Multistrain probiotics fail to modulate the asthmatic phenotype, respiratory microbiota, and immune responses in cats

Julia D. Remaks, DVM, MS1,2,3; Aida I. Vientos-Plotts, DVM, PhD, DACVIM1,2,4; Hansjorg Rindt, PhD1,4; Zachary McAdams, BS1,5,6; Aaron C. Ericsson, DVM, PhD, DACLAM1,5,6; Carol R. Reinero, DVM, PhD, DACVIM1,2,4*

1College of Veterinary Medicine, University of Missouri, Columbia, MO
2Department of Veterinary Medicine and Surgery, College of Veterinary Medicine, University of Missouri, Columbia, MO
3Garden State Veterinary Specialists, Tinton Falls, NJ
4Comparative Internal Medicine Laboratory, College of Veterinary Medicine, University of Missouri, Columbia, MO
5University of Missouri Metagenomics Center, University of Missouri, Columbia, MO
6Department of Veterinary Pathobiology, College of Veterinary Medicine, University of Missouri, Columbia, MO

*Corresponding author: Dr. Reinero (reineroc@missouri.edu)

OBJECTIVE
To determine if multistrain probiotics administered to asthmatic cats treated with anti-inflammatory glucocorticoids would attenuate the asthmatic phenotype and beneficially alter respiratory, blood, and oropharyngeal (OP) microbial communities and immune parameters versus placebo.

ANIMALS
13 client-owned asthmatic cats.

METHODS
A randomized, blinded, placebo-controlled clinical trial of asthmatic cats receiving anti-inflammatory glucocorticoids with oral multistrain probiotics or placebo assessed owner-perceived improvement and airway eosinophilia at baseline and after 2 weeks of treatment. Bronchoalveolar lavage fluid (BALF), blood, OP, and rectal microbial communities were compared using 16S rRNA amplicon sequencing. Real-time PCR for transcription factors, activation markers and cytokines, and IgA ELISAs were evaluated. Statistical analyses used 2-way repeated-measures ANOVA or permutational ANOVA (significance, P < .05).

RESULTS
After treatment, there were no significant differences in owner-perceived clinical signs or mean ± SEM BALF eosinophils between groups. There was a significant decrease in rectal α-diversity but not in α- or β-diversity in BALF, blood, or OP between groups or over time. There were no significant differences in CD25, FoxP3, GATA, Helios, IL-4, IL-5, IL-10, IL-13, IL-17, IFN-γ mRNA, or serum or BALF IgA between groups or over time.

CLINICAL RELEVANCE
In asthmatic cats, oral multistrain probiotics failed to improve owner-perceived signs, reduce airway eosinophilia, modify microbial community composition, or alter assessed immune responses versus placebo or over time. Longer treatment, different probiotic composition or delivery (eg, aerosolized), or larger number of cats would represent the next stages of study.

Keywords: cytokines, dysbiosis, 16S rRNA amplicon sequencing, One Health, inflammatory airway disease

Feline asthma is characterized by airway eosinophilia, intermittent bronchospasm, airway hyperresponsiveness, mucus hypersecretion, and architectural remodeling. Currently, there is no cure, and lifelong administration of glucocorticoids is required to suppress ongoing airway inflammation. Feline asthma is believed to be allergic in etiology, with both a genetic predisposition and certain environmental exposures inducing a T-helper 2 (Th2)-mediated response against specific allergens.1,2 Allergen-specific Th2 cells produce cytokines such as IL-4, IL-5, and IL-13 leading to sustained local inflammation. In people, other Th1 subsets including

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T-regulatory cells (Tregs) and T_{h1} and T_{h17} populations are decreased in favor of the T_{h2}-allergic response.\textsuperscript{3} Tregs are critical to promote tolerance.\textsuperscript{4} Many environmental influences tip the balance from a tolerant to an inflammatory environment. Recent studies\textsuperscript{5–7} in human asthmatics have investigated the role of the airway microbiota in the pathogenesis of various respiratory diseases, including asthma. Of interest, cats and humans share very similar features of allergic asthma,\textsuperscript{4} making the study of the feline microbiota relevant to One Health initiatives.

When using 16S rRNA amplicon sequencing, it is recognized that the airways of people and cats are not sterile but are comprised of distinct, complex bacterial populations.\textsuperscript{8,9} Respiratory microbiota play a key role in establishing health versus disease states.\textsuperscript{10–12} The microbiota have a symbiotic relationship with the host providing a barrier against pathogenic species, regulating cell growth, promoting healing, and modulating immune responses.\textsuperscript{12} Dysbiosis, or disruption of the commensal microbiota, has consequences including overgrowth of pathogens or pathobionts that compete with commensal microbes for nutrients and host binding sites, loss of commensal microbial diversity, and a host inflammatory response contributing to disease development.\textsuperscript{13} Chronic inflammation further disrupts the microbiota to maintain a persistent inflammatory state.

Experimental and spontaneously asthmatic cats have evidence of respiratory dysbiosis.\textsuperscript{14,15} Significant changes in microbial community structure, decreased richness, and increased \( \alpha \)-diversity were seen in the lower airways of experimental and spontaneously asthmatic cats.\textsuperscript{14,15} Additionally, the presence of suspected pathobionts or opportunistic pathogens was documented in cats with spontaneous asthma.\textsuperscript{14,15} To investigate the modulation of local bacterial communities, a study\textsuperscript{16} in healthy cats administered oral multistrain probiotics showed probiotic species were detectable in the lower airways. Microbial community composition of the lower airways was significantly different before and after probiotic administration, suggesting multistrain probiotics may hold promise to target respiratory dysbiosis in feline asthma. The objective of this study was to investigate the effects of oral multistrain probiotics versus placebo in asthmatic cats treated with anti-inflammatory doses of glucocorticoids. We hypothesized that asthmatic cats receiving multistrain probiotics would have an attenuated asthmatic phenotype (improvement in owner-perceived clinical signs and airway eosinophilia); increased richness and diversity of airway, blood, oropharyngeal (OP), and rectal microbial communities; and altered immune markers.

**Methods**

**Criteria for enrollment**

Thirteen client-owned cats were enrolled with informed client consent at the Veterinary Health Center, University of Missouri between August 2020 and November 2022. The clinical trial was a prospective double-blinded study evaluating the use of multistrain probiotics with standard-of-care glucocorticoids (prednisolone) for feline asthma. Inclusion criteria were a CBC, routine administration of heartworm prevention or a negative feline heartworm antibody test, Baermann fecal or fenbendazole trial (50 mg/kg/day for 7 days), thoracic radiographs or thoracic CT, and cytology of bronchoalveolar lavage fluid (BALF) with greater than 10% eosinophils. The percent BALF eosinophil cutoff for asthmatic cats was derived from a study\textsuperscript{17} comparing BALF and lung tissue in healthy and asthmatic pet cats, which established that healthy cats should have BALF eosinophils less than 5%. As the dose of prednisolone was anti-inflammatory (with the intent to minimally impact the immune effects of the probiotic), cats with clinical evidence of moderate to severe asthma including audible wheezing without a stethoscope, increased expiratory effort (abdominal "push"), or respiratory distress were excluded. Other exclusion criteria were the administration of antibiotics or glucocorticoids within the month before enrollment or concerns about undergoing general anesthesia safely. This study protocol was reviewed and approved by the University of Missouri Animal Care and Use Committee No. 9802.

All cats received prednisolone 0.5 mg/kg/day and were randomized to receive either a daily capsule of a probiotic swallowed whole (PRO group; Visbiome Vet capsules; ExeGi Pharma; 112.5 billion live beneficial bacteria per 0.475 gm) or placebo (CONTROL group; anhydrous lactose) using a table of random numbers. At baseline (DO) and week 2 (W2), a visual analog scale (VAS) score was used to provide a subjective quantitative measure of owner-assessed severity in respiratory clinical signs.\textsuperscript{18,19} The VAS used a 10-cm scale to reflect observed respiratory signs ranging from no clinical signs (0 cm) to extreme respiratory distress (10 cm). Owners were trained on the use of the VAS and marked a vertical line along the sliding scale (subsequently measured by investigators) to indicate their perception of clinical sign severity within the preceding 2- to 4-week period.

**Sample collection**

Anesthetic protocols were tailored to each cat by a board-certified anesthesiologist. Cats were intubated with sterile endotracheal tubes (3.5 to 4 mm) while taking care to minimize OP contamination. A sterile 8-Fr red rubber catheter was inserted through the endotracheal tube and gently wedged in an airway; BALF was collected by instilling and then aspirating 20 mL of sterile 0.9% saline as a single bolus. The BALF was distributed into aliquots for cytology, culture (at the attending clinician’s discretion), and microbiota analyses. Whole blood in EDTA, serum, OP, and rectal samples were also collected. The OP swab was collected by brushing the caudodorsal oropharynx while reducing contamination by the oral cavity. A sterile cotton swab was inserted into the rectum while avoiding the perianal region for the
rectal samples. After collection, the samples were stored either on ice or refrigerated at 1 to 4°C until processing. Samples were then centrifuged to pellet bacterial cells. Samples were collected at D0 and W2 after starting medications.

**DNA extraction, 16S rRNA library preparation, sequencing, and informatics**

For PRO and CONTROL groups at D0 and W2, BALF, OP, and rectal samples were pelleted separately and stored at -80°C resuspended in lysis buffer adapted from Yu and Morrison20 (4% SDS, 50 mM EDTA, 500 mM NaCl, and 50 mM Tris-HCl pH 8.0). Red blood cells were lysed, and the remaining contents were pelleted before resuspension in lysis buffer as previously described.16 DNA extraction was then performed with a DNeasy blood and tissue kit (Qiagen) as previously described.14-16 Controls included 0.9% NaCl and 0.9% NaCl collected from a sterile 8-Fr red rubber catheter, MB lysis buffer, 10 mM Tris-EDTA solution, and 2 Visbiome capsules. DNA from BALF, OP, and rectum was extracted using the column method as previously described.14-16 Library construction and sequencing were completed at the University of Missouri DNA Core Facility as previously described.8,14-16 Assembly, filtering, binning, and annotation of DNA sequences was performed at the MU Informatics using Quantitative Insights into Microbial Ecology 2 (QIIME 2) v2021.2.21 For sequencing data, paired-end reads were trimmed of the universal primers and illumina adapters using cutadapt.22 Untrimmed sequences and indels were discarded. Forward and reverse reads were truncated to 150 bp before denoising with DADA2 using default settings. DADA2-generated amplicon sequence variants (ASVs) were filtered to between 249 and 257 base pairs in length. Given that some sampled tissues yielded relatively low biomass, contaminating features were removed from the feature table using the decontam24 package. The ASVs recovered from EDTA, buffer, and saline samples were designated as negative controls. Decontam identified 22 ASVs as contaminating features that were subsequently filtered from the feature table. The filtered table was rarefied to 1,169 features per sample for all downstream analyses using the rarefy function within the vegan library.25,26 Taxonomic classifications were assigned to each ASV using the sklearn algorithm trained on the SILVA 138 reference database trimmed to the V4 region of the 16S rRNA gene (515 to 806).

**Real-time PCR**

White blood cells were isolated by lysing RBCs with a hypotonic buffer and then flash-freezing them before storage at -80°C for subsequent immune analysis utilizing real-time PCR (RT-PCR) assays. Primers were optimized to detect mRNA expression relevant to Treg cells (CD25, foxP3, GATA, IFZF2 [Helios], IL-10), T_{h}2 cells (IL-4, IL-5, IL-13), T_{h}17 cells (IL-17), and T_{h}1 cells (IFN-γ). The PCR primers were designed from the reference sequences in the NCBI database using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast), with at least one intron between the primer sites to decrease the probability that genomic DNA contaminant was present in RNA preparation. The generated primer sequences are available (Supplementary Table S1). The RNA from each cat at D0 and W2 was isolated using a commercial kit (RNAqueous-4PCR; Invitrogen) and then quantified using a Qubit 3 Fluorometer (Invitrogen). This RNA was converted to cDNA with the use of SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer’s instructions. Each 384-well plate contained a housekeeping gene, tubulin A, along with targets of interest (CD25, foxP3, GATA, IFZF2 [Helios], IL-4, IL-5, IL-10, IL-13, IL-17, and IFN-γ) for relative mRNA quantification in the blood. Each well comprised a 10-µL reaction using SYBR green (Roche Diagnostics), 0.3 µM of forward and reverse primer, and 2 µL of cDNA. The RT-PCR assays were performed with a BioRad CFX384. The RT-PCR method was as follows: stage 1: following 1 cycle; and stage 2: 95°C for 10 minutes (cycle); and stage 2: 94°C for 15 seconds, 62°C for 30 seconds (except for IL-5, which required an annealing temperature of 55°C), 72°C for 30 seconds (40 cycles). A melt curve analysis was performed for every run. All samples were run in triplicate. The cycle threshold (C_{t}) for each reaction was calculated and outliers with > 15% coefficient of variance were discarded. Triplicate values were averaged for each cat and compared to the housekeeping gene values to obtain the ΔC. The ΔΔC was calculated by determining the relative differences between D0 and W2 for each immune target. Three cats were missing data from 1 time point due to errors in the collection and isolation of RNA; thus, a comparison between time points (ΔΔC) could not be performed for those cats.

**Serum and BALF IgA quantification**

Banked aliquots of serum and BALF supernatant were thawed for quantification of IgA using a commercially available ELISA (Cat IgA ELISA Kit; ICL) according to manufacturer instructions with provided standards. Serum samples were diluted 1:10,000, 1:30,000, and 1:100,000, and BALF samples were diluted 1:7,500 and 1:10,000 as needed to fall on the standard curve. Standards were run in duplicate and serum and BALF samples were run in triplicate. Results were reported using a Synergy H1 Microplate Reader (BioTek) set at a 450-nm absorbance. A Bradford protein assay on BALF supernatant was performed to normalize IgA to total protein as previously described.28 Values with a greater than or equal to 15% coefficient of variance were discarded as outliers.

**Statistical analysis**

Statistical analysis for clinical and immunologic data was performed using Sigma Plot 14.0 (Systat Software Inc). Repeated measures 2-way ANOVA
was used to compare VAS score, BALF eosinophils, and neutrophils, α-diversity within samples, RT-PCR targets of interest, and serum and BALF IgA between both treatment groups and time points. Results for eosinophil and neutrophil counts are reported as mean ± SEM. Post hoc analyses were accomplished with a Tukey test. A t test was used to detect statistical significance (P < .05) between patient weights and ages.

All microbiome analyses were performed using the open-source R Statistical Software v4.2.2. Chao1 and Shannon diversity indices were calculated using the `microbiome`29 and `vegan`25,26 libraries, respectively. To determine differences in microbial composition between samples (β-diversity), weighted (Bray-Curtis) and unweighted (Jaccard) distance matrices were generated using the `vegan` library from a quarter-root transformed feature table. Principal coordinate analysis of distance matrices was performed using the `ape`30 library with a Calliez correction.

Differences in α-diversity were determined using a 2-sample t test within sample type. Differences in community composition were determined using the `adonis2` function (vegan) using a 2-way permutational ANOVA (PERMANOVA) with sample type and time point as main effects. Pairwise comparisons were made using the pairwiseAdonis library.31 To identify differentially abundant taxa between pre- and post-Visbiome treatment, the conservative analysis of composition of microbiome with bias correction 2 (ANCOM-BC2)32 was applied to each sample type. ANCOM-BC2 identifies structural zeros features present in at least one group and absent in one group before determining differentially abundant taxa using a linear regression model.

Results

Clinical findings

Nineteen cats were screened, and 6 cats were excluded due to a lack of owner compliance and concerns about safely undergoing anesthesia for reevaluations. Thirteen cats met all inclusion criteria. Individual cat data are provided (Supplementary Table S2). Of those cats, 10 were castrated males and 3 were spayed females. Breeds were comprised of domestic shorthairs (7), mixed breed (4), Bobtail (1), and Siamese (1). Nine cats were newly diagnosed asthmatics. Of the remaining 4, 1 was previously definitively diagnosed with asthma via a compatible clinical picture and diagnostics including BALF cytology, and 3 were presumptively diagnosed with asthma based on clinical signs and thoracic radiographs. None of the previously diagnosed cats were receiving glucocorticoids on a regular

![Figure 1](image-url)
basis. The primary presenting clinical sign present in all cats was cough (n = 13) with secondary signs being increased respiratory rate (6) and auscultable wheeze (4). Historical labored respiration noted by the owner or primary care veterinarian (n = 3) and vomiting or retching (2) were lesser reported clinical signs. On physical examination, increased bronchovesicular sounds were the most common finding (n = 9). Six of 13 were receiving monthly heartworm prevention (Revolution Plus [Zoetis Inc], Advantage Multi [Elanco], and Interceptor [Elanco]) at the time of enrollment.

All 13 cats had negative feline heartworm antibody tests. Twelve of 13 cats had fenbendazole trials, and the 13th cat received monthly Revolution Plus (Zoetis Inc) year round. Eleven cats had negative fecal examinations. Twelve cats had thoracic radiographs. The most common finding was a diffuse bronchial pattern in nine cats, with 2 cats having unremarkable radiographs and 1 cat having a diffuse unstructured interstitial pattern. One cat had a thoracic CT performed with peribronchial cuffing and peribronchovascular thickening and bronchiolectasis noted.

For owner-perceived clinical signs, there was a significant improvement in VAS over time (P = .008) but not time within group (P = .386), meaning that regardless of treatment group, cats had a lower mean ± SEM VAS posttreatment (PRO: D0 40 ± 9, W2 32 ± 9; CONTROL: D0 48 ± 13, CONTROL W2 1 ± 13). There was no significant change in the percentage of BALF eosinophils or neutrophils between groups, over time, or in groups over time (Figure 1).

Sequencing results

Due to some sites having lower biomass, the Good coverage (C) was used in each sample that passed rarefaction (Figure 2). Rarefaction curves showing the number of unique ASVs are available (Supplementary Figure S1). This method indicated that the expected proportion of true ASVs within a sample was sequenced. Resolved to the taxonomic level of family (phylum), at baseline, BALF was predominated by Chitinophagaceae (Bacteroidota), Beijerinckiacaeae (Pseudomonadota), and Sphingomonadaceae (Pseudomonadota) (mean ± SEM: 43.90 ± 1.62%, 12.25 ± 0.90%, and 11.24 ± 0.77%, respectively); blood was predominated by Pasteurellaceae (Pseudomonadota), Porphyromonadaceae (Bacteroidota), and Chitinophagaceae (Bacteroidota) (22.35 ± 1.00%, 17.82 ± 1.04%, and 10.52 ± 0.43%, respectively); OP was predominated by Pasteurellaceae (Pseudomonadota), Porphyromonadaceae (Bacteroidota), and Chitinophagaceae (Bacteroidota) (21.39 ± 0.54%, 13.63 ± 0.21%, and 9.76 ± 0.57%, respectively); and rectum was predominated by Bacteroidaceae (Bacteroidota),
Lachnospiraceae (Bacillota), and Tissierellales (Bacillota) (17.46 ± 0.37%, 10.51 ± 0.40%, and 9.85 ± 0.50%, respectively). Further information on specific phyla and families from each sample location is available (Supplementary Figures S2 and S3).

Probiotic administration did not result in significant changes in α-diversity within BALF, blood, and OP samples, based on Chao1 and Shannon indices (Figure 3). However, there was a significant decrease in richness and evenness in rectal microbial communities over time (P = .048). While there were overall differences in community composition between sample types, there was no effect of treatment, based on a 2-way PERMANOVA. In addition, PERMANOVA and principal coordinate analysis of Bray-Curtis and Jaccard distances were used to assess the β-diversity of the microbial communities found at each site. Administration of multistrain probiotics did not result in significant changes in microbial community composition and structure in BALF, blood, OP, or rectal sites of asthmatic cats.

Sequencing of the probiotic capsules generated 12 ASVs. Considering that sequencing of the 16S rRNA V4 subregion yields limited species-level resolution, the most prevalent genera were Streptococcus, Lactobacillus, and Bifidobacterium (mean ± SEM: 53.67 ± 0.12%, 17.14 ± 0.61%, and 16.36 ± 0.76%, respectively) and these taxa from the probiotic were compared between the different microbial community sites studied. There was no significant difference in the relative abundance of the 12 ASVs after probiotic administration in any site (Figure 5).

**Immune analyses**

There was no significant difference in mRNA expression of CD25, foxP3, GATA, IFZF2 (Helios), IL-4, IL-5, IL-10, IL-13, IL-17, and IFN-γ between PRO or CONTROL groups or over time in the blood. Further information is provided (Supplementary Figure S4). When looking at interactions of group by time, only IFN-γ was significantly different (P = .006); however, post hoc analysis showed IFN-γ was significantly lower at W2 versus D0 in only the CONTROL group, which was not interpreted as biologically relevant. No significant differences in concentration of serum IgA or BALF IgA normalized to BALF total protein were detected between groups, over time, or groups by time.

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**Figure 4**—Principal coordinate (PCo) analysis of Bray-Curtis similarity index (measurement of β-diversity) in bronchoalveolar lavage fluid (BALF), blood, oropharyngeal (OP), and rectal communities compared to Visbiome control groups in 13 asthmatic cats. One-way permutational ANOVA showed no significant difference in community composition between collection sites. D0 = Baseline. Post = W2 (triangles). Pre = D0 (circles). W2 = 2-week recheck.
Discussion

In asthmatic cats treated with anti-inflammatory doses (0.5 mg/kg/day) of glucocorticoids, oral multistrain probiotics failed to attenuate the asthmatic phenotype; significantly alter local or distant microbial communities; modify mRNA expression for assessed transcription factors, activation markers, and cytokines relevant to allergic asthma; or increase serum or BALF IgA compared to placebo. While glucocorticoids, which are considered standard of care for asthma therapy, could not be withheld for ethical reasons, a conservative anti-inflammatory dose was used to test the impact of multistrain probiotics versus placebo. Understanding that feline asthma is associated with respiratory dysbiosis and that oral multistrain probiotics in healthy cats significantly altered respiratory microbial communities and were associated with the detection of probiotic species in upper and lower airways, targeting the microbiota is a logical next therapeutic step for this disease. However, the current study suggests that treatment with one type of oral probiotic in unopened capsule form failed to address the complexity of factors at play affecting asthma phenotypes, respiratory dysbiosis, and immunopathogenesis.

There is no single test to confirm the diagnosis of feline asthma. In the current study, diagnosis of feline asthma was based on the presence of eosinophilia (≥ 10%) on BALF cytology, after reducing suspicion for feline heartworm-associated respiratory disease (HARD), *Aelurostrongylus abstrusus* or *Toxocara cati* infection, and chronic bronchitis (predominant non-degenerate neutrophilia in BALF with no concurrent airway eosinophilia). The cutoff of percent BALF eosinophils was lower than in prior studies based on findings from a landmark manuscript comparing BALF and lung tissue eosinophils from healthy and asthmatic client-owned cats. In contrast to all earlier studies that established reference ranges for healthy cat BALF percent eosinophils based solely on the absence of clinical signs and radiographic abnormalities in client-owned and research cats, this study confirmed that in the healthy client-owned cats, the lung tissue examined microscopically did not have features of asthma and established that healthy cats should have a BALF percent eosinophils less than 5%. Absence of clinical signs in asthmatic cats with documented airway eosinophilia by BALF analysis, ie, so-called subclinical inflammation, has been previously documented. This brings into question the assumption that the absence of clinical signs implies “health.” Similarly, thoracic radiography may be an insensitive tool to diagnose asthma with prior studies showing between 6% and 23% of radiographs show no abnormalities. Histologic examination, as was only performed in the study of Shibly et al, should be perceived as the criterion standard.

Traditional therapy for feline asthma has centered on the lifelong administration of glucocorticoids. Glucocorticoids modulate transcription of many genes, including inflammatory cytokines and chemokines relevant to the pathogenesis of asthma such as IL-4, IL-5, and IL-13. Both oral and inhaled glucocorticoids administered to cats with experimental or naturally occurring asthma significantly decrease airway eosinophilia. For the current study, we elected to treat cats that were not
receiving steroids at the time of diagnosis with lower anti-inflammatory doses of oral glucocorticoids to better facilitate observation of changes in microbial composition and immune parameters from the addition of a probiotic as higher doses may have masked effects of the probiotic. Regardless of the treatment group, owners perceived a significant improvement in clinical signs, which may indicate that glucocorticoids alone are responsible for alleviating clinical signs, as has been documented in previous studies. Importantly, this study supported prior research demonstrating that despite improvement in clinical signs, subclinical airway inflammation may persist in some cats. This holds clinical significance, as underlying airway inflammation can lead to undetected architectural changes of airways and worsening of microscopic disease, despite the owner perception of improved clinical signs. As with airway eosinophilia, there was no statistical difference in the magnitude of airway neutrophilia between treatment groups. Investigation of airway neutrophilia in the study cats was performed to determine if there were parallels to human asthmatics wherein different endotypes (mechanistic pathways) of asthma exist. Enotypic differences may explain variable responses to therapy. While overall in our population airway eosinophilia predominated over airway neutrophilia, mixed inflammation or neutrophil-predominating inflammation was noted in some cats. This may be evidence that cats may have differing asthmatic endotypes prompting the need for further studies in feline asthma to perhaps explain variable responses to treatment.

Novel therapies to improve clinical signs and minimize the constellation of abnormalities found in allergic asthma are needed. Documenting respiratory dysbiosis in experimental and spontaneously asthmatic cats brings into question the potential for modulating the microbiota as a novel therapy. Sensitized mice administered prebiotics and probiotics had significantly improved airway hyperresponsiveness, decreased eosinophil recruitment to the airways, and reduction in IL-4, IL-5, and IL-13 in BALF. In healthy cats administered the same probiotic orally, increased microbial richness and diversity in the airways were noted. Additionally, some probiotic species were absent in the airways at baseline but detected after 4 weeks of oral probiotic administration. Speculative mechanisms of immigration of respiratory microbes include mucosal dispersion, microaspiration, and inhalation. Subclinical microaspiration serves as a potential avenue for seeding and subsequent colonization of the lower airways. In the cats of the current study, when looking at the presence and absence of taxa, none of the live probiotic taxa in the probiotic were sequenced in the airways. Potential reasons for these contrasting results could include differences in the way the probiotic was administered (opened capsule sprinkled on food in healthy cats vs whole capsule in the current study), the expertise of the person administering the probiotic (veterinarians versus clients), client compliance, the possibility that bacterial strains found in this probiotic may not be optimal to support changes in asthmatic airways, or the environments substantially differed between client-owned cats and research colony cats.

In this study, cats were administered the capsule in its entirety, whereas in the previous study, the probiotic capsule was opened and mixed in with food. Administration of a whole oral capsule may have hindered probiotic species from entering the airways if microaspiration serves as the primary mode of immigration of bacterial species into the airways. In future iterations of this study, alternative methods of delivery of the probiotic, such as mixing the contents of the capsule in food or inhaled or intranasal administration, could be considered.

Recent studies in asthmatic humans and cats have documented respiratory dysbiosis with a shift toward having an increased abundance of pathobionts (organisms found in health that could contribute to disease if the conditions are supportive) or pathogens. These may compete for resources with commensal organisms, leading to a loss of diversity and a propensity for inflammatory responses. The presence of pathogens and overgrowth of pathobionts demonstrated in those affected may make it more challenging for beneficial probiotic species to gain a foothold and change the microbial composition in the dysbiotic microbial ecosystem. Contrary to our hypothesis, there was no significant change in α- or β-diversity between PRO and CONTROL groups in BALF, blood, and OP microbial communities. In fact, there was a significant decrease in α-diversity in the rectal microbiota with the administration of multistrain probiotics, but this was only seen when using the Shannon index. No significant changes to β-diversity were seen.

Feline asthma is thought to develop due lack of immunologic tolerance to benign inhaled antigens (aeroallergens) and is attributed to a strongly polarized Th2 response and functional impairment of Treg cells. With Th2 polarization, a resultant decrease in Th1 and Th17 responses should occur. However, while yet to be characterized in cats, in humans, there is recognition that asthma is not a single entity but consists of distinct phenotypes (variable clinical presentations) and endotypes (mechanistic pathways). The type 2 (T2-high) endotype encompasses innate and adaptive immune cells both generally expressing the master transcription factor GATA3 producing type 2 cytokines including IL-4, IL-5, and IL-13, among others. The canonical marker of T2-high asthma is the eosinophil. The T2-low endotype encompasses Th1-mediated and Th17-mediated signatures, which may lead to severe asthma refractory to typical steroid administration. The T2-low asthma endotypes are characterized by airway neutrophilia and the absence of airway eosinophilia. The current
study explored T H1 and T H17 markers (IFN-γ and IL-17, respectively) in the context of feline asthma, thus offering more insight into possible endotypes within our feline patients. We chose RT-PCR to quantify cytokine mRNA rather than ELISAs to measure protein concentration, as prior studies using BALF supernatant for ELISAs have shown cytokines are often below the limits of detection making interpretation of data challenging. The lack of significant difference in mRNA expression of T H1, T H2, T H17, and Treg markers (CD25, foxP3, Helios, and IL-10) between the treatment groups may have been due to a small sample size or dissimilar asthma endotypes in cats as compared with people.

The mechanisms by which oral probiotics exert distant effects in allergic asthma by inducing Tregs, altering T H2 cytokine profiles, and modulating mucosal IgA antibody production. Most studies of feline asthma have primarily focused on the role of IgE production in immunopathogenesis. However, studies exist in both human and veterinary literature that document IgA-mediated changes in asthmatic phenotypes. The IgA possesses both immunosuppressive and proinflammatory properties and helps to maintain homeostasis at mucosal barriers. At respiratory mucosal sites, IgA acts as a neutralizing antibody and plays a role in the induction of tolerance to benign antigens. Specific immunotherapy for atopic disease leads to increases in allergen-specific IgA, which competes with IgE for binding to allergens, thus offering a possible therapeutic target for feline asthma and attenuation of clinical signs. We were unable to prove our hypothesis that the administration of multistrain probiotics would increase serum and BALF IgA. However, the IgA ELISA measured total serum and BALF IgA and not allergen-specific IgA, so it is still possible probiotic treatment could have had an unrecognized effect.

There were several limitations to this study. First, the subjects enrolled were client-owned cats with naturally occurring asthma having no standardization of environment or nutrition and varied genetics making the study complex. However, in comparison to experimentally asthmatic cats, they represent the target population that veterinarians will encounter in practice. Second, the accepted dosage range in asthmatic cats for prednisolone is 0.5 to 2 mg/kg/day, and doses in the current study were 0.5 mg/kg/day, which may have been suboptimal to gain control of airway inflammation. As previously mentioned, the low dose was intentional to not mask the effects of the probiotic. Third, owner compliance may have limited appropriate administration of prednisolone, probiotic, or placebo as prescribed. The multistrain probiotics and placebo were large capsules, making administration challenging. Fourth, our study had a small sample size that may have hindered the ability to discern statistical significance. Finally, the timing of the collection of repeat samples (2 weeks) may have been too short in duration to see an appreciable difference between PRO and CONTROL groups.

In conclusion, we determined that oral multistrain probiotics versus placebo in asthmatic cats treated with anti-inflammatory doses of glucocorticoids failed to improve owner-perceived respiratory clinical signs and airway eosinophilia, the latter of which is a key marker for ensuring control of the disease. Additionally, administration of oral multistrain probiotics did not increase the richness and diversity of microbial communities at any site evaluated; after mRNA expression of transcription factors, cytokines, or receptors relevant to the immunopathogenesis of asthma; or increase the quantity of IgA antibody in serum or BALF. Future studies could consider using a larger number of cats or longer treatment duration, using a different formulation or route of probiotic (e.g., inhaled), opening the capsule on food to better disperse the probiotic to the airways during consumption, and assessing steroid-sparing effects of a multistrain probiotic after controlling asthma with high-dose steroids.

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Disclosures

Drs. Reinero and Vientos-Plotts speak at conferences on respiratory disease and may receive honoraria and reimbursement for travel and accommodations. These presentations may encompass topics on feline allergic asthma, but compensation is independent of and unrelated to the current study. Drs. Reinero and Vientos-Plotts have received gifts for respiratory research, but this study was not funded by those gifts. No other authors have conflicts of interest to disclose.

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ORCID

Julia D. Remaks https://orcid.org/0009-0002-5295-6234
Carol R. Reinero https://orcid.org/0000-0002-6382-5582

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Supplementary Materials

Supplementary materials are posted online at the journal website: avmajournals.avma.org.