects on the skin microbiota and ultimately treatment success. As such, these are important in stratifying participants of future microbiome studies testing these effects. The most robust studies would include both genetic, physical skin barrier, immune system and microbiota evaluations simultaneously.

Progress has quickly been made in the study of the canine gastrointestinal microbiome over the last decade. Leading research groups have quickly moved beyond the study of bacterial microbiota to the functional aspects of dysbiosis such as bile acid metabolism.[189,190] We hope to move the study of the skin microbiome in veterinary medicine similarly to investigate the functional aspects of dysbiosis in dermatologic conditions. Specifically lipidomics could provide revolutionary insight into the presence and distribution of skin commensals on healthy and allergic canine skin. These studies might also direct us towards the development of pre-biotics that include specific proportions of triglycerides that favor the growth of healthy commensals and prevent the overgrowth of virulent opportunistic pathogens like *Malassezia pachydermatis*. We have recently tested the effect of an essential oil and essential fatty acid topical product on the skin of bloodhounds, and found that by qPCR the abundance of *Malassezia* increased after weekly application.[191] We have not carried out species identification yet for these samples, but

these results do demonstrate the potential for topicals to influence the composition of the mycobiota.

Descriptive studies were our initial goal of the works presented in this thesis, but our findings have quickly propelled us forward into investigations of functionality of skin mycobiota in canine allergic dermatitis. Importantly, recent studies have reproduced our results lending accuracy to these findings.[87,192] These and future studies may change the way we approach treatment or prevention of secondary infections in allergic dogs. Other yet unexplored effects of these results are how the skin mycobiota of companion animals influence the skin microbiota of co-habiting people and their health.

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APPENDIX A

SUPPLEMENTARY TABLES

Supplementary table S1. Overall effect of body site, dog, and health status on alpha diversity. Fungal richness was estimated with observed species and Chao1 in Mothur, fungal diversity was estimated using the non-parametric Shannon and Inverse Simpson in Mothur, and overall effect of body site, dog and health status was calculated with Kruskal-Wallis tests performed in JMP. P-values are listed for each factor on each group of samples including healthy only body sites, allergic only body sites, and body sites shared between health status groups (Meason-Smith, 2015).

Group	**Factor**	**Observed Species**	**Chao1**	**Shannon**	**Inverse Simpson**
Healthy	Body Site	0.0002	0.0004	0.0283	0.1380
	Dog	<0.0001	<0.0001	0.0003	0.0088
Allergic	Body Site	<0.0001	0.0030	0.0297	0.1657
	Dog	0.9480	0.8437	0.0900	0.0455
Shared Sites	Health Status	0.0005	0.0047	0.0807	0.3965

Supplementary table S2. Alpha diversity calculations by site in healthy skin group. Fungal richness was estimated with observed species and Chao1 in Mothur, and fungal diversity was estimated using the non-parametric Shannon and Inverse Simpson in Mothur. The median, minimum and maximum values were calculated for each healthy body site (Meason-Smith, 2015).

Skin Site	**Observed Species**		**Chao1**		**Shannon**		**Inverse Simpson**	
	Median	Min-Max	Median	Min-Max	Median	Min-Max	Median	Min-Max
Axilla	46	24-87	66	40-140	1.9	1.3-2.4	1.9	2.1-6.4
Conjunctiva	26	18-41	40	29-55	1.8	1.5-2.3	1.8	3.1-6.8
Dorsal Nose	51	23-110	75	40-157	2.0	1.5-2.7	2.0	2.6-5.7
Ear	37	20-71	52	32-88	2.2	1.7-3.1	2.2	3.3-10.0
Groin	44	21-89	63	36-135	2.1	1.5-2.6	2.1	3.0-6.8
Interdigital	43	25-86	64	40-116	1.9	1.3-2.4	1.9	1.9-7.0
Lip	49	23-110	68	40-151	2.0	1.4-2.6	2.0	2.9-6.6
Lumbar	39	11-54	56	19-73	1.9	0.1-2.6	1.9	1.0-8.5
Nostril	24	16-41	41	30-54	1.2	0.2-2.1	1.2	1.1-5.5
Pinna	45	24-86	67	40-146	2.0	1.5-2.3	2.0	2.3-7.5

Supplementary table S3. Alpha diversity calculations by dog in healthy skin group. Alpha diversity calculations by dog in healthy skin group. Fungal richness was estimated with observed species and Chao1 in Mothur, and fungal diversity was estimated using the non-parametric Shannon and Inverse Simpson in Mothur. The median, minimum and maximum values were calculated for each healthy dog (Meason-Smith, 2015).

Dog Number	**Observed Species**		**Chao1**		**Shannon**		**Inverse Simpson**	
	Median	Min-Max	Median	Min-Max	Median	Min-Max	Median	Min-Max
1	23	16-47	40	29-63	1.8	0.9-2.4	4.7	2.1-7.6
2	24	11-44	40	19-58	1.6	0.1-1.9	3.7	1.0-4.5
3	37	23-58	56	38-79	2.1	0.9-2.4	4.8	1.6-6.7
4	36	22-48	51	34-70	2.0	1.2-2.2	4.1	2.5-5.4
5	29	22-42	47	41-65	2.0	0.3-2.3	5.1	1.1-7.5
6	39	23-58	53	32-87	2.1	1.6-2.4	4.9	3.0-6.8
7	41	28-49	53	42-66	2.0	1.6-2.4	3.5	2.9-7.2
8	45	19-89	61	43-135	1.9	0.2-2.6	3.8	1.1-6.3
9	45	22-62	64	38-85	1.7	1.1-2.2	3.0	1.8-5.3
10	86	41-110	116	54-157	2.3	1.6-3.1	4.8	2.1-10.0

Supplementary table S4. Dissimilarity of fungal community membership and structure between dogs. The median R-value was calculated from R values only for comparisons that were significant. The minimum and maximum R-values for this subset of significant R-values were also calculated and reported within the table. ANOSIM was performed on distance matrices produced using the three beta diversity metrics on two groups of samples: healthy only and allergic only. If there were no significant comparisons then n/a was listed in the table. All P-values were corrected using the FDR equation (Meason-Smith, 2015).

		Healthy Dogs	**Allergic Dogs**
Bray Curtis	Median R (min-max)	0.338 (0.11-0.667)	0.306 (0.176-0.559)
	Median P value (FDR) (min-max)	0.045 (0.003-0.049)	0.023 (0.016-0.036)
	Number of Significant Comparisons (total comparisons)	38 (45)	11 (28)
Jaccard	Median R (min-max)	0.535 (0.147-0.836)	n/a
	Median P value (FDR) (min-max)	0.045 (0.006-0.049)	n/a
	Number of Significant Comparisons (total comparisons)	23 (45)	0
Theta YC	Median R (min-max)	0.381 (0.108-0.954)	0.297 (0.178-0.683)
	Median P value (FDR) (min-max)	0.045 (0.004-0.049)	0.038 (0.019-0.04)
	Number of Significant Comparisons (total comparisons)	41 (45)	8 (28)

Supplementary table S5. Combined and filtered relative abundance table for 193 fungal taxa that were tested for significant differences between body sites, dogs, and health status. For each test, the Kruskal-Wallis P-values are listed in columns following the taxa names. Classification of each sample within body site, dog, and health status are found in rows above the sample identifiers (Meason-Smith, 2015).

Phylum Class Order Family Genus	**Healthy (Dog)**	**Healthy (Body Site)**	**Allergic (Dog)**	**Allergic (Body Site)**	**Shared Sites (Health Status)**
Ascomycota	**0.0012**	0.5617	0.1749	0.4812	0.9898
Ascomycota_class_incertae_sedis	**0.0350**	0.4549	0.3241	0.6614	0.1631
Ascomycota_order_incertae_sedis	**0.0379**	0.5118	0.4102	0.7441	0.1398
Ascomycota_family_incertae_sedis	**0.0305**	0.5328	0.6147	0.7567	0.2343
Dothideomycetes	**0.0290**	0.4073	0.1037	0.6580	0.9746
Capnodiales	**0.0000**	0.1200	0.0543	0.5415	0.8457
Capnodiales_family_incertae_sedis	**0.0031**	0.0669	0.3477	0.5351	0.4063
Capnobotryella	0.6575	0.2633	0.9521	0.3389	**0.0006**
Cladosporium	**0.0080**	0.0562	0.2259	0.5272	0.3215
Dissoconium	**0.0000**	0.3329	0.6609	0.3989	0.2692
Ramichloridium	**0.0010**	0.7842	0.9718	0.3233	**0.0095**
Mycosphaerellaceae	**0.0000**	0.1038	0.1626	0.2639	0.7247
Cercospora	**0.0001**	0.7459	0.6544	0.3082	0.8293
Mycosphaerella	**0.0027**	0.3541	0.5455	0.3707	**0.0014**
unclassified_Mycosphaerellaceae_genus	**0.0000**	0.1069	0.1623	0.3951	0.7128
unclassified_Capnodiales_family	**0.0351**	0.0748	0.8141	0.2057	**0.0019**
unclassified_Capnodiales_genus	**0.0367**	0.0936	0.8141	0.3214	**0.0016**
Dothideales	0.0583	**0.0348**	0.7476	0.5865	0.9618
Dothioraceae	**0.0250**	**0.0354**	0.9635	0.4691	0.7191
unclassified_Dothioraceae_genus	0.0561	0.0589	0.9419	0.3625	0.3099
Dothideomycetes_order_incertae_sedis	**0.0446**	0.9517	0.2221	0.7672	0.5571
Dothideomycetes_family_incertae_sedis	**0.0430**	0.9251	0.2946	0.7615	0.6040
Epicoccum	**0.0452**	0.9283	0.2946	0.7490	0.5575
Pleosporales	**0.0000**	0.9630	0.2679	0.8058	0.5338
Montagnulaceae	**0.0037**	0.7200	0.7616	0.7741	0.2930
unclassified_Montagnulaceae_genus	**0.0075**	0.7516	0.6781	0.6744	0.1785
Phaeosphaeriaceae	**0.0174**	0.4555	0.8601	0.7779	**0.0006**
Pleosporaceae	**0.0000**	0.9220	0.2285	0.7720	**0.0369**
Alternaria	**0.0000**	0.9422	0.1271	0.7149	0.0510

Phylum Class Order Family Genus	Healthy (Dog)	Healthy (Body Site)	Allergic (Dog)	Allergic (Body Site)	Shared Sites (Health Status)
Cochliobolus	**0.0001**	0.7721	0.5482	0.4740	**0.0000**
Exserohilum	**0.0014**	0.6062	0.7101	0.7086	**0.0001**
Lewia	0.1398	0.7730	0.9998	0.7996	0.6724
Stagonospora	**0.0038**	0.3333	0.3116	0.9419	0.9369
Pleosporales_family_incertae_sedis	**0.0000**	0.4950	0.1313	0.7538	0.7531
Ascochyta	**0.0008**	0.9336	0.0587	0.9941	0.1543
Leptosphaerulina	0.4286	0.0666	0.9699	0.5129	0.3035
Phoma	**0.0000**	0.7698	0.1896	0.6975	0.0968
unclassified_Pleosporales_family	**0.0000**	0.9077	0.1283	0.2525	0.5199
unclassified_Pleosporales_genus	**0.0000**	0.9145	0.1283	0.3156	0.4773
unclassified_Dothideomycetes_order	**0.0002**	0.2754	0.2231	0.7278	**0.0006**
unclassified_Dothideomycetes_family	**0.0002**	0.2504	0.2656	0.7464	**0.0005**
unclassified_Dothideomycetes_genus	**0.0002**	0.2459	0.2324	0.7135	**0.0005**
Eurotiomycetes	**0.0000**	0.5979	0.2456	0.1213	**0.0092**
Chaetothyriales	**0.0041**	0.6878	0.7999	0.5146	**0.0005**
Herpotrichiellaceae	**0.0048**	0.8447	0.9560	0.4630	**0.0014**
unclassified_Chaetothyriales_family	**0.0057**	0.1468	0.8742	0.7677	0.0541
unclassified_Chaetothyriales_genus	**0.0059**	0.1501	0.9518	0.7852	0.0516
Eurotiales	**0.0000**	0.9337	0.2410	0.0928	**0.0367**
Trichocomaceae	**0.0000**	0.9259	0.2812	0.1160	**0.0349**
Aspergillus	**0.0002**	0.9243	0.2885	0.5112	0.2612
Penicillium	**0.0014**	0.7607	0.9969	0.3440	**0.0015**
unclassified_Trichocomaceae_genus	**0.0003**	0.9327	0.8712	0.5862	**0.0001**
Lecanoromycetes	**0.0017**	0.2251	0.8360	0.4780	**0.0244**
Leotiomycetes	**0.0358**	0.4503	0.1580	0.3347	**0.0006**
Helotiales	**0.0251**	0.1977	0.2731	0.5594	0.4233
Helotiaceae	0.3777	0.1135	0.4003	0.6162	0.7460
Articulospora	**0.0280**	0.1036	0.3333	0.7147	0.2223
Sclerotiniaceae	**0.0046**	0.6983	0.6894	0.7478	**0.0002**
unclassified_Sclerotiniaceae_genus	**0.0042**	0.6609	0.6744	0.6816	**0.0001**
Leotiomycetes_order_incertae_sedis	**0.0147**	0.4885	0.6958	0.8131	**0.0000**
Erysiphaceae	**0.0185**	0.5353	0.7018	0.7601	**0.0000**
Blumeria	**0.0021**	0.8089	0.7194	0.7962	**0.0000**
Golovinomyces	**0.0098**	**0.0475**	0.6863	0.5598	**0.0000**
Podosphaera	**0.0010**	0.7618	0.9999	0.7789	0.0890
Pezizomycetes	**0.0007**	0.2667	0.6075	0.6606	**0.0005**

Phylum Class Order Family Genus	Healthy (Dog)	Healthy (Body Site)	Allergic (Dog)	Allergic (Body Site)	Shared Sites (Health Status)
Pezizales	**0.0005**	0.2627	0.5895	0.7260	**0.0004**
unclassified_Pezizales_family	**0.0001**	0.4901	0.8877	0.7711	**0.0002**
unclassified_Pezizales_genus	**0.0001**	0.4648	0.9641	0.6940	**0.0002**
Saccharomycetes	**0.0146**	0.5894	0.0801	0.5224	0.1478
Saccharomycetales	**0.0135**	0.6621	0.1803	0.5567	0.1108
Saccharomycetaceae	**0.0357**	0.9158	0.3604	0.9771	**0.0124**
Saccharomycetales_family_incerta_sedis	**0.0237**	0.4405	0.1507	0.5682	**0.0020**
Candida	**0.0173**	0.5950	0.1451	0.6395	**0.0014**
Sordariomycetes	0.2881	0.3269	0.2731	0.1351	0.5468
Glomerellales	**0.0088**	**0.0188**	0.9538	0.5077	**0.0005**
Glomerellales_family_incertae_sedis	**0.0002**	0.4989	0.8363	0.7143	0.2873
Plectosphaerellaceae	**0.0046**	0.0801	0.9423	0.4680	**0.0004**
Plectosphaerella	**0.0049**	0.0916	0.9405	0.3716	**0.0010**
Hypocreales	**0.0087**	0.2736	0.6133	0.0767	0.2805
Bionectriaceae	**0.0055**	0.4392	0.8686	0.5152	0.1149
Hydropisphaera	0.1597	0.3533	0.5457	0.4012	**0.0033**
Clavicipitaceae	**0.0001**	0.6948	0.7772	0.1347	**0.0002**
Claviceps	**0.0000**	0.7728	0.6874	0.2892	**0.0002**
Hypocreales_family_incertae_sedis	**0.0021**	0.0962	0.6056	0.2733	0.8023
Fusarium	**0.0010**	0.1086	0.7275	0.3775	0.3147
Myrothecium	**0.0164**	0.1485	0.9756	0.3224	0.2075
Sarocladium	**0.0166**	0.2437	0.1436	0.6888	0.4185
unclassified_Hypocreales_family	**0.0002**	0.2341	0.4727	0.1742	**0.0368**
unclassified_Hypocreales_genus	**0.0002**	0.2341	0.5252	0.2177	**0.0335**
Microascales	**0.0000**	0.2532	0.2405	0.5063	**0.0001**
Halosphaeriaceae	**0.0000**	0.7699	0.6194	0.5469	**0.0000**
Periconia	**0.0000**	0.7812	0.6750	0.4625	**0.0000**
Sordariales	0.0858	0.3011	0.4976	0.6379	0.1058
Chaetomiaceae	0.2769	0.0766	0.8717	0.4957	0.2901
Chaetomium	0.5055	0.0566	0.9395	0.3636	0.3267
Sordariaceae	**0.0214**	0.7725	0.2388	0.5482	**0.0120**
Gelasinospora	**0.0134**	0.8033	0.3318	0.3825	**0.0081**
Sordariomycetes_order_incertae_sedis	**0.0000**	0.9384	0.1973	0.7177	**0.0017**
Magnaporthaceae	**0.0000**	0.7571	0.1421	0.7803	0.6100
Magnaporthe	**0.0000**	0.7337	0.1171	0.9354	0.9896
Phialophora	**0.0004**	0.7299	0.7168	0.5080	0.1649

Phylum Class Order Family Genus	**Healthy (Dog)**	**Healthy (Body Site)**	**Allergic (Dog)**	**Allergic (Body Site)**	**Shared Sites (Health Status)**
Sordariomycetes_family_incertae_sedis	**0.0093**	0.6970	0.4004	0.3548	**0.0002**
Acremonium	**0.0092**	0.7437	0.4004	0.3411	**0.0001**
Trichosphaeriales	0.9909	0.1596	0.7492	0.6001	0.3456
unclassified_Sordariomycetes_order	0.1164	0.2628	0.7664	0.4912	**0.0261**
unclassified_Sordariomycetes_family	0.1231	0.3212	0.8211	0.5668	**0.0235**
unclassified_Sordariomycetes_genus	0.1231	0.3170	0.7983	0.3543	**0.0202**
Xylariales	**0.0005**	0.4756	0.6538	0.5695	0.2086
Amphisphaeriaceae	0.5628	0.2641	0.4741	0.4549	0.2571
Pestalotiopsis	0.6466	0.3145	0.6994	0.3849	0.2654
unclassified_Xylariales_family	0.2854	0.5063	0.8605	0.4542	**0.0010**
unclassified_Xylariales_genus	0.2803	0.5063	0.9322	0.3650	**0.0009**
Xylariaceae	**0.0000**	0.6987	0.5380	0.7173	0.7921
unclassified_Ascomycota_class	0.0924	0.2859	0.8409	0.0596	0.4205
unclassified_Ascomycota_order	0.0910	0.2859	0.7568	0.1341	0.4055
unclassified_Ascomycota_family	0.0951	0.3064	0.8226	0.2235	0.4113
unclassified_Ascomycota_genus	0.0963	0.2979	0.8446	0.2794	0.3877
Basidiomycota	**0.0009**	0.8417	0.2303	0.3913	1.1801
Agaricomycetes	**0.0008**	0.6656	0.1815	0.3777	0.9390
Agaricales	**0.0006**	0.6864	0.9271	0.5457	0.1355
Agaricales_family_incertae_sedis	**0.0000**	0.9116	0.6315	0.7417	0.1400
Psathyrellaceae	**0.0019**	0.7344	0.5334	0.5467	0.2371
Coprinellus	0.0712	0.7759	0.6878	0.3865	0.7973
unclassified_Psathyrellaceae_genus	**0.0007**	0.7713	0.5526	0.7153	0.9682
Schizophyllaceae	0.4748	0.9379	0.6039	0.9790	**0.0002**
Schizophyllum	0.2798	0.9368	0.6672	0.9797	**0.0002**
unclassified_Agaricales_family	0.0578	0.1806	0.6301	0.7298	**0.0155**
unclassified_Agaricales_genus	0.0592	0.1736	0.6632	0.7057	**0.0127**
Agaricomycetes_order_incertae_sedis	**0.0003**	0.6643	0.4680	0.9197	0.8748
Corticiaceae	**0.0004**	0.8797	0.9479	0.7402	0.6067
Peniophoraceae	**0.0041**	0.7819	0.8786	0.7335	0.8150
Peniophora	**0.0057**	0.8152	0.7116	0.7399	0.9402
Auriculariales	**0.0000**	0.9995	0.5531	0.5858	0.1068
Cantharellales	**0.0075**	0.6450	0.7711	0.8314	**0.0012**
Ceratobasidiaceae	0.4079	0.6024	0.5924	0.7751	0.7808
Hydnaceae	**0.0000**	0.9972	0.3466	0.7771	**0.0000**
Hydnum	**0.0000**	0.9972	0.3507	0.7369	**0.0000**

Phylum Class Order Family Genus	Healthy (Dog)	Healthy (Body Site)	Allergic (Dog)	Allergic (Body Site)	Shared Sites (Health Status)
Polyporales	**0.0005**	0.7433	0.7418	0.7187	0.4230
Polyporales_family_incertae_sedis	**0.0032**	0.7164	0.8215	0.7881	0.2861
Irpex	**0.0000**	0.8087	0.7062	0.9227	**0.0031**
Trametes	0.0979	0.5037	0.6774	0.3449	**0.0305**
Basidiomycota_class_incertae_sedis	**0.0008**	0.6310	0.7988	0.4996	0.2759
Basidiomycota_order_incertae_sedis	**0.0007**	0.7027	0.3742	0.7414	**0.0003**
Basidiomycota_family_incertae_sedis	**0.0009**	0.7353	0.4252	0.7479	**0.0002**
Cerinosterus	**0.0002**	0.8613	0.7108	0.5969	**0.0000**
Wallemia	**0.0000**	0.8556	0.3587	0.7954	**0.0022**
Entylomatales	**0.0000**	0.9982	0.4933	0.9688	**0.0021**
unclassified_Entylomatales_family	**0.0000**	0.9508	0.5851	0.9733	**0.0019**
unclassified_Entylomatales_genus	**0.0000**	0.9476	0.6206	0.9864	**0.0016**
Malasseziales	**0.0491**	0.7898	0.5119	0.9775	0.3096
Malasseziales_family_incertae_sedis	**0.0484**	0.7953	0.6003	0.9891	0.3000
Malassezia	0.0502	0.7697	0.6331	0.9752	0.3043
Pucciniales	0.1532	0.6740	0.2342	0.7959	**0.0000**
Pucciniastraceae	**0.0343**	0.7176	0.1882	0.7643	**0.0000**
Pucciniastrum	**0.0360**	0.7662	0.1177	0.7287	**0.0000**
Sporidiobolales	**0.0043**	0.4381	0.7232	0.4927	0.0691
Sporidiobolales_family_incertae_sedis	**0.0027**	0.4608	0.8307	0.5192	**0.0366**
Rhodotorula	**0.0485**	0.3564	0.7740	0.4093	0.1312
Sporobolomyces	**0.0002**	0.7595	0.3268	0.4636	**0.0001**
unclassified_Sporidiobolales_family	0.2733	0.4264	0.9414	0.5908	**0.0196**
unclassified_Sporidiobolales_genus	0.2662	0.3553	0.9779	0.5043	**0.0163**
Tremellomycetes	**0.0079**	0.6093	0.3217	0.3105	0.1561
Filobasidiales	0.9636	0.3700	0.2608	0.5012	0.2034
Filobasidiaceae	0.9321	0.6890	0.3477	0.4594	0.2365
Filobasidium	0.9321	0.7509	0.3431	0.3961	0.2094
Tremellales	**0.0005**	0.7956	0.2591	0.4853	0.1108
Tremellaceae	**0.0037**	0.5953	0.4053	0.7157	0.1233
Tremellales_family_incertae_sedis	**0.0056**	0.6909	0.3334	0.4441	0.1037
Cryptococcus	**0.0042**	0.7766	0.3561	0.4140	0.0837
unclassified_Tremellales_family	**0.0002**	0.4065	0.8451	0.5855	0.7601
unclassified_Tremellales_genus	**0.0002**	0.3455	0.8594	0.5053	0.7436
unclassified_Basidiomycota_class	**0.0046**	0.4873	0.2681	0.9499	0.6863
unclassified_Basidiomycota_order	**0.0043**	0.4797	0.2585	0.9770	0.6551

Phylum Class Order Family Genus	Healthy (Dog)	Healthy (Body Site)	Allergic (Dog)	Allergic (Body Site)	Shared Sites (Health Status)
unclassified_Basidiomycota_family	**0.0044**	0.4920	0.3351	0.9826	0.7065
unclassified_Basidiomycota_genus	**0.0043**	0.5158	0.3427	0.9895	0.6723
Ustilaginomycetes	**0.0000**	0.6175	0.8904	0.1708	0.3354
Ustilaginales	**0.0000**	0.6385	0.8217	0.2609	0.3279
unclassified_Ustilaginales_family	**0.0000**	0.7361	0.9735	0.3847	0.3609
unclassified_Ustilaginales_genus	**0.0000**	0.7729	0.9876	0.3306	0.3308
Ustilaginaceae	**0.0000**	0.7854	0.7909	0.3119	0.2873
Pseudozyma	**0.0001**	0.7941	0.8725	0.4301	0.3832
Sporisorium	**0.0000**	0.5984	0.4710	0.3324	**0.0000**
unclassified_Ustilaginaceae_genus	**0.0031**	0.6630	0.4016	0.7883	0.3122
Glomeromycota	0.1166	1.2023	0.8817	0.5524	0.4158
Glomeromycetes	0.1244	0.4809	0.8817	0.4420	0.2661
unclassified_Fungi_phylum	**0.0003**	0.4225	0.2804	0.6427	**0.0412**
unclassified_Fungi_class	**0.0003**	0.6760	0.2243	0.5142	**0.0264**
unclassified_Fungi_order	**0.0002**	0.6613	0.2524	0.5784	**0.0247**
unclassified_Fungi_family	**0.0002**	0.6852	0.3059	0.5713	**0.0215**
unclassified_Fungi_genus	**0.0002**	0.7369	0.3004	0.5211	**0.0182**
Total number of significant taxa (p<0.05)	153	4	**0**	**0**	85

Supplementary table S6. Alpha diversity calculations by site in allergic skin group. Fungal richness was estimated with Observed species and Chao1 in Mothur, and fungal diversity was estimated using the non-parametric Shannon and Inverse Simpson in Mothur. The median, minimum and maximum values were calculated for each allergic body site (Meason-Smith, 2015).

Skin Site	**Observed Species**		**Chao1**		**Shannon**		**Inverse Simpson**	
	Median	Min-Max	Median	Min-Max	Median	Min-Max	Median	Min-Max
Axilla	34	20-52	48	42-69	1.8	1.4-2.3	3.6	2.7-6.2
Ear	21	12-31	39	22-59	1.4	0.1-1.9	2.6	1.0-5.4
Groin	27	23-47	48	43-56	1.9	0.3-2.5	3.7	1.1-7.5
Interdigital	38	31-45	52	46-59	2.1	1.0-5.6	4.7	1.8-9.1
Lumbar	27	21-37	47	31-63	2.0	1.2-2.4	4.8	2.5-7.4
Nostril	19	16-21	38	29-42	1.2	0.2-1.9	2.8	1.1-5.8

Supplementary table S7. Alpha diversity calculations by dog in allergic skin group. Fungal richness was estimated with Observed species and Chao1 in mothur, and fungal diversity was estimated using the non-parametric Shannon and Inverse Simpson in mothur. The median, minimum and maximum values were calculated for each allergic dog (Meason-Smith, 2015).

Dog Number	**Observed Species**		**Chao1**		**Shannon**		**Inverse Simpson**	
	Median	Min-Max	Median	Min-Max	Median	Min-Max	Median	Min-Max
11	31	16-52	43	29-69	2.0	1.5-2.5	4.7	3.2-7.7
12	31	20-43	42	37-54	2.1	1.4-2.3	5.1	2.6-6.2
13	23	20-37	46	39-51	1.6	0.3-2.6	3.8	1.1-9.1
14	26	21-45	49	38-59	1.6	0.4-2.6	3.1	1.1-7.5
15	22	20-39	40	31-52	1.9	1.4-2.1	4.9	2.8-7.1
16	31	16-40	47	32-56	1.4	0.7-1.5	2.6	1.5-3.1
17	37	12-39	48	22-51	1.6	0.1-1.9	3.0	1.0-3.7
18	24	19-38	46	38-63	1.8	1.2-2.2	4.2	2.8-4.8

Supplementary table S8. Dissimilarity of fungal community membership and structure between body sites. R values from global test of the factor body site performed with ANOSIM on distance matrices generated from only allergic body sites using the three beta diversity metrics Bray Curtis, Jaccard, and Yue-Clayton theta coefficient (mothur) were listed for significant comparisons (*P<0.05 after FDR correction). R values from global test of the factor health status performed with ANOSIM on distance matrices generated from only body sites shared between the two health status groups using the three beta diversity metrics Bray Curtis, Jaccard, and Yue-Clayton theta coefficient (mothur) were listed for significant comparisons (*P<0.05 after FDR correction) (Meason-Smith, 2015).

		Bray Curtis	**Jaccard**	**Theta YC**
Allergic Dogs	Axilla	0.331*	0.207	0.294*
	Groin	0.227	0.151	0.213*
	Interdigital	0.441*	0.176	0.300*
Comparison of Shared Sites between Healthy and Allergic Dogs	Ear	0.093	0.249*	0.092
	Groin	0.013	0.264*	0.052
	Interdigital	-0.022	0.402*	-0.033

Supplementary table S9. Alpha diversity averages for healthy cats. Average values are listed with the standard deviation in parentheses (Meason-Smith, 2017).

	Chao1		Observed OTUs		Shannon	
Body Site						
Axilla	625	(215)	121	(42)	3.07	(0.83)
Chin	693	(219)	144	(56)	3.66	(1.18)
Conjunctiva	430	(152)	92	(51)	1.36	(0.65)
Dorsal Nose	764	(470)	146	(62)	3.95	(0.88)
Dorsum	647	(187)	116	(26)	3.35	(0.76)
Ear Canal	617	(150)	146	(32)	2.85	(1.33)
Groin	681	(188)	158	(39)	3.17	(1.28)
Interdigital	693	(127)	179	(49)	4.15	(0.60)
Nostril	657	(255)	165	(40)	2.21	(0.98)
Oral	754	(275)	163	(30)	3.81	(0.91)
Preaural space	684	(321)	192	(44)	4.58	(0.93)
Prepuce/Labia	730	(210)	137	(48)	2.01	(1.09)
Cat						
C1	617	(251)	141	(32)	3.74	(1.00)
C2	612	(216)	140	(37)	3.15	(1.41)
C3	844	(605)	121	(13)	2.39	(1.08)
C4	737	(207)	163	(28)	3.38	(1.02)
C5	659	(258)	161	(46)	4.14	(1.20)
C6	648	(188)	163	(52)	4.17	(1.62)
C7	756	(201)	165	(41)	3.17	(0.88)
C8	590	(341)	116	(30)	2.97	(1.10)
C9	763	(115)	211	(48)	3.89	(0.93)
C10	501	(162)	120	(44)	2.24	(0.92)
C11	677	(73)	144	(29)	3.53	(0.97)
Skin Physiology						
Haired	679	(269)	148	(47)	3.51	(1.04)
Mucosal	585	(236)	120	(47)	1.96	(1.04)
Oral	754	(275)	163	(30)	3.81	(0.91)
Sebaceous	693	(219)	144	(56)	3.66	(1.18)

Supplementary table S10. Average R statistic and range of P-values for significant pairwise comparisons (Meason-Smith, 2017).

		Jaccard			Bray Curtis			Pearson		
	Factor	**N**	**R**	**P-value**	**N**	**R**	**P-value**	**N**	**R**	**P-value**
Healthy	Cat	20	0.223	0.003-0.025	19	0.215	0.003-0.038	26	0.227	0.004-0.041
	Skin Physiology	1	0.282	0.003	1	0.317	0.003	1	0.316	0.003
Allergic	Cat	2	0.413	0.041-0.050	6	0.37	0.041-0.041	0	n/a	n/a

N- number of significant pairwise comparisons

Supplementary table S11. Fungal taxa from filtered relative abundance table for healthy cat samples (Meason-Smith, 2017).

									Body Site					Cat				
Kingdom	Phylum	Class	Order	Family	Genus	Average Relat	Standard Devi	Number of ta	Kruskal-Wallis	Degrees of Fre	P-value	Rank	Adjusted P	Kruskal-Wallis	Degrees of Fre	P-value	Rank	Adjusted P
Fungi	Ascomycota					78.56	17.74	4	5.92663122	12	0.91973522	4	0.91973522	22.2215974	10	0.01401465	1	0.05605861
		Dothideomycetes				62.01	24.19	13	14.2726947	12	0.28363457	6	0.61454156	24.5345638	10	0.00630113	3	**0.02730491**
			Capnodiales			20.56	20.93	27	25.3004765	12	0.01346146	4	0.09086486	44.9665748	10	2.2051E-06	2	**2.9769E-05**
				Capnodiales family incertae sedis		19.04	19.74	36	24.6326764	12	0.01666269	4	0.14996422	43.2640786	10	4.4604E-06	2	**8.0288E-05**
					Cladosporium	13.85	13.51	48	22.1368658	12	0.0360142	10	0.17286816	26.8541797	10	0.00274628	14	**0.00941583**
					Devriesia	0.24	1.01	48	7.40902594	12	0.82944302	44	0.90484693	25.1982448	10	0.00498214	15	**0.01594284**
					Other Capnodiales family incertae sedis	4.83	15.62	48	27.926746	12	0.00567017	3	0.09072272	41.6755735	10	8.5637E-06	4	**0.00010276**
				Mycosphaerellaceae		1.00	5.51	36	12.8942263	12	0.37677546	25	0.54255666	33.5961041	10	0.00021616	8	**0.00097271**
					Cercospora	0.66	5.44	48	13.3393188	12	0.34486747	32	0.51730121	47.7506375	10	6.8924E-07	2	**1.6542E-05**
					Mycosphaerella	0.22	0.97	48	10.9707082	12	0.53143066	37	0.68942356	29.5098972	10	0.00102983	12	**0.00411932**
				Unclassified Capnodiales family		0.45	1.15	36	6.46928422	12	0.89060508	36	0.89060508	28.5378904	10	0.00147964	10	**0.00532671**
					Unclassified Capnodiales genus	0.45	1.15	48	6.46928422	12	0.89060508	47	0.90955412	28.5378904	10	0.00147964	13	**0.00546329**
			Dothideales			1.31	4.26	27	22.1701712	12	0.03565627	6	0.16045324	16.2960814	10	0.09146463	17	0.14526736
				Dothioraceae		1.28	4.26	36	24.4874764	12	0.01744726	5	0.12562028	14.8771816	10	0.13660416	23	0.21381521
					Unclassified Dothioraceae genus	1.15	4.19	48	18.2050716	12	0.10960424	20	0.26305017	17.9550293	10	0.05572699	22	0.12158616
			Dothideomycetes order incertae sedis			12.29	19.45	27	27.5563516	12	0.0064204	1	0.17335076	42.0162915	10	7.4488E-06	3	**6.7039E-05**
				Dothideomycetes family incertae sedis		12.25	19.46	36	27.6042088	12	0.0063184	2	0.11373117	40.3847567	10	1.4495E-05	3	**0.00017394**
					Epicoccum	12.25	19.46	48	27.6042088	12	0.0063184	4	0.07582078	40.3847567	10	1.4495E-05	5	**0.00013915**
			Other Dothideomycetes			0.32	1.48	27	15.7145128	12	0.20466455	10	0.55259429	15.8071469	10	0.10528773	19	0.14961941
				Other Dothideomycetes		0.32	1.48	36	15.7145128	12	0.20466455	16	0.46049524	15.8071469	10	0.10528773	20	0.18951792
					Other Dothideomycetes	0.32	1.48	48	15.7145128	12	0.20466455	24	0.4093291	15.8071469	10	0.10528773	30	0.16846037
			Pleosporales			27.49	23.53	27	10.8302932	12	0.54351593	21	0.6988062	35.1036853	10	0.00011988	6	**0.00053945**
				Pleosporaceae		15.78	17.72	36	15.0935944	12	0.2363579	17	0.50052262	26.5844809	10	0.00302874	11	**0.00991223**
					Alternaria	12.66	17.11	48	12.8449955	12	0.38040709	33	0.55331941	31.7207288	10	0.00044567	10	**0.00213923**
					Cochliobolus	0.22	1.11	48	20.5168451	12	0.05791917	15	0.18534133	13.2167063	10	0.21180668	37	0.27477624
					Exserohilum	0.31	1.17	48	20.1968452	12	0.06345274	17	0.17916068	13.6826446	10	0.18796545	35	0.25778118
					Other Pleosporaceae	0.19	0.87	48	15.6012965	12	0.21018721	25	0.40355944	22.1241705	10	0.01448438	17	**0.04089706**
					Stagonospora	0.28	0.82	48	30.561408	12	0.002297	2	0.05512793	9.56845577	10	0.47913349	44	0.52269108
					Unclassified Pleosporaceae genus	1.89	4.57	48	27.0821475	12	0.00751948	6	0.06015584	7.9033098	10	0.63828104	47	0.65186149
				Pleosporales family incertae sedis		2.28	6.68	36	22.3347777	12	0.03393462	6	0.20360771	11.3335787	10	0.33212647	30	0.39855177
					Ascochyta	1.17	6.04	48	9.49092459	12	0.66052493	38	0.83434727	18.5581098	10	0.04624789	21	0.10570946
					Other Pleosporales family incertae sedis	0.21	1.47	48	17.5471613	12	0.13014797	21	0.29748107	12.4939538	10	0.25335656	39	0.31182346
					Phoma	0.60	1.72	48	27.5737284	12	0.00638318	5	0.06127855	16.6111635	10	0.08342304	25	0.16017224
				Unclassified Pleosporales family		9.02	16.78	36	20.3772589	12	0.06027765	9	0.24111059	30.5168043	10	0.00070478	9	**0.00281911**
					Unclassified Pleosporales genus	9.02	16.78	48	20.3772589	12	0.06027765	16	0.18083294	30.5168043	10	0.00070478	11	**0.00307539**
		Eurotiomycetes				1.76	4.83	13	15.8769707	12	0.19693624	4	0.64004278	20.5922131	10	0.02412355	5	0.06272122
			Eurotiales			1.67	4.83	27	14.4279991	12	0.27421576	14	0.52884469	21.1848895	10	0.01984058	13	**0.04120736**
				Trichocomaceae		1.67	4.83	36	14.4279991	12	0.27421576	21	0.47008417	21.1848895	10	0.01984058	17	**0.04201534**
					Aspergillus	0.45	1.56	48	20.7544995	12	0.05409308	14	0.185462	13.7085914	10	0.18670337	34	0.26358123
					Other Trichocomaceae	0.47	4.20	48	14.3269821	12	0.28031698	28	0.48054339	17.6556948	10	0.06105685	23	0.12742299
					Penicillium	0.73	2.11	48	12.1323545	12	0.43510951	34	0.61427225	20.3948292	10	0.02573184	20	0.06175641
		Leotiomycetes				1.15	4.68	13	12.2725232	12	0.42404868	9	0.61251475	20.5618154	10	0.02436496	6	0.05279076
			Helotiales			1.14	4.68	27	7.99042513	12	0.78587818	25	0.84874844	26.5654728	10	0.00304967	8	**0.01029263**
				Other Helotiales		0.25	1.72	36	7.17654558	12	0.84572945	33	0.92261394	36.6632773	10	6.4707E-05	6	**0.00038824**
					Other Helotiales	0.25	1.72	48	7.17654558	12	0.84572945	45	0.90211141	36.6632773	10	6.4707E-05	7	**0.00044371**
				Sclerotiniaceae		0.87	4.39	36	7.59112154	12	0.81621153	31	0.94785855	33.6697941	10	0.00021005	7	**0.00108026**
					Other Sclerotiniaceae	0.82	4.11	48	7.59112154	12	0.81621153	43	0.91111984	33.6697941	10	0.00021005	8	**0.0012603**
		Other Ascomycota				1.41	6.98	13	13.8215827	12	0.31224657	7	0.57988648	14.8525879	10	0.13752938	10	0.17878819
			Other Ascomycota			1.41	6.98	27	13.8215827	12	0.31224657	15	0.56204382	14.8525879	10	0.13752938	20	0.18566466
				Other Ascomycota		1.41	6.98	36	13.8215827	12	0.31224657	22	0.51094893	14.8525879	10	0.13752938	24	0.20629407
					Other Ascomycota	1.41	6.98	48	13.8215827	12	0.31224657	30	0.49959451	14.8525879	10	0.13752938	33	0.20004273
		Saccharomycetes				4.36	13.99	13	11.2381485	12	0.50862717	11	0.60110484	25.3978125	10	0.00464035	2	**0.03016226**
			Saccharomycetales			4.36	13.99	27	11.2381485	12	0.50862717	20	0.68664668	25.3978125	10	0.00464035	9	**0.01392104**
				Saccharomycetaceae		2.54	9.50	36	20.5608674	12	0.05719266	8	0.25736697	26.3662187	10	0.00327764	12	**0.00983293**
					Saccharomyces	1.17	5.40	48	22.2745536	12	0.03455548	9	0.1842959	33.2145729	10	0.00025067	9	**0.00133688**
					Unclassified Saccharomycetaceae genus	0.98	6.77	48	9.20083615	12	0.68568737	39	0.84392292	14.8652258	10	0.13705328	32	0.20557993
				Saccharomycetales family incertae sedis		1.60	10.57	36	7.24550646	12	0.84097207	32	0.94609358	10.3168788	10	0.41314608	33	0.45070482
					Candida	1.59	10.57	48	6.06868512	12	0.91258012	48	0.91258012	11.1848405	10	0.3432998	42	0.39234263
		Sordariomycetes				7.40	13.71	13	30.9786511	12	0.00198483	2	**0.01290142**	11.6872589	10	0.30653221	11	0.36226534
			Glomerellales			0.27	1.29	27	12.3594489	12	0.41726059	17	0.662708	16.330261	10	0.09056057	16	0.15282095
				Plectosphaerellaceae		0.25	1.27	36	9.75909168	12	0.63708609	28	0.81911068	13.4724215	10	0.19844385	25	0.28575915
					Plectosphaerella	0.10	0.47	48	11.0014865	12	0.5287913	36	0.70505507	12.62448	10	0.24543174	38	0.31001904
			Hypocreales			3.41	6.38	27	23.9587005	12	0.02060571	5	0.11127084	14.0589261	10	0.17032074	21	0.21898381
				Hypocreales family incertae sedis		2.09	3.99	36	18.5605149	12	0.09970096	11	0.32629406	15.1108681	10	0.12807178	22	0.20957201
					Fusarium	1.65	3.52	48	22.1080717	12	0.03632629	11	0.15851472	16.0536849	10	0.09810589	28	0.16818152
					Myrothecium	0.17	0.86	48	19.5549643	12	0.07598691	18	0.20263175	2.65926077	10	0.98834614	48	0.98834614
				Nectriaceae		0.23	1.18	36	18.0936988	12	0.11287498	13	0.31257687	10.6010559	10	0.38943159	32	0.43811053
					Gibberella	0.21	1.16	48	14.0223731	12	0.29928185	29	0.49536306	17.5421104	10	0.06319659	24	0.12639318
				Other Hypocreales		0.61	4.02	36	18.5379564	12	0.10030584	12	0.30091751	15.4172979	10	0.11757234	21	0.20155259
					Other Hypocreales	0.61	4.02	48	18.5379564	12	0.10030584	19	0.25340421	15.4172979	10	0.11757234	31	0.1820475
				Unclassified Hypocreales family		0.28	0.81	36	21.6033521	12	0.04221333	7	0.21709712	22.0719879	10	0.01474204	14	**0.0379081**
					Unclassified Hypocreales genus	0.28	0.81	48	21.6033521	12	0.04221333	13	0.1558646	22.0719879	10	0.01474204	18	**0.0393121**
			Microascales			0.27	1.96	27	14.6300313	12	0.26229497	12	0.59016368	26.6173648	10	0.00299285	7	**0.01154386**
				Halosphaeriaceae		0.27	1.96	36	15.0893009	12	0.23658913	18	0.47317827	21.1868301	10	0.01982781	16	**0.04461256**
			Trichosphaeriales			2.88	10.97	27	15.9941378	12	0.19150471	9	0.57451413	9.54614944	10	0.48117084	26	0.49967741
				Trichosphaeriales family incertae sedis		2.88	10.97	36	16.1357774	12	0.18509606	15	0.44423056	9.82913875	10	0.45560939	34	0.48240994
					Nigrospora	2.88	10.97	48	16.1357774	12	0.18509606	23	0.38628744	9.82913875	10	0.45560939	43	0.50858722
		Unclassified Ascomycota class				0.30	0.87	13	13.5745869	12	0.32869471	8	0.53412891	16.5312009	10	0.08540256	7	0.15860475
			Unclassified Ascomycota order			0.30	0.87	27	13.5745869	12	0.32869471	16	0.55467233	16.5312009	10	0.08540256	15	0.15372461
				Unclassified Ascomycota family		0.30	0.87	36	13.5745869	12	0.32869471	23	0.51447868	16.5312009	10	0.08540256	18	0.17080512
					Unclassified Ascomycota genus	0.30	0.87	48	13.5745869	12	0.32869471	31	0.50894665	16.5312009	10	0.08540256	26	0.15766626
	Basidiomycota					9.02	9.79	4	10.7483802	12	0.55059679	2	1.10119357	15.2041776	10	0.12479333	4	0.12479333
		Agaricomycetes				0.71	1.35	13	31.5165316	12	0.00164234	1	**0.02135037**	9.63792395	10	0.47281261	12	0.51221366
			Agaricales			0.31	0.92	27	25.3583181	12	0.01321326	3	0.11891935	11.2579454	10	0.33777805	24	0.38000031
				Psathyrellaceae		0.12	0.69	36	6.70494244	12	0.87648051	35	0.90152281	12.8176243	10	0.23404818	26	0.32406671
			Agaricomycetes order incertae sedis			0.14	0.50	27	7.52650944	12	0.82095221	26	0.85252729	22.0463038	10	0.01487044	11	**0.03650018**
			Polyporales			0.20	0.79	27	12.0340103	12	0.44295211	18	0.66442816	12.8370054	10	0.23292837	22	0.28586664
				Polyporales family incertae sedis		0.13	0.68	36	13.2331977	12	0.35232126	24	0.52848189	5.40163993	10	0.86278581	36	0.86278581
		Agaricostilbomycetes				0.91	2.99	13	6.91332018	12	0.86328813	13	0.86328813	59.7555266	10	4.0315E-09	1	**5.241E-08**
			Agaricostilbomycetes order incertae sedis			0.91	2.99	27	6.91332018	12	0.86328813	27	0.86328813	59.7555266	10	4.0315E-09	1	**1.0885E-07**
				Agaricostilbomycetes family incertae sedis		0.91	2.99	36	6.91332018	12	0.86328813	34	0.91406979	59.7555266	10	4.0315E-09	1	**1.4514E-07**
					Sterigmatomyces	0.90	2.99	48	6.49688356	12	0.88899569	46	0.92764767	63.1283498	10	9.229E-10	1	**4.4299E-08**
		Basidiomycota class incertae sedis				2.59	6.31	13	12.071154	12	0.43998223	10	0.5719769	14.9371906	10	0.13436861	9	0.19408799
			Basidiomycota order incertae sedis			1.06	5.13	27	9.31474139	12	0.67583814	23	0.79337521	39.2288432	10	2.3149E-05	4	**0.00015626**
				Basidiomycota family incertae sedis		1.06	5.13	36	9.31474139	12	0.67583814	29	0.83897149	39.2288432	10	2.3149E-05	4	**0.00020834**
					Wallemia	1.03	5.13	48	8.93724649	12	0.70828088	41	0.82920688	42.2519221	10	6.7629E-06	3	**0.00010821**
			Leucosporidiales			0.18	1.29	27	16.521382	12	0.16850526	8	0.56870525	18.5104483	10	0.04693979	14	0.09052674
			Malasseziales			0.42	3.12	27	11.3104628	12	0.502516	19	0.71410169	36.9451479	10	5.7843E-05	5	**0.00031235**
				Malasseziales family incertae sedis		0.42	3.12	36	11.3104628	12	0.502516	26	0.69579139	36.9451479	10	5.7843E-05	5	**0.00041647**
					Malassezia	0.42	3.12	48	11.3104628	12	0.502516	35	0.6891648	36.9451479	10	5.7843E-05	6	**0.00046275**
			Sporidiobolales			0.91	2.34	27	17.8683983	12	0.11974522	7	0.46187443	12.7720667	10	0.23669653	23	0.27786114
				Sporidiobolales family incertae sedis		0.86	2.31	36	20.1582018	12	0.06415188	10	0.23094675	12.7088854	10	0.24040685	27	0.32054247
					Rhodotorula	0.30	1.30	48	22.7099552	12	0.03029172	8	0.18175029	9.03808704	10	0.52849284	46	0.55147078
					Sporobolomyces	0.51	1.75	48	21.6459803	12	0.04168446	12	0.16673784	13.5959088	10	0.19223393	36	0.25631191
		Tremellomycetes				4.59	7.36	13	24.61616	12	0.01675024	3	0.07258438	8.39552756	10	0.59026182	13	0.59026182
			Cystofilobasidiales			0.17	0.64	27	10.3223273	12	0.58770577	22	0.72127526	10.6591144	10	0.38468527	25	0.41546009
				Cystofilobasidiaceae		0.17	0.63	36	10.3715147	12	0.58340307	27	0.77787076	10.6207267	10	0.38781967	31	0.45037123
					Cystofilobasidium	0.17	0.63	48	8.11838835	12	0.77581163	42	0.88664186	12.4055083	10	0.25883341	40	0.3106001
			Filobasidiales			0.21	0.93	27	15.0135284	12	0.24069745	11	0.59080283	24.1522232	10	0.00720643	10	**0.01945737**
				Filobasidiaceae		0.21	0.93	36	15.0135284	12	0.24069745	19	0.45605833	24.1522232	10	0.00720643	13	**0.01995628**
					Filobasidium	0.21	0.93	48	15.0135284	12	0.24069745	26	0.44436452	24.1522232	10	0.00720643	16	**0.0216193**
			Tremellales			4.21	7.25	27	26.7129236	12	0.0084967	2	0.11470543	8.04523839	10	0.62441799	27	0.62441799
				Other Tremellales		0.24	1.32	36	17.1049176	12	0.1456931	14	0.37463941	9.35559868	10	0.49872098	35	0.51297015
					Other Tremellales	0.24	1.32	48	17.1049176	12	0.1456931	22	0.31787586	9.35559868	10	0.49872098	45	0.53196905
				Tremellales family incertae sedis		3.63	7.16	36	30.0102001	12	0.00278257	1	0.10017265	12.1250233	10	0.27677632	28	0.35585527

Supplementary Table S11. Continued

Kingdom	Phylum	Class	Order	Family	Genus	Average Relat	Standard Devi	Number of tax	Body Site Kruskal-Wallis	Degrees of Fre	P-value	Rank	Adjusted P	Cat Kruskal-Wallis	Degrees of Fre	P-value	Rank	Adjusted P
					Cryptococcus	3.48	7.15	48	30.6780581	12	0.00220526	1	0.10585226	16.4243584	10	0.08811203	27	0.1566436
				Unclassified Tremellales family		0.29	1.00	36	26.5824175	12	0.00887006	3	0.10644068	11.4653582	10	0.32242899	29	0.40025668
					Unclassified Tremellales genus	0.29	1.00	48	26.5824175	12	0.00887006	7	0.06082324	11.4653582	10	0.32242899	41	0.37747784
	Other Fungi					0.17	1.13	4	14.6154194	12	0.26314455	1	1.0525782	15.8750991	10	0.10326409	3	0.13768545
		Other Fungi	Other Fungi			0.17	1.13	27	14.6154194	12	0.26314455	13	0.54653099	15.8750991	10	0.10326409	18	0.15489614
				Other Fungi		0.17	1.13	36	14.6154194	12	0.26314455	20	0.47366019	15.8750991	10	0.10326409	19	0.19565828
					Other Fungi	0.17	1.13	48	14.6154194	12	0.26314455	27	0.46781253	15.8750991	10	0.10326409	29	0.17091987
						0.17	1.13	13	14.6154194	12	0.26314455	5	0.68417583	15.8750991	10	0.10326409	8	0.16780415
Unassigned	Unassigned					12.00	14.47	4	9.11043085	12	0.69347035	3	0.92462713	21.4118482	10	0.01839789	2	**0.03679579**
		Unassigned				12.00	14.47	13	9.11043085	12	0.69347035	12	0.75125954	21.4118482	10	0.01839789	4	0.05979315
			Unassigned			12.00	14.47	27	9.11043085	12	0.69347035	24	0.78015414	21.4118482	10	0.01839789	12	**0.04139526**
				Unassigned		12.00	14.47	36	9.11043085	12	0.69347035	30	0.83216442	21.4118482	10	0.01839789	15	**0.04415494**
					Unassigned	12.00	14.47	48	9.11043085	12	0.69347035	40	0.83216442	21.4118482	10	0.01839789	19	**0.04647889**
Counts													2					53

Ave. Rel. Abund.- average relative abundance, Std. Dev. Rel. Abund.- average standard deviation of the relative abundance, N-number of taxa included within the phylogentic level (class, order, family, genus), Kruskall-Wallis Stat.- Kruskall-Wallis statistic, DF-degrees of freedom, Adjusted P-value- corrected with the Benjamini Hochberg equation.

Average relative abundance and standard deviation calculated across all healthy cat samples. The number of taxa present within a level (e.g. phylum) that was used for Benjamini and Hochberg False Discovery Rate (FDR) correction listed, along with the adjusted P-value. P-values less than or equal to 0.05 are bolded.

Supplementary Table S12. Alpha diversity averages for allergic cats. Average values listed with standard deviation in parentheses (Meason-Smith, 2017).

	Chao1		Observed OTUs		Shannon	
Body Site						
Axilla	881	(288)	192	(36)	3.42	(0.49)
Dorsum	1015	(249)	211	(44)	3.95	(0.99)
Ear Canal	683	(166)	153	(32)	2.81	(0.94)
Groin	764	(341)	225	(54)	3.52	(0.32)
Interdigital	748	(285)	209	(31)	3.96	(0.77)
Nostril	729	(153)	164	(48)	2.31	(0.85)
Cat						
C12	729	(152)	179	(55)	2.99	(1.00)
C13	742	(240)	204	(42)	3.87	(1.07)
C14	775	(122)	212	(14)	3.41	(0.56)
C15	535	(160)	164	(49)	3.42	(1.23)
C16	941	(265)	223	(51)	3.86	(0.73)
C17	797	(235)	210	(29)	3.87	(0.85)
C18	1010	(480)	183	(79)	2.99	(0.57)
C19	689	(146)	194	(43)	3.03	(0.63)
C20	751	(114)	199	(51)	2.58	(0.68)
Skin Physiology						
Haired	757	(274)	205	(43)	3.44	(0.73)
Mucosal	729	(153)	164	(48)	2.31	(0.85)
Steroid Usage						
Yes	766	(778)	211	(197)	3.46	(3.30)
No	750	(278)	195	(53)	3.04	(0.85)

Supplementary table S13. Fungal taxa from filtered relative abundance table for allergic cat samples (Meason-Smith, 2017).

Phylum	Class	Order	Family	Genus	Ave. Rel. Abund.	Std. Dev. Rel. Abunc	N	Body Site Kruskal-wallis	DF	P-value	Rank	Adjusted P	Cat Kruskall-Walli	DF	P-value	Rank	Adjusted P
Ascomycota					77.48	20.99	3	8.64	5	0.1244	1	0.3733	8.22	8	0.4118	2	0.6178
	Ascomycota class incertae sedis				1.10	4.54	12	6.98	5	0.2223	3	0.8892	9.03	8	0.3399	5	0.8158
		Ascomycota order incertae sedis			1.10	4.54	21	6.98	5	0.2223	5	0.9336	9.03	8	0.3399	13	0.5491
			Ascomycota family incertae sedis		1.10	4.54	28	6.98	5	0.2223	5	1.2448	9.03	8	0.3399	18	0.5287
				Coniosporium	0.87	4.52	41	8.33	5	0.1387	6	0.9479	11.51	8	0.1744	14	0.5108
	Dothideomycetes				46.55	26.86	12	4.17	5	0.5247	6	1.0495	8.45	8	0.3909	6	0.7818
		Capnodiales			20.47	17.99	21	9.69	5	0.0845	3	0.5918	11.16	8	0.1929	10	0.4052
			Capnodiales family incertae sedis		18.14	16.05	28	8.76	5	0.1191	2	1.6668	8.66	8	0.3717	19	0.5477
				Cladosporium	17.50	16.02	41	8.42	5	0.1346	5	1.1039	7.43	8	0.4915	34	0.5926
				Other Capnodiales family incerta sedis	0.62	2.02	41	5.90	5	0.3164	11	1.1794	2.47	8	0.9632	40	0.9873
			Mycosphaerellaceae		1.72	4.87	28	2.83	5	0.7258	21	0.9677	15.25	8	0.0544	5	0.3046
				Cercospora	1.27	4.11	41	4.67	5	0.4577	17	1.1039	8.41	8	0.3945	30	0.5391
			Unclassified Capnodiales family		0.52	2.36	28	2.21	5	0.8190	33	1.0175	8.69	8	0.3691	28	0.5405
				Unclassified Capnodiales genus	0.52	2.36	41	2.21	5	0.8190	33	1.0175	8.69	8	0.3691	28	0.5405
		Dothideomycetes order incertae sedis			4.79	9.66	21	3.38	5	0.6409	15	0.8972	8.47	8	0.3887	14	0.5831
			Botryosphaeriaceae		0.38	1.52	28	5.80	5	0.3266	8	1.1432	9.50	8	0.3020	14	0.6039
			Dothideomycetes family incertae sedis		4.41	9.37	28	4.89	5	0.4293	13	0.9246	7.68	8	0.4650	20	0.6509
				Epicoccum	4.41	9.37	41	4.89	5	0.4293	14	1.2571	7.68	8	0.4650	32	0.5957
		Other Dothideomycetes			1.66	9.46	21	10.06	5	0.0735	2	0.7717	13.13	8	0.1074	7	0.3223
			Other Dothideomycetes		1.66	9.46	28	10.06	5	0.0735	1	2.0579	13.13	8	0.1074	10	0.3008
				Other Dothideomycetes class	1.66	9.46	41	10.06	5	0.0735	4	0.7533	13.13	8	0.1074	10	0.4405
		Pleosporales			19.39	20.08	21	5.68	5	0.3386	7	1.0157	10.36	8	0.2405	12	0.4208
			Pleosporaceae		14.26	17.12	28	5.47	5	0.3616	11	0.9203	7.13	8	0.5224	21	0.6965
				Alternaria	10.02	13.10	41	4.17	5	0.5250	19	1.1329	8.23	8	0.4113	31	0.5439
				Cochliobolus	0.60	2.60	41	1.43	5	0.9213	35	1.0792	9.82	8	0.2777	19	0.5992
				Other Pleosporaceae family	0.83	3.48	41	4.83	5	0.4372	15	1.1951	8.82	8	0.3576	26	0.5639
				Unclassified Pleosporaceae genus	2.19	8.40	41	2.90	5	0.7155	29	1.0115	12.77	8	0.1201	11	0.4476
			Pleosporales family incertae sedis		0.70	2.40	28	3.63	5	0.6037	17	0.9944	9.28	8	0.3189	15	0.5953
			Unclassified Pleosporales family		4.16	10.34	28	2.40	5	0.7917	31	1.0471	9.52	8	0.3000	20	0.6150
				Unclassified Pleosporales genus	4.16	10.34	41	2.40	5	0.7917	31	1.0471	9.52	8	0.3000	20	0.6150
	Eurotiomycetes				4.19	10.26	12	10.28	5	0.0677	2	0.4060	12.53	8	0.1292	3	0.5168
		Eurotiales			2.54	5.54	21	7.42	5	0.1911	4	1.0033	13.14	8	0.1071	6	0.3748
			Trichocomaceae		2.54	5.54	28	7.42	5	0.1911	4	1.3377	13.14	8	0.1071	9	0.3331
				Aspergillus	0.57	1.36	41	4.78	5	0.4432	16	1.1356	8.96	8	0.3455	25	0.5666
				Eurotium	1.09	4.78	41	3.78	5	0.5817	21	1.1357	10.19	8	0.2518	18	0.5735
				Penicillium	0.87	3.11	41	3.84	5	0.5731	20	1.1748	13.92	8	0.0839	7	0.4913
		Onygenales			1.48	8.42	21	3.48	5	0.6259	14	0.9389	28.34	8	0.0004	1	**0.0087**
			Arthrodermataceae		1.48	8.42	28	3.48	5	0.6259	19	0.9224	28.34	8	0.0004	1	**0.0116**
				Arthroderma	1.48	8.42	41	3.48	5	0.6259	26	0.9870	28.34	8	0.0004	1	**0.0170**
	Leotiomycetes				0.45	2.10	12	3.32	5	0.6501	8	0.9751	5.18	8	0.7385	9	0.9847
	Other Ascomycota				0.44	2.04	12	5.59	5	0.3487	4	1.0461	2.41	8	0.9659	11	1.0537
		Other Ascomycota			0.44	2.04	21	5.59	5	0.3487	8	0.9153	2.41	8	0.9659	20	1.0142
			Other Ascomycota		0.44	2.04	28	5.59	5	0.3487	9	1.0849	2.41	8	0.9659	27	1.0017
			Other Ascomycota	Other Ascomycota	0.44	2.04	41	5.59	5	0.3487	12	1.1914	2.41	8	0.9659	41	0.9659
	Saccharomycetes				1.06	3.58	12	2.56	5	0.7679	10	0.9215	14.25	8	0.0754	2	0.4527
		Saccharomycetales			1.06	3.58	21	2.56	5	0.7679	19	0.8488	14.25	8	0.0754	4	0.3961
			Saccharomycetaceae		0.42	1.56	28	2.58	5	0.7651	23	0.9315	22.03	8	0.0049	2	0.0680
	Sordariomycetes				22.71	24.50	12	4.16	5	0.5271	7	0.9036	6.50	8	0.5919	8	0.8878
		Hypocreales			11.46	18.50	21	3.12	5	0.6821	18	0.7958	10.70	8	0.2192	11	0.4185
			Hypocreales family incertae sedis		8.13	18.16	28	1.66	5	0.8938	26	0.9625	13.63	8	0.0919	7	0.3676
				Fusarium	3.16	5.96	41	0.88	5	0.9719	37	1.0770	25.20	8	0.0014	2	**0.0295**
				Myrothecium	1.23	5.80	41	6.64	5	0.2490	7	1.4585	8.79	8	0.3607	27	0.5478
				Sarocladium	2.40	14.15	41	0.96	5	0.9657	36	1.0999	7.53	8	0.4805	33	0.5970
			Nectriaceae		0.54	2.26	28	1.73	5	0.8850	25	0.9912	7.03	8	0.5334	22	0.6789
				Gibberella	0.52	2.27	41	0.41	5	0.9951	41	0.9951	9.45	8	0.3061	22	0.5704
			Other Hypocreales		0.41	1.63	28	3.59	5	0.6096	18	0.9482	12.19	8	0.1430	11	0.3641
				Other Hypocreales order	0.41	1.63	41	3.59	5	0.6096	25	0.9997	12.19	8	0.1430	13	0.4512
			Unclassified Hypocreales family		1.81	4.17	28	0.60	5	0.9879	39	1.0385	20.17	8	0.0097	4	0.0995
				Unclassified Hypocreales genus	1.81	4.17	41	0.60	5	0.9879	39	1.0385	20.17	8	0.0097	4	0.0995
		Sordariomycetes order incertae sedis			3.88	11.88	21	1.75	5	0.8829	20	0.9270	19.28	8	0.0134	2	0.1408
			Magnaporthaceae		2.68	9.70	28	0.79	5	0.9774	28	0.9774	21.94	8	0.0050	3	**0.0469**
				Magnaporthe	2.68	9.70	41	0.79	5	0.9774	38	1.0545	21.94	8	0.0050	3	0.0686
		Trichosphaeriales			5.18	15.62	21	5.16	5	0.3965	10	0.8327	15.13	8	0.0566	3	0.3962
			Trichosphaeriales family incertae sedis		5.18	15.62	28	5.16	5	0.3965	12	0.9252	15.13	8	0.0566	6	0.2641
				Nigrospora	5.18	15.62	41	5.16	5	0.3965	13	1.2506	15.13	8	0.0566	6	0.3867
		Unclassified Sordariomycetes order			0.98	5.16	21	3.64	5	0.6023	16	1.0540	9.10	8	0.3340	23	0.5954
			Unclassified Sordariomycetes family		0.98	5.16	28	3.64	5	0.6023	23	1.0736	9.10	8	0.3340	23	0.5954
				Unclassified Sordariomycetes genus	0.98	5.16	41	3.64	5	0.6023	23	1.0736	9.10	8	0.3340	17	0.5501
	Unclassified Ascomycota class				0.97	2.78	12	6.54	5	0.2571	6	0.8998	13.52	8	0.0952	8	0.4877
		Unclassified Ascomycota order			0.97	2.78	21	6.54	5	0.2571	6	1.1998	13.52	8	0.0952	8	0.4877
			Unclassified Ascomycota family		0.97	2.78	28	6.54	5	0.2571	8	1.3176	13.52	8	0.0952	8	0.3331
				Unclassified Ascomycota genus	0.97	2.78	41	6.54	5	0.2571	8	1.3176	13.52	8	0.0952	5	0.3997
Basidiomycota					10.78	16.61	3	6.61	5	0.2516	2	0.3773	7.85	8	0.4480	3	0.4480
	Agaricomycetes				4.64	8.49	12	1.12	5	0.9523	11	1.0388	16.02	8	0.0421	1	0.5051
		Agaricales			2.02	7.15	21	0.74	5	0.9808	21	0.9808	11.98	8	0.1523	8	0.3997
			Physalacriaceae		1.48	7.10	28	1.38	5	0.9261	27	0.9604	21.20	8	0.0066	4	**0.0465**
				Armillaria	0.34	1.44	41	3.75	5	0.5859	22	1.0920	11.03	8	0.2002	17	0.4829
			Psathyrellaceae		0.30	1.18	28	2.07	5	0.8399	24	0.9799	5.30	8	0.7246	26	0.7803
				Coprinellus	0.15	0.59	41	3.02	5	0.6967	28	1.0201	6.51	8	0.5900	35	0.6911
		Agaricomycetes order incertae sedis			0.47	1.62	21	3.36	5	0.6452	16	0.8468	6.17	8	0.6280	18	0.7327
		Polyporales			1.68	3.99	21	5.03	5	0.4119	11	0.7864	8.05	8	0.4287	16	0.5627
			Ganodermataceae		0.49	2.04	28	2.65	5	0.7531	22	0.9585	11.45	8	0.1776	12	0.4144
				Ganoderma	0.49	2.04	41	2.65	5	0.7531	30	1.0293	11.45	8	0.1776	15	0.4855
		Polyporales	Polyporales family incertae sedis		0.78	2.47	28	8.42	5	0.1346	3	1.2563	9.25	8	0.3219	16	0.5632
	Agaricostilbomycetes				0.15	0.56	12	3.15	5	0.6769	9	0.9026	6.51	8	0.5900	7	1.0113
		Agaricostilbomycetes order incertae sedis			0.15	0.56	21	3.15	5	0.6769	17	0.8362	6.51	8	0.5900	17	0.7288
			Agaricostilbomycetes family incertae sedis	*Sterigmatomyces*	0.15	0.56	41	3.15	5	0.6769	27	1.0279	6.51	8	0.5900	35	0.6911
					0.15	0.56	28	3.15	5	0.6769	20	0.9477	6.51	8	0.5900	24	0.6883
	Basidiomycota class incertae sedis				1.52	6.07	12	0.82	5	0.9758	12	0.9758	3.66	8	0.8866	10	1.0639
		Sporidiobolales			1.14	5.81	21	4.69	5	0.4546	12	0.7956	8.41	8	0.3942	15	0.5518
			Sporidiobolales family incertae sedis		1.11	5.81	28	4.04	5	0.5434	15	1.0143	6.99	8	0.5380	23	0.6550
				Sporobolomyces	0.13	0.45	41	6.16	5	0.2911	10	1.1936	12.55	8	0.1284	12	0.4386
	Tremellomycetes				4.47	7.98	12	11.81	5	**0.0375**	1	0.4501	1.76	8	0.9874	12	0.9874
		Cystofilobasidiales			0.29	1.59	21	5.53	5	0.3551	9	0.8285	5.70	8	0.6808	19	0.7525
			Cystofilobasidiaceae		0.29	1.59	28	5.53	5	0.3551	10	0.9942	5.70	8	0.6808	25	0.7625
		Tremellales			4.15	7.81	21	11.40	5	**0.0440**	1	0.9237	1.75	8	0.9879	21	0.9879
			Tremellales family incertae sedis		2.53	5.96	28	5.99	5	0.3072	7	1.2286	1.85	8	0.9853	28	0.9853
				Cryptococcus	2.28	5.66	41	10.14	5	0.0712	3	0.9735	4.86	8	0.7720	39	0.8116
			Unclassified Tremellales family		1.33	5.60	28	16.01	5	**0.0068**	1	0.2790	5.45	8	0.7091	37	0.7857
				Unclassified Tremellales genus	1.33	5.60	41	16.01	5	**0.0068**	1	0.2790	5.45	8	0.7091	37	0.7857
Unassigned					11.46	14.10	3	4.55	5	0.4729	3	0.4729	11.20	8	0.1904	1	0.5713
	Unassigned				11.46	14.10	12	4.55	5	0.4729	5	1.1348	11.20	8	0.1904	4	0.5713
		Unassigned			11.46	14.10	21	4.55	5	0.4729	13	0.7638	11.20	8	0.1904	9	0.4443
			Unassigned		11.46	14.10	28	4.55	5	0.4729	14	0.9457	11.20	8	0.1904	13	0.4101
				Unassigned	11.46	14.10	41	4.55	5	0.4729	18	1.0771	11.20	8	0.1904	16	0.4880
Counts <0.05										4.0000							6.0000

Ave. Rel. Abund.- average relative abundance, Std. Dev. Rel. Abund.- average standard deviation of the relative abundance, N-number of taxa included within the phylogentic level (class, order, family, genus), Kruskall-Wallis Stat.- Kruskall-Wallis statistic, DF-degrees of freedom, Adjusted P-value- corrected with the Benjamini Hochberg equation.

Average relative abundance and standard deviation calculated across all healthy cat samples. The number of taxa present within a level (e.g. phylum) used for Benjamini and Hochberg false discovery rate (FDR) correction listed, along with the adjusted P-value. P-values less than or equal to 0.05 are bolded.

Supplementary table S14. Global R statistics for beta diversity analysis for healthy and allergic cats (Meason-Smith, 2017).

		Jaccard		**Bray Curtis**		**Pearson**	
Factor		**R**	**P-value**	**R**	**P-value**	**R**	**P-value**
Healthy	Cat	0.225	0.001	0.324	0.001	0.304	0.001
	Body Site	0.07	0.003	0.083	0.001	0.079	0.002
	Skin Physiology	0.208	0.002	0.213	0.003	0.212	0.001
Allergic	Cat	0.364	0.001	0.324	0.001	0.304	0.001
	Body Site	-0.048	0.806	-0.109	0.992	-0.104	0.988
	Skin Physiology	0.073	0.225	0.208	0.047	0.233	0.031
	Steroids	0.036	0.163	0.1	0.016	0.1	0.005

Supplementary table S15. Fungal taxa from filtered relative abundance table for six sites (Meason-Smith, 2017).

Phylum	Class	Order	Family	Genus	Ave. Rel. Abu	Std. Dev. Rel.	N	Kruskall-Walli	DF	P-value	Rank	Adjusted P
Ascomycota					78.62	17.22	5	0.00	1	0.9816	5	0.9816
	Ascomycota class incertae sedis				0.46	2.81	15	5.31	1	0.0212	5	0.0637
		Ascomycota order incertae sedis			0.46	2.81	31	5.31	1	0.0212	9	0.0732
			Ascomycota family incertae sedis		0.46	2.81	45	5.31	1	0.0212	11	0.0869
				Coniosporium	0.37	2.78	58	4.93	1	0.0264	9	0.1701
	Dothideomycetes				57.21	25.82	15	12.71	1	0.0004	2	**0.0027**
		Capnodiales			21.17	20.48	31	0.00	1	0.9602	30	0.9922
			Capnodiales family incertae sedis		19.55	19.66	45	0.01	1	0.9297	41	1.0204
				Cladosporium	15.55	15.42	58	1.09	1	0.2966	26	0.6616
				Other Capnodiales family incertae sedis	3.81	14.43	58	11.93	1	0.0006	1	**0.0321**
			Mycosphaerellaceae		1.05	3.48	45	0.09	1	0.7664	34	1.0143
				Cercospora	0.60	2.82	58	0.56	1	0.4531	36	0.7300
				Mycosphaerella	0.23	1.04	58	0.81	1	0.3673	32	0.6656
			unclassified Capnodiales family		0.47	1.78	45	2.87	1	0.0903	16	0.2540
				unclassified Capnodiales genus	0.47	1.78	58	2.87	1	0.0903	16	0.3273
		Dothideales			0.61	1.94	31	5.81	1	0.0160	7	0.0707
			Dothioraceae		0.60	1.94	45	6.01	1	0.0142	7	0.0916
				unclassified Dothioraceae genus	0.49	1.73	58	6.76	1	0.0093	5	0.1080
		Dothideomycetes order incertae sedis			10.62	16.80	31	9.64	1	0.0019	2	**0.0295**
			Botryosphaeriaceae		0.19	1.01	45	0.54	1	0.4622	23	0.9043
				unclassified Botryosphaeriaceae genus	0.16	0.98	58	0.19	1	0.6627	45	0.8542
			Dothideomycetes family incertae sedis		10.43	16.79	45	11.28	1	0.0008	1	**0.0352**
				Epicoccum	10.43	16.79	58	11.28	1	0.0008	2	**0.0227**
		Other Dothideomycetes			0.83	6.15	31	0.62	1	0.4294	18	0.7395
			Other Dothideomycetes		0.83	6.15	45	0.62	1	0.4294	22	0.8783
				Other Dothideomycetes	0.83	6.15	58	0.62	1	0.4294	34	0.7325
		Pleosporales			23.96	21.86	31	3.73	1	0.0536	11	0.1511
			Leptosphaeriaceae		0.09	0.45	45	0.01	1	0.9395	42	1.0066
				Leptosphaeria	0.09	0.45	58	0.01	1	0.9395	55	0.9908
			Pleosporaceae		15.47	17.50	45	0.35	1	0.5517	25	0.9930
				Alternaria	12.09	16.07	58	0.67	1	0.4132	33	0.7262
				Cochliobolus	0.38	1.93	58	0.97	1	0.3256	28	0.6745
				Other Pleosporaceae	0.42	2.31	58	0.04	1	0.8373	49	0.9910
				Stagonospora	0.14	0.61	58	1.32	1	0.2505	22	0.6604
				unclassified Pleosporaceae genus	2.06	6.20	58	1.55	1	0.2130	20	0.6178
			Pleosporales family incertae sedis		2.04	7.07	45	7.05	1	0.0079	5	0.0714
				Ascochyta	1.19	6.64	58	1.00	1	0.3169	27	0.6807
				Other Pleosporales family incertae sedis	0.28	1.75	58	1.29	1	0.2569	23	0.6478
				Phoma	0.41	1.64	58	5.10	1	0.0239	8	0.1734
			unclassified Pleosporales family		6.18	11.41	45	7.37	1	0.0066	3	0.0993
				unclassified Pleosporales genus	6.18	11.41	58	7.37	1	0.0066	3	0.1280
	Eurotiomycetes				2.50	7.13	15	0.91	1	0.3393	10	0.5089
		Chaetothyriales			0.10	0.57	31	0.18	1	0.6684	23	0.9008
		Eurotiales			1.75	4.05	31	0.20	1	0.6564	22	0.9250
			Trichocomaceae		1.75	4.05	45	0.20	1	0.6564	30	0.9846
				Aspergillus	0.32	0.97	58	0.03	1	0.8674	51	0.9864
				Eurotium	0.57	3.15	58	0.03	1	0.8661	50	1.0047
				Penicillium	0.81	2.61	58	0.02	1	0.8874	52	0.9898
		Onygenales			0.65	5.67	31	0.72	1	0.3976	17	0.7250
			Arthrodermataceae		0.64	5.67	45	0.39	1	0.5325	24	0.9985
				Arthroderma	0.64	5.67	58	0.39	1	0.5325	38	0.8128
	Leotiomycetes				0.99	3.05	15	0.16	1	0.6890	13	0.7950
		Helotiales			0.84	2.81	31	1.80	1	0.1794	13	0.4279
			Other Helotiales		0.26	1.89	45	1.10	1	0.2953	20	0.6643
				Other Helotiales	0.26	1.89	58	1.10	1	0.2953	24	0.7135
			Sclerotiniaceae		0.58	2.12	45	0.19	1	0.6599	31	0.9579
				Other Sclerotiniaceae	0.54	1.98	58	0.18	1	0.6699	46	0.8446
	Other Ascomycota				0.26	1.37	15	2.32	1	0.1278	7	0.2738
		Other Ascomycota			0.26	1.37	31	2.32	1	0.1278	12	0.3301
			Other Ascomycota		0.26	1.37	45	2.32	1	0.1278	18	0.3195
				Other Ascomycota	0.26	1.37	58	2.32	1	0.1278	18	0.4118
	Saccharomycetes				2.36	9.00	15	0.22	1	0.6414	12	0.8017
		Saccharomycetales			2.36	9.00	31	0.22	1	0.6414	21	0.9468
			Saccharomycetaceae		1.21	6.34	45	0.01	1	0.9202	40	1.0352
				Saccharomyces	0.19	0.83	58	0.36	1	0.5510	39	0.8195
				unclassified Saccharomycetaceae genus	0.58	4.08	58	0.20	1	0.6573	44	0.8664
			Saccharomycetales family incertae sedis		1.09	6.51	45	0.09	1	0.7635	33	1.0411
				Candida	1.06	6.51	58	0.62	1	0.4303	35	0.7131
	Sordariomycetes				14.22	21.89	15	15.89	1	0.0001	1	**0.0010**
	Sordariomycetes	Glomerellales			0.39	2.06	31	0.01	1	0.9104	29	0.9732
			Plectosphaerellaceae		0.38	2.06	45	0.18	1	0.6683	32	0.9398
				Other Plectosphaerellaceae	0.37	2.06	58	0.21	1	0.6442	43	0.8689
		Hypocreales			6.95	14.28	31	10.12	1	0.0015	1	**0.0455**
			Hypocreales family incertae sedis		4.55	12.96	45	5.41	1	0.0200	10	0.0900
				Fusarium	2.30	5.09	58	2.98	1	0.0845	15	0.3268
				Myrothecium	0.52	3.68	58	2.00	1	0.1578	19	0.4816
				Sarocladium	1.11	9.82	58	0.81	1	0.3671	31	0.6868
			Nectriaceae		0.42	1.80	45	0.02	1	0.8818	37	1.0725
				Gibberella	0.40	1.80	58	0.00	1	0.9543	57	0.9710
			Other Hypocreale	Other Hypocreale	0.72	4.53	58	0.02	1	0.8952	54	0.9615
					0.72	4.53	45	0.02	1	0.8952	39	1.0329
			unclassified Hypocreales family		0.87	2.76	45	3.83	1	0.0503	14	0.1618
				unclassified Hypocreales genus	0.87	2.76	58	3.83	1	0.0503	12	0.2434
		Sordariomycetes order incertae sedis			1.93	8.30	31	6.72	1	0.0095	5	0.0591
			Magnaporthaceae		1.42	6.89	45	5.06	1	0.0245	12	0.0921
				Magnaporthe	1.41	6.89	58	5.16	1	0.0231	7	0.1913
		Trichosphaeriales			4.19	14.87	31	0.94	1	0.3325	16	0.6442
			Trichosphaeriales family incertae sedis		4.19	14.87	45	0.94	1	0.3325	21	0.7125
				Nigrospora	4.19	14.87	58	0.94	1	0.3325	29	0.6650
		unclassified Sordariomycetes order			0.43	3.24	31	0.32	1	0.5735	19	0.9357
			unclassified Sordariomycetes family		0.43	3.24	45	0.32	1	0.5735	26	0.9926
				unclassified Sordariomycetes genus	0.43	3.24	58	0.32	1	0.5735	40	0.8316
	unclassified Ascomycota class				0.59	2.05	15	1.42	1	0.2340	8	0.4388
		unclassified Ascomycota order			0.59	2.05	31	1.42	1	0.2340	14	0.5182
			unclassified Ascomycota family		0.59	2.05	45	1.42	1	0.2340	19	0.5543
				unclassified Ascomycota genus	0.59	2.05	58	1.42	1	0.2340	21	0.6464
Basidiomycota					9.86	13.18	5	0.00	1	0.9449	4	1.1811
	Agaricomycetes				2.25	5.85	15	10.68	1	0.0011	3	**0.0054**
		Agaricales			0.99	4.68	31	6.38	1	0.0115	6	0.0596
			Physalacriaceae		0.62	4.60	45	7.36	1	0.0067	4	0.0750
				Armillaria	0.15	1.02	58	4.32	1	0.0376	10	0.2182
			Psathyrellaceae		0.20	0.83	45	0.28	1	0.5953	29	0.9237
				Coprinellus	0.13	0.60	58	0.18	1	0.6704	47	0.8273
				Coprinopsis	0.07	0.43	58	0.82	1	0.3643	30	0.7043

Phylum	Class	Order	Family	Genus	Ave. Rel. Abu	Std. Dev. Rel.	N	Kruskall-Walli	DF	P-value	Rank	Adjusted P
Ascomycota					78.62	17.22	5	0.00	1	0.9816	5	0.9816
		Agaricomycetes order incertae sedis			0.31	1.14	31	0.06	1	0.8091	26	0.9647
			Peniophoraceae		0.12	0.53	45	0.01	1	0.9395	43	0.9832
				Peniophora	0.12	0.53	58	0.01	1	0.9395	56	0.9731
		Hymenochaetales			0.10	0.50	31	0.00	1	0.9921	31	0.9921
			Hymenochaetaceae		0.10	0.50	45	0.00	1	0.9921	45	0.9921
		Polyporales			0.74	2.70	31	8.72	1	0.0031	3	**0.0325**
			Ganodermataceae		0.21	1.34	45	4.32	1	0.0376	13	0.1302
				Ganoderma	0.21	1.34	58	4.32	1	0.0376	10	0.2182
			Polyporales family incertae sedis		0.34	1.64	45	8.09	1	0.0044	2	0.0999
	Agaricostilbomycetes				0.80	2.85	15	5.64	1	0.0176	4	0.0658
		Agaricostilbomycetes order incertae sedis			0.80	2.85	31	5.64	1	0.0176	8	0.0680
			Agaricostilbomycetes family incertae sedis		0.80	2.85	45	5.64	1	0.0176	9	0.0878
				Sterigmatomyces	0.80	2.85	58	5.64	1	0.0176	6	0.1697
	Basidiomycota class incertae sedis				1.58	4.54	15	4.74	1	0.0295	6	0.0738
		Basidiomycota order incertae sedis			0.31	1.16	31	7.04	1	0.0080	4	0.0618
			Basidiomycota family incertae sedis		0.31	1.16	45	7.04	1	0.0080	6	0.0598
				Wallemia	0.31	1.16	58	7.04	1	0.0080	4	0.1157
		Sporidiobolales			1.14	4.34	31	0.09	1	0.7621	24	0.9844
			Sporidiobolales family incertae sedis		1.10	4.34	45	0.30	1	0.5817	27	0.9694
				Rhodotorula	0.57	3.95	58	0.25	1	0.6146	42	0.8487
				Sporobolomyces	0.50	1.57	58	1.09	1	0.2961	25	0.6869
	Tremellomycetes				5.08	8.76	15	1.02	1	0.3122	9	0.5204
		Cystofilobasidiales			0.22	1.25	31	0.05	1	0.8182	27	0.9394
			Cystofilobasidiaceae		0.22	1.25	45	0.05	1	0.8182	36	1.0227
				Cystofilobasidium	0.22	1.25	58	0.47	1	0.4947	37	0.7755
		Filobasidiales			0.14	0.80	31	4.35	1	0.0370	10	0.1146
			Filobasidiaceae		0.14	0.80	45	3.62	1	0.0572	15	0.1717
				Filobasidium	0.14	0.80	58	3.62	1	0.0572	14	0.2371
		Tremellales			4.71	8.68	31	1.03	1	0.3093	15	0.6392
			Other Tremellales		0.28	1.51	45	2.80	1	0.0944	17	0.2498
				Other Tremellales	0.28	1.51	58	2.80	1	0.0944	17	0.3219
			Tremellales family incertae sedis		3.64	8.10	45	5.90	1	0.0151	8	0.0851
				Cryptococcus	3.43	8.00	58	3.82	1	0.0508	13	0.2265
			unclassified Tremellales family		0.73	3.77	45	0.00	1	0.9704	44	0.9924
				unclassified Tremellales genus	0.73	3.77	58	0.00	1	0.9704	58	0.9704
Other Fungi					0.08	0.31	5	0.07	1	0.7886	2	1.9716
	Other Fungi				0.08	0.31	15	0.07	1	0.7886	14	0.8450
		Other Fungi			0.08	0.31	31	0.07	1	0.7886	25	0.9779
			Other Fungi		0.08	0.31	45	0.07	1	0.7886	35	1.0140
				Other Fungi	0.08	0.31	58	0.07	1	0.7886	48	0.9529
Unassigned					11.28	12.45	5	0.30	1	0.5832	1	2.9161
	Unassigned				11.28	12.45	15	0.30	1	0.5832	11	0.7953
		Unassigned			11.28	12.45	31	0.30	1	0.5832	20	0.9040
			Unassigned		11.28	12.45	45	0.30	1	0.5832	28	0.9373
				Unassigned	11.28	12.45	58	0.30	1	0.5832	41	0.8250
unclassified Fungi phylum					0.13	0.60	5	0.02	1	0.8898	3	1.4830
	unclassified Fungi class				0.13	0.60	15	0.02	1	0.8898	15	0.8898
		unclassified Fungi order			0.13	0.60	31	0.02	1	0.8898	28	0.9852
			unclassified Fungi family		0.13	0.60	45	0.02	1	0.8898	38	1.0537
				unclassified Fungi genus	0.13	0.60	58	0.02	1	0.8898	53	0.9738
counts												9.0000

Average relative abundance and standard deviation calculated across all samples that were included in the comparison between health statues (number of body sites sampled was six). The number of taxa present within a level (e.g. phylum) that was used for Benjamini and Hochberg False Discovery Rate (FDR) correction listed, along with the adjusted P-value. P-values less than or equal to 0.05 are bold.

Supplementary table S16. Alpha diversity averages for health status (Meason-Smith, 2017).

	Chao1		Observed OTUs		Shannon	
Healthy	666	(208)	150	(42)	3.20	(1.02)
Allergic	657	(258)	141	(29)	3.26	(0.79)

Values listed represent averages with standard deviations in parentheses

Supplementary table S17. Kruskal-wallis p values for relative abundance of *Malassezia* species between individual atopic dogs and between time points.

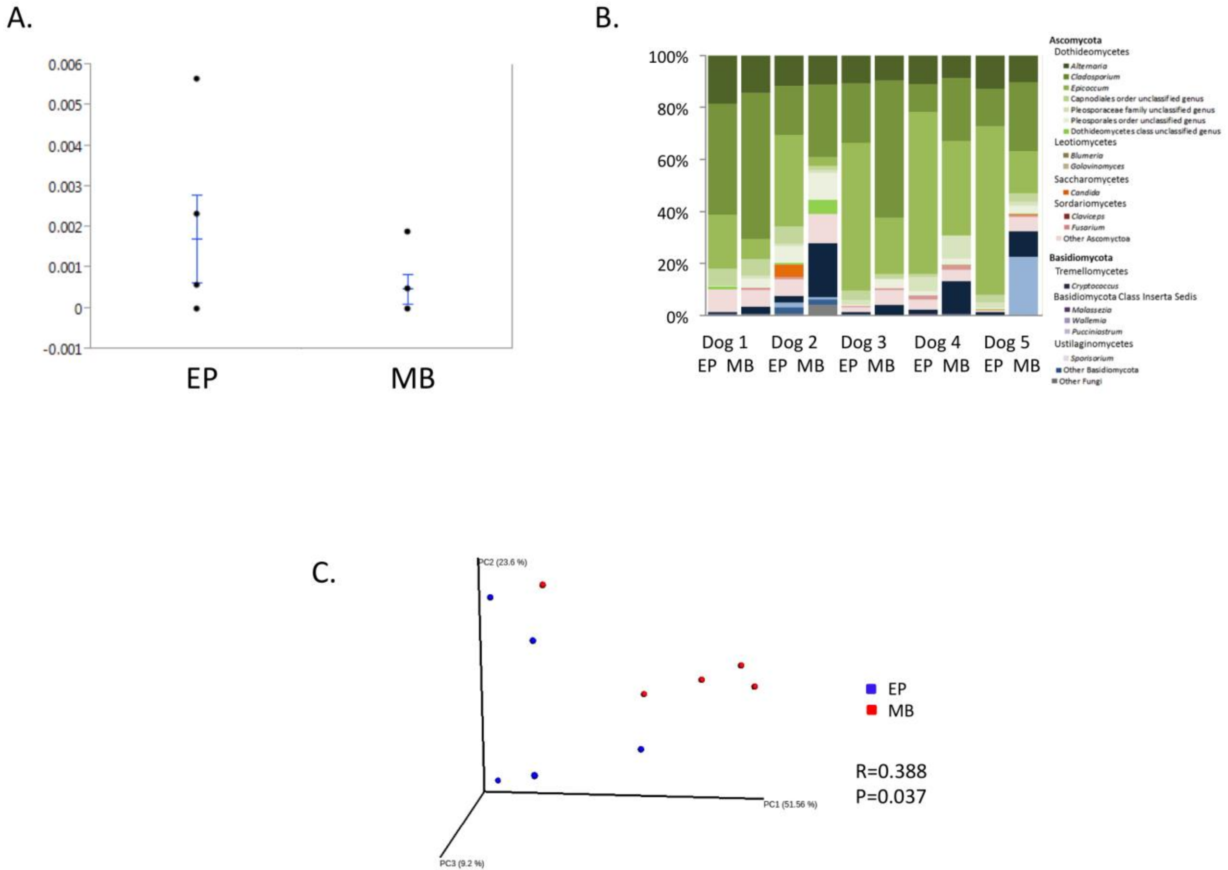
NGS	**Individual**	**Time point**
Malassezia sp.	**0.0070**	0.9608
M. furfur	**0.0153**	0.9115
M. globosa	0.1853	0.3390
M.obtusa	**0.0095**	0.934
M. pachydermatis	0.2546	0.4317
M. restricta	**0.0437**	0.8208
M. slooffiae	0.1425	0.3432
M. sympodialis	0.2914	0.9763
M. yamatoensis	**0.0251**	0.9536

Supplementary table S18. Kruskal-wallis p values for genome equivalents of *Malassezia* species between individual atopic dogs and between time points.

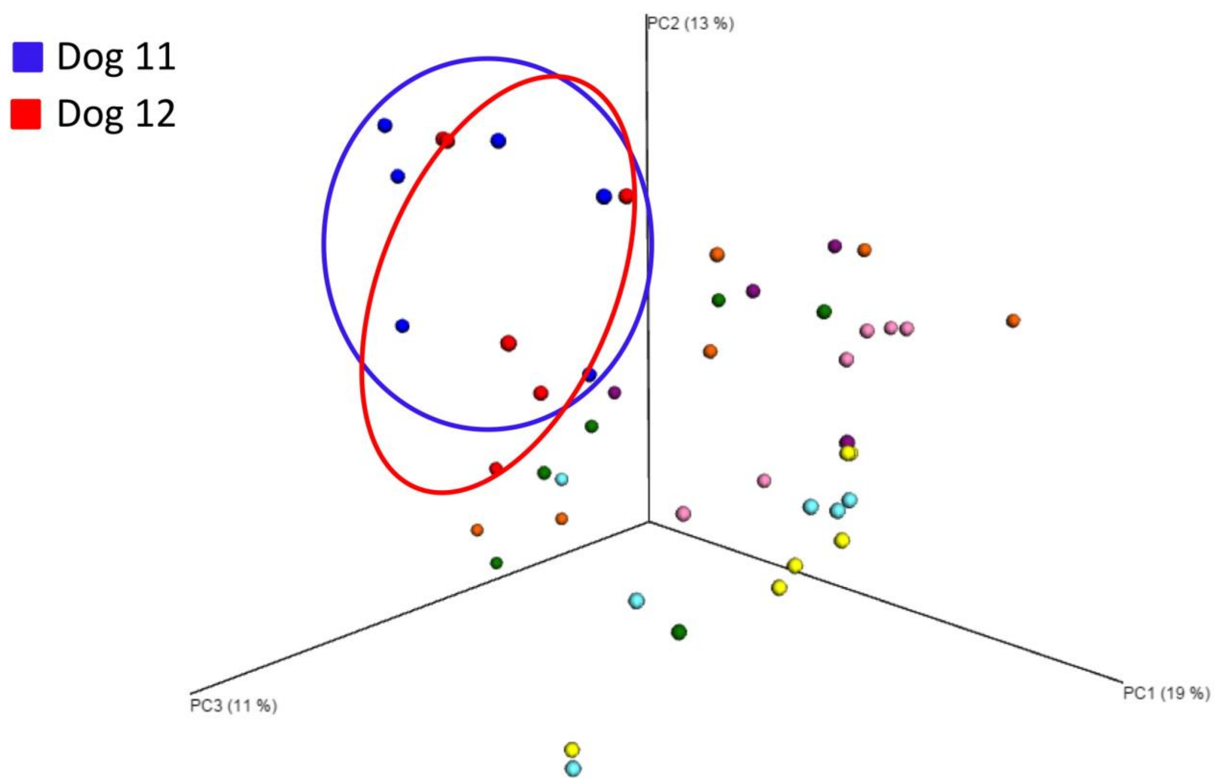
qPCR	**Individual**	**Time point**
M. pachydermatis	**0.0063**	0.9489
M. restricta	0.1307	0.0750

APPENDIX B

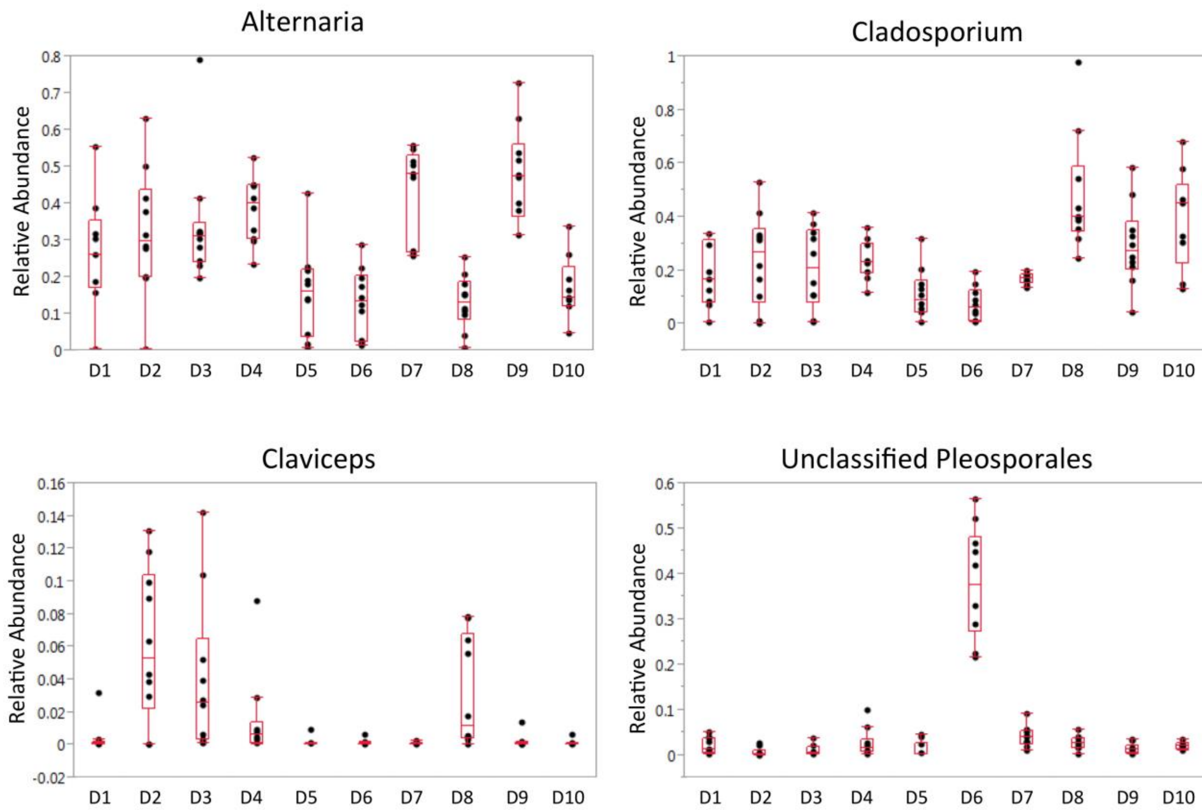
SUPPLEMENTARY FIGURES



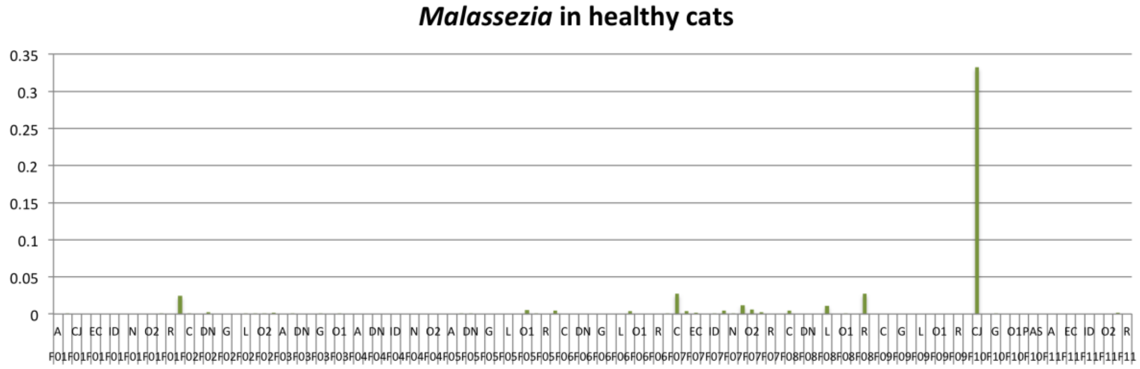
Supplementary Figure S1 Results from comparison of two extraction protocols. (a) Relative abundances of *Malassezia* in the ear canal of five dogs were plotted in JMP with mean error bars. EP (Epicentre MasterPure Yeast Lysis Kit) signifies the samples extracted using the Findley *et. al.* 2013 protocol, and MB (MoBio) signifies the samples extracted following the Rodrigues *et. al.* 2014 protocol. (b) Stacked bar plot of predominant fungal taxa for each sample, grouped by dog number and extraction protocol. (c) PCoA plot of Bray Curtis calculated pairwise distances between samples. Samples are colored by kit (Meason-Smith, 2015).

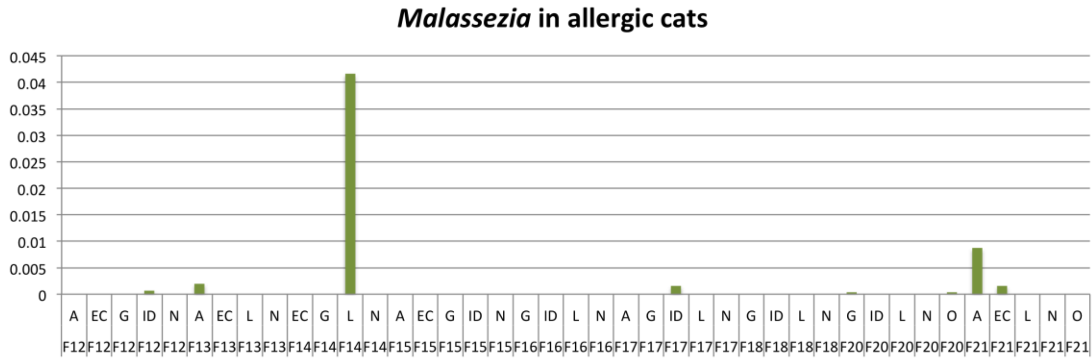


Supplementary Figure S2. Dissimilarity of fungal community membership between allergic dogs. PCoA plot of Bray Curtis calculated pairwise distances between body sites of only allergic dogs. The two dogs in red and blue cluster together and come from the same household. Each color represents samples from different sites from each allergic dog (Meason-Smith, 2015).

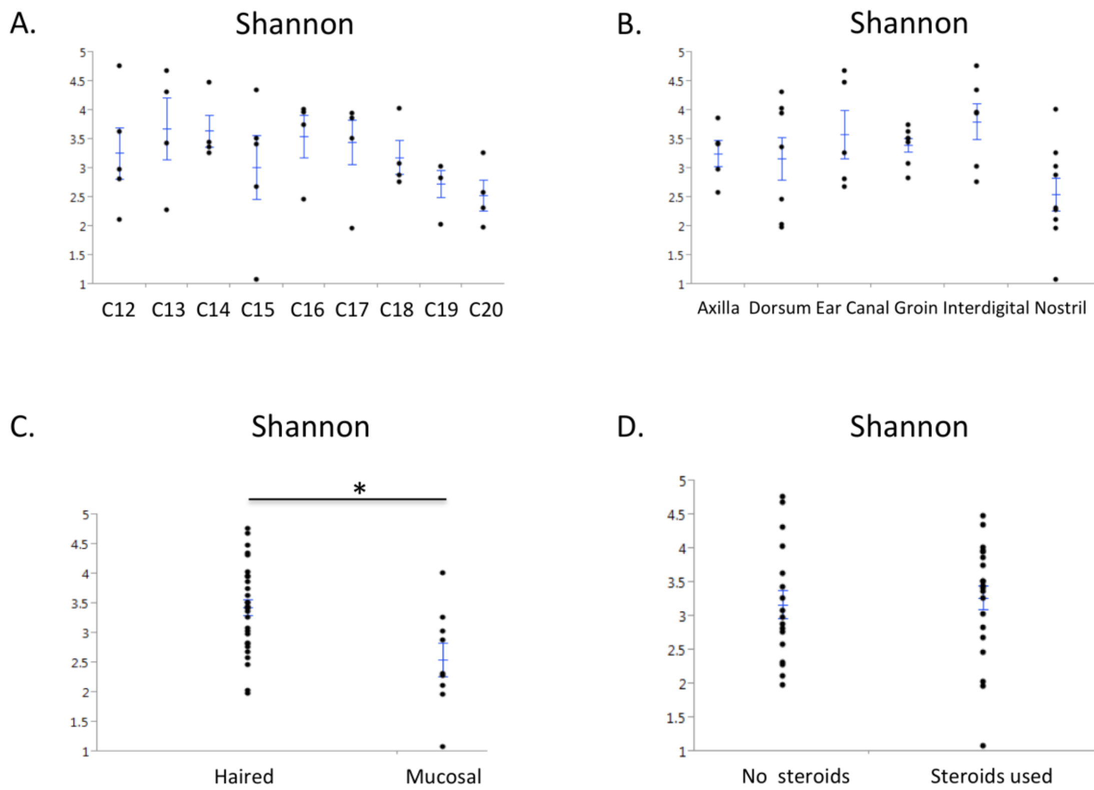


Supplementary Figure S3. Relative abundances of fungal taxa that were significantly different between dogs. Dots represent body sites and are grouped by dog (Meason-Smith, 2015).



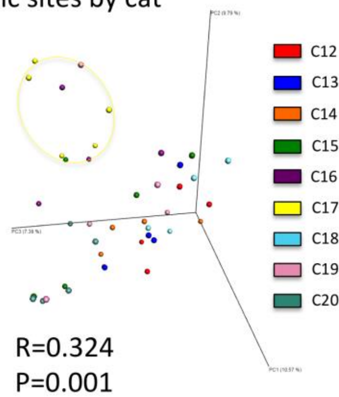


Supplementary figure S4. Relative abundance of *Malassezia* in healthy and allergic feline skin samples. The relative abundance of *Malassezia* is plotted for each skin sample from healthy and allergic cats. A-axilla, C-chin, CJ-conjunctiva, DN-dorsal nose, D-dorsum, EC-ear canal, G-groin, ID-interdigital space, N-nostril, O-oral, PAS-preaural space, R-reproductive tract (Meason-Smith, 2017).

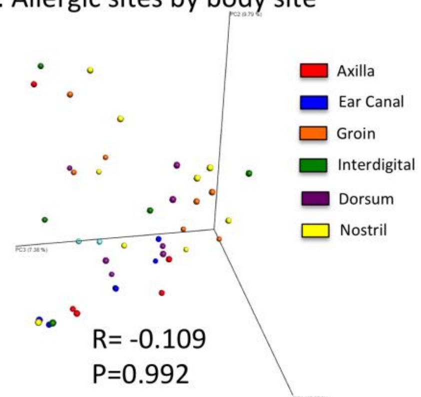


Supplementary figure S5. Alpha diversity of allergic cats. Alpha diversity estimated with Shannon diversity metric, and samples grouped by (a) cat, (b) body site, (c) skin physiology, and (d) steroids usage. Means and mean error bars are plotted in blue for each group. Groups with a mean significantly different from other means are denoted by asterisks, with associated P-values (Steel-dwas multiple comparisons test, of *<0.05, **<0.01, ***<0.001 (Meason-Smith, 2017).

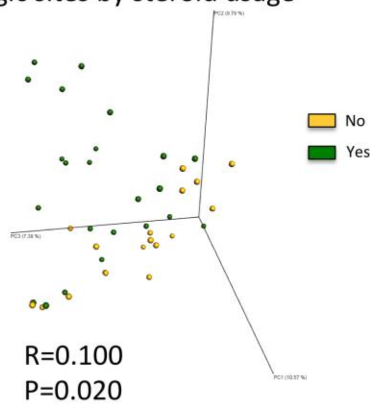
A. Allergic sites by cat



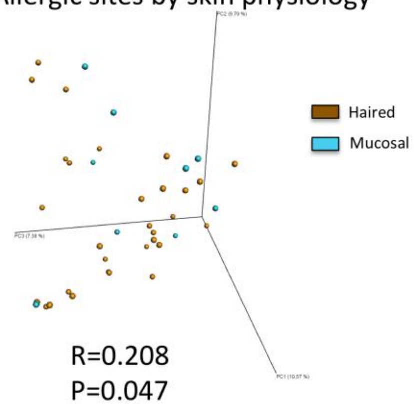
B. Allergic sites by body site



C. Allergic sites by steroid usage

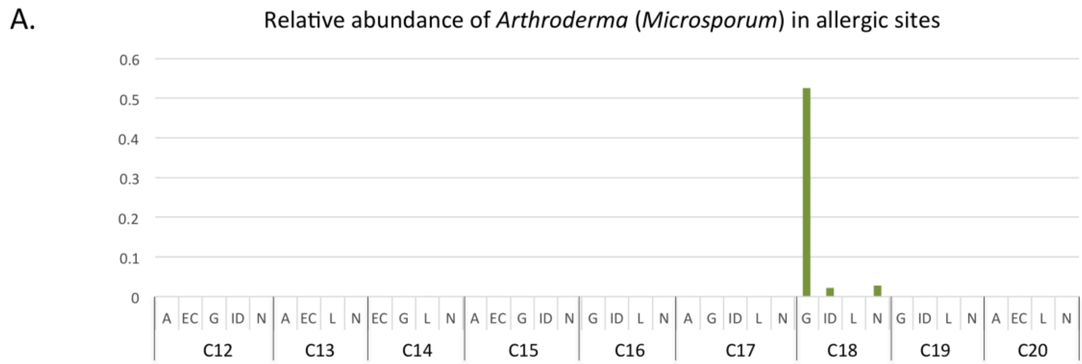


D. Allergic sites by skin physiology

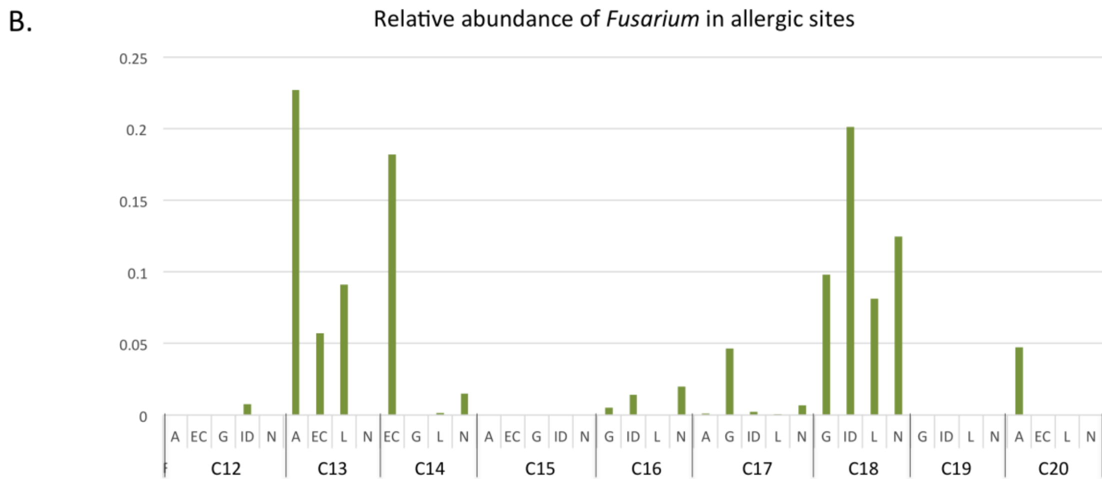


Supplementary figure S6. Beta diversity of allergic cats. PCoA plot of Bray Curtis pairwise distances for healthy cat samples, with associated ANOSIM global R statistic, and P-value; colored by (a) cat, (b) body site, (c) steroids usage, and (d) skin physiology (Meason-Smith, 2017).

A.

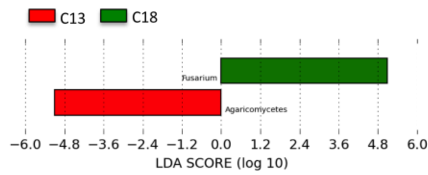


B.

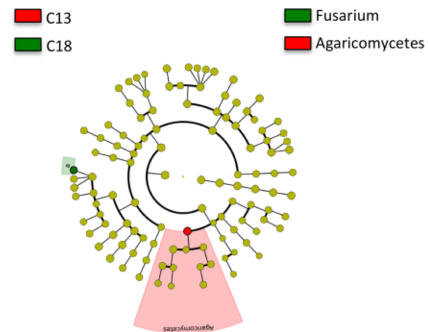


Supplementary figure S7. Relative abundance of *Arthroderma* and *Fusarium* in allergic feline skin samples. The relative abundance of (a) *Arthroderma* and (b) *Fusarium* is plotted for each skin sample from allergic cats (Meason-Smith, 2017).

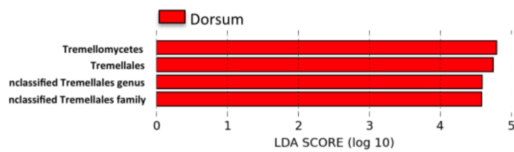
A. LEfSe significant taxa for allergic cats



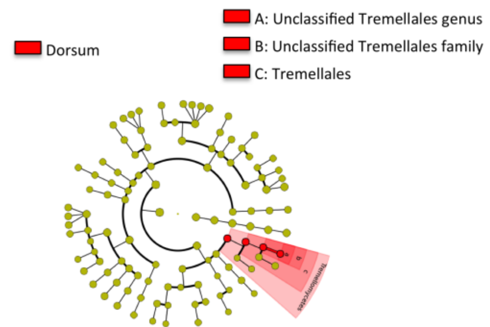
B. LEfSe cladogram for allergic cats



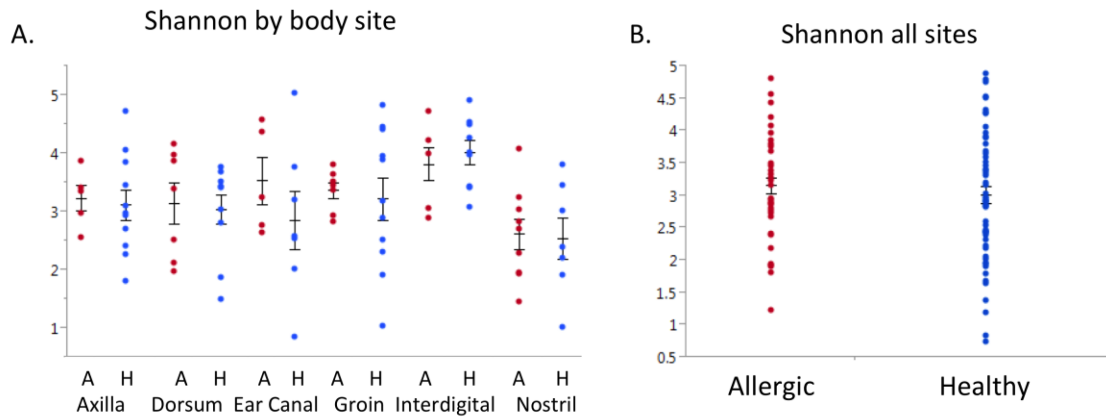
C. LEfSe significant taxa for allergic body sites



D. LEfSe cladogram for allergic body sites



Supplementary figure S8. LDA effect size (LEfSe) analysis of allergic cats. Fungal taxa that are significantly increased or decreased in allergic (a-b) cats or (c-d) body sites are presented in two forms- as bar blots showing the LDA score, and as a cladogram demonstrating the phylogenetic relationships. Taxa are colored according to cat or body site in which they are increased in abundance (Meason-Smith, 2017).



Supplementary figure S9. Comparison of alpha diversity between healthy and allergic feline skin for six sites. Alpha diversity estimated with Shannon diversity metric, and samples grouped by (a) body site and health status, and (b) health status only, Means and mean error bars are plotted in blue for each group. Means were not significantly different for any group (Meason-Smith, 2017).