

Characterization of the cutaneous mycobiota in healthy and allergic cats using next generation sequencing

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Background – Next generation sequencing (NGS) studies have demonstrated a diverse skin-associated microbiota and microbial dysbiosis associated with atopic dermatitis in people and in dogs. The skin of cats has yet to be investigated using NGS techniques.

Hypothesis/Objectives – We hypothesized that the fungal microbiota of healthy feline skin would be similar to that of dogs, with a predominance of environmental fungi, and that fungal dysbiosis would be present on the skin of allergic cats.

Animals – Eleven healthy cats and nine cats diagnosed with one or more cutaneous hypersensitivity disorders, including flea bite, food-induced and nonflea nonfood-induced hypersensitivity.

Methods – Healthy cats were sampled at twelve body sites and allergic cats at six sites. DNA was isolated and Illumina sequencing was performed targeting the internal transcribed spacer region of fungi. Sequences were processed using the bioinformatics software QIIME.

Results – The most abundant fungal sequences from the skin of all cats were classified as *Cladosporium* and *Alternaria*. The mucosal sites, including nostril, conjunctiva and reproductive tracts, had the fewest number of fungi, whereas the pre-aural space had the most. Allergic feline skin had significantly greater amounts of Agaricomycetes and Sordariomycetes, and significantly less *Epicoccum* compared to healthy feline skin.

Conclusions – The skin of healthy cats appears to have a more diverse fungal microbiota compared to previous studies, and a fungal dysbiosis is noted in the skin of allergic cats. Future studies assessing the temporal stability of the skin microbiota in cats will be useful in determining whether the microbiota sequenced using NGS are colonizers or transient microbes.

Introduction

Next generation sequencing (NGS) techniques have provided a methodology to characterize host-associated microbial communities (microbiota) more comprehensively and have consequently revealed a much more diverse microbiota than was previously thought to exist.¹ In humans, NGS studies have shown that skin-associated bacterial microbiota are distributed according to physiological niches,² such as dry, moist and sebaceous skin microenvironments,³ whereas the distribution of the fungal microbiota (mycobiota) is more dependent upon body site location such as core body versus feet.⁴ In contrast to what is observed in humans, the bacterial microbiota of canine skin are influenced by body site rather than physiological niches.⁵ The mycobiota are more likely to be distributed evenly across body sites within a dog and

significant differences in mycobiota are observed between dogs.⁶

The specific bacterial and fungal taxa of canine skin differ from those of human skin. Canine skin is dominated by bacteria in the phyla Proteobacteria, Firmicutes and Actinobacteria,⁵ and environmental fungi such as *Alternaria* and *Cladosporium*,⁶ whereas human skin is colonized more abundantly by bacteria in the phyla Actinobacteria and Firmicutes,³ and the fungal genus *Malassezia*.⁴ Hygiene practices and environmental exposures are thought to contribute to the differences in diversity and taxa between host species,⁶ although studies are still required to better investigate their influence on the microbiome. The microbial communities present on feline skin have only been investigated using culture dependent methods.^{7–16} The results of these studies are variable and fungal genera commonly isolated include *Penicillium*, *Cladosporium*, *Aspergillus*, *Alternaria* and *Malassezia*.

Bacterial and fungal dysbiosis (alteration to the normal microbiota) has been identified in human atopic dermatitis (AD)^{17,18} and canine allergic dermatitis.^{5,6} The lesional skin of atopic human patients exhibits reduced bacterial diversity with proportionate increases in *Staphylococcus*

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species¹⁷ and increased fungal diversity.¹⁸ By contrast nonlesional skin of allergic dogs possessed reduced diversity of both bacterial and fungal microbiota.^{5,6}

Cats suffer from an allergic dermatitis sometimes resembling human and canine AD, referred to as nonflea nonfood-induced hypersensitivity dermatitis (NFNFIHD), which suggests that environmental allergens are triggers for these cases.¹⁹ However, the pathogenesis of NFNFIHD is incompletely understood and lacks some of the defining characteristics of human and canine AD.^{20,21} These include absence of a proven genetic predisposition for any subgroup of NFNFIHD (with the exception of a report of three affected littermates),²² clinical presentation²³ and uncertainty as to whether the skin barrier is impaired in NFNFIHD. Furthermore, there have been variable reports on the role of allergen-specific IgE in cats with NFNFIHD.^{24,25} The skin microbiota of cats with NFNFIHD has yet to be investigated with either NGS or culture-dependent methods. Only a single study using cytological examination of tape strips demonstrated an overgrowth of *Malassezia* in allergic cats compared to control cats.¹³

The goals of this study were to characterize the microbiota of feline skin using NGS and to determine whether alterations to the microbiota exist in feline allergic skin diseases. It was hypothesized that the microbiota of feline skin would be similar to canine skin and that fungal dysbiosis would also exist in feline allergic dermatitis. Similar to previous studies, the influence of individual characteristics such as the type of body site (haired, moist, oral, sebaceous) and body site location were assessed. Overall fungal diversity and relative abundance of select taxa (i.e. the amount of a fungal taxon sequenced in a sample relative to the total amount of fungal DNA sequenced for that sample) were compared between healthy and allergic feline skin.

Materials and methods

Subject recruitment

All samples for this study were collected following a protocol approved by the College of Veterinary Medicine, Texas A&M University, Institutional Animal Care and Use Committee. Eleven cats (numbered C1–C11) were enrolled in this study on the basis of no current or prior dermatological conditions and were assigned to the healthy group (Table 1). There were five castrated males and six spayed females ranging in age from two to 17 years. Six cats were domestic short hair, two were domestic medium hair and three were domestic long hair cats.

Nine cats (C12–C20) were included in the allergic group (Tables 1 and 2). This group ranged in age from 4 to 11 years of age and included four castrated males and five spayed females. There were six domestic shorthairs, two Siamese and one Persian cat represented. All were diagnosed with a hypersensitivity dermatitis (HD) after exclusion of other pruritic dermatoses such as ectoparasitism and bacterial or fungal infections.

The classification of HD for each cat is presented in Table 2: flea bite hypersensitivity (FBH, $n = 8$), food-induced hypersensitivity dermatitis (FIHD, $n = 1$) and nonflea nonfood-induced hypersensitivity dermatitis (NFNFIHD, $n = 4$). Four cats had received a diagnosis of more than one type of HD and one cat, which had failed to respond to an appropriate trial with a flea preventative, but which had not completed a dietary elimination trial at the time of sampling, was classified as having nonflea bite hypersensitivity (NFBH). The age of onset ranged from three to six years of age, although two cats had

experienced a gradual progression of clinical signs with the exact age of onset unknown. Seven of ten cats had no seasonal exacerbations of clinical signs, whereas one had flares during the summer only and two experienced flares during the spring and summer. The most common clinical signs included pruritus and alopecia. There was a wide range of lesion distribution (Table 2). Six cats had documented steroid administration, but only two (C14 and C15) were receiving steroids at the time of sample collection. Additionally, three cats were receiving therapies including oral ciclosporin (C15 and C16), sublingual immunotherapy (C17) and oral antihistamines (C16).

Exclusion criteria included exposure to systemic antimicrobial drugs within the six months (healthy control group) or 30 days (HD group) prior to sampling. Bathing was not allowed during the week prior to sampling. None of the cats exhibited 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 the axilla, chin, conjunctiva, dorsal nose, dorsum, ear canal, groin, interdigital space, nostril, oral cavity, preaural space and vulva or prepuce. Six sites commonly affected by HD were sampled from the allergic group, including the 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.⁶

ITS sequencing and sequence analysis

Illumina sequencing (Illumina Inc.; San Diego CA, USA) of all samples was performed on an Illumina MiSeq instrument at the University of Minnesota Genomics Center using ITS1F (5'-CTTGGTCATTTAGA GGAAGTAA-3') and ITS2R (5'-GCTGCGTTCTTCATCGATGC-3') primers that amplified the internal transcribed spacer (ITS-1) region, a noncoding 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.²⁶ 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 ITS sequence database.⁴ Taxonomic assignments were made with a formatted version of the ITS taxonomy file.⁴ OTU tables were rarefied at 3,100 sequences for healthy only samples, 5,000 for allergic only samples and 3,300 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. To determine whether fungal richness and diversity of skin microbiota were different between cats, body sites or type of body site, the alpha diversity measures for each metric were analysed across all body sites within a cat ("Cat"), across all cats at one body site ("Body Site") or for all body sites within a particular type of body site ["Skin Type" included haired (axilla, dorsal nose, dorsum, ear canal, groin, interdigital space, preaural space), mucosal (conjunctiva, nostril, prepuce and vulva), oral (cavity) and sebaceous (chin)]. Beta diversity was measured using weighted Jaccard, Bray Curtis and Pearson metrics. These calculations are performed for each possible pair of samples and the distance matrix generated was then used to create 3D PCoA plots. Analysis of similarities (ANOSIM) was performed on the distance matrices to determine statistical significance of a factor (cat, body site, skin type) on the dissimilarity between samples.

Statistical analysis

All statistical analyses were completed as described previously,⁶ 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 three samples for allergic cats, or five samples for healthy cats. Using the statistical software JMP Pro 11, (SAS Institute, Inc.; Cary, NC, USA) data were tested for normality and Kruskal–Wallis tests were performed to determine whether the mean value (relative abundance or

Table 1. Signalment and medical histories of twenty cats enrolled in the study

Cat number	Health status	Breed	Age	Sex	Fleas	Time indoors	Indoor environment	Outdoor environment	Previous antibiotic 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
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.

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.

Table 2. Hypersensitivity classification, age of onset, seasonality, clinical signs and distribution, and treatments for nine allergic cats

Cat number	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	Ciclosporin	Y
C16	DSH	5	FBH, NFNFIHD	4	N	Pruritus, self-induced alopecia, excoriations	Chest, ventral abdomen, dorsum, tail, limbs	Y	Ciclosporin, 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

Allergen 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 dermatophyte infection, treated with lime sulfur dips and lesions resolved 3 months prior to sample collection.

DSH domestic short hair, Per Persian, Sia Siamese, FBH flea bite hypersensitivity, FIHD food-induced hypersensitivity dermatitis, NFNFIHD non-flea nonfood-induced hypersensitivity dermatitis, NFBH nonflea bite hypersensitivity, G gradual, Y yes, N no.

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. A Wilcoxon–Mann–Whitney U -test was

performed to determine significant differences between health statuses.

In order to determine whether the beta diversity of samples was significantly influenced by cat, body site, skin type, steroids or health

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. *R* values were calculated for each pairwise comparison between groups (significant comparisons summarized in Table S2) and a global *R* statistic was calculated for the factor under study (cat, body site, skin type) (ANOSIM, PRIMER 6). The combined and filtered relative abundance tables were also used in linear discriminant analysis (LDA) effect size (LEfSe)²⁷ 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.²⁸

Results

One hundred and thirty two samples were collected from healthy cats and 54 from allergic cats. Due to low numbers of sequences (less than 3,000), 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. All median alpha diversity measurements can be found in Table S1. 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 harboured a more rich and diverse mycobiota than the other cats (Figure 1). The conjunctiva and reproductive tract sites of healthy cats were the least diverse body sites, whereas the preaural space was the most rich and diverse (Figure 1). Fungal diversity was also significantly different between skin types (Shannon, $P < 0.0001$), with the mucosal sites (including conjunctiva, nostril and reproductive tract sites) being significantly less diverse than oral, sebaceous (chin) and haired sites (Figure 1).

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, whereas the Bray Curtis and Pearson estimators further account for differences in amounts of fungal taxa between samples. The results of performing ANOSIM on the distance matrices generated by all three metrics produced comparable results, as demonstrated in

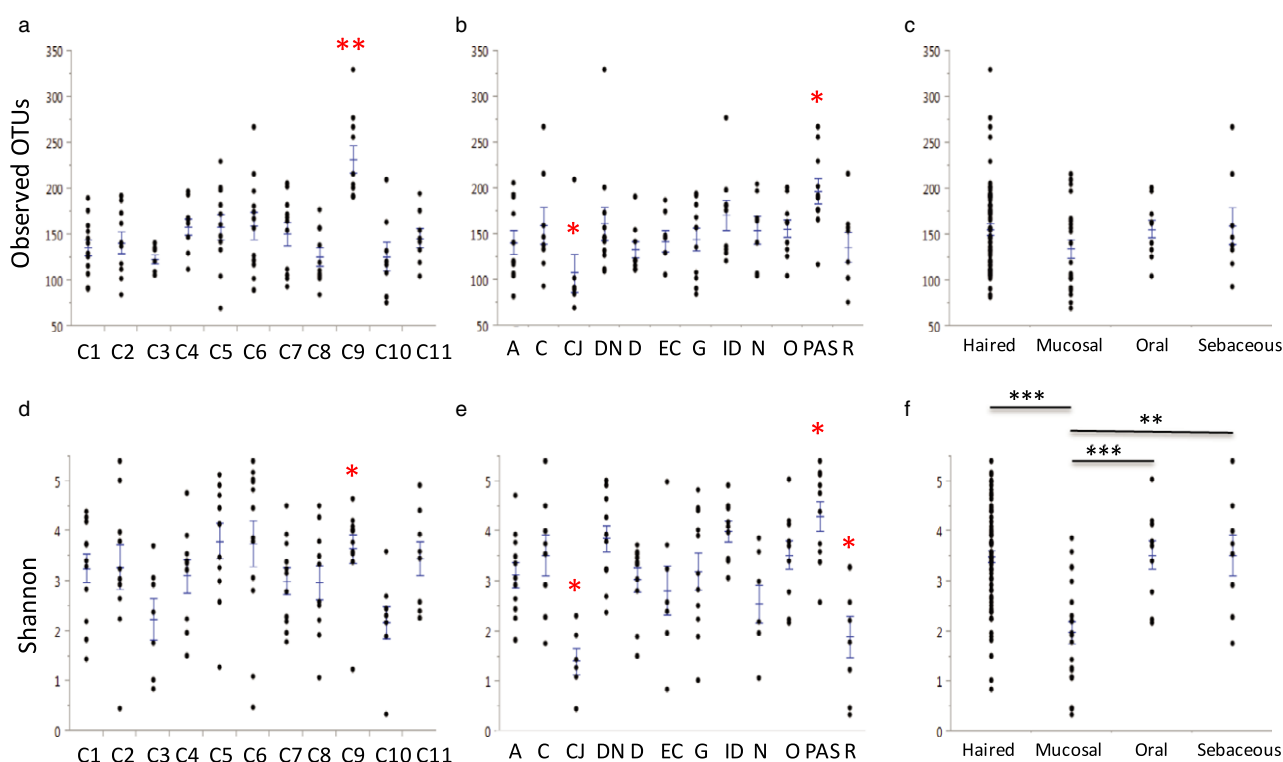


Figure 1. Alpha diversity of healthy cats. (a–c) Alpha diversity estimated with observed OTU's and samples grouped by (a) cat, (b) body site and (c) skin type. (d–f) Alpha diversity estimated with Shannon diversity metric and samples grouped by (d) cat, (e) body site and (f) skin type. Means and mean 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–Dwass 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. Haired (axilla, dorsal nose, dorsum, ear canal, groin, interdigital space, preaural space), mucosal (conjunctiva, nostril, prepuce and vulva), oral (cavity) and sebaceous (chin).

Figure 2. The R statistic indicated the effect that 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 was the factor). An R value of one would indicate that the factor has a very strong influence on the presence and/or abundance of mycobiota. Some clustering of healthy cat samples ($n = 108$) by cat was 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 2; 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 (Table S2). Clustering was less apparent by skin type (Figure 2; ANOSIM global $R = 0.208$; $P = 0.002$) and absent by body site (Figure 2; ANOSIM global $R = 0.083$; $P = 0.001$).

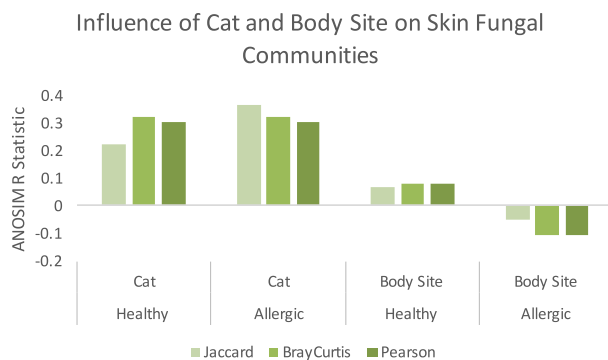
Skin fungal taxonomic composition of healthy cats

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

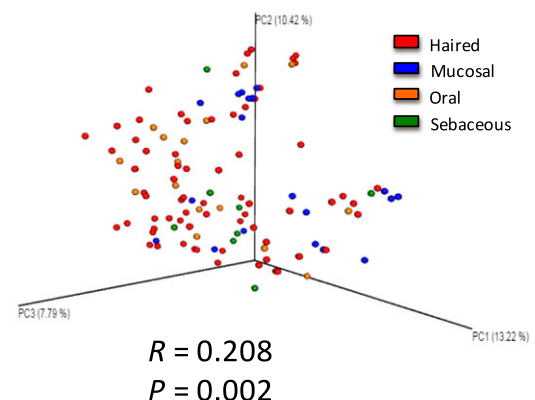
this class were *Cladosporium*, *Alternaria* and *Epicoccum* (Figure 3). There was also a remarkable proportion of fungal sequences (21%) that were classified within the Ascomycota phylum but could not be classified further (Figures 3 and 4; Other Ascomycota). The most abundant genus within the Basidiomycota 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 4. *Malassezia* was sequenced from 30% of healthy cat samples ($n = 35$) but was present at greater than 1% relative abundance in only 5% ($n = 6$) of samples (Figure S1). The median relative abundance of unassigned sequences was 6%; however, there were several samples that had greater than 50% unassigned sequences. Due to the fact that fungal databases are still undergoing curation, these sequences may be assigned to fungal taxa in future studies.

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 abundances of 53 taxa were significantly different between cats (Table S3; FDR adjusted $P < 0.05$); only two taxa were significantly different between body

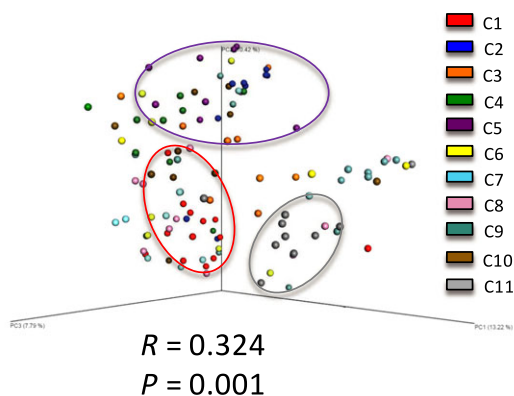
a Comparison of beta diversity metrics



b Healthy sites by skin type



c Healthy sites by cat



d Healthy sites by body site

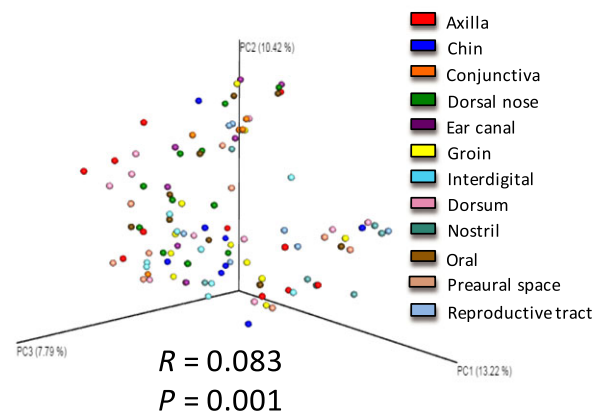


Figure 2. Beta diversity of healthy cats. (a) Comparison of ANOSIM global R statistic between three metrics, Jaccard, Bray Curtis and Pearson, for the factors of cat and body site in both health status groups. (b–d) PCoA plot of Bray Curtis pairwise distances for healthy cat samples, with associated ANOSIM global R statistic and P -value; coloured by (b) skin type, (c) cat and (d) body site. Haired (axilla, dorsal nose, dorsum, ear canal, groin, interdigital space, preaural space), mucosal (conjunctiva, nostril, prepuce and vulva), oral (cavity) and sebaceous (chin).

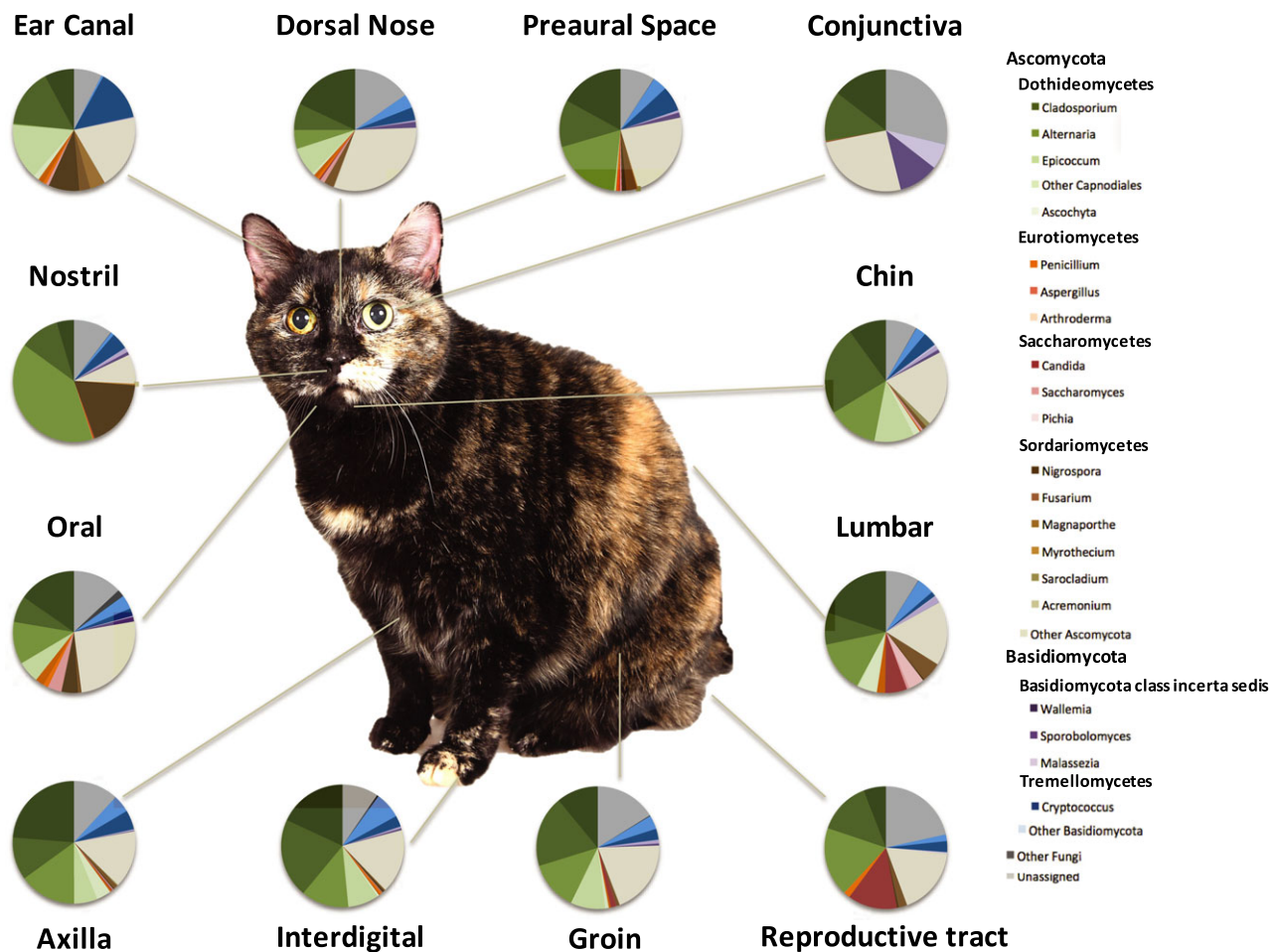


Figure 3. Fungal taxonomic composition of healthy cat body sites. The relative abundances of the most common taxa were averaged by body site and are represented by pie charts.

sites and eight taxa were different between skin type. 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 (Table S3). 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 reported in Table S4. No significant differences in fungal richness or diversity between allergic cats, nor between allergic body sites (Figure S2) were identified with Kruskal–Wallis tests. Similar to healthy cats, allergic cats possessed reduced fungal diversity at mucosal sites (conjunctiva, nostril and reproductive; Figure S2; Kruskal–Wallis, $P < 0.05$). No differences in fungal richness nor diversity were identified between allergic cats that had received or were currently receiving steroids and allergic cats that had never received steroids (Figure S2).

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 type and steroid

usage. PCoA plots revealed some clustering of sites by cat (Figure S3; 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 was visually apparent in the PCoA plot of Bray Curtis pairwise distances between allergic cat samples. Skin type did not have a major effect on differences in beta diversity between allergic samples (Figure S3; 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 (Table S2). 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 3 and 4). 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 (Figure S1).

Kruskal–Wallis tests identified six taxa that were differentially abundant between allergic cats, but no taxa were identified as significantly different between body sites (Table S5). Two of the genera that were significantly different between cats were *Arthroderma* (sexual stage of *Microsporum*, causative agent for dermatophytosis) and *Fusarium* (Figure S4). *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 (Figure S5; LDA score of 5). LEfSe analysis also showed that an unclassified *Tremellales* genus, phylum basidiomycete, was more abundant on the dorsum of allergic cats than on other body sites of allergic cats (Figure S5; 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 (Figure S6 and Table S6) and neither were the estimated beta diversities

influenced by health status overall (Table S7). 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 5).

The Kruskal–Wallis tests revealed that nine taxa were significantly different between groups including the genus *Epicoccum* and nonclassified Capnodiales order (Table S8), which were also identified as significantly more abundant in the healthy group by LEfSe analysis (Figure 6; LDA score of 4 to 5). The classes Agaricomycetes and Sordariomycetes were also identified as significantly different between groups (Table S8) and LEfSe analysis showed these classes to be significantly more abundant in the allergic group (Figure 6; LDA score of -3 to -4). Figure 3 visually demonstrates differences in averages of fungal taxa between healthy and allergic groups at each of the six sites common to the two groups.

Discussion

This study has demonstrated that fungi colonizing the skin of cats tend to be similar across the entire body of

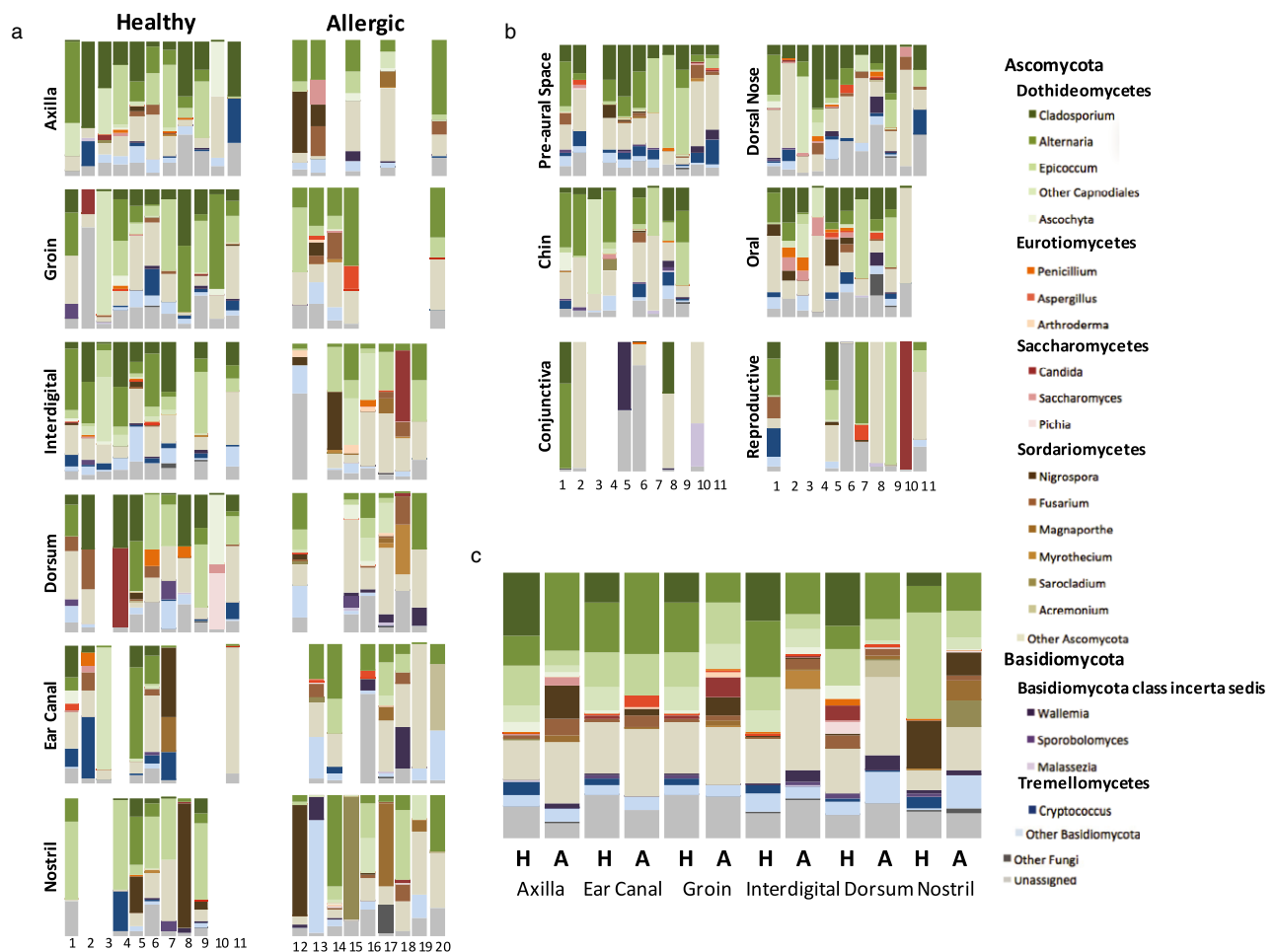


Figure 4. Fungal taxonomic composition of healthy and allergic feline skin. (a–b) Relative abundance of fungal taxa are presented for each sample and coloured by fungal genus. (c) Comparison of most abundant fungal taxa between healthy and allergic skin, averaged for each of six sites.

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.

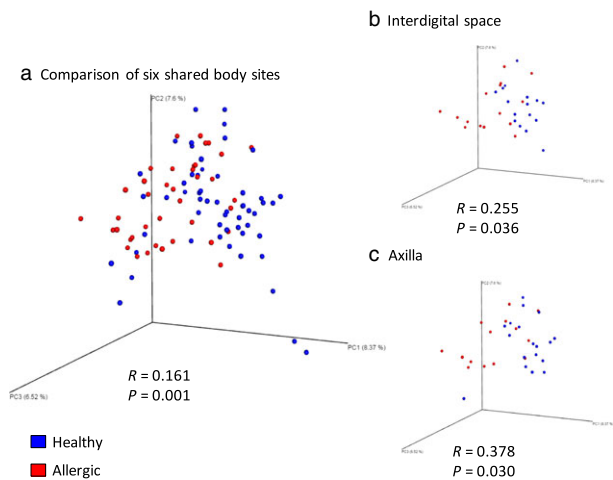
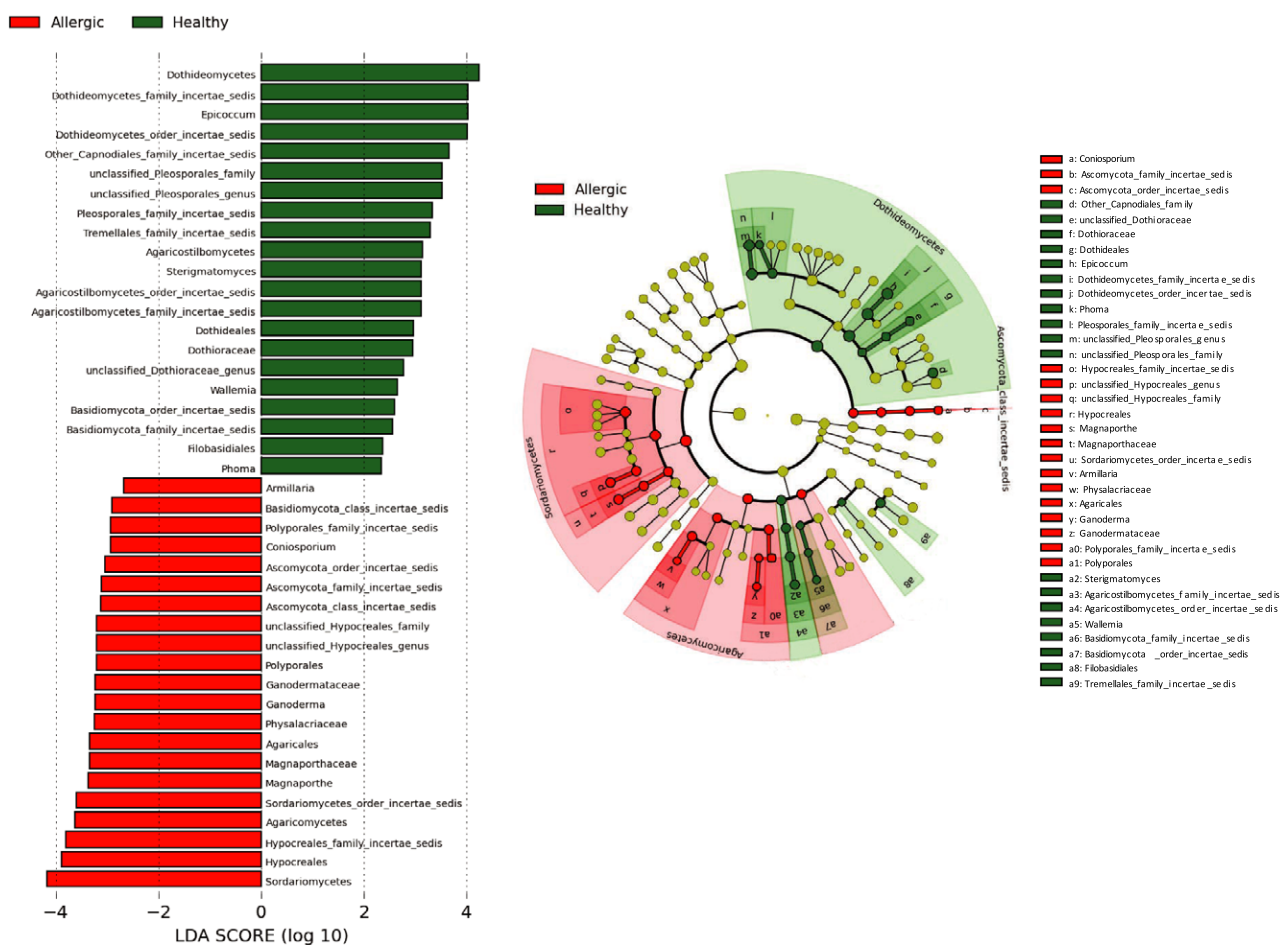


Figure 5. Comparison of beta diversity between healthy and allergic skin. PCoA plots of Jaccard pairwise distances for healthy and allergic feline skin samples, with associated ANOSIM global *R* statistic and associated *P*-value for (a) six sites, (b) only the interdigital spaces and (c) only the axillae. Coloured by health status.

This study also identified reduced diversity at mucosal sites and a predominance of Dothideomycetes (*Cladosporium*, *Alternaria* and *Epicrocorm*) similar to what has been reported for canine skin.⁶ Although it is not possible to compare the results of two NGS studies quantitatively, the qualitative diversity of fungi sequenced from feline skin appears to be comparable to that of canine skin⁶ and much more diverse than what has been found on the human body (with the exception of pedal sites⁴). A previous study suggested that outdoor exposure might explain the predominance of environmental fungi sequenced from the skin of dogs;⁶ however, the same taxa of fungi were also abundant on these cats, many of which (13 of 20) were housed strictly indoors. Further studies are warranted to evaluate how outdoor exposure might influence the carriage of fungi on the skin of companion animals.

Aside from the influences on diversity of fungi inhabiting the skin of people and animals, many questions remain 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. 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, the clinical history of this case suggests the possibility that dermatophytosis could have a long-standing effect on the skin mycobiota across the entire cat. This finding also raises continued concern regarding a potential carrier state for dermatophytosis in cats²⁹ 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 cutaneous fungal microbiota and a carrier state following resolution of lesions. Interestingly, this cat (C18) also had a significant increase in *Fusarium* DNA across all of its body sites, compared to other cats. The potential relationship between colonization of *Fusarium* and *Arthroderma* may be of interest for future studies.

Malassezia has been implicated as a significant allergen in human and canine AD,^{30–32} whereas it has yet to be associated with feline HD. Several studies have cultured *Malassezia* spp. from the skin of healthy cats^{9,33} and cats with otitis.^{8,10,11,14} In one of these studies, *Malassezia* was cultured from approximately 40% of healthy cats.⁸ 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 also have 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.³³ Another study identified an overgrowth of *Malassezia* spp. from the skin of allergic cats using cytological examination of tape strips.¹³ 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 groups. A previous NGS study of healthy and allergic canine skin also reported an unexpectedly low abundance of *Malassezia*.⁶ 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 lesions. Lesion distributions varied amongst study participants, but in accordance with typical cutaneous reaction patterns associated with these types of HD. However, 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,^{6,18} and fungal richness and diversity have differed between species (increased diversity in human AD patients and reduced richness in allergic dogs). Unlike in dogs with allergic dermatitis,⁶ there was not an overall reduction in fungal diversity in the allergic cat group. Some factors that might explain this finding include the differences in distribution and phenotypic presentation of

lesions between canine AD and NFNFIHD in cats,³⁴ or differences in immune regulation of the skin in these two species. Another possible explanation could be a lack of skin barrier impairment in allergic cats, as is often described in atopic dogs³⁵ and people.³⁶ There have yet to be any studies to provide evidence for or against impairment of the skin barrier in allergic cats, nor have there been any studies comparing transepidermal water loss between healthy and allergic cats.

A complex dialogue between skin microbiota and host immune systems is known to occur.^{37,38} For instance, the host commensal microbiota is capable of inducing expression of antimicrobial peptides,³⁹ which can then alter or modulate the presence and abundance of certain skin microbes. There is still debate as to whether the microbial dysbiosis observed in inflammatory skin disorders is a cause or 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 dialogue 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 a predominance of environmental fungi such as *Cladosporium* and *Alternaria*. These findings correlate well with what has been shown through culture-dependent studies of feline skin^{7,8,12,15,16} and NGS studies of canine skin.⁶ Further studies with larger numbers of animals are needed to confirm the present findings, and to evaluate the role of the environment on the skin microbiota. 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.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Figure S1. 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.

Figure S2. Alpha diversity of allergic cats. Alpha diversity estimated with Shannon diversity metric and samples grouped by (a) cat, (b) body site, (c) skin type and (d) steroid 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–Dwass multiple comparisons test, of * <0.05 , ** <0.01 , *** <0.001).

Figure S3. 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; coloured by (a) cat, (b) body site, (c) steroids usage and (d) skin type.

Figure S4. 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.

Figure S5. 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 plots showing the LDA score and as a cladogram demonstrating the phylogenetic relationships. Taxa are coloured according to cat or body site in which they are increased in abundance.

Figure S6. 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.

Table S1. Alpha diversity median for healthy cats. The median alpha diversity was calculated for each body site, cat and skin type in the group of healthy cats using the three metrics: Chao1, observed OTUs and Shannon.

Table S2. Average *R* statistic and range of *P*-values for significant pairwise comparisons. The average *R* statistic and *P*-values were reported for only the significant ($P < 0.05$) pairwise comparisons between cats and between skin type groups. All *P*-values were adjusted for multiple comparisons.

Table S3. Fungal taxa from filtered relative abundance table for healthy cat samples. The average relative abundances of fungal taxa sequenced from healthy cats are reported. Results from testing that the relative abundance of each taxon was significantly different for at least one body site or cat are included on respective rows.

Table S4. Alpha diversity median for allergic cats. The median alpha diversity was calculated for each body site, cat, skin type and steroid usage in the group of allergic cats using the three metrics: Chao1, observed OTUs and Shannon.

Table S5. Fungal taxa from filtered relative abundance table for allergic cat samples. The average relative abundances of fungal taxa sequenced from allergic cats are reported. Results from testing that the relative abundance of each taxon was significantly different for at least one body site or cat are included on respective rows.

Table S6. Alpha diversity median for health status. The median alpha diversity was calculated for health status group using the three metrics: Chao1, observed OTUs and Shannon.

Table S7. Global *R* statistics for beta diversity analysis for healthy and allergic cats. Beta diversity of samples was calculated using Jaccard, Bray Curtis and Pearson metrics. ANOSIM was performed on all three metrics to determine significant differences in fungal communities between healthy and allergic cats using the factors Cat, Body Site and Skin Type. The global *R* value is representative of all members within a factor and is distinct from previously reported pairwise comparisons.

Table S8. Fungal taxa from filtered relative abundance table for shared skin sites for healthy and allergic cats. The average relative abundances of fungal taxa sequenced from only the sites that were sampled in both healthy and allergic cats are reported. Results from testing that the relative abundance of each taxon was significantly different for at least one body site or cat are included on respective rows.

Résumé

Contexte – Les études de séquençage de dernière génération (NGS) ont mis en évidence des microbiotes cutanés variés et une dysbiose microbienne associée à la dermatite atopique de l'homme et du chien. La peau des chats doit encore être étudiée par des techniques NGS.

Hypothèses/Objectifs – Nous supposons que le microbiote fongique de peau féline saine pourrait être identique à celle du chien avec une prédominance de champignons environnementaux et que la dysbiose fongique pourrait être présente sur la peau des chats allergiques.

Sujets – Onze chats sains et neuf chats diagnostiqués avec un ou plus d'une hypersensibilité, y compris l'hypersensibilité aux piqûres de puces, l'hypersensibilité liée à l'alimentation et non liée à l'alimentation et non liée aux puces.

Méthodes – Les chats sains ont été prélevés sur douze sites corporels et les chats allergiques à six sites. L'ADN a été isolé et un séquençage Illumina a été réalisé ciblant la région de l'espace interne transcrit des champignons. Les séquences ont été réalisées par le logiciel bioinformatique QIIME.

Résultats – Les séquences fongiques les plus abondantes de la peau de tous les chats ont été identifiées comme *Cladosporium* et *Alternaria*. Les muqueuses, comme les narines, les conjonctives et le tractus urinaire, présentaient le plus faible nombre de champignons tandis que l'espace pré-auriculaire en présentait le plus. La peau allergique féline avait significativement plus de quantité d'Agaricomycètes et de Sordariomycètes et significativement moins d'*Epicoccum* comparé à la peau saine.

Conclusions – La peau des chats sains semble avoir un microbiote fongique plus diversifié comparé aux études antérieures et une dysbiose fongique est notée sur la peau des chats allergiques. De futures études évaluant la stabilité du microbiote cutané des chats seront utiles pour déterminer si les microbiotes séquencés par NGS sont des colonisateurs ou des microbes transitoires.

Resumen

Introducción – los estudios de secuenciación de próxima generación (NGS) han demostrado una diversa microbiota asociada a la piel y disbiosis microbiana asociada con la dermatitis atópica en las personas y en los perros. La piel de los gatos aún no se ha investigado con el uso de técnicas de NGS.

Hipótesis/Objetivos – Nuestra hipótesis era que la microbiota fúngica de la piel sana felina sería similar a la de los perros, con un predominio de hongos del medio ambiente, y que existiría disbiosis de hongos en la piel de gatos alérgicos.

Animales – Once gatos sanos y nueve gatos diagnosticados con uno o más trastornos de hipersensibilidad cutánea, incluyendo picadura de pulgas e hipersensibilidad no inducida ni por alimentos ni por pulgas.

Métodos – Muestras de gatos sanos se obtuvieron en doce sitios del cuerpo y de seis en gatos alérgicos. Se aisló el DNA y la secuenciación se realizó con Illumina dirigida a la región espaciadora transcrita interna de hongos. Las secuencias fueron procesadas con el software de bioinformática QIIME.

Resultados – Las secuencias fúngicas más abundantes de la piel de todos los gatos se clasificaron como *Cladosporium* y *Alternaria*. Los sitios de mucosas, incluidas las fosas nasales, la conjuntiva y tracto reproductivo, tuvieron menor número de hongos, mientras que el espacio pre-auricular tuvo la mayoría. La piel de gatos alérgicos tuvo cantidades significativamente mayores de *Agaricomycetes* y *Sordariomycetes*, y significativamente menores de *Epicoccum* en comparación con la piel sana felina.

Conclusiones – La piel de gatos sanos parece tener una microbiota fúngica más diversa en comparación con estudios anteriores, y se observa una disbiosis fúngica en la piel de los gatos alérgicos. Serán útiles más estudio evaluando la estabilidad temporal de la microbiota de la piel en gatos para determinar de si la microbiota secuenciada utilizando NGS son colonizadores o microbios transitorios.

Zusammenfassung

Hintergrund – Die „Next Generation Sequencing“ (NGS) Studien haben eine vielfältige Haut-bezogene Biozönose und eine mikrobielle Dysbiose im Zusammenhang mit atopischer Dermatitis bei Menschen und Hunden gezeigt. Die Haut von Katzen muss noch mittels NGS Techniken untersucht werden.

Hypothese/Ziele – Wir hypothesierten, dass die Biozönose der Pilze auf der gesunden Haut der Katzen ähnlich wie die der Hunde sein würde, mit einer Dominanz der Umweltpilze und dass eine fungale Dysbiose auf der Haut allergischer Katzen bestehen würde.

Tiere – Elf gesunde Katzen und neun Katzen, die mit einer oder mehreren kutanen Hypersensibilitäten, wie Flohstichallergie, Futter-induzierter und weder durch Floh noch durch Futter induzierter Hypersensibilität diagnostiziert worden waren.

Methoden – Bei den gesunden Katzen wurde an zwölf Körperstellen und bei den allergischen Katzen an sechs Körperstellen Proben entnommen. Es wurde DNA isoliert und Illumina Sequenzierung durchgeführt, welche auf die internen transkribierten Spacerregionen der Pilze abzielte. Die Sequenzen wurden mittels Bioinformatics Software QIIME verarbeitet.

Ergebnisse – Die häufigsten Pilzsequenzen aus der Haut der Katzen wurden als *Cladosporidium* und *Alternaria* klassifiziert. Die Schleimhäute, inklusive Nase, Konjunktiva und Reproduktionstrakt hatten die geringste Zahl an Pilzen, während prä-aural die meisten auftraten. Allergische Katzenhaut wies im Vergleich zur gesunden Katzenhaut signifikant mehr *Agaricomycetes* und *Sordariomycetes* und signifikant weniger *Epicoccum* auf.

Schlussfolgerungen – Die Haut von gesunden Katzen scheint eine vielfältigere Pilzbiozönose aufzuweisen als in früheren Studien beschrieben wurde und eine Dysbiose der Pilze wird auf der Haut von allergischen Katzen gesehen. Zukünftige Studien, die die temporale Stabilität der Mikrobiota der Haut von Katzen erfassen werden nützlich sein, um festzustellen ob die mittels NGS sequenzierten Mikrobiota Kolonien bilden oder nur vorübergehende Mikroben darstellen.

要約

背景 – 次世代シーケンシング(NGS)を用いた研究によって、皮膚微生物叢の多様性や、人や犬のアトピー性皮膚炎に関連した微生物叢バランス失調が明らかにされてきた。しかし、猫の皮膚に関しては、NGSを用いた研究はいまだされていない。

仮説/目的 – 健康猫の真菌微生物叢は犬と同様に、環境性の真菌が大部分を占めており、また、アレルギー猫では真菌叢バランス失調が認められると仮説を立てた。

供与動物 – 健康猫11頭および1つ以上の皮膚過敏症を持つ猫9頭。皮膚過敏症には、咬傷過敏症、食物誘発性過敏症、非食物誘発性過敏症が含まれる。

方法 – 健康猫からは12箇所、アレルギー猫からは6箇所の体部位よりサンプルを採取した。DNAを抽出し、真菌の内部転写スペーサー(internal transcribed spacer)領域を標的としたIlluminaシーケンシングを実施した。シーケンス解析にはバイオインフォマティクスのQIIMEソフトウェアを用いた。

結果 – すべての猫の皮膚において、*Cladosporium*および*Alternaria*に属する真菌が最も豊富に認められた。鼻孔、結膜、生殖器系含む粘膜部位では真菌数は最も少なく、一方で、耳介前部では最も多く認められた。アレルギー猫の皮膚では健康猫と比べて、*Agaricomycetes*および*Sordariomycetes*が有意に多く、*Epicoccus*が有意に少なかった。

結論 – 健康猫の皮膚の真菌叢は、過去の報告よりも多様性に富んでおり、アレルギー猫の皮膚における真菌叢バランス失調が認められた。今後は、猫の皮膚微生物叢の経時的な安定性を評価することによって、NGSで得られた微生物叢のシーケンス結果が生着菌なのか、一過性の微生物なのかを明らかにすることができる。

摘要

背景 — 新一代测序(NGS)研究显示皮肤存在多种微生物群,以及人和犬异位性皮炎时存在微生物菌群失调。然而猫皮肤尚未使用NGS技术研究。

假设/目的 — 我们假设猫皮肤真菌微生物群与犬相似,如果出现环境真菌优势,过敏症的猫也会存在皮肤真菌失调。

动物 — 十一只健康猫,九只诊断出一种或多种皮肤过敏症的患猫,包括跳蚤叮咬、食物过敏和非跳蚤叮咬非食物过敏的过敏症。

方法 — 从健康猫身体十二个位点取样,过敏症患猫的六个位点取样。针对真菌内部转录间隔区进行DNA分离和Illumina测序。序列使用生物信息学软件QIIME处理。

结果 — 所有猫身上最丰富的真菌序列为枝孢菌和链格孢霉。粘膜位点,包括鼻孔、结膜和生殖道真菌最少,耳道前部最多。过敏症患猫与健康猫比较伞菌属和粪壳菌属显著增多,附球菌属显著降低。

总结与临床意义 — 本次研究中健康猫皮肤与之前的研究相比,有着更丰富的真菌微生物群,过敏症患猫存在真菌失调。未来研究评估皮肤暂居微生物群的稳定性,将有助于判断使用NGS测出的真菌是定殖还是临时的。

Resumo

Contexto — Estudos de sequenciamento de nova geração (SNG) tem demonstrado diversa microbiota associada a pele e disbiose microbiana associada à dermatite atópica em humanos e cães. A pele de gatos ainda não foi investigada por SNG.

Hipóteses/Objetivos — A nossa hipótese foi de que a microbiota fúngica de pele felinos saudáveis seria similar àquela de cães, com predominância de fungos ambientais, e que disbiose fúngica estaria presente na pele de gatos alérgicos.

Animais — Onze gatos saudáveis e nove gatos diagnosticados com uma ou mais distúrbios de hipersensibilidade, incluindo picada de pulgas, induzida por alimentos e hipersensibilidade não induzida por pulgas e alimentos.

Métodos — Os gatos saudáveis foram amostrados em 12 áreas corpóreas e os gatos alérgicos em seis locais. O DNA foi isolado e sequenciamento por Illumina foi realizado tendo como alvo a região interna transcrita de espaçamento do fungo. As sequências foram processadas utilizando o *software* de bioinformática QIIME.

Resultados — As sequências de fungo mais abundantes da pele de todos os gatos foram classificadas como *Cladosporium* e *Alternaria*. As regiões mucosas, incluindo as narinas, conjuntivas e trato reprodutivo, apresentaram o menor número de fungos, enquanto a região pré-auricular apresentou o maior. A pele de felinos alérgicos apresentou quantidades significativamente maiores de Agaricomycetes e Sordariomycetes, e significativamente menos *Epicoccum* comparado com a pele do felino saudável.

Conclusões — A pele de gatos saudáveis apresentou aparentemente menor diversidade da microbiota fúngica comparada a estudos anteriores, e a disbiose fúngica foi notada na pele de gatos alérgicos. Estudos futuros avaliando a estabilidade ao longo do tempo da microbiota cutânea de gatos será útil para determinar se a microbiota sequenciada por SNG é residente ou temporária.