

# Longitudinal Evaluation of the Skin Microbiome and Association with Microenvironment and Treatment in Canine Atopic Dermatitis

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Host-microbe interactions may play a fundamental role in the pathogenesis of atopic dermatitis, a chronic relapsing inflammatory skin disorder characterized by universal colonization with *Staphylococcus* species. To examine the relationship between epidermal barrier function and the cutaneous microbiota in atopic dermatitis, this study used a spontaneous model of canine atopic dermatitis. In a cohort of 14 dogs with canine atopic dermatitis, the skin microbiota were longitudinally evaluated with parallel assessment of skin barrier function at disease flare, during antimicrobial therapy, and post-therapy. Sequencing of the bacterial 16S ribosomal RNA gene showed decreased bacterial diversity and increased proportions of *Staphylococcus* (*S. pseudintermedius* in particular) and *Corynebacterium* species compared with a cohort of healthy control dogs (n = 16). Treatment restored bacterial diversity with decreased proportions of *Staphylococcus* species, concurrent with decreased canine atopic dermatitis severity. Skin barrier function, as measured by corneometry, pH, and transepidermal water loss also normalized with treatment. Bacterial diversity correlated with transepidermal water loss and pH level but not with corneometry results. These findings provide insights into the relationship between the cutaneous microbiome and skin barrier function in atopic dermatitis, show the impact of antimicrobial therapy on the skin microbiome, and highlight the utility of canine atopic dermatitis as a spontaneous nonrodent model of atopic dermatitis.

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## INTRODUCTION

Atopic dermatitis (AD) is a chronic inflammatory skin disorder that affects approximately 10% of children (Spergel, 2010) and is commonly associated with *Staphylococcus aureus* colonization (Leyden et al., 1974). Genetic risk conferred by mutations in the gene encoding the epidermal barrier protein filaggrin suggests that barrier dysfunction in part contributes to the disease (O'Regan et al., 2009). Environmental factors, including staphylococcal colonization and infection, may also contribute to disease etiology and/or severity. Recent studies have highlighted the dysbiotic nature of the AD skin

microbiome, including a predominance of *S. aureus* during active flares, suggesting a role for *S. aureus* and the skin microbiome in atopic inflammation (Kong et al., 2012).

Mouse models have highlighted the effects of specific genetic changes in AD, but they are limited in their clinical similarity and do not recapitulate the complexity of the human disease (Scharschmidt and Segre, 2008; Marsella and Girolomoni, 2009). Canine atopic dermatitis (cAD) occurs spontaneously and exhibits similar immunological and clinical features of human AD, therefore providing a useful intermediate model (Marsella and Girolomoni, 2009). cAD affects approximately 10% of dogs and presents with similar lesion distribution, life stage of onset, IgE-specific immune responses, and predisposition to chronic and recurrent superficial bacterial dermatitis and folliculitis (Hillier and Griffin, 2001; Santoro et al., 2015). Epidermal barrier function is impaired in cAD, suggesting a route for epicutaneous sensitization (Santoro et al., 2015). The pathogenesis of cAD is multifactorial, and complex interactions between genetics and environment are hypothesized, as in human AD (Bizikova et al., 2015).

Flare states in atopic humans and dogs are associated with colonization and/or superficial infection by *Staphylococcus* species: *S. aureus* in humans and *S. pseudintermedius* or *S. schleiferi* in dogs (Fazakerley et al., 2009; Furiani et al., 2011; Kong et al., 2012; Leyden et al., 1974; Santoro et al., 2015). Recent studies in an *Adam17*-deficient mouse model

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Abbreviations: AD, atopic dermatitis; ANOSIM, analysis of similarity; cAD, canine atopic dermatitis; DNA, deoxyribonucleic acid; rRNA, ribosomal ribonucleic acid; TEWL, transepidermal water loss

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**Table 1. Signalment data of study cohorts**

Characteristic	cAD	Control
n	14	16
Median age, mo (range)	71.0 (10–120)	79.5 (8–144)
Ratio male:female	5:9	1:1
Spayed/neutered	13	15
Average cumulative lesion score		
Visit 1	22.00	0
Visit 2	14.92	0
Visit 3	15.93	0

suggest that *S. aureus* drives lesion formation (Kobayashi et al., 2015). Toxins produced by *S. aureus* are hypothesized to trigger or exacerbate inflammation in AD (Williams and Gallo, 2015), such as the  $\delta$ -toxin recently shown to induce mast cell degranulation and promote inflammatory skin disease (Nakamura et al., 2013). Further understanding of host-microbe dynamics during flare, treatment, and resolution is critical for improved therapies to manage atopic inflammation.

Herein we report an integrated analysis of the canine cutaneous microbiome and the skin barrier in cAD. The skin microbiome was defined using culture-independent sequencing of the 16S ribosomal RNA (rRNA) gene before, during, and after antimicrobial treatment. In parallel, quantitative assessment of the skin barrier was measured using transepidermal water loss (TEWL), epidermal moisture content (corneometry), and pH. The results of this study should inform future studies of the functional relationship between host cutaneous barrier function and the skin microbiome of humans and dogs.

## RESULTS

### Summary of study participants and design

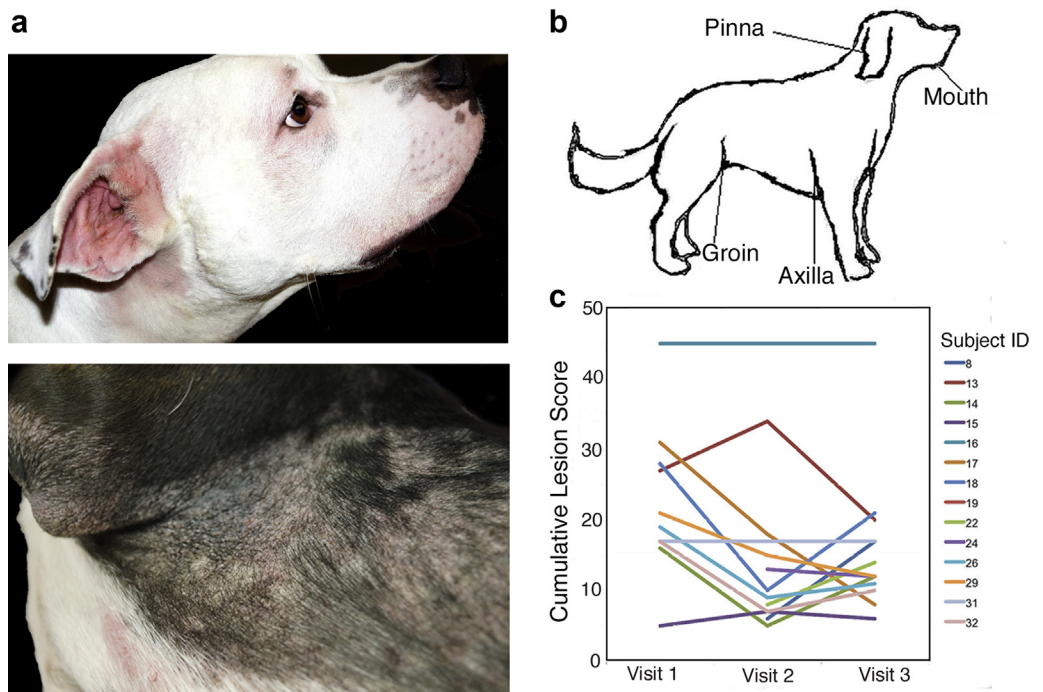
Thirty-two dogs (n = 15 affected by cAD, n = 17 unaffected) were enrolled from September 3, 2013 to March 7, 2014 at the University of Pennsylvania Matthew J. Ryan Veterinary Hospital (Table 1, see Supplementary Table S1 online). One dog in each cohort was excluded because of unrelated medical problems. All cAD subjects had active lesions of superficial bacterial dermatitis and folliculitis at enrollment (Figure 1a).

The skin was swabbed to sample microbiota at anatomic sites with a predilection for cAD lesions: the pinna, axilla, and groin (Figure 1b). The mouth was also sampled, because licking of the skin is a manifestation of pruritus in dogs. Assessment and sampling occurred at three study visits: visit 1 at initial presentation with a flare of cAD and concurrent bacterial dermatitis, visit 2 at the conclusion of 4–6 weeks of culture- and susceptibility-directed oral antimicrobial therapy, and visit 3 at 4–6 weeks after the conclusion of antimicrobial therapy. Healthy dogs were assessed and sampled contemporaneously.

Site-specific (pinna, axilla, and groin) assessment and semiquantitative scoring of lesion severity were performed based on the parameters used for the Canine Atopic Dermatitis Extent and Severity Index (Olivry et al., 2007). This scoring was performed to assess the relationship between microbiota, skin barrier, and clinical signs at a given site. Cumulative site-specific lesion scores varied widely in dogs with cAD (range = 5–45) (Figure 1c). The components of the site-specific lesion scores—erythema, lichenification, and alopecia—were strongly positively correlated with each other (see Supplementary Table S2 online). Cumulative lesion scores decreased with antimicrobial treatment from median ( $\pm$  standard deviation) of 19 ( $\pm$ 12.1) at visit 1 to a median of

### Figure 1. Canine atopic dermatitis.

(a) Typical clinical findings of cAD include alopecia and erythema of the periocular region and muzzle as well as evidence of chronic dermatitis and folliculitis in regions such as the pinna, axilla, and groin. (b) The anatomic sites sampled for microbiomic analysis included the mouth, axilla, groin, and concave pinna. (c) Lesion scores (x-axis) measuring cumulative site-specific lesion severity at each study visit (y-axis). Each line represents one subject with cAD (n = 14). ID, identification.

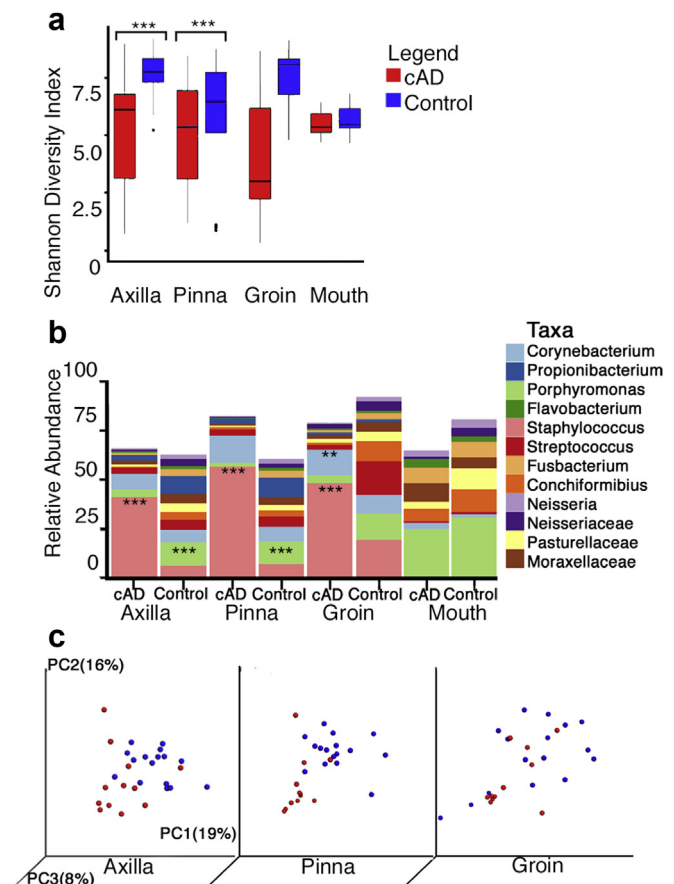


10 ( $\pm 13.1$ ) at visit 2, although this decrease was not significant ( $P = 0.56$ ). Subject 16 was identified as a minor outlier, with a cumulative lesion score of 45 and a severe phenotype of chronic cAD in which 4 weeks of therapy was unlikely to alter the degree of chronic dermatitis and scarring. Upon exclusion of this subject from this particular analysis, the median cumulative lesion score significantly dropped with treatment, from 18 ( $\pm 7.5$ ) at visit 1 to 9.5 ( $\pm 8.1$ ) at visit 2 ( $P = 0.035$ ) (Table 1 and Figure 1c, see Supplementary Table S1). This minor outlier was not removed from any of the subsequent analyses. Five of 14 subjects had an elevated cumulative lesion score at visit 3 compared with visit 2, corresponding to recrudescence of bacterial dermatitis in the posttreatment interval, but this change was not significant across the entire cohort ( $P = 0.26$ ).

### The microbiome of cAD skin differs from that of normal canine skin

To analyze microbial communities in cAD and control dogs, the V1–V3 region of the 16S rRNA gene was amplified and sequenced from skin swabs. The skin microbiome of cAD and unaffected control dogs differed at visit 1, before treatment. The Shannon Diversity Index score, an alpha diversity metric that takes into account the number of taxa present and their abundance in the community, was significantly lower in the cAD group compared with the control group ( $P = 0.0001$ ; Figure 2a, see Supplementary Figure S1 online). These differences were significant at the pinna and axilla but not at the groin or mouth (pinna,  $P = 5.0 \times 10^{-4}$ ; axilla,  $P = 0.006$ ; groin,  $P = 0.075$ ; mouth,  $P = 0.363$ ; Figure 2a) and reiterated with numerous other alpha diversity metrics (see Supplementary Figure S1).

The predominant bacteria on healthy canine skin were *Porphyromonas*, *Staphylococcus*, *Streptococcus*, *Propionibacterium*, and *Corynebacterium* species and genera belonging to the families Neisseriaceae and Moraxellaceae (Figure 2b, see Supplementary Figure S2 online). Although the same taxa were present in cAD and control dogs, the relative abundance of taxa varied dramatically between the two groups. Dogs with cAD flares had significantly increased relative abundance of *Staphylococcus* species (cAD median =  $45 \pm 29\%$ , control median =  $5 \pm 18\%$ ,  $P < 1.0 \times 10^{-4}$ ) across all skin sites. There was a decreased relative abundance of *Porphyromonas* species in the pinna (cAD median =  $1 \pm 3\%$ , control median =  $10 \pm 6\%$ ,  $P = 1.0 \times 10^{-4}$ ) and axilla (cAD median =  $2 \pm 5\%$ , control median =  $10 \pm 7\%$ ,  $P = 1.0 \times 10^{-4}$ ). In the groin, there was a significant median increase in the relative abundance of *Corynebacterium* species in cAD (cAD median =  $9 \pm 13\%$ , control median =  $1 \pm 16\%$ ,  $P = 0.003$ ) (Figure 2b, see Supplementary Figure S2). This trend was similar in the axilla and pinna, although it did not reach significance. In the oral cavity the most abundant taxa did not differ significantly between control and cAD dogs and consisted of many anaerobes including *Porphyromonas*, *Conchiformibius*, *Fusobacterium*, unclassified Moraxellaceae, *Flavobacterium*, and unclassified Prevotellaceae species (Figure 2b, see Supplementary Figure S2). Skin microbial communities of cAD dogs were significantly different from those of control dogs as determined by the weighted UniFrac metric, a



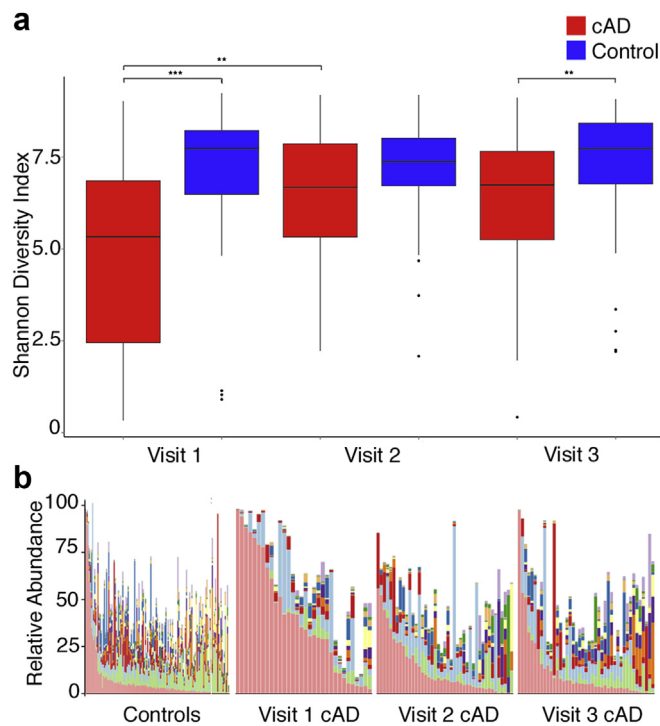
**Figure 2. The microbiome of cAD during flares and prior to treatment.**

(a) Median Shannon Diversity Index score for each anatomical site sampled in cAD dogs (red) compared with unaffected dogs (blue). Line in box represents median, boxes represent interquartile range, whiskers represent lowest and highest values within 1.5 times the interquartile range, and dots represent outliers. (b) Median taxonomic relative abundance (y-axis) of the 12 genera present in greatest abundance. cAD and control dogs are partitioned by anatomical site sampled (x-axis). (c) Principle coordinates analysis of the weighted UniFrac metric comparing cAD (red) and unaffected controls (blue) in the axilla and pinna. Clustering is significant as assessed by the analysis of similarities test (axilla:  $R = 0.403$ ,  $P = 0.001$ ; pinna:  $R = 0.639$ ,  $P = 0.001$ ). Percent variability explained by each axis is given. \*\*\* $P < 0.001$ , \*\* $P < 0.01$ . cAD, canine atopic dermatitis; PC, principle coordinate.

distance metric that takes into account shared phylogeny and is weighted for abundance of observed organisms in the community ( $R = 0.445$ ,  $P = 0.001$ ; Analysis of Similarity (ANOSIM) test). Similar differences were observed when examining each anatomical site individually and visualizing clustering using principle coordinates analysis (axilla:  $R = 0.403$ ,  $P = 0.001$ ; pinna:  $R = 0.639$ ,  $P = 0.001$ ; groin:  $R = 0.297$ ,  $P = 0.002$ ; ANOSIM test; Figure 2c). Significant clustering was not associated with patient sex, breed, or age or with antimicrobial class used during treatment (see Supplementary Figure S3 online).

### Treatment normalizes the cAD microbiome

The Shannon Diversity Index score increased across skin sites during treatment of dogs with cAD between visits 1 and 2 ( $P = 0.004$ ; Figure 3a) and approached mean Shannon Diversity Index values observed in the control group at visit 2 (control =  $7.17 \pm 1.44$ ; cAD =  $6.53 \pm 1.74$ ; Figure 3a).



**Figure 3. Skin microbiome changes during treatment of cAD.** (a) Change in Shannon Diversity Index scores longitudinally with treatment. Line in box represents median, boxes represent interquartile range, whiskers represent lowest and highest values within 1.5 times the interquartile range, and dots represent outliers. (b) Median taxonomic relative abundance. See Figure 2 for the taxonomic legend. \*\*\* $P < 0.001$ , \*\* $P < 0.01$ .

There was no longer a statistically significant difference in Shannon Diversity Index score between control and cAD dogs at visit 2 ( $P = 0.090$ ), but significant differences re-emerged at visit 3 during the posttreatment interval ( $P = 0.008$ ; Figure 3a). These findings suggest that antimicrobial therapy restores diversity of the skin microbiome in cAD but that the effects may dissipate once treatment is withdrawn or if bacterial dermatitis recurs.

To further examine the relationship between treatment, disease severity, and microbial diversity, correlations between microbial diversity and lesion scores were analyzed. The Shannon Diversity Index score was significantly inversely correlated with site-specific erythema ( $R = -0.467$ ,  $P < 1.0 \times 10^{-4}$ ), alopecia ( $R = -0.379$ ,  $P < 1.0 \times 10^{-4}$ ), lichenification ( $R = -0.454$ ,  $P < 1.0 \times 10^{-4}$ ), and total lesion scoring ( $R = -0.458$ ,  $P = 1.0 \times 10^{-4}$ ) (see Supplementary Table S2), suggesting that decreased microbial diversity is associated with lesion severity at cAD predilection sites.

At the taxonomic level, concurrent with increased diversity, relative abundance of *Staphylococcus* species decreased a median of 45% to  $6 \pm 16\%$  ( $P < 0.001$ ) across all skin sites of cAD dogs from visits 1 to 2. *Corynebacterium* species increased in cAD dogs (median =  $8 \pm 9\%$ ) compared with the control group ( $4 \pm 4\%$ ) at visit 3 ( $P = 0.007$ ) relative to control dogs (Figure 3b, see Supplementary Figure S2). Clustering by the weighted UniFrac metric also decreased with treatment when comparing dogs with cAD to control dogs ( $R = 0.0502$ ,  $P = 0.002$  at visit 3;  $R = 0.445$ ,  $P = 0.001$  at visit 1; ANOSIM test). Together, these data indicate that

antimicrobial therapy normalizes the skin microbiome of cAD.

### Skin barrier dysfunction correlates with cAD severity and skin microbiome

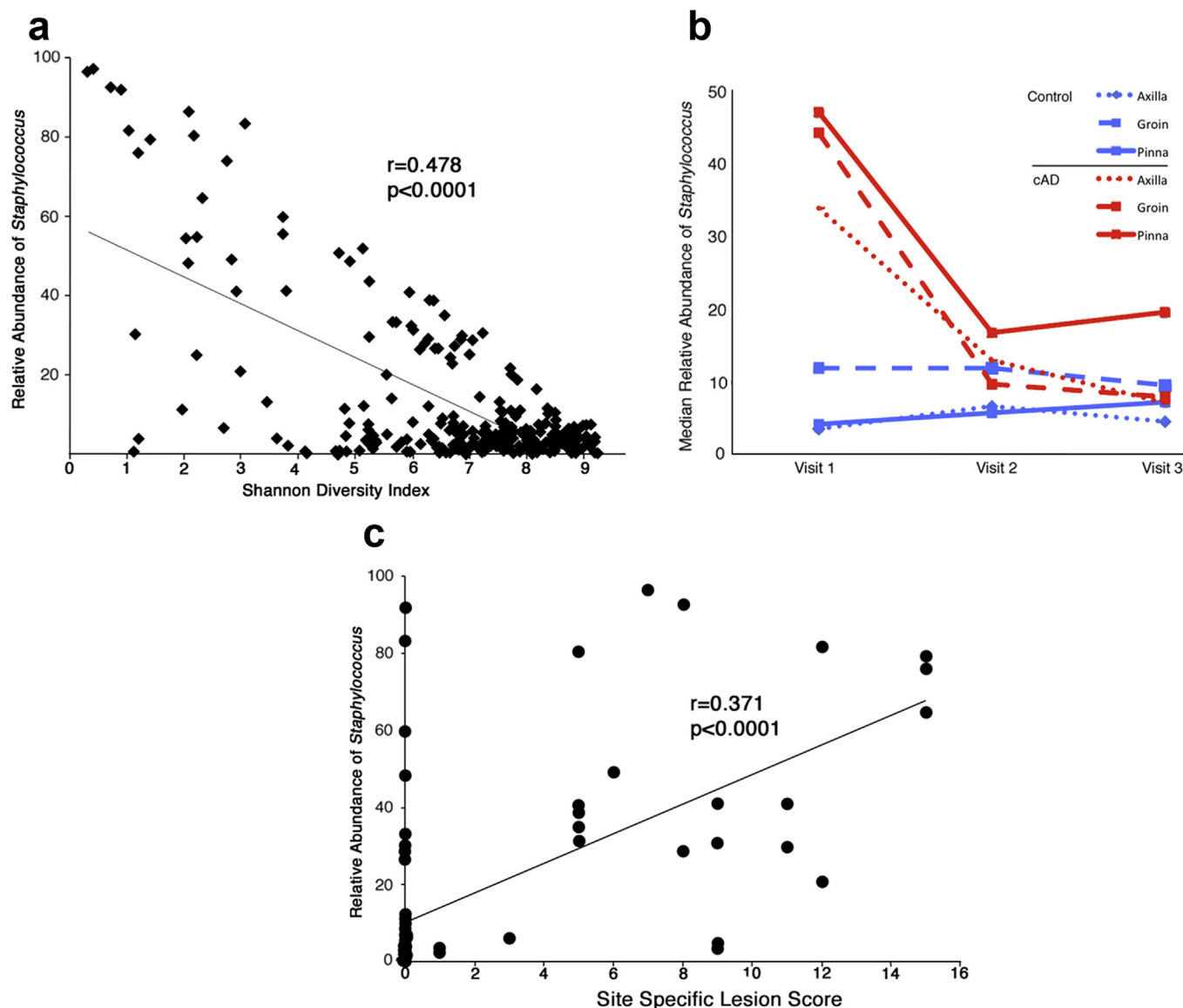
In parallel with sampling the skin microbiome, barrier function was assessed by TEWL, hydration (corneometry), and pH (see Supplementary Tables S2 and S3 and Supplementary Figure S4 online). Site-specific lesion scores were positively correlated with TEWL ( $R = 0.365$ ,  $P = 0.0001$ ), suggesting that more severe cAD is associated with impaired barrier function, and were negatively correlated with pH ( $R = -0.194$ ,  $P = 0.03$ ). Similarly, Shannon Diversity Index score was negatively correlated with TEWL ( $R = -0.249$ ,  $P = 0.006$ ) but showed a weakly positive correlation with pH ( $R = 0.18$ ,  $P = 0.05$ ). Additional alpha diversity metrics followed similar trends and were also statistically significant ( $P < 0.05$ ; see Supplementary Table S2). Skin moisture (corneometry) did not correlate with site-specific lesion scores or alpha diversity metrics. These results show that concurrent with cAD flares, alpha diversity decreases and is correlated with disease severity, TEWL, and pH.

During the treatment of cAD, TEWL and corneometry scores decreased and trended toward the distribution seen in the control group but were not statistically different between visits 1 and 2 (see Supplementary Table S3 and Supplementary Figure S4). The proportional change in lesion scores in patients with cAD correlated with the change in TEWL from visit 1 to visit 2 ( $R = 0.682$ ,  $P = 0.02$ ). There was no correlation between changes in lesion scores and TEWL between visits 1 and 3, likely because of recrudescence of bacterial dermatitis in some cAD dogs. The proportional change in corneometry did not correlate with the change in lesion scores from visit 1 to visit 2, but there was a significant correlation between these parameters at visits 1 and 3 ( $R = 0.624$ ,  $P = 0.03$ ). Skin pH did not differ significantly between dogs with cAD and healthy controls across the three visits.

### *Staphylococcus pseudintermedius* predominates in cAD

The relationships between *Staphylococcus* species, cAD severity, and treatment in the canine skin microbiome were correlated. Relative abundance of *Staphylococcus* species was significantly inversely correlated with alpha diversity ( $R = -0.686$ ,  $P < 1.0 \times 10^{-4}$ ; Figure 4a) and decreased in mean relative abundance with treatment (45%, 15%, and 18% at visits 1, 2, and 3, respectively), in contrast to control dogs in which mean relative abundance of *Staphylococcus* species remained low across skin sites at each visit (10%; Figure 4b). There was a positive correlation with the relative abundance of *Staphylococcus* species and site-specific lesion scoring ( $R = 0.609$ ,  $P < 1.0 \times 10^{-4}$ ; Figure 4c), suggesting that the amount of *Staphylococcus* species increases as disease severity increases.

Because *Staphylococcus* species in humans can be commensal (*S. epidermidis*) or potential pathogens (*S. aureus*), species identification of *Staphylococcus* was determined. One microbial culture from a lesional site and axilla in control dogs or when lesions had resolved were performed contemporaneously with microbiome swabs at each visit. Most of the isolates were identified as *S. pseudintermedius* in both dogs with cAD and healthy controls, followed by



**Figure 4. *Staphylococcus* species and cAD.** (a) Correlation between relative abundance of *Staphylococcus* species (y-axis) and Shannon Diversity Index score (x-axis). (b) Change in *Staphylococcus* species relative abundance (y-axis) longitudinally at each skin site in cAD (red lines) and control (blue lines) dogs. (c) Correlation between relative abundance of *Staphylococcus* species (y-axis) and lesion severity (x-axis) as measured by site-specific lesion score. Species-level relative abundance (y-axis) of (d) cultured *Staphylococcus* isolates and (e) 16S rRNA sequences using phylogenetic placement. cAD, canine atopic dermatitis.

*S. epidermidis* (a coagulase-negative *Staphylococcus* species) and *S. schleiferi* (a coagulase-variable *Staphylococcus* species) (Figure 4d). To speciate and compare 16S rRNA sequence data to cultures, we used a most recent common ancestor analysis of phylogenetic placement using the pplacer algorithm (Matsen et al., 2010) and a curated database of *Staphylococcus* species genomes (Conlan et al., 2012). Most of the sequences identified across all samples were attributed to *S. pseudintermedius* (59.4%), with lesser contributions from *S. aureus*, *S. epidermidis*, *S. haemolyticus*, *S. hominis*, *S. lugdunensis*, and *S. saprophyticus* (Figure 4e). *S. schleiferi* was not detected, suggesting that the region of the 16S rRNA gene used in this study may not be able to reliably classify *S. schleiferi* to the species level.

## DISCUSSION

Approximately 70 million dogs live in 40 million households across the United States (American Veterinary Medical Association, 2012), and 10% are afflicted with cAD (Hillier and Griffin, 2001). In this spontaneous large animal model of AD, alterations in skin microbiome parallel those observed in AD patients, including increased relative abundance of *Staphylococcus* spp. and decreased microbial diversity compared to healthy controls. The longitudinal dynamics of the skin microbiome of cAD during flare and treatment correlate with changes in cutaneous barrier function. This work unveils the dynamic relationship between cutaneous barrier function and the skin microbiome in mammalian health and disease states.

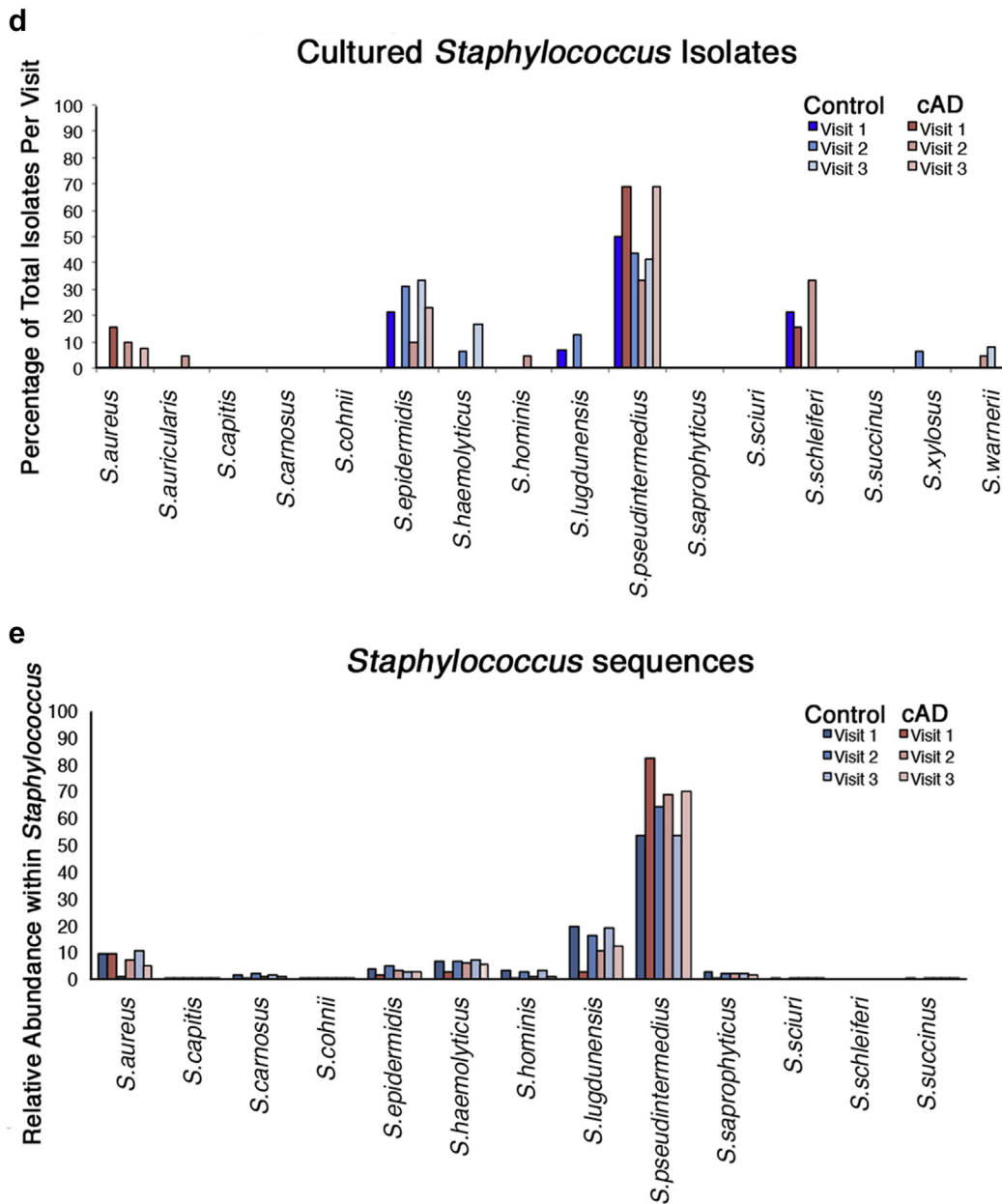


Figure 4. Continued

The cutaneous microbiome in cAD has been previously reported in a small cross-sectional study of allergic ( $n = 6$ ) and healthy dogs ( $n = 12$ ), with a similar decrease in microbial diversity, but there were no differences in the relative abundance of *Staphylococcus* organisms between the two cohorts (Rodrigues Hoffmann et al., 2014). The contrast may be due to different methodologies used, geography, and differing selection criteria (with dogs in the present study presenting with evidence of bacterial dermatitis and disease flare). Our results are harmonious with longitudinal studies in human AD, where microbial diversity is decreased and *Staphylococcus* and *Corynebacterium* species predominate during flare states (Kong et al., 2012). Furthermore, the *Adam17<sup>fl/fl</sup>Sox9<sup>-Cre</sup>* murine model of AD is characterized by high relative abundance of cutaneous *Staphylococcus* and

*Corynebacterium* organisms that normalized after antimicrobial therapy (Kobayashi et al., 2015).

In cAD there is a predisposition to the development of coagulase-positive *Staphylococcus* species colonization and dermatitis as in AD. *S. aureus* is the primary coagulase-positive *Staphylococcus* species of human skin and mucosal sites. *S. pseudintermedius* is a skin and mucosal commensal in the dog and, as we substantiate independent of culture, the most frequent pathogen isolated from dogs with skin or ear canal infections (Bannoehr and Guardabassi, 2012). Human *S. pseudintermedius* colonization is rare and primarily restricted to those with regular contact with dogs and cats (Talan et al., 1989; Goodacre et al., 1997; Guardabassi et al., 2004). By the same token, *S. aureus* is infrequently isolated from infection and carriage sites of dogs

in clinical practice and in epidemiological surveys, and it is considered a comparatively infrequent canine pathogen (Beck et al., 2012; Morris, Rook, et al., 2006; Morris, Boston, et al., 2010). The dog may act as a potential vector of *S. aureus*, which raises zoonotic and anthrozoönotic concerns for potential transfer of pathogens, drug resistance, and genetic elements (Boag et al., 2004; Bramble et al., 2011; Misisic et al., 2015; Song et al., 2013).

Mechanisms of staphylococcal perturbation of the epidermal barrier are still unclear and may be through direct or indirect (immunostimulatory) means. Mitigation of staphylococcal overgrowth clearly ameliorates disease severity, and the epidermal barrier normalizes. However, methicillin and multidrug resistance are now commonplace in both veterinary and human medicine. The findings presented here may inform future efforts to develop alternative (nonantibiotic) approaches to controlling staphylococcal burden in skin disease.

## MATERIALS AND METHODS

### Animal subjects

Dogs with cAD ( $n = 16$ ) and healthy dogs ( $n = 14$ ) were prospectively enrolled in this pilot study at the University of Pennsylvania Matthew J. Ryan Veterinary Hospital, after examination by a board-certified veterinary dermatologist (CC, EM, DM). The same dermatologist examined each patient across all time points. Dogs with cAD were included in the study by fulfilling standardized criteria (five criteria of Favrot et al., 2010) and by ruling out dermatoses with similar presentations as depicted by Hensel et al. (2015) based on dermatologic examination and clinical histories. Dermatologic examination in all patients involved, but was not limited to, skin and otic cytology, flea combing/direct examination, and skin scraping. Dogs with evidence of other underlying systemic disease, an atopic history that was not clearly documented, or evidence of active ecto-parasitic (including *Demodex* species, *Sarcoptes* species, and *Ctenocephalides felis*) infestations were excluded. All subjects were on a strict flea control regimen. Four dogs with cAD had a history of antibiotic exposure within 45 days before enrollment. Therapy was prescribed as indicated by the attending veterinary dermatologist. Systemic antimicrobial therapy was prescribed based on aerobic culture and sensitivity at enrollment. See Supplementary Table S4a and b for specific therapies prescribed for each patient. Healthy dogs were enrolled in the study during the same time period. A subset of dogs was owned by veterinarians and veterinary technicians. Informed consent was obtained from all owners before enrollment. All experiments were carried out according to approved Institutional Animal Care and Use Committee protocols. Site-specific assessment and semi-quantitative scoring of lesion severity were performed and included erythema, lichenification, and self-induced alopecia, each on a 0–5 scale (0 = no lesions to 5 = most severe). The same dermatologist (CC and DM) assessed/scored each patient at each time point. This scoring system is based on Canine Atopic Dermatitis Extent and Severity Index (Olivry et al., 2007), because of familiarity of the dermatologists, akin to the Scoring Atopic Dermatitis (SCORAD) metric used in human dermatology. The lesion score was used to assess for changes at a given site that might correlate with microbial shifts or skin barrier function and not as a means for documenting changes in disease flare.

### Skin microenvironmental assessment

Noninvasive probes for measuring subsurface-epidermal water content by the capacitance method (Corneometer CM 825, Courage+Khazaka, Cologne, Germany), skin pH (Skin-pH-Meter, PH 905, Courage+Khazaka, Cologne, Germany), and TEWL by diffusion (Tewameter TM300, Courage+Khazaka, Cologne, Germany) were used at each visit according to the manufacturer's instructions. Sampling sites included the axilla, groin, and concave aspect of the pinna. These sites were chosen because they are sparsely haired, allowing for corneometry and tewametry measurements, and are cAD predilection sites. Dogs were sampled in lateral recumbency with minimal physical restraint. The order of measurements at each site was corneometry, TEWL, and pH. TEWL measurements were averaged at 1-second intervals for a 30-second period. Five corneometry measurements were performed over a 3cm<sup>2</sup> area and were averaged. Three consecutive pH readings were averaged. Measurements were performed in the dermatology clinic treatment area. During sampling and assessment, the mean indoor ambient temperature was 22.2°C (range = 18.3–23.9°C) and the mean relative humidity was 25.5% (range, = 16–68%).

### Microbiomic sampling

The oral cavity, axilla, concave pinna, and groin were swabbed (3cm<sup>2</sup> region) using Catch-All Sample Collection Swabs (Epicentre Biotechnologies, Madison, WI). Swabs were rubbed vigorously over the skin site or mouth for 10–15 second intervals. Swabs exposed to air in the treatment room and laboratory at extraction were used as negative control samples. Swabs were placed in 300 µl of Yeast Cell Lysis Solution (Epicentre Biotechnologies, Madison, WI), and the tip of the swab was aseptically cut from the handle and stored at –80°C until extraction.

Contemporaneous swabs were taken from a single lesional site of dogs with dermatitis and submitted for aerobic bacterial culture. Swabs were processed using standard laboratory protocols, and isolates were identified as *Staphylococcus* species by use of a conventional biochemical identification system (MicroScan Walkaway 40 PC20 Gram-positive combo-panel, Dade Behring, Sacramento, CA) as described by the manufacturer. The results of the culture and antimicrobial sensitivity testing were used to direct 4–6 weeks of systemic (oral) antimicrobial therapy (see Supplementary Table S4a).

### DNA extraction and sequencing

DNA extractions were performed as previously described (Misisic et al., 2015). The V1–V3 hypervariable region of the 16S rRNA gene was amplified using a barcoding strategy as described (Fadrosh et al., 2014) and primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 534R (5'-ATTACCGCGGCTGCTGG-3'). Sequencing was performed on the Illumina MiSeq instrument (Illumina, San Diego, CA) using 300–base pair paired-end chemistry at the University of Maryland Institute for Genome Sciences.

### 16S rRNA gene analysis

Paired-end reads were demultiplexed by Flexbar (Dodt et al., 2012) and assembled using Pear (Zhang et al., 2014), resulting in 17,065,344 sequences. Sequences less than 465 base pairs and greater than 535 base pairs and sequences with 10 or more homopolymers were removed, resulting in 13,023,611 sequences. QIIME version 1.8 (Caporaso et al., 2010) was used for further downstream processing and analyses. Sequences were aligned to the Greengenes database, and taxonomy was assigned using the RDP classifier (Wang et al., 2007). Operational taxonomic units were picked using

97% sequence similarity with cd-hit (Li and Godzik, 2006; Fu et al., 2012), and a representative sequence based on the most abundant sequence was used. Each sample was rarified to 4,000 sequences for alpha- and beta-diversity analyses. The pplacer binary (Matsen et al., 2010) was used as previously described (Gardner et al., 2013) for species level assessment of *Staphylococcus* organisms using a *Staphylococcus* reference database (Conlan et al., 2012). Water and processed blank samples were sequenced and processed in parallel, and contaminants were identified and removed as previously reported (Misic et al., 2015). Published best practices were used as guidelines (Bokulich et al., 2012). Sequences were deposited in the National Center for Biotechnology Information (NCBI) Short Read Archive under BioProject Accession No. PRJNA302288.

### Statistics

The R Statistical Package (R Core Team, 2015) was used for all computations. Nonparametric Wilcoxon rank sum tests were used to compare differences between groups. For within-subject comparisons, paired Wilcoxon signed rank tests were used. Pearson product moment correlation coefficients were calculated for correlations and tested using the Student *t* test.

### CONFLICT OF INTEREST

The authors state no conflict of interest.

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### SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at [www.jidonline.org](http://www.jidonline.org), and at <http://dx.doi.org/10.1016/j.jid.2016.01.023>.

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