USING PROBIOTICS TO MODULATE THE ASTHMATIC PHENOTYPE, RESPIRATORY MICROBIOTA, AND IMMUNE RESPONSES IN CATS

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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

USING PROBIOTICS TO MODULATE THE ASTHMATIC PHENOTYPE, RESPIRATORY MICROBIOTA, AND IMMUNE RESPONSES IN CATS

Presented by Julia Remaks,

A candidate for the degree of Master of Science,

And hereby certify that, in their opinion, it is worthy of acceptance.

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DEDICATION

I would like to thank my family for their unwavering support (both mental and financial) during the years of higher education I chose to pursue. Thank you for sticking with me when I decided to get yet another degree.

And to Groot, my best asthmatic boy.

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1. Antigen-presenting cell (APC)
2. Bronchoalveolar lavage (BAL)
3. Bronchoalveolar lavage fluid (BALF)
4. Placebo (CONTROL)
5. Domestic shorthair (DSH)
6. Oropharyngeal (OP)
7. Polymerase chain reaction (PCR)
8. Probiotics (PRO)
9. T helper 1 (T_H1)
10. T helper 2 (T_H2)
11. T helper 17 (T_H17)
12. T regulatory cell (Treg)
13. Visual analog score (VAS)

ABSTRACT

Feline asthma is associated with respiratory dysbiosis, correction of which could serve as novel treatment. We hypothesized that in asthmatic cats treated with anti-inflammatory glucocorticoids, probiotics would attenuate the asthmatic phenotype and beneficially alter respiratory, blood and oropharyngeal (OP) microbial communities and immune parameters versus placebo. 13 client-owned asthmatic cats were enrolled. A randomized, blinded, placebo-controlled clinical trial of asthmatic cats receiving anti-inflammatory glucocorticoids with oral 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 fecal 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 two-way RM-ANOVA or PERMANOVA (significance, $p < 0.05$). 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 fecal 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. In asthmatic cats, oral 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 composition or delivery (e.g., aerosolized) of probiotic, or larger number of cats would represent next stages of study.

CHAPTER 1

INTRODUCTION & LITERATURE REVIEW

Feline allergic asthma is a naturally occurring respiratory disorder characterized by lower airway inflammation, intermittent bronchospasm and airway hyperreactivity, overproduction of mucus, and remodeling and narrowing of the airways [1]. Clinical signs include cough, increased respiratory effort, wheeze, exercise intolerance, and respiratory distress [1,2]. In severe cases, if untreated or inadequately managed, asthma can lead to death via airway obstruction. Cats diagnosed with asthma are predominantly younger, with a median age of 4 to 5 years at diagnosis [3]. The current standard of therapy for feline asthma is a combination of corticosteroids to decrease lower airway inflammation and minimize architectural remodeling of the airways, bronchodilators to alleviate bronchospasm, environmental modifications, and weight management [1]. Asthma in genetically predisposed cats with certain environmental influences is believed to be allergic in etiology. In sensitized cats, T_H2-mediated immunologic responses with subsequent production of cytokines and allergen-specific IgE develop against what should be benign inhaled substances (aeroallergens) [1]. Pet cats and humans share environments and thus, exposure to aeroallergens [2]. This has importance when considering the One Health perspective and future directions of therapy targeting allergic asthma as cats provide a useful large-animal model for clinical research.

Cats with asthma have immune responses to environmental allergens which would otherwise be tolerated in healthy cats [1] stemming from environmental and genetic factors predisposing them to allergic disease. Examples of environmental factors that may contribute to development of asthma include exposure to air pollutants, respiratory

viruses, environmental tobacco smoke, and aerosolized allergens [4]. Common allergens identified via allergen-specific IgE testing in naturally occurring asthmatic cats have been used to reproduce an allergic asthmatic phenotype in research cats, reinforcing the allergic nature of this disease. House dust mites and Bermuda grass allergens were utilized, and those cats developed respiratory clinical signs, eosinophilic airway inflammation, allergen-specific hyperresponsiveness, a T_H2 cell cytokine profile in blood and bronchoalveolar lavage fluid (BALF), induction of allergen-specific IgE, and airway remodeling all consistent with a feline asthma phenotype and endotype [5]. Genetic factors include predisposition to hypersensitivity reactions, also known as atopy, which has been documented in many studies to be hereditary in nature [4]. Elevated concentrations of IgE have been detected in multiple atopic diseases [4] and feline asthmatics have been shown to have IgE reactivity [6], thus providing a basis for potential immunotherapeutic interventions [6] and more insight into etiopathogenesis.

Inhalation of allergens leads to their uptake by antigen-presenting cells (APCs) closely associated with M cells of the respiratory mucosa and dendritic cells with dendrites intercalating between respiratory epithelial cells [7,8]. Antigens are constantly being sampled by immune cells in mucosal sites (immune surveillance) to determine if there are threats. Responses of the mucosal immune system include immune exclusion, immune elimination, and tolerance. Antigens are presented with major histocompatibility complex class II (MHC II) to naïve T helper 0 (T_H0) cells, which will then ultimately differentiate into T helper 1 (T_H1) T helper 2 (T_H2), T helper 17 (T_H17), or Treg cells [1].

The T_H2-mediated response is the key driver in the pathogenesis of feline asthma, subsequently leading to production of effector cytokines IL-4, IL-5, and IL-13, among

others which orchestrate the allergic inflammatory response and production of IgE [1,2,8]. These pro-inflammatory cytokines lead to harmful bystander local tissue immune responses when targeting what should be benign inhaled substances that characterize allergy and allergic asthma [8]. IL-4 is produced by T_H2 cells and induces differentiation of T_H0 cells (naïve cells) into T_H2 cells, thus propagating a positive feedback loop of inflammation [8]. IL-4 is also crucial for inducing class switching of B lymphocytes to IgE, the classic antibody present in allergic responses. After allergen challenge, IL-5 is responsible for eosinophil recruitment to inflamed pulmonary tissues, leading to the key airway eosinophilia and subsequent bronchospasm characteristic of feline allergic asthma [8]. IL-13 is a central regulator in synthesis of IgE and plays a role in hypersecretion of mucus and airway hyperresponsiveness. Mast cells avidly bind IgE with high affinity Fcε receptors and then upon subsequent exposure to specific allergen, the bound IgE is cross-linked, leading to degranulation of mast cells and further exacerbation of inflammation [4].

Although the majority of studies have focused on the role of IgE in asthma, IgA also has functions in both feline and human asthma [9-11]. IgA plays a dichotomous role, with both immunosuppressive and pro-inflammatory pathways in constant balance between health and disease states [10]. IgA is important at mucosal barriers, acting as a neutralizing antibody in the respiratory system that helps promote tolerance of inhaled benign antigens [10], thus dampening inflammation. As discussed previously, APCs sample luminal antigens and lead to production of effector B cells (i.e., plasma cells) that produce allergen-specific antibodies, including IgA [10]. IgA can scavenge allergens, preventing their binding to IgE and subsequent degranulation and the allergic response [10], dampening inflammation.

With polarization toward a T_H2 population in allergic asthma, T_H1, T_H17 and Treg populations are not produced [12]. The cytokines IFNγ and IL-17 are derived from T_H1 and T_H17-mediated responses (respectively). These alternative T_H pathways may be associated with variable clinical presentations (phenotypes) and mechanistic pathways (endotypes) of asthma and are worth further investigation. A decrease or functional impairment of T regulatory cells (Tregs), critical in mediating tolerance of the immune system [13], may allow for persistence of the T_H2 inflammatory response characteristic of feline allergic asthma. Markers for Tregs include CD25, FoxP3, Helios (IKFZ2), and IL-10. Expression of these T_H1, T_H17 and Treg markers and cytokines may offer value in further understanding of molecular mechanisms of allergic asthma including dysregulation.

As described above, feline asthma is thought to be allergic in etiology with excessive T_H2 cell cytokines driving pathologic changes. However, several phenotypes and endotypes are included under the umbrella of the syndrome of asthma in people [14]. Conventional phenotypes or clinical presentations are divided into non-atopic (intrinsic) and atopic (extrinsic) asthma, with atopic asthma predominating in younger cohorts of people. Asthma is most commonly diagnosed in younger cats, similarly to the atopic asthma phenotype seen in children. In addition to clinical phenotypes, there is increasing importance placed on recognition of molecular mechanism known as endotypes. The current proposed classification of asthma endotypes is T_H2-high (eosinophilic) and T_H2-low (non-eosinophilic), with the T_H2-high category being most similar to the canonical model of allergic asthma [14]. The T_H2-high endotype can be further subdivided into early-onset (extrinsic) asthma and late-onset asthma phenotypes. However, there is much

overlap when utilizing these categorization schemes, as patients vary in age of onset, inflammatory patterns, and disease severity [14]. Although patients may have similar clinical signs, response to therapy is largely dictated by the predominant asthmatic endotype. T_H2-low endotypes have been linked with activation of T_H1 and T_H17 cells and this may lead to severe, neutrophilic, and/or corticosteroid-resistant asthma [14]. Although the immunopathogenesis of asthma has centered on T_H2-mediated immunologic responses, T_H2-skewed inflammation was observed in only half of the human patients with asthma in one study [15], thus reiterating that asthma in humans is not a single disorder. There has also been a push in equine medicine to identify different phenotypes and endotypes in asthmatic patients, as this may offer more targeted treatments or management strategies [16]. As this has not previously been studied in our feline asthmatic patients, we may be missing differing inflammatory pathways present, which may affect response to therapy.

There is no cure for feline allergic asthma and current therapy centers on regular administration of glucocorticoids to minimize airway inflammation [1,2]. Steroids have significant side effects including polyuria, polydipsia, polyphagia, weight gain, behavior changes, and predisposition to diabetes mellitus. [17] They are administered lifelong to suppress eosinophilic inflammation present in the airways [1,2]. Additionally, while treatment may cause resolution of clinical signs, subclinical eosinophilic inflammation may still be present, which leads to persistent and irreversible airway remodeling [18]. This has clinical significance in how we treat feline asthma and how aggressive we should be in rechecking bronchoalveolar lavage fluid (BALF) to target decreasing eosinophilic inflammation within the airways. Thus, it is important to explore novel therapeutic

options for feline asthma to improve quality of life and prevent progression of architectural changes to the airways that occurs with prolonged inflammation. One promising target, the host microbiome, offers a potential avenue of exploration for novel therapies.

A balance between a tolerogenic and inflammatory environment within the body is modulated by the microbiome, with a symbiotic relationship between the host and microbes [19]. Comprehensive studies of the gastrointestinal tract microbiome in humans and in veterinary species are well-documented. However, fewer studies have explored bacterial communities within the respiratory tract as the airways have conventionally been considered a sterile environment when using traditional laboratory culture methods. However, next-generation techniques utilizing 16S rRNA amplicon sequencing have demonstrated distinct, complex bacterial populations in the lungs of healthy cats and humans [20,21]. Although the lungs in humans have a low biomass in comparison to the colon, the number of bacteria is comparable to the duodenum [22]; thus, it is crucial not to underestimate the significance of the respiratory microbiome. Respiratory bacterial communities play a large role in equilibrium between systemic health and disease [22-24] where the microbiota have a symbiotic relationship with the host, with regulation of cellular growth, maintenance of innate barriers, stimulation of healing, and adaptation of systemic immune responses in humans [19].

The composition of the respiratory microbiota is affected by immigration and elimination of airway microbes as well as local reproduction. Proposed mechanisms of immigration, or the entrance of respiratory microbes, relies on three modalities: mucosal dispersion, microaspiration, and inhalation [20,22,25]. It is important to consider that there are

anatomic differences between species which may affect inhalation [26]; regardless, it is an important route of entry of microbes into the respiratory tract. Elimination of microbes occurs via mucociliary movement, coughing, and the host's immune system. In healthy humans, the microbiome is influenced by continuous fluctuation of the immigration and elimination of various microbes [26]. Overgrowth of a singular bacterial species is more consistent with pathologic processes, with a varied and complex microbiome being associated with a healthier individual [24,26]. High variability within the microbiome between individuals within a species leads to a lack of consensus on what constitutes a normal or typical microbiota; however, many definitions agree that a healthy microbiome confers development of immune tolerance [27].

It is important to consider that environmental factors have effects on a host microbiome [20] and subsequent development of allergic asthma. In mouse models studying germ-free mice and neonates, the respiratory microbiome develops shortly after birth while the lungs are in the final developmental stages [26]. The diversity of the microbiome increases with age in mice, which is consistent with human microbiota in the first three years of life [26]. Similarly, a previous study documented significant changes in lower airway microbiota as healthy kittens aged from 14 weeks to 25 weeks and 24 weeks to 35 weeks of age [20]. Several bacterial species are needed within this early developmental period to ensure a "normal" pattern of host immunity, with colonization of the neonatal mice with conventional microbiota being protected from the effects of asthma, versus germ-free mice being more susceptible to the asthma phenotype [26,28].

There are other proposed mechanisms for how microbiota influence allergy and asthma. Mucosal bacteria can modulate innate lymphoid cells (ILC2 cells), which lead to

production of T_H2 effector cytokines and direct actions on Treg cells in mice. Commensal organisms can also promote differentiation of T_H0 cells into Treg cells and they can aid in prevention of colonization and proliferation of pathogenic bacteria by competing with known pathogens for shared resources [29].

The microbiota are generally considered symbiotic, where they confer benefits to the host. An imbalance of bacteria is known as dysbiosis, and pathologic shifts in the microbiota may be associated with disease development and progression [25,30]. Disease in humans results from competition between commensal and pathologic microbes, loss of bacterial diversity, and pronounced host inflammatory responses [30]. Dysbiosis within the airways is speculated to predispose to development and progression of an asthma phenotype in humans and cats [29,31]. Thus, targeting dysbiosis may offer a novel therapy for respiratory diseases, in particular, asthma.

Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host [32] and they are a consideration when attempting to modulate the host microbiome [27] and targeting dysbiosis. The specific mechanisms for alteration of the respiratory tract microbiome via administration of oral probiotics and alteration of the gastrointestinal microbiota are not well-understood. However, probiotics have been demonstrated to exert systemic effects via induction of Tregs in mice [33,34], alteration of T_H2 cytokine profiles in murine models and humans [35,36], and modulation of mucosal IgA antibody production in children [37]. These alterations in commensal bacteria are likely in part due to direct interaction with the host microbiome and the common mucosal immune system (CMIS) [19,20].

The allowance of crosstalk via the CMIS has been dubbed the gut-lung axis, where the anatomic, systemic, and nervous systems all mediate the exchange of microbial signals between the gastrointestinal and respiratory tracts [23,26]. This may provide explanation of how microbes present in the gut can influence immune functions in other mucosal sites, such as the airways. The CMIS involves circulating lymphocytes among various mucosal sites. Induction, or initiation of the immune response, takes place in mucosa-associated lymphoid tissue, such as gut-associated lymphoid tissue (GALT) or bronchus-associated lymphoid tissue (BALT) and distant inductive sites include the draining lymph nodes. Lymphocytes are imprinted to allow them to home to mucosal tissues; this is done via expression of various chemokine receptors that will allow them to bind to cell adhesion molecules and chemokine receptors in mucosal tissues. In this manner, activated lymphocytes circulate via lymphatics and vasculature from one mucosal surface to another (i.e. gut to respiratory tract) and affect induction of Treg cells and alteration of T_H2 cells at distant sites of the body.

Many animal models have explored the immunologic implications of administering probiotics and their effects on the respiratory tract in health and disease [20,29,31]. Orally administered probiotics have been found to modify the respiratory microbiota of healthy cats by populating the lungs with different bacteria and altering preexisting bacterial populations [38]. At the conclusion of the trial, oral probiotics were associated with significant changes and increased diversity to the lower airway microbiota [38]. Probiotic administration for treatment of inflammatory respiratory disease in equine patients also serves as a translational model for human asthma [39]. A previous study demonstrated that horses administered pre- and probiotics had significantly

decreased airway eosinophil count on tracheal wash than the control group [39]. Studies with experimentally sensitized mice that received oral *Lactobacillus reuteri* demonstrated attenuated clinical signs (decreased airway hyperresponsiveness), and reduction in eosinophilic influx to airways and peripheral eosinophilia (via decreased IL-5). This same organism induced Treg cells that subsequently suppressed airway eosinophilia, hypersecretion of mucus, and airway hyperresponsiveness [33,35]. These studies have potential clinical implications: probiotics may alter aberrant immunologic pathways or modulate clinical signs associated with asthma. It merits study to determine if restoration of a healthy microbiota may help attenuate the pathologic immunologic changes in asthma.

We predict that asthmatic cats receiving probiotics will have an attenuated asthmatic phenotype with improved clinical signs and airway eosinophilia; increased richness and diversity of airway, blood, oropharyngeal (OP) and fecal microbial communities; altered T_H1, T_H2, T_H17, and Treg immune markers; and increased serum and BALF IgA. Utility of oral probiotics in feline asthmatic patients could be a useful large-animal model to explore its potential utility in humans with allergic asthma, as humans and pet cats typically share more similar environments than humans and horses [2].

CHAPTER 2

USING PROBIOTICS TO MODULATE THE ASTHMATIC PHENOTYPE, RESPIRATORY MICROBIOTA, AND IMMUNE RESPONSES IN CATS

Introduction

Feline asthma is characterized by airway eosinophilia, intermittent bronchospasm and 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 (T_H2)-mediated response against specific aeroallergens. [1,2] Allergen-specific T_H2 cells produce cytokines such as IL-4, IL-5, and IL-13 leading to sustained local inflammation. Other T_H subsets including T regulatory cells (Tregs) and T_H1 and T_H17 populations are decreased in favor of the T_H2 allergic response. [12] Tregs are critical to promote tolerance. [13] Many environmental influences tip the balance from a tolerogenic to inflammatory environment. Recent studies in human asthmatics have investigated the role of the airway microbiota in the pathogenesis of various respiratory diseases, including asthma. [21,40-42] Of interest, cats and humans share very similar features of allergic asthma, making study of the feline microbiota of One Health relevance.

When using 16S rRNA amplicon sequencing, it is recognized that lungs of people and cats are not sterile but are comprised of distinct, complex bacterial populations. [20,21] Respiratory microbiota play a key role in establishing health versus disease states. [22-24] The microbiota have a symbiotic relationship with the host providing a barrier against pathogenic species, regulating cell growth, promoting healing, and modulating immune

responses. [19] 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 that contributes to disease development. [30] Chronic inflammation further disrupts the microbiota to maintain a persistent inflammatory state.

Both experimental and spontaneously asthmatic cats have evidence of respiratory dysbiosis. [29,31] Significant changes in microbial community structure, decreased richness, and increased α-diversity were seen in the lower airways for experimental and spontaneously asthmatic cats. [29,31] Additionally, the presence of suspected pathobionts or opportunistic pathogens was documented in cats with spontaneous asthma. [29,31] To investigate modulation of local bacterial communities, a study in healthy cats administered oral probiotics showed probiotic species were detectable in the lower airways. [38] Microbial community composition of the lower airways was significantly different before and after probiotic administration [38], suggesting probiotics may hold promise to target respiratory dysbiosis in feline asthma. The objective of this study was to investigate the effects of oral probiotics versus placebo in asthmatic cats treated with anti-inflammatory doses of glucocorticoids. We hypothesized that asthmatic cats receiving 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 fecal microbial communities; and altered immune markers.

Methods and Materials

Criteria for Enrollment

Thirteen client-owned cats were enrolled with informed client consent at the *blinded for review* between August 2020-November 2022. The clinical trial was a prospective double-blinded study evaluating use of probiotics with standard-of-care glucocorticoids for feline asthma. Inclusion criteria were a complete blood count, routine administration of heartworm prevention or a negative feline heartworm antibody test, Baermann fecal or fenbendazole trial, thoracic radiographs or thoracic computed tomography (CT), and cytology of bronchoalveolar lavage fluid (BALF) with >7% eosinophils. Exclusion criteria included administration of antibiotics or glucocorticoids within the month prior to enrollment or concerns of undergoing general anesthesia safely. Definitive diagnosis of feline asthma was based on the presence of eosinophilia (≥ 7%) on BALF cytology, after ruling out feline heartworm-associated respiratory disease (HARD), *Toxocara cati* infection, and chronic bronchitis.

All cats received prednisolone (0.5 mg/kg/day) and were randomized to receive either a probiotic (PRO group; Visbiome Vet capsules, ExeGi Pharma, Rockville, MD; 112.5 billion live beneficial bacteria per 0.475 gm [a]) or placebo (CONTROL group; anhydrous lactose) using a table of random numbers. At baseline (D0) and week 2 (W2), a visual analog score (VAS) ranging from 0-100, with 0 being absent clinical signs and 100 being the most severe clinical signs was completed by each client, to provide owner perception of clinical signs.

Sample Collection

Anesthetic protocols were tailored to each cat by a board-certified anesthesiologist. Cats were intubated with sterile endotracheal tubes (3.5-4 Fr) while taking care to minimize oropharyngeal 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, then aspirating 20 mL of sterile 0.9% saline. The BALF was distributed into aliquots for cytology, culture (at the attending clinician's discretion), and microbiota analyses. Whole blood in EDTA, serum, and oropharyngeal (OP) and fecal 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 fecal 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 pre-treatment (D0) and 2 weeks after starting medications (W2).

DNA Extraction, 16S rRNA Library Preparation, Sequencing, and Informatics

For PRO and CONTROL groups at D0 and W2, BALF, OP and fecal samples were pelleted separately and stored at -80 °C resuspended in lysis buffer adapted from Yu et al. (4% sodium dodecyl sulfate, 50 mM EDTA, 500 mM NaCl, and 50 mM Tris-HCl pH 8.0). [43] Red blood cells were lysed, and the remaining contents were pelleted prior to re-suspension in lysis buffer as previously described. [17] DNA extraction was then performed with a DNeasy blood and tissue kit (Qiagen, Hilden, Germany) as previously described. [20,29,31,38] Controls included 0.9% NaCl, 0.9% NaCl collected from a sterile 8

Fr red rubber catheter, MB lysis buffer, 10 mM Tris-EDTA solution, and two Visbiome capsules.

DNA from BALF, OP and feces was extracted using the column method as previously described. [20,29,31,38] Library construction and sequencing was completed at the *blinded for review* DNA Core facility as previously described. [20,29,31,38] Assembly, filtering, binning, and annotation of DNA sequences was performed at the *blinded for review* using Quantitative Insights into Microbial Ecology 2 (QIIME 2) v2021.2. [44] For sequencing data, paired-end reads were trimmed of the universal primers and Illumina adapters using *cutadapt.* [45] Untrimmed sequences and indels were discarded. Forward and reverse reads were truncated to 150 bp before denoising with DADA2 [46] 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 *decontam*[47] package. 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. [48,49] Taxonomic classifications were assigned to each ASV using the *sklearn* algorithm trained on the SILVA 138[50] reference database trimmed to the V4 region of the 16S rRNA gene (515-806).

Real-time PCR

White blood cells were isolated by lysing red blood cells with a hypotonic buffer and then flash-freezing them prior to 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_H2 cells (IL-4, IL-5, IL-13), T_H17 cells (IL-17), and T_H1 cells (IFN-γ). 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 in Table 1. RNA from each cat at D0 and W2 was isolated using a commercial kit (RNAqueous-4PCR, Invitrogen, Vilnus, Lithuania) and then quantified using a Qubit 3 Fluorometer (Invitrogen, Malaysia). This RNA was converted to cDNA with use of SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Each 384-well plate contained a housekeeping gene, tubulin A (Tub 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. Each well comprised a 10-microliter reaction using SYBR green (Roche Diagnostics, Mannheim, Germany), 0.3 uM of forward and reverse primer, and 2 microliters of cDNA. The PCR assays were performed with a BioRad CFX384. RT-PCR method was as follows: Stage 1: 95 °C for 10 minutes (1 cycle); 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_t. The $\Delta\Delta C_t$ was calculated by determining relative differences between D0 and W2 for each immune target. Three cats were missing data from one time point due to errors in collection and isolation of RNA, thus comparison between time points ($\Delta\Delta C_t$) 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, Portland, OR) 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 1:7500 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, Winooski, VT) set at 450 nm absorbance. A Bradford protein assay on BALF supernatant was performed to normalize IgA to total protein as previously described. [51] Values with ≥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., Carlsbad, CA). 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 timepoints. Results for eosinophil and neutrophil counts were reported as mean ± SEM. Post-hoc analyses were accomplished with a Tukey test. A t-test was used to detect statistical significance between patient weights and ages.

All downstream analyses were performed using the open-source R statistical software v4.2.2 (Vienna, Austria). Chao1 and Shannon diversity indices were calculated using the *microbiome*[52] and *vegan*[48,49] libraries, respectively. To determine differences in microbial composition between samples (β diversity), weighted and unweighted distance matrices were generated using the *vegan* library from a quarter-root transformed feature table. Principal coordinate analysis (PCA) of distance matrices were performed using the *ape*[53] library with a Calliez correction.

Differences in α-diversity were determined using a two-sample T test within sample type. Differences in community composition were determined using the *adonis2* function (*vegan*) using a two-way permutational analysis of variance (PERMANOVA) with sample type and timepoint as main effects. Pairwise comparisons were made using the *pairwiseAdonis* library. [54] To identify differentially abundant taxa between pre- and post-Visbiome treatment, the conservative analysis of composition of microbiome with bias correction 2 (ANCOM-BC2) [55] 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 for enrollment. Six cats were excluded due to lack of owner compliance and concerns for safely undergoing anesthesia for re-evaluations. Thirteen cats met all inclusion criteria. Of those cats, 10 were castrated males and 3 were spayed females. Breeds were comprised of domestic shorthairs (6), mixed breed (4), Bobtail (1), and Siamese (1). Nine cats were newly diagnosed asthmatics. Of the remaining four, one was previously definitively diagnosed with asthma via a compatible clinical picture and diagnostics including BALF cytology, and three were presumptively diagnosed with asthma based on clinical signs and thoracic radiographs; however, none of these cats were receiving glucocorticoids on a regular basis. Clinical signs included cough (n = 13), increased respiratory effort (n = 6), wheeze (n = 4), respiratory distress (n = 3), and true vomiting/retching (n = 2). No cat had a reported history of exercise intolerance. 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., Kalamazoo, MI) or Advantage Multi (Elanco, Shawnee, KS), Interceptor (Elanco, Greenfield, IN)) 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., Kalamazoo, MI) 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 two cats having unremarkable radiographs and one cat having a diffuse

unstructured interstitial pattern. One cat had a thoracic CT performed with peribronchial cuffing/peribronchovascular thickening and bronchiolectasis noted.

For owner perceived clinical signs, there was a significant improvement in VAS over time (p=0.008) but not time within group (p = 0.386), meaning that regardless of treatment group, cats had a lower mean ± SEM VAS post-treatment (PRO: D0 40 ± 9, W2 32 ± 9; CONTROL: D0 48 ± 13, CONTROL W2 1 ± 13). There was no significant change in 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, Good's coverage (*C*) was used in each sample that passed rarefaction (Figure 2). Rarefaction curves showing the number of unique ASVs were generated (Figure 3). This method indicated that the expected proportion of true ASVs within a sample were sequenced. Resolved to the taxonomic level of family (phylum), at baseline, BALF was predominated by *Chitinophagaceae* (*Bacteroidota), Beijerinckiaceae (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%, 10.52 ± 0.43%, respectively); OP was predominated by *Pasteurellaceae (Pseudomonadota), Porphyromonadaceae (Bacteroidota),* and *Chitinophagaceae (Bacteroidota)* (21.39 ± 0.54%, 13.63 ± 0.21%, 9.76 ± 0.57%, respectively); and feces was predominated by *Bacteroidaceae (Bacteroidota),*

Lachnospiraceae (Bacillota), and *Tissierellales (Bacillota)* (17.46 ± 0.37%, 10.51 ± 0.40%, 9.85 ± 0.50%, respectively). Specific phyla and families from each sample location were compared (Figures 4 and 5).

Probiotic administration did not result in significant changes in richness or α-diversity within BALF, blood and OP samples, based on Chao1 and Shannon indices, respectively (Figure 6). However, there was a significant decrease in α-diversity in fecal microbial communities over time when using a Shannon index ($p = 0.048$). While there were overall differences in community composition between sample types, there was no effect of treatment, based on a two-way PERMANOVA. In addition, PERMANOVA and principal coordinate analysis (PCA) of Bray Curtis (Figure 7) and Jaccard distances were used to assess β-diversity of the microbial communities found at each site. Administration of probiotics did not result in significant changes in microbial community composition and structure in BALF, blood, OP, or fecal sites of asthmatic cats.

Sequencing of the probiotic capsules generated 12 ASVs. While species-level resolution was not achieved, 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 were compared between the different microbial community sites studied. There was no significant difference in relative abundance of the 12 ASVs after probiotic administration in any site (Figure 8).

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 (Figure 9). When looking at interactions of group by time, only IFN-γ was significantly different (p=0.006); 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.

Discussion

In asthmatic cats treated with anti-inflammatory doses of glucocorticoids, oral 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, lower doses than generally recommended with randomization to probiotic or placebo were used to test impact of probiotics. Understanding that feline asthma is associated with respiratory dysbiosis[29,31] and that oral probiotics in healthy cats significantly altered respiratory microbial communities and was associated with detection of probiotic species in upper and lower airways[38] 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 failed to address the complexity of factors at play affecting asthma phenotypes, respiratory dysbiosis, and immunopathogenesis.

Traditional therapy for feline asthma has centered on administration of glucocorticoids. [17,56,57] 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. [17,58] Both oral and inhaled steroids administered to cats with experimental or naturally occurring asthma significantly decrease airway eosinophilia. [17,57] Several studies have compared inhaled versus oral steroids; however, no studies to date have evaluated differing doses of oral glucocorticoids for treatment of feline asthma. For the current study, we elected to use lower anti-inflammatory doses of oral steroids to better facilitate observation of changes in microbial composition and immune parameters from addition of a probiotic. Regardless of treatment group, owners perceived a significant improvement in clinical signs, which may indicate that steroids alone are responsible for alleviating clinical signs, as has been documented in previous studies. [18,56,57] Alternatively, these findings may highlight the caregiver placebo effect documented in veterinary medicine. [59,60] Importantly, prior research has demonstrated that despite improvement in clinical signs, subclinical airway inflammation may persist in some cats[18,57], which was evident in the current study. This holds clinical significance, as underlying airway inflammation can lead to undetected architectural changes of airways and worsening of microscopic disease, despite owner perception of improved clinical signs. Evaluation of response to therapy based on clinical signs alone is not recommended in asthmatic patients. [18,57] As with airway eosinophilia, there was no statistical difference in 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. [14,61] Endotypic 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 airway eosinophilia are needed. 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. [62] In healthy cats administered oral probiotics, increased microbial richness and diversity in the airways was noted. [38] Additionally, some probiotic species were absent in the airways at baseline but detected after 4 weeks of oral probiotic administration. [38] 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. [22,25,38] In the cats of the current study, when looking at 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 versus whole capsule), expertise of the person administering the probiotic (veterinarians versus clients), client compliance, or the possibility that bacterial strains found in this probiotic may not be optimal to support changes in asthmatic airways.

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.[38] Administration of an 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.[22,26] Studies in murine models and people have demonstrated immunomodulatory effects of intranasally-administered probiotics with decreased severity of chronic respiratory diseases such as asthma[63-65] that may offer an alternative method of administration to modify the microbial composition of 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[25,29,31] with a shift towards having 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 loss of diversity and propensity for inflammatory responses.[29-31] Presence of pathogens and overgrowth of pathobionts demonstrated in those affected may prevent ability of beneficial probiotic species to gain a foothold and change the microbial composition in the preexisting 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 fecal microbiota with administration of 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 tolerance to benign inhaled substances (aeroallergens) and is attributed to a strongly polarized T_H2 response and functional impairment of Treg cells. With T_H2 polarization, a resultant decrease in T_H1- and T_H17-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. [14] The canonical marker of T2-high asthma is the eosinophil. The T2-low endotypes include T_H1-mediated and T_H17-mediated signatures, which may lead to severe asthma refractory to typical steroid administration. [14] T2-low asthma endotypes are characterized by airway neutrophilia and absence of airway eosinophilia. [14,61] 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. [66] The lack of significant difference in mRNA expression of T_H1, T_H2 and T_H17 and Treg markers (CD25, foxP3, Helios, 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 are through induction of Tregs, [33,34] altering T_H2 cytokine profiles [35,36]and modulating mucosal IgA

antibody production. [37] 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. [9-11] IgA possesses both immunosuppressive and pro-inflammatory properties and helps to maintain homeostasis at mucosal barriers. [10] At respiratory mucosal sites, IgA acts as a neutralizing antibody, and plays a role in induction of tolerance to benign antigens. [10] 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. [11] We were unable to prove our hypothesis that administration of 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. 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 0.5-2 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. [17] Third, owner compliance may have limited appropriate administration of prednisolone, probiotic, or placebo as prescribed. The 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 may have

been too short in duration. The recheck timepoint chosen for our study was 2 weeks after administration of study drugs, which may have been too little time to see an appreciable difference between PRO and CONTROL groups.

In conclusion, we determined that oral 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 disease. Additionally, administration of oral probiotics did not increase richness and diversity of microbial communities at any site evaluated, or alter mRNA expression of transcription factors, or cytokines or receptors relevant to the immunopathogenesis of asthma, or increase quantity of IgA antibody in serum or BALF. Future studies could consider a different formulation or route of probiotic (e.g., inhaled), a larger number of cats, and longer durations of treatment.

CHAPTER 3
CONCLUSIONS & FUTURE DIRECTIONS

The focus of this thesis was to determine if administration of oral probiotics could modulate the clinical asthmatic phenotype, dysbiotic microbiota, and aberrant immune responses in pet cats with allergic asthma. We proposed to investigate the effects of oral probiotics versus placebo in asthmatic cats treated with anti-inflammatory doses of glucocorticoids. We hypothesized that asthmatic cats receiving probiotics would have improvement in owner-perceived clinical signs and airway eosinophilia; increased richness and diversity of airway, blood, OP, and fecal microbial communities; and altered immunologic markers. Although probiotics did not significantly alter any of the tested outcome parameters, results provided a basis of exploring novel therapeutic options for asthmatic cats to help improve quality of life, clinical signs, and airway inflammation associated with their disease. Additionally, this study was important in characterizing various microbial communities for comparison to previous and future studies evaluating the feline respiratory, blood, and gastrointestinal microbiome. Lastly, to our knowledge, this study was the first of its kind to attempt to document potential differences in endotypes of feline asthma by examining T_H1, T_H17, and Treg markers.

Glucocorticoids are the current standard-of-care in treatment of feline asthma due to their ability to regulate gene transcription of characteristic inflammatory cytokines of asthma [17,58]. Unfortunately, glucocorticoids have the potential to cause significant adverse effects in our feline patients such as polyuria, polydipsia, polyphagia, behavior changes, and increased susceptibility to diabetes mellitus; thus, discovery of alternative therapies is necessary. In the current study, owner perception of clinical signs improved regardless of treatment group over time, indicating that administration of steroids alone could improve

clinical signs [18] or emphasizing a potential caregiver placebo effect [59,60], or a combination thereof. Future studies should investigate a larger population of cats administered higher doses of corticosteroids (1 to 2 mg/kg/day) in addition to administration of probiotics as the dose of steroid chosen for our study (0.5 mg/kg/day) may have been too low to appreciably decrease airway inflammation [17]. Airway eosinophilia and neutrophilia did not differ between treatment groups or over time, despite owner-perceived improvement in clinical signs, which is also documented in a prior study of feline asthma investigating subclinical inflammation [18].

Previous studies have documented bacterial overgrowth of potential pathogens or pathobionts in asthmatic cats, leading to competition for nutrients with commensal organisms, causing subsequent loss of diversity and worsening inflammation [29-31]. The most abundant phyla seen in BALF, blood, OP and fecal samples in the current study were *Pseudomonadota* (formerly *Proteobacteria*), *Bacteroidota* (formerly *Bacteroidetes*), and *Bacillota* (formerly *Firmicutes*). In a previous study, both healthy cats and asthmatic cats had the same predominant phyla (under previous name classifications), but there were significant differences in relative abundance between the two groups of cats with a more variable microbial composition in asthmatic cats [29]. Unfortunately, no significant difference in α-diversity or β-diversity of the species recovered from BALF, blood, OP, or fecal communities was seen between treatment groups (PRO and CONTROL) or between timepoints (D0 and W2) in the current study. Potential limitations include the re-evaluation timepoint chosen for our study. We repeated BAL and sample collection 2 weeks after initiation of treatment, which may have been too short a course of

glucocorticoids and/or probiotics to see appreciable differences between PRO and CONTROL groups.

Other limitations include the composition of the probiotic microbial species, potential competition between commensal and pathologic microbiota, and route of administration of the probiotic chosen for our study. Future studies should consider utilizing probiotics with a wider variety of commensal bacterial species. Moreover, respiratory dysbiosis occurs in humans and cats with asthma [22,29,31] which can lead to overgrowth of pathogens and pathobionts, thus resulting in competition for resources and possible impedance of growth of commensal species. This competition may reduce the ability of commensal species to impact beneficial changes to the microbial ecosystem. The modality of administration of the probiotic may also have affected the results of the study, as it was administered in large capsules, which may decrease the ease of administration in pet cats. The capsule formulation may also have hindered the ability for the bacterial species to enter the respiratory tract. As previously discussed, one of the proposed mechanisms of immigration of bacterial species into the respiratory tract is via microaspiration [38]. In a previous study performed in healthy research cats, the capsules were opened and then given to the cats in their diet [38], potentially offering more opportunity for microaspiration of the bacterial species. Similarly, inhaled or intranasally-administered probiotics may be evaluated in future studies. Studies in humans and murine models have evaluated effects of intranasal probiotics and have documented attenuation of clinical signs associated with asthma and other chronic respiratory diseases [63-65]. Direct respiratory inoculation may more significantly alter microbial composition of the airways.

The current study had a small number of enrolled cats and was not powered to detect significant differences in asthmatic endotypes. A larger population of asthmatic cats with heterogeneous clinical signs and presentations such as cough alone versus those with bronchospasm may also help to ascertain whether various phenotypes and endotypes exist (T_H1 versus T_H17-mediated, differences in concentration of IgA) in the context of feline asthma. Although most feline asthma studies have focused on airway eosinophilia, we evaluated neutrophil counts in lower airways to explore potential endotypic differences in cats, similar to the T_H2-low (non-eosinophilic) category that has been documented in humans [14]. The majority of enrolled cats had airway eosinophilia, but many cats had mixed inflammation or neutrophilic inflammation present, which may provide support for distinct feline asthmatic endotypes and potential for polarization toward T_H1 and T_H17 immunologic profiles [14]. Alternatively, cats may have mixed inflammation due to persistent endothelial damage from chronic asthma or chronic asthmatic bronchitis, where eosinophils and non-degenerate neutrophils predominate [3]. Neutrophils also express pattern recognition receptors that can recognize pathogen-activated molecular patterns which may be present on pathogens [67] and pathobionts present in respiratory dysbiosis, which perpetuate further neutrophilic inflammation. Thus, overgrowth of respiratory pathogens and pathobionts mediated by T_H1 and T_H17 immunologic responses may contribute to a T_H2-low (non-eosinophilic) endotype of asthma which may be more responsive to administration of probiotics. Prior to further studies investigating probiotic administration in cats, identification of endotypes is crucial, as a heterogeneous population of cats may not uniformly respond to probiotic therapy.

The traditional pathogenesis of asthma focuses on T_H2-mediated immunologic responses, with effector cytokines IL-4, IL-5, and IL-13 being important in perpetuating inflammation and causing hallmark clinical signs of asthma [1,2,8]. With T_H2-polarized responses, T_H1 and T_H17 immune responses are lessened [12]. However, as discussed previously, T_H2-low endotypes of asthma exist in people, where T_H1 and T_H17-mediated responses may predominate and mRNA expression of IFNγ and IL-17 cytokines may consequently be increased. To our knowledge, this was the first study exploring T_H1 and T_H17 markers in feline asthma. Although there was no significant difference in mRNA expression of T_H1, T_H2 and T_H17 cytokines and Treg markers between the treatment groups in the current study, future studies should continue to explore a variety of transcription factors, markers, and cytokines in feline asthma, as we may be underestimating the variety of endotypes in our pet cats. Moreover, very few studies regarding these markers in any feline disease process exist, prompting further need for ongoing research in our veterinary species [68,69].

We originally hypothesized that administration of probiotics would increase serum and BALF total IgA and found no significant difference compared with placebo. In future studies, it may be more rewarding to specifically quantify allergen-specific IgA. Allergen-specific IgA acts as a scavenger, competing with IgE for binding of allergens, preventing them from binding to mast cell-bound IgE, allowing for potential therapeutic interventions [11].

In conclusion, oral probiotics administered to asthmatic cats treated with anti-inflammatory doses of glucocorticoids were not successful in improvement of owner-perceived clinical signs and or reduction of airway eosinophilia. Moreover, oral

probiotics did not impact richness and diversity of BALF, serum, OP and fecal microbiota. Probiotics failed to influence mRNA expression of transcription factors, markers, cytokines applicable to asthma immunopathogenesis or increase quantity of serum and BALF IgA antibody. Future directions include larger studies; increased dosages of corticosteroids; further exploration of mixed inflammation and airway neutrophilia in BALF to characterize possible phenotypic and endotypic differences in feline asthma; a longer course of glucocorticoids and probiotics; and alternative probiotic species and/or route of administration of probiotics.

APPENDIX 1: FIGURES

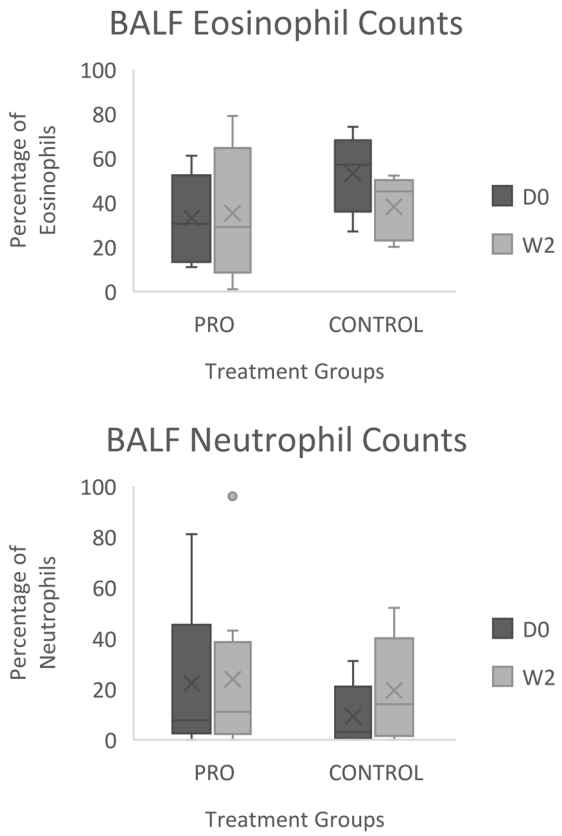


Figure 1: Box-and-whiskers plot demonstrating airway eosinophilia and neutrophilia in 13 asthmatic cats at D0 and W2 in both PRO and CONTROL treatment groups. The top end of the box represents the 75th percentile and the bottom end of the box indicates the 25th percentile. The line within the box represents the median. The whiskers on the top and the bottom of the boxes indicate the 95th and 5th percentiles, respectively. The filled-in circles represent outliers.

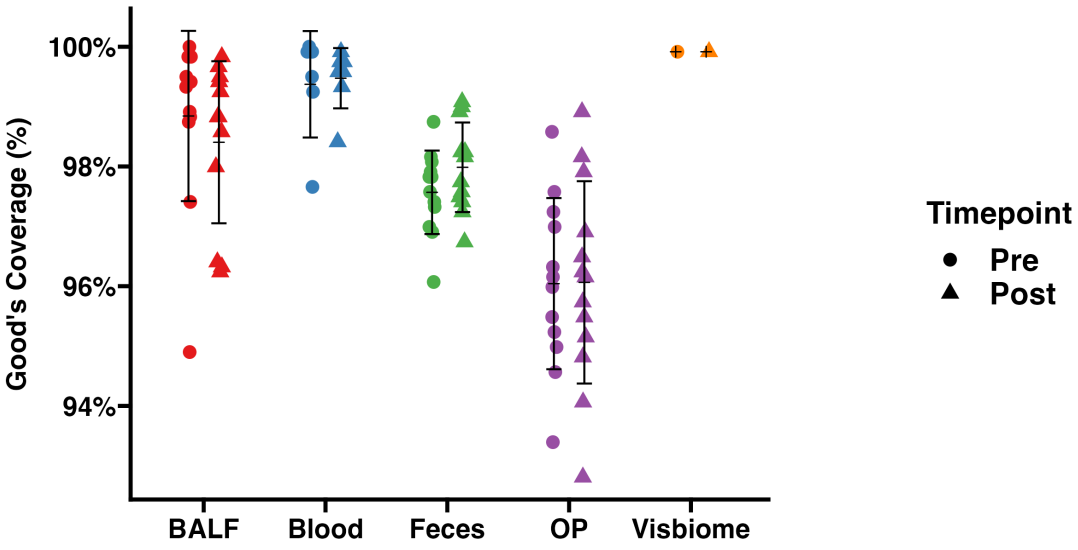


Figure 2: Good's coverage (*C*) of each sample obtained from 13 asthmatic cats that passed rarefaction. *C* indicates the expected proportion of true ASVs within a sample that were sequenced. n_1 = # of singletons N = # of total features. $C = 1 - \frac{n_1}{N}$ This is a method of estimating what percent of the total sequences is represented in a sample and is used for samples with lower biomass. Pre=D0 (circles), Post=W2 (triangles), BALF (red circles), Blood (blue circles), Feces (green circles), OP (purple circles), Visbiome (orange circles)

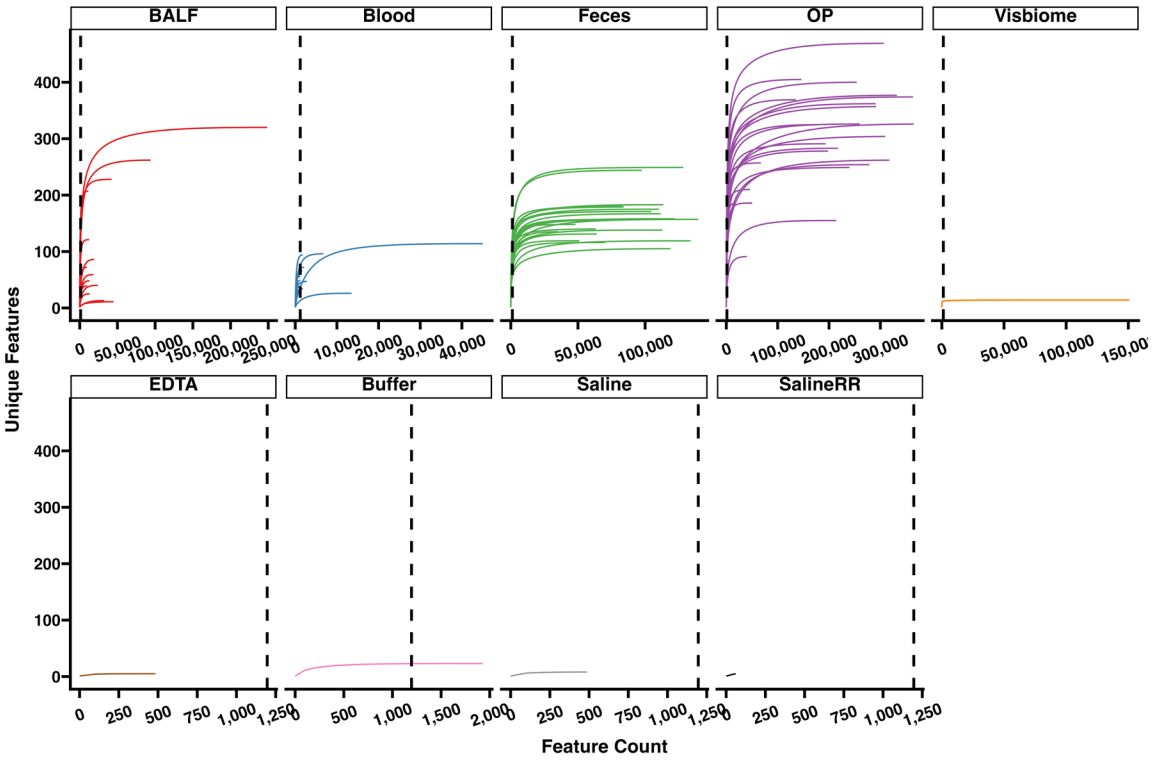
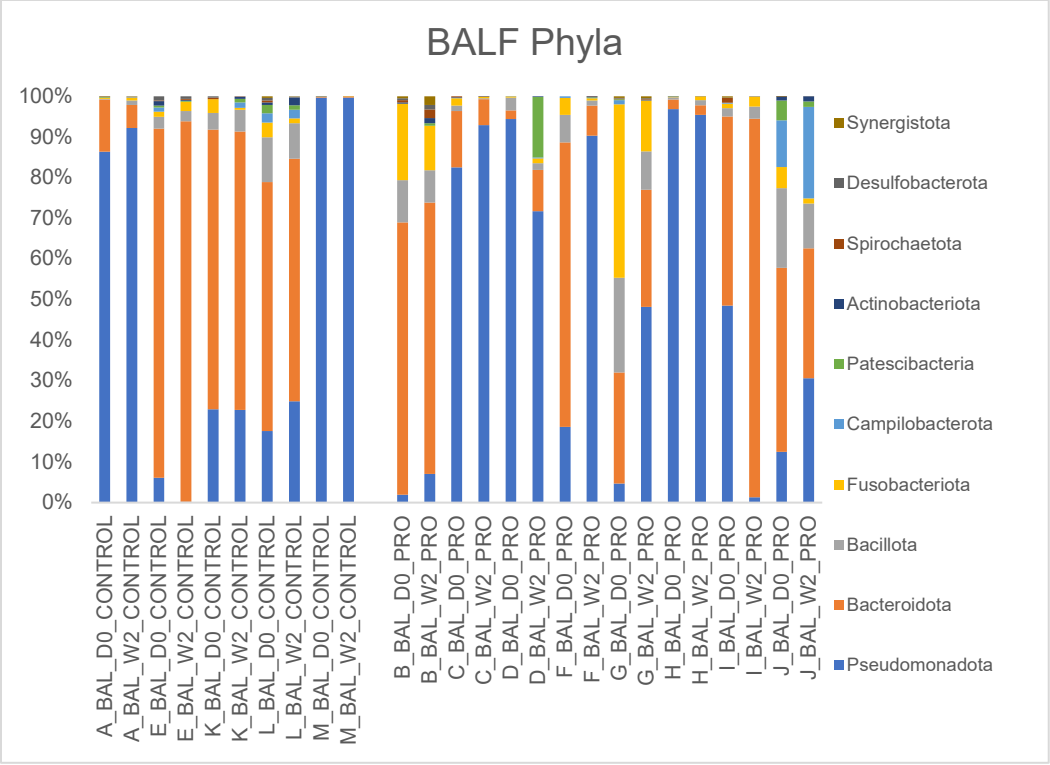
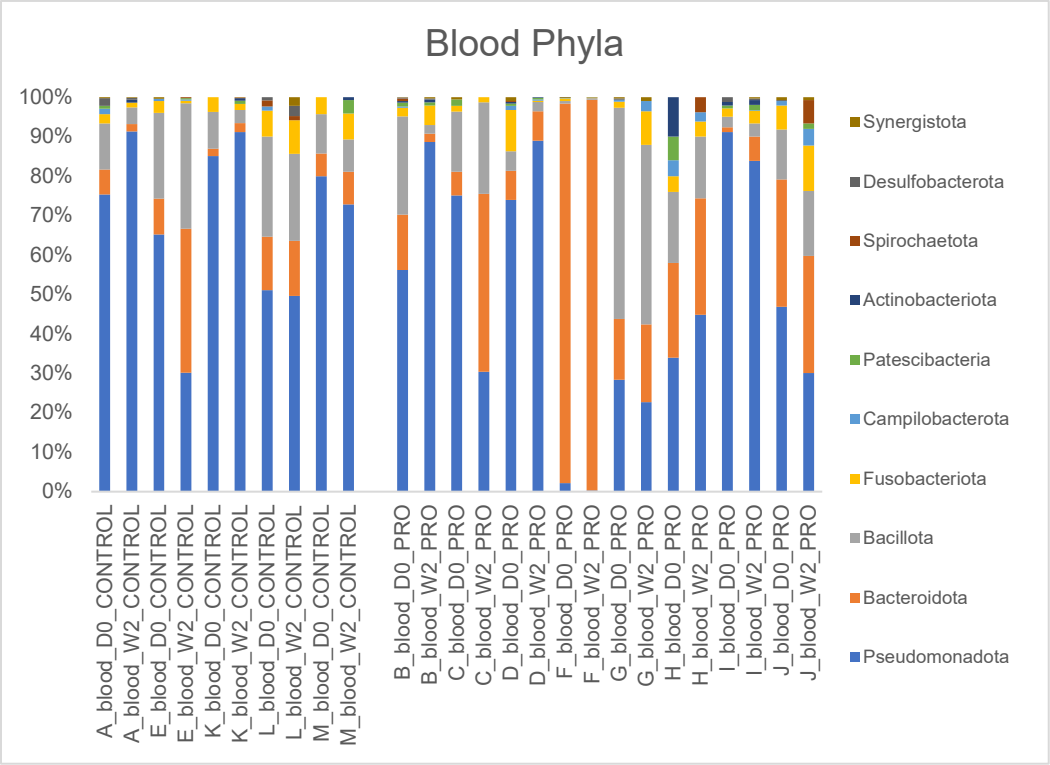


Figure 3: Rarefaction curves showing number of unique ASVs as sampling depth increases for all samples. Feature table rarefied to 1196 feature counts/sample. Dashed line = 1196 features.


BALF Phyla
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
A_BAL_D0_CONTROL
A_BAL_W2_CONTROL
E_BAL_D0_CONTROL
E_BAL_W2_CONTROL
K_BAL_D0_CONTROL
K_BAL_W2_CONTROL
L_BAL_D0_CONTROL
L_BAL_W2_CONTROL
M_BAL_D0_CONTROL
M_BAL_W2_CONTROL
B_BAL_D0_PRO
B_BAL_W2_PRO
C_BAL_D0_PRO
C_BAL_W2_PRO
D_BAL_D0_PRO
D_BAL_W2_PRO
F_BAL_D0_PRO
F_BAL_W2_PRO
G_BAL_D0_PRO
G_BAL_W2_PRO
H_BAL_D0_PRO
H_BAL_W2_PRO
I_BAL_D0_PRO
I_BAL_W2_PRO
J_BAL_D0_PRO
J_BAL_W2_PRO
Synergistota
Desulfobacterota
Spirochaetota
Actinobacteriota
Patescibacteria
Campilobacterota
Fusobacteriota
Bacillota
Bacteroidota
Pseudomonadota


Blood Phyla
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
A_blood_D0_CONTROL
A_blood_W2_CONTROL
E_blood_D0_CONTROL
E_blood_W2_CONTROL
K_blood_D0_CONTROL
K_blood_W2_CONTROL
L_blood_D0_CONTROL
L_blood_W2_CONTROL
M_blood_D0_CONTROL
M_blood_W2_CONTROL
B_blood_D0_PRO
B_blood_W2_PRO
C_blood_D0_PRO
C_blood_W2_PRO
D_blood_D0_PRO
D_blood_W2_PRO
F_blood_D0_PRO
F_blood_W2_PRO
G_blood_D0_PRO
G_blood_W2_PRO
H_blood_D0_PRO
H_blood_W2_PRO
I_blood_D0_PRO
I_blood_W2_PRO
J_blood_D0_PRO
J_blood_W2_PRO
Synergistota
Desulfobacterota
Spirochaetota
Actinobacteriota
Patescibacteria
Campilobacterota
Fusobacteriota
Bacillota
Bacteroidota
Pseudomonadota

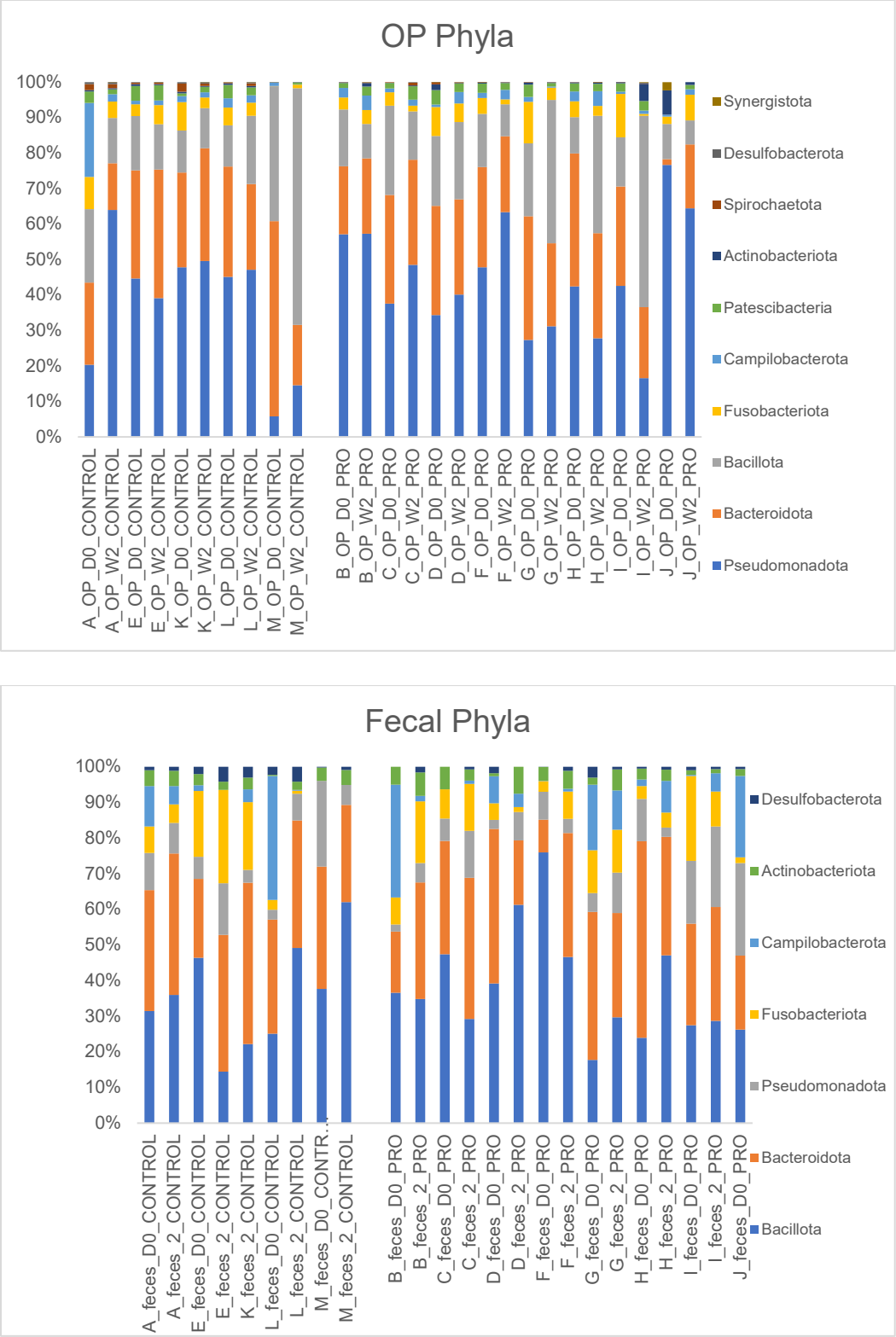
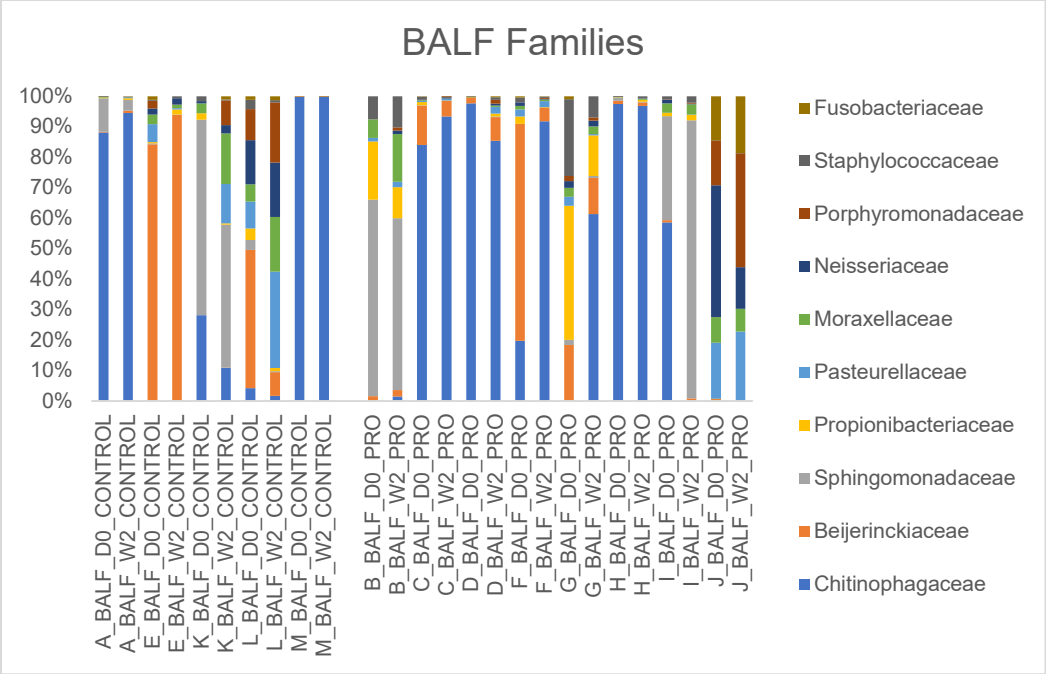
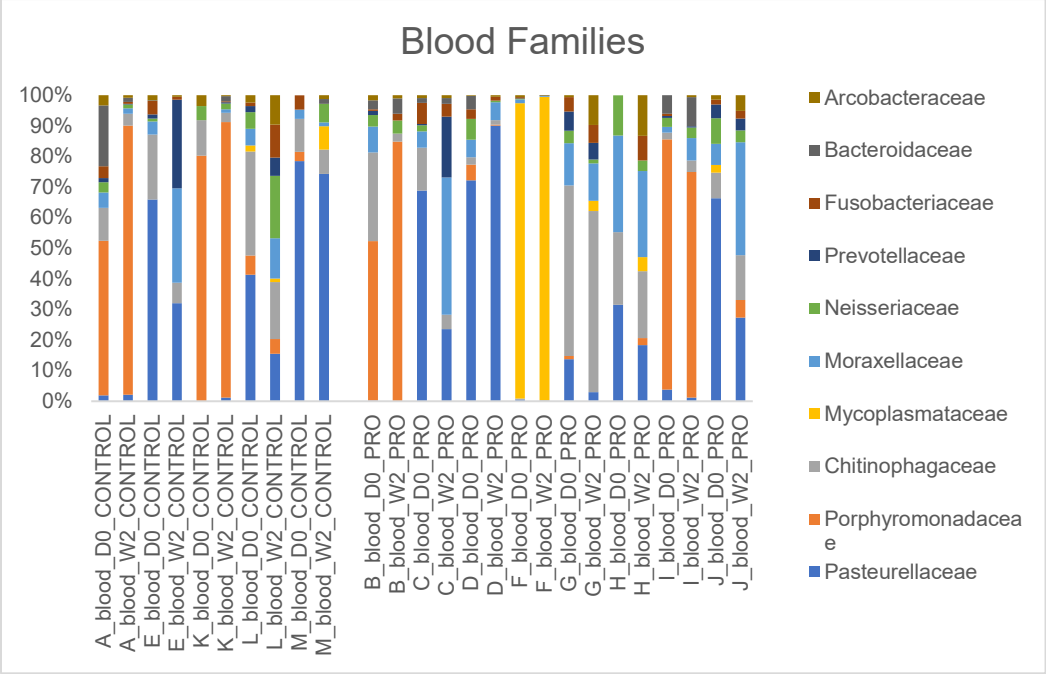


Figure 4: Stacked bar graphs showing the abundance of the microbial communities at the phyla taxonomic level in BALF, blood, OP and fecal sites in PRO and CONTROL

groups at time D0 and W2 for 13 cats (labeled A-M)**.** For the fecal microbial community at the phyla taxonomic level, 2 samples did not meet rarefaction criteria (< 1196 distinct features) and were discarded.


BALF Families
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
A_BALF_D0_CONTROL
A_BALF_W2_CONTROL
E_BALF_D0_CONTROL
E_BALF_W2_CONTROL
K_BALF_D0_CONTROL
K_BALF_W2_CONTROL
L_BALF_D0_CONTROL
L_BALF_W2_CONTROL
M_BALF_D0_CONTROL
M_BALF_W2_CONTROL
B_BALF_D0_PRO
B_BALF_W2_PRO
C_BALF_D0_PRO
C_BALF_W2_PRO
D_BALF_D0_PRO
D_BALF_W2_PRO
F_BALF_D0_PRO
F_BALF_W2_PRO
G_BALF_D0_PRO
G_BALF_W2_PRO
H_BALF_D0_PRO
H_BALF_W2_PRO
I_BALF_D0_PRO
I_BALF_W2_PRO
J_BALF_D0_PRO
J_BALF_W2_PRO
Fusobacteriaceae
Staphylococcaceae
Porphyromonadaceae
Neisseriaceae
Moraxellaceae
Pasteurellaceae
Propionibacteriaceae
Sphingomonadaceae
Beijerinckiaceae
Chitinophagaceae


Blood Families
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
A_blood_D0_CONTROL
A_blood_W2_CONTROL
E_blood_D0_CONTROL
E_blood_W2_CONTROL
K_blood_D0_CONTROL
K_blood_W2_CONTROL
L_blood_D0_CONTROL
L_blood_W2_CONTROL
M_blood_D0_CONTROL
M_blood_W2_CONTROL
B_blood_D0_PRO
B_blood_W2_PRO
C_blood_D0_PRO
C_blood_W2_PRO
D_blood_D0_PRO
D_blood_W2_PRO
F_blood_D0_PRO
F_blood_W2_PRO
G_blood_D0_PRO
G_blood_W2_PRO
H_blood_D0_PRO
H_blood_W2_PRO
I_blood_D0_PRO
I_blood_W2_PRO
J_blood_D0_PRO
J_blood_W2_PRO
Arcobacteraceae
Bacteroidaceae
Fusobacteriaceae
Prevotellaceae
Neisseriaceae
Moraxellaceae
Mycoplasmataceae
Chitinophagaceae
Porphyromonadacea e
Pasteurellaceae

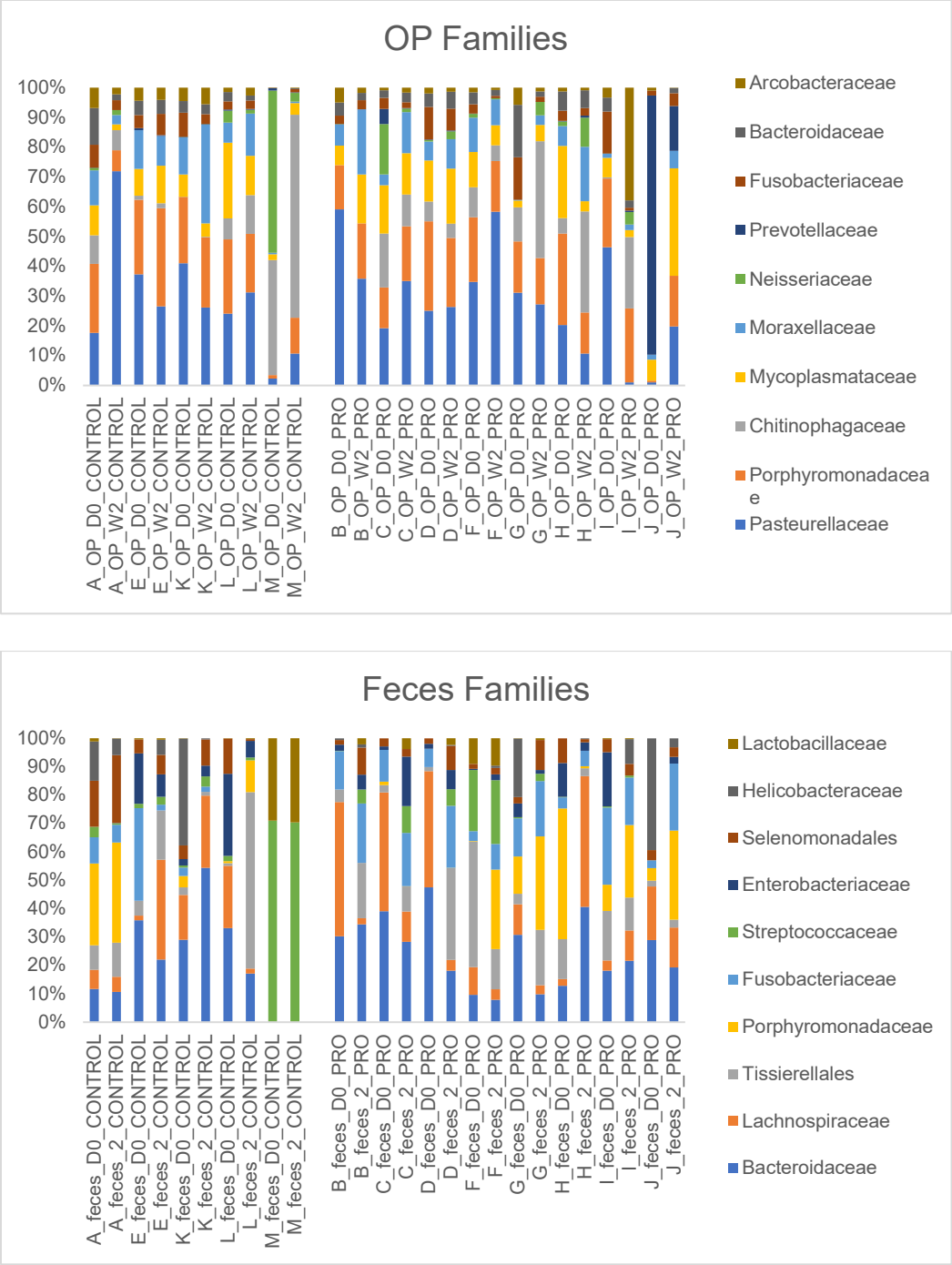


Figure 5: Stacked bar graphs showing the abundance of the microbial communities at the family taxonomic level in BALF, blood, OP and fecal sites in PRO and CONTROL groups at time D0 and W2 for 13 cats (labeled A-M)**.** For the fecal microbial community

at the family taxonomic level, 2 samples did not meet rarefaction criteria (< 1196 distinct features) and were discarded.

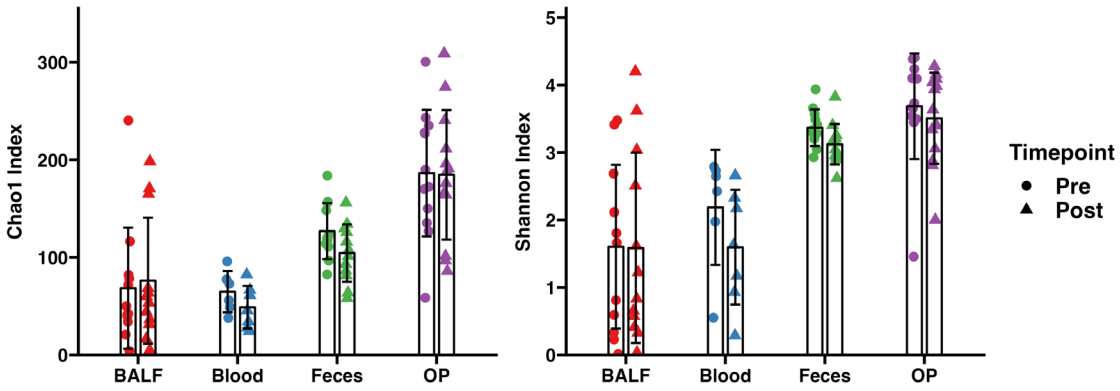


Figure 6: Alpha diversity metrics depicting richness (left) and diversity (right) in 13 asthmatic cats. Pre=D0 (circles), Post=W2 (triangles), BALF (red circles), Blood (blue circles), Feces (green circles), OP (purple circles).

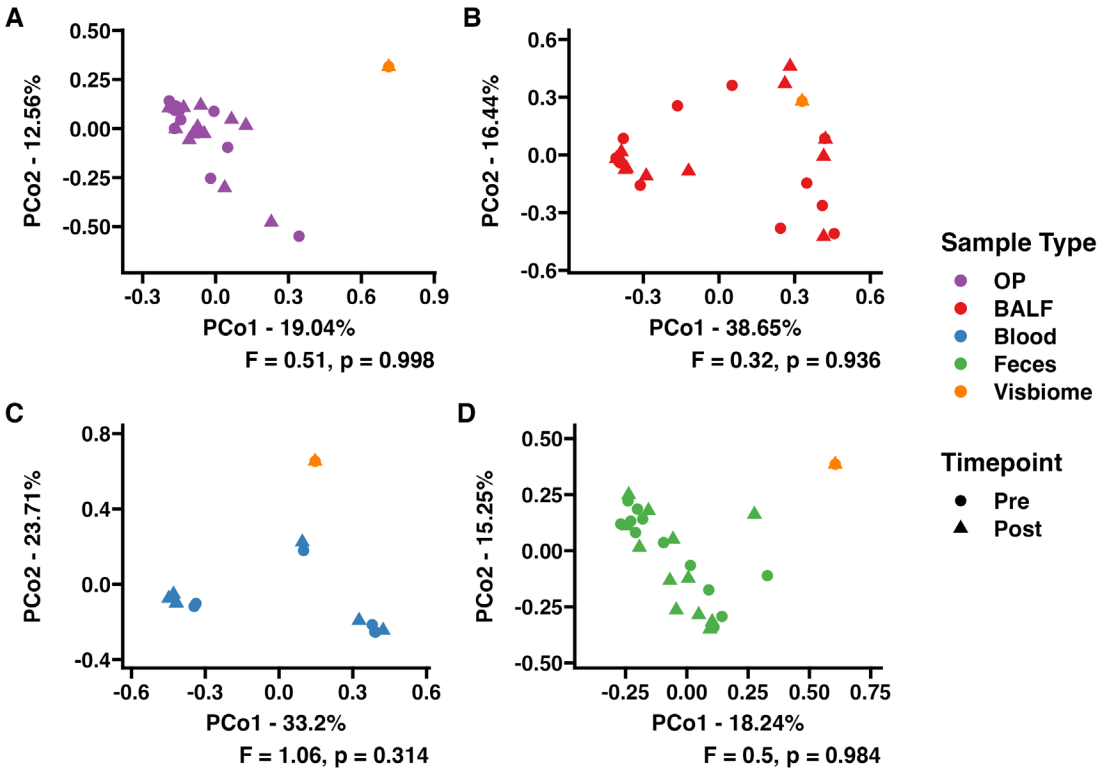


Figure 7: Principal coordinate analysis (PCA) of Bray Curtis similarity index (measurement of β-diversity) in BALF, blood, OP and fecal communities compared to Visbiome control groups in 13 asthmatic cats. Pre=D0 (circles), Post=W2 (triangles), OP (purple circles), BALF (red circles), Blood (blue circles), Feces (green circles), Visbiome (orange circles). One-way PERMANOVA showed no significant difference in community composition between collection sites.

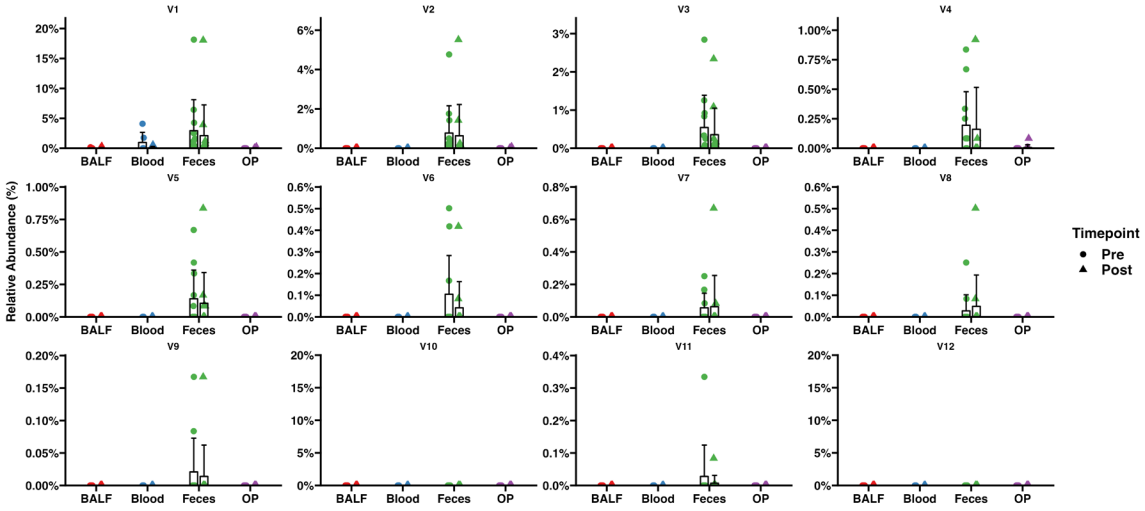


Figure 8: Scatter plot showing the relative abundance of the 12 amplicon sequence variants sequenced from the Visbiome positive controls. The lowest taxonomic level sequenced for each ASV is shown. Pre=D0 (circles), Post=W2 (triangles), BALF (red), Blood (blue), Feces (green), OP (purple); V1: species: *Streptococcus salivarius*; V2: genus: *Lactobacillus*; V3: species: *Bifidobacterium animalis*; V4: genus: *Bifidobacterium*; V5: species: *Lactobacillus helveticus*; V6: genus: *Streptococcus*; V7: genus: *Lactobacillus*; V8: genus: *Lactobacillus*; V9: genus: *Lactobacillus*; V10: genus: *Bifidobacterium*; V11: genus: *Lactobacillus*; V12: genus: *Lactobacillus*

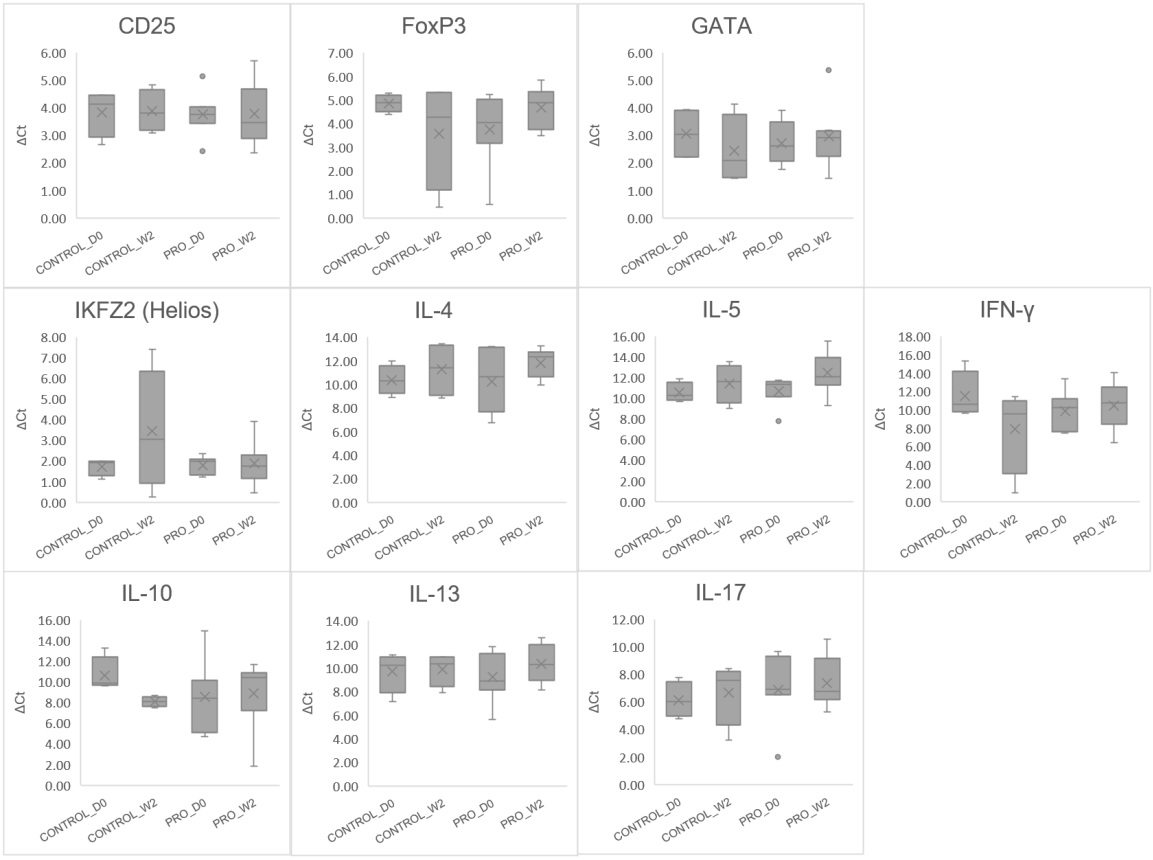


Figure 9: Box-and-whiskers plots of the ΔCt of the transcription factors, activation markers, and cytokines for relative quantification of mRNA when compared to Tub A (housekeeping gene value). The top end of the box represents the 75th percentile and the bottom end of the box indicates the 25th percentile. The line within the box represents the median. The whiskers on the top and the bottom of the boxes indicate the 95th and 5th percentiles, respectively. The filled-in circles represent outliers.

APPENDIX 2: TABLES

Target	Forward and reverse (5' to 3')	Length (bp)	Accession number
Tub A	Tgccctatccccgcatccac ccatctggttggctggYtcaaag	115	XM_045061749.1
CD25	Gctatgaccacggagtcattcgt cccgctcagaaggacgatactg	96	XM_019833862.3
FoxP3	TACGCCACCCTCATCCGCTGG CGCCACCCCAAAGGACCTCACC	144	XM_045050292.1
GATA3	Agaaccgacccctcatcaagc tctccgttggcatttcttctcc	118	XM_023256321.2
IKFZ2 (Helios)	Ggtgccttttgagagacctgct tctgggtagctgaatcgcatga	117	XM_019838622.3
IL-4	CAGGAACCTCAGCAGCATGG GCATGATCGCTTTTAGCCTTTCC	98	NM_001043339.1
IL-5	GAACTCTACTGATAGGCGACGG AGCTTTTCCACAGCATCCCC	139	NM_001009845.2
IL-10	Cgaggacccagacatcaaacagc actcttcacctgctccaccacc	132	AF060520.1
IL-13	GGTCATTGCTCTCACCTGCC TGACGCTCCACACCATGC	143	XM_006927586.3
IL-17	GTGGCTGTCCAACAAGAGGAGTCTGTCG GGGGATGCGGCTGGGGTCG	70	XM_019810830.3
IFN-γ	Aaacgggatgacttcctcaagc actttgaagagttcatttattgctttgc	86	NM_001009873.1

Table 1: Forward and reverse primer sequences for targets of interest designed and purchased using NCBI database with Primer-BLAST.

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