

Lung Microbiome Is Influenced by the Environment and Asthmatic Status in an Equine Model of Asthma

Gabrielle Fillion-Bertrand^{1*}, Robert P. Dickson^{2*}, Roxane Boivin¹, Jean-Pierre Lavoie¹, Gary B. Huffnagle², and Mathilde Leclerc¹

¹Department of Clinical Sciences, Faculty of Veterinary Medicine, University of Montreal, St. Hyacinthe, Quebec, Canada; and ²Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, Michigan

ORCID IDs: 0000-0003-4655-2102 (J.-P.L.); 0000-0002-3910-0370 (M.L.).

Abstract

There is evidence that the lung microbiome differs between patients with asthma and healthy humans, but the effect of environmental conditions and medication is unknown and difficult to study. Equine asthma is a naturally occurring chronic airway disease characterized by reversible airway inflammation and bronchoconstriction upon exposure to inhaled antigens. In the present study, we evaluated the effect that environmental conditions and disease status have on pulmonary, nasal, and oral microbiomes. Six asthmatic and six healthy horses were studied while at pasture ("low antigen exposure"), as well as when being housed indoors and fed good-quality hay ("moderate exposure") and poor-quality hay ("high exposure"). At each time point, lung function was recorded; BAL, oral, and nasal rinses were collected; and *16S rRNA* gene sequencing was performed. Asthmatic horses developed airway obstruction and

inflammation under moderate and high antigen exposure conditions, whereas nonasthmatic horses showed mild inflammation under high antigen exposure, without bronchoconstriction. Lung, oral, and nasal communities clustered by environmental condition, but only lung communities were different between healthy and asthmatic horses. The association between asthma and lung microbiome was strongest in horses under moderate antigen exposure. Pulmonary, oral, and nasal microbiomes are influenced by environmental conditions, but only the pulmonary microbiome differs between horses with and without asthma. This difference, seen mainly when airway inflammation was present in horses with asthma but not in control animals, suggests that the altered lung microbiome in asthma might not be inherent but coincident with inflammation.

Keywords: *16S rRNA* gene; microbiota; asthma; horses; pulmonary disease

The term *microbiome* refers to all the microbes (bacteria, viruses, fungi) found in a particular habitat (1). Although the skin and the gastrointestinal tract are well known to harbor trillions of bacteria, it was only with the development of culture-independent techniques that the lung, long considered a sterile organ, was found to have a diverse, yet low-biomass, microbiome (2, 3). Of known bacterial phyla, three (Proteobacteria, Firmicutes,

and Bacteroidetes) make up the majority of communities found in the healthy lung (4–10). It is speculated that these bacteria participate in maintaining a healthy environment by limiting the expansion of pathogens and by modulating the innate immune system (1, 11).

The lung microbiome of patients with asthma has been shown to differ in its diversity and composition (5, 9, 10, 12) and, in one study, in its bacterial load (12) when

compared with that of healthy individuals. Bacterial diversity has been associated with airway hyperresponsiveness (12) and airflow limitation (10). Both diversity and community composition also differ between patients with asthma showing a positive response to clarithromycin (12) or oral corticosteroids (9) and those who do not. This supports the hypothesis that the pulmonary microbiome contributes to asthma pathophysiology and could explain,

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*These authors contributed equally to this work.

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Correspondence and requests for reprints should be addressed to Mathilde Leclerc, D.V.M., Department of Clinical Sciences, Faculty of Veterinary Medicine, University of Montreal, 3200 Rue Sicotte, St-Hyacinthe, QC, J2S 2M2 Canada. E-mail: mathilde.leclerc@umontreal.ca.

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Clinical Relevance

Some studies have shown that patients with asthma have distinct lung microbiomes compared with those of healthy individuals, but numerous potential confounders, such as environment and medication, are intrinsic to such studies in humans. To address this, we compared the pulmonary, oral, and nasal microbiomes of horses with and without equine asthma receiving no medication and housed together in three different controlled environments. Lung, oral, and nasal communities clustered by environmental condition, but only pulmonary communities were different between healthy and asthmatic horses. Also, the lung microbiome was distinct from the nasal and oral microbiomes. The difference between asthmatic and healthy horses was present mainly when airway inflammation was present in horses with asthma but not in control horses, suggesting that the altered lung microbiome observed with asthma might not be inherent but coincident with inflammation. Further studies will determine if this altered lung microbiome contributes to persistent inflammation in equine and human asthma.

in part, the different phenotypes of asthma, including response to treatment. However, in these studies, between 50% and 100% of the patients were receiving corticosteroids (inhaled, oral, or both) (5, 9, 10, 12), which is an important confounding factor, especially because oral and, to a lesser extent, inhaled corticosteroids affect the lung microbiome (10). Other unresolved questions include the effect that the environment has on healthy and unhealthy pulmonary microbiomes, as well as the interactions between oral, nasal, and pulmonary bacterial communities of patients with asthma, in the absence of medication. These issues cannot be easily answered in humans, owing to ethical considerations, making the control of environmental conditions difficult and the intake of medication inevitable.

Equine asthma is a naturally occurring disease of horses affecting approximately

15% of the adult population in the northern hemisphere (13). It is characterized by reversible bronchospasm and airway inflammation (eosinophilic, mastocytic, or neutrophilic in milder forms, predominantly neutrophilic in severely affected horses) when exposed to inhaled antigens (14, 15). The use of an equine model of asthma has the advantage of allowing for multiple lung sampling in standing animals that can be kept in the same environments as healthy control animals for longitudinal studies (16).

We hypothesized that the pulmonary bacterial microbiome is influenced by the surrounding environment and differs between healthy and untreated asthmatic horses. The twofold aim of this study was to compare the bacterial pulmonary microbiome of healthy and asthma-affected horses in three distinct environments and to compare the pulmonary microbiome with oral and nasal microbiomes in those same environments (17). Part of this work was presented as a poster at the 113th American Thoracic Society International Conference in May 2017 (18).

Methods

Twelve adult horses were studied (10 mares, 2 geldings). Horses with equine asthma ($n = 6$; weight, 523.67 ± 76.65 kg; age, 16.67 ± 5.43 yr [mean $\pm$ SD]) had a well-

characterized history of reversible episodes of airway obstruction and inflammation with stabling and hay exposure. Control horses ($n = 6$; weight, 509.33 ± 27.17 kg; age, 12.83 ± 3.06 yr) had no history of respiratory obstruction with stabling and hay feeding. Horses were otherwise deemed healthy on the basis of physical examination and complete blood count. They did not receive antimicrobials or corticosteroids for at least 4 weeks before enrollment. All experimental procedures were performed in accordance with the Canadian Council for Animal Care guidelines and were approved by the animal care committee of the University of Montréal (15Rech1760).

Experimental Design

Healthy and asthma-affected horses were initially on pasture for at least 3 weeks before lung function was recorded and BAL fluid (BALF) collected ("low antigen exposure"). They were then exposed to indoor allergens in a crossover design by being housed in a barn and fed low-dust, good-quality hay ("moderate antigen exposure") and poor-quality hay (hay harvested wet to mold growth) ("high antigen exposure") for 3 weeks each.

Lung Function and Sample Collection and Processing

Lung function was measured on standing, unsedated horses, using an impulse oscillometry system, as described previously

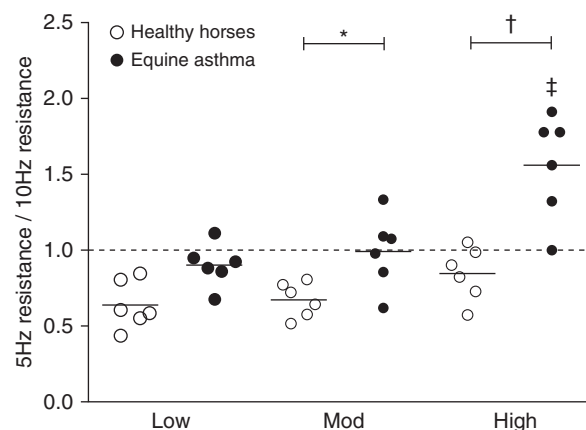


Figure 1. Lung function. Resistance at 5 Hz over resistance at 10 Hz (R5/R10 ratio) in horses with asthma (solid circles) and healthy horses (open circles) under three environmental conditions. Black lines represent the mean. A ratio superior to 1 (dashed line) is considered abnormal and reflects pulmonary obstruction. *Difference between groups ($P = 0.01$); †difference between groups ($P < 0.0001$); ‡different from low antigen exposure ($P < 0.0001$). High = high antigen exposure; Low = low antigen exposure; Mod = moderate antigen exposure.

(19) (see data supplement). Once the horses were sedated, nasal and oral cavities were sequentially rinsed with 50 ml of sterile isotonic saline. Nasal and oral washes were collected via a sterilized funnel in a conical tube and immediately placed on ice and processed within 2 hours of collection. A 5-ml aliquot was used for DNA extraction. BALF was collected on standing, sedated horses using a 2.5-m videoendoscope (Olympus Medical Systems Corp.). To avoid upper airway contamination, the videoendoscope was first advanced in a protective sheath through the nasal passages and down to the trachea. Then the videoendoscope was further advanced out of the sheath and into the right main bronchi until it lodged in a 10-mm-diameter bronchi. Additional details about the BALF collection and control specimens are provided in the data supplement. Total DNA was extracted from 5 ml of each sample using the DNeasy Blood and Tissue Kit (QIAGEN) following the manufacturer's instructions with minor modifications. Sequencing was performed using the MiSeq IEMFileVersion 4 platform with a MiSeq Reagent Kit V2 (500 cycles) (Illumina) according to the manufacturer's instructions with modifications for low-biomass samples (see data supplement).

Data Analysis

Sequence data were processed using the software mothur v.1.27.0 according to the standard operating procedure for MiSeq sequence data using a minimum sequence length of 250 bp (20). Sequences are available via the National Center for Biotechnology Information (NCBI) Sequence Read Archive (accession number PRJNA361129). We performed microbial ecology analysis using the vegan package 2.0-4 (21) in R (version 3.2.3 [2015-12-10] "Wooden Christmas-Tree") (see data supplement).

Results

Animals

There was no significant difference between groups in terms of age and weight ($P > 0.1$). As expected, only horses with equine asthma developed clinical signs of respiratory disease with antigen exposure (cough and increased respiratory efforts).

Lung Function

Asthmatic horses under moderate antigen exposure and high antigen exposure developed airway obstruction, whereas healthy horses did not (Figure 1).

Total and Differential Cell Counts

As expected, horses with equine asthma developed airway neutrophilia (14, 22, 23) with significantly higher neutrophil percentages in their BALF under moderate antigen exposure and high antigen exposure than healthy horses under the same conditions and compared with low antigen exposure. There was also an increase of eosinophils between low and moderate antigen exposure in the asthmatic group only. Also as expected, healthy horses had a modest increase in neutrophils when housed indoors, without change in function or clinical signs. The percentage of return was

lower in the equine asthma group under low and high antigen exposure, but this did not affect the total cell count or the cell count per milliliter (Figure 2; see also Table E1 in the data supplement).

Reagents and Videoendoscope Contamination

Bacterial DNA is detectable even in sterile reagents, and sequencing the contamination of low-biomass microbiome studies is a major technical challenge. Taxa detected in videoendoscope rinse specimens were indistinguishable from those detected in reagent control specimens ($P = 0.685$), indicating no appreciable contaminating signal introduced via the videoendoscope. By contrast, taxa detected in scope rinse specimens were significantly distinct from those detected in BALF specimens ($P = 0.007$) and oral and nasal specimens

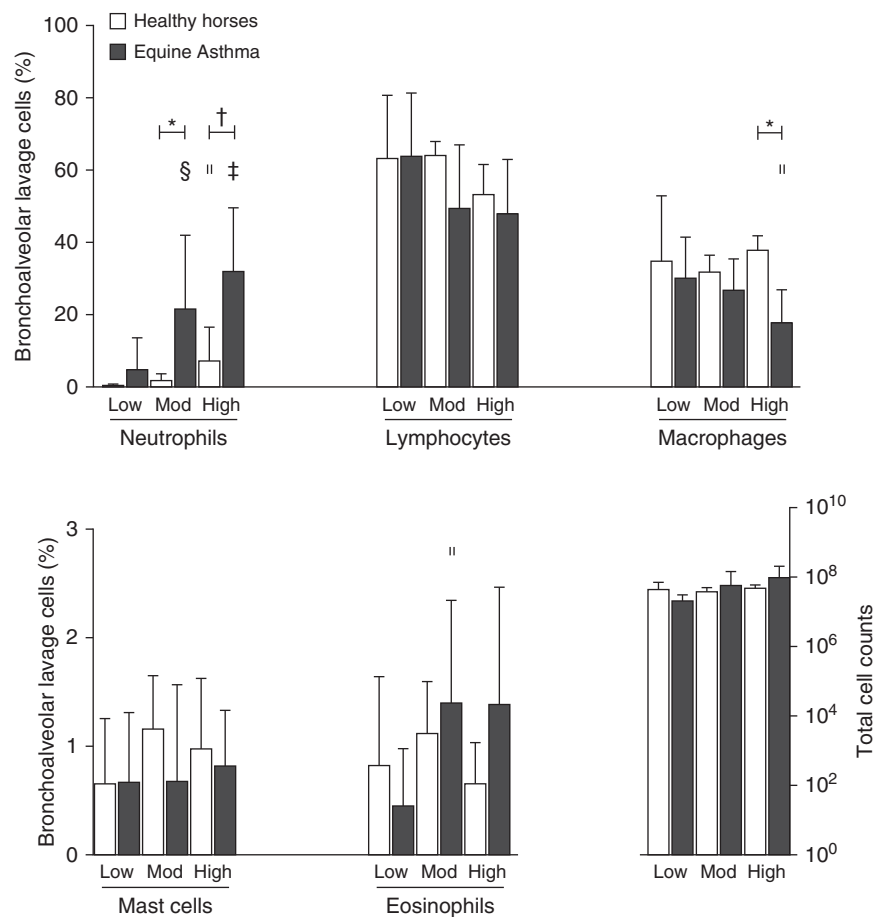


Figure 2. BAL cytology. BAL cell differentials and total cell counts in horses with asthma (solid bars) and healthy horses (open bars) under three environmental conditions. Data presented are mean $\pm$ SD. *Different between groups ($P = 0.0007$); †different between groups ($P = 0.001$); ‡different from low antigen exposure ($P < 0.0001$); §different from low antigen exposure ($P = 0.0008$); ‖different from low antigen exposure ($P \leq 0.02$).

($P = 0.001$). Similarly, taxa detected in reagent control specimens were distinct from those detected in BALF and oral and nasal specimens ($P \leq 0.001$ for all). The five most abundant operational taxonomic units (OTUs) detected in scope rinse specimens, comprising 11.9% of all sequences, were classified as *Planctomycetaceae* (OTU303), *Bacteroides* (OTU029), *Enterobacteriaceae* (OTU006), unclassified Alphaproteobacteria (OTU617), and Anaerolineaceae (OTU087). Collectively, these OTUs comprised only 1.6% of sequences detected in BALF specimens. The five most abundant OTUs detected in reagent control specimens, comprising 23.1% of all sequences, were classified as *Bacteroides* (OTU029), *Prevotella* (OTU315), *Acinetobacter* (OTU190), *Saprosiraceae* (OTU787), and *Porticoccus* (OTU350). Collectively, these OTUs comprised only 0.49% of sequences in BALF specimens, 0.13% of sequences in oral specimens, and 0.23% of sequences in nasal specimens. We thus found minimal evidence of sequencing contamination in our experimental specimens. No OTUs were excluded from analysis of BALF, oral, or nasal specimens.

Bacterial DNA Burden

There was significantly more bacterial DNA, as measured using quantitative PCR of the

16S *rRNA* gene, in oral than in nasal samples. Both oral and nasal samples contained more bacterial DNA than BALF. There were no significant differences between groups or environmental conditions (Figure 3).

Sequencing Results

Means of 45,561, 46,170, and 9,449 high-quality reads were obtained for oral, nasal, and BALF specimens, respectively. Five samples had fewer than 500 sequences and were excluded from the analysis. These represented one scope rinse, two BALF samples from control horses in the low antigen exposure environment, and two BALF samples from asthmatic horses in the high antigen exposure environment.

Pulmonary versus Nasal and Oral Microbiomes

Oral and nasal communities had greater richness (number of detectable OTUs) than BALF communities. Shannon diversity indexes (taking into account richness and evenness across the community) also showed greater diversity in oral and nasal specimens than in BALF samples (Figure 4). BALF specimens had significantly greater richness and diversity than videoendoscope rinse specimens ($P = 0.03$ and 0.002 , respectively). Sequences derived from oral, nasal, and BALF specimens represented 871 different OTUs from at least 23 families.

Bacterial taxa were first studied in anatomic sites. Pulmonary, oral, and nasal specimens contained distinct microbiome, whether studied collectively ($P < 0.001$) (Figure 5) or stratified by environmental conditions at the time of bronchoscopy ($P < 0.01$ for all comparisons). This site specificity of respiratory microbiome remained significant when analysis was restricted to either healthy or asthmatic horses ($P < 0.01$ for all comparisons), and even when restricted by groups and environments ($P < 0.05$ for all comparisons with the single exception of nasal vs. BALF microbiome in asthmatic horses in the low antigen exposure environment [$P = 0.16$]).

BiPlot and mvabund analyses both identified a *Pasteurella*-classified OTU (OTU001) as relatively enriched in nasal specimens. This OTU, which was aligned with 100% coverage and identity via NCBI BLAST with *Pasteurella multocida*, comprised $39.5 \pm 2.7\%$ of sequences in nasal specimens. By contrast, the same techniques identified an *Actinobacillus*-classified OTU (OTU002) as relatively enriched in oral rinse specimens ($23.2 \pm 11.3\%$ of sequences). Both of these site-defining OTUs were detected in BALF specimens, but they comprised only $3.0 \pm 13.1\%$ and $1.9 \pm 5.8\%$ of BALF sequences, respectively. BALF communities were composed of a large number of low-abundance sequences and were not dominated by any specific site-defining OTU. The most abundant taxa detected in BALF specimens, compared with their representation in other specimens, are shown in Figure 6.

Influence of the Environment on Pulmonary, Nasal, and Oral Microbiomes

Richness and Shannon diversity index of pulmonary, nasal, and oral microbiomes did not vary significantly with environmental conditions. Only an increase in richness was observed for the videoendoscope specimens sampled under moderate and high antigen exposure. In a given horse, there was little temporal stability of the pulmonary microbiome over time in the three different environments. Lung communities clustered strongly by environmental condition ($P = 0.004$ by permutational multivariate analysis of variance) (Figure E1), but they did not cluster by individual horse ($P = 0.75$). In a direct comparison of Bray-Curtis community dissimilarity, lung communities

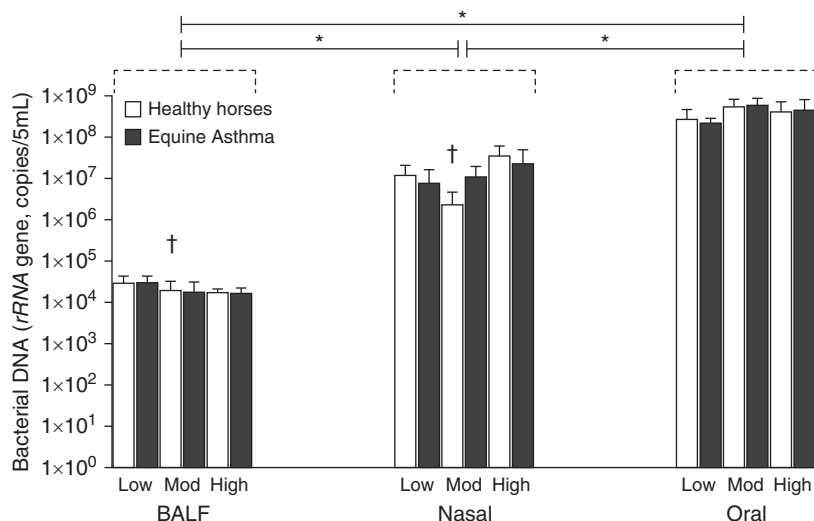


Figure 3. Bacterial 16S *rRNA* gene quantification by PCR. 16S *rRNA* gene quantification by PCR in BAL fluid (BALF), nasal washes, and oral washes of horses with asthma (solid bars) and healthy horses (open bars) under three environmental conditions. Data presented are mean $\pm$ SD. *Difference between dashed brackets ($P \leq 0.01$); †no significant difference between these groups. No significant difference was found in 16S quantification between asthmatic and control animals in all environments for each of the three sites. *rRNA* = ribosomal RNA.

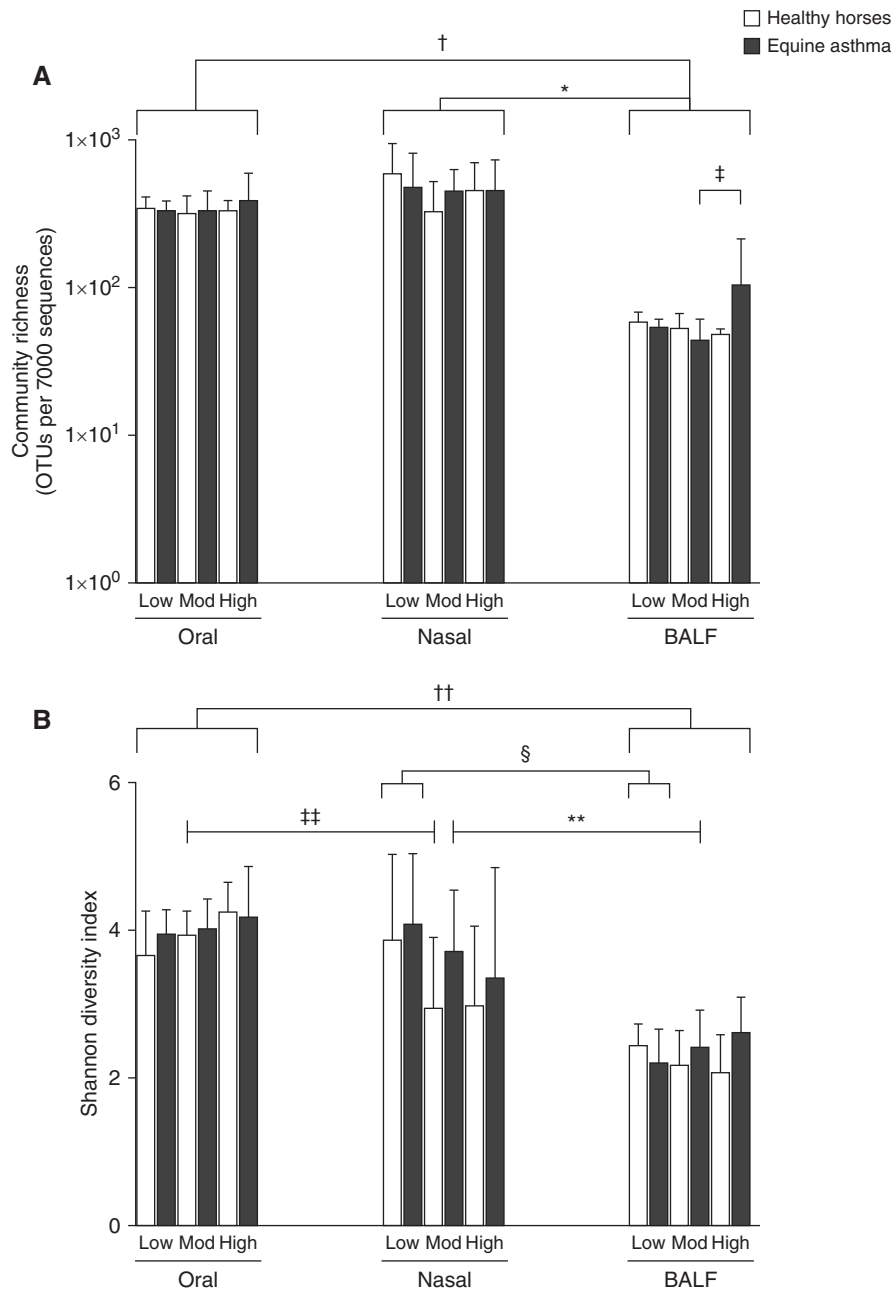


Figure 4. Richness and diversity. (A) Community richness and (B) Shannon diversity indexes in BALF, nasal washes, and oral washes of horses with asthma (solid bars) and healthy control horses (open bars) under three environmental conditions. Data presented are mean $\pm$ SD. *Difference between groups ($P \leq 0.0002$) for all antigen exposure; †difference between groups ($P \leq 0.0003$) for all antigen exposure; ‡difference between conditions ($P = 0.002$); §difference between groups ($P \leq 0.002$) for all comparisons; **difference between nasal and BALF ($P = 0.002$); ††difference between groups ($P \leq 0.008$) for all comparisons except for oral versus BALF for equine asthma under high antigen exposure; ‡‡difference between oral and nasal ($P = 0.01$). OTU = operational taxonomic unit.

were more similar in horses in the same environment than across environments (Bray-Curtis indices comparison; $P < 0.01$). Importantly, this clustering by environmental condition was not present in the taxa detected

in scope rinse specimens ($P = 0.58$), confirming that clustering by environment was not attributable to batch processing or procedural bias. No OTUs in BALF specimens were identified via BiPlot or

mvabund as specific to an environmental condition, but a trend was found for decreased Gammaproteobacteria among high antigen exposure specimens ($P = 0.07$). Oral and nasal communities also clustered by environmental condition ($P < 0.001$ and $P = 0.02$, respectively). The effect of environment on the lung microbiome was more strongly significant in healthy ($P = 0.004$) than in asthmatic horses ($P = 0.035$).

Influence of Health Status on Pulmonary, Nasal, and Oral Microbiomes

No difference in richness or Shannon diversity index was observed between microbiome detected in the lung, mouth, or nose of healthy and asthmatic horses (Figure 4). Pulmonary communities of healthy and asthmatic horses were significantly different at the family level of taxonomic designation ($P = 0.006$ by permutational multivariate analysis of variance) (Figure 7A), but not at the OTU level of taxonomy. No specific bacterial family drove this difference, but there was a trend for an overrepresentation of the Pasteurellaceae family in asthmatic horses ($P = 0.06$). Interestingly, when restricting analysis by environment, the difference between asthmatic and healthy lung microbiome was significant only in the moderate antigen exposure environment ($P = 0.015$) (Figure 7B), with still no specific OTUs being over- or underrepresented. Oral and nasal microbiomes were not significantly different between healthy and asthmatic horses (adonis function; $P = 0.68$ and $P = 0.23$, respectively), regardless of the environmental conditions.

In summary, bacterial communities were different between lung, nose, and mouth, and they changed with antigen exposure at the OTU level of taxonomic designation and were not tested at the other levels. Bacterial communities were also different in the lungs (but not in the nose and mouth) between asthmatic and healthy horses at the family level of taxonomy but not at the OTU or phylum level.

Discussion

To the best of our knowledge, this is the first report of sequential analysis of pulmonary bacterial microbiome in sick and healthy animals housed in different environments. Because asthmatic and healthy horses

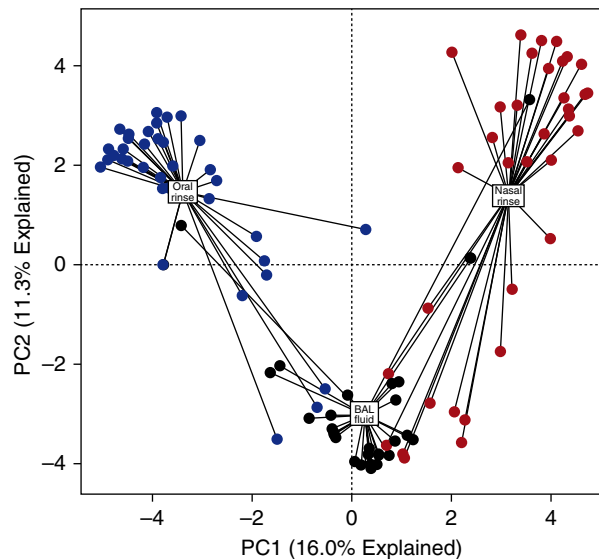


Figure 5. Principal component (PC) analysis of BALF (black circles), nasal rinses (red circles), and oral rinses (blue circles) of asthmatic and healthy horses. Specimens from the three environmental conditions (low antigen exposure, moderate antigen exposure, and high antigen exposure) are represented at the family level of taxonomic designation. $P < 0.001$ for all comparisons.

respond differently to antigen exposure (airway inflammation and obstruction with low degree of antigenic exposure in horses with asthma vs. mild inflammation without obstruction with high degree of antigenic exposure in healthy individuals), this study offers a unique opportunity to look at the effect that the environment, with or without concurrent inflammation, has on the lung microbiome. The main findings of this study are that the pulmonary microbiome 1) is influenced by environmental conditions, more so in health than in asthma, and 2) differs between horses with and without asthma, but mainly when horses with asthma have antigen-induced airway inflammation and obstruction. In addition to this, oral and nasal microbiomes are influenced by the surrounding environment, but not by disease state. Finally, in horses, the oral, nasal, and pulmonary microbiomes are distinct from one another, regardless of the environmental condition or disease status.

Pulmonary, Oral, and Nasal Microbiomes Are Influenced by the Environment

We found that oral, nasal, and pulmonary communities clustered by environment but not by individual or order of sampling. Because “environment” in this context involves a change in both housing and forage type, variation in the oral

microbiome was expected. In humans, variation in the oral microbiome over time was reported in some (24) but not all studies (25, 26), and even when stability was observed, external changes such as living abroad had repercussions on the oral microbiome (25). The clustering of pulmonary communities by environment was less expected. The fact that short-term changes in housing and forage type can alter the pulmonary bacterial microbiome of mature, healthy animals could have important consequences on future experiments. Of note, the indoor environmental conditions chosen for this experiment were known to have the potential of inducing pulmonary inflammation (severe in horses with asthma, mild and transient in healthy horses [14]) but were also representative of normal housing conditions for horses. It is therefore difficult to separate the effect of environment by itself (exposure to different bacteria via inhalation and microaspirations) from changes in microbial populations secondary to the host developing airway inflammation. The fact that variation with environmental conditions is more significant in healthy horses indirectly supports the direct role of the environment, but the fact that group discrimination is present only when there is the largest difference in BALF inflammation supports the prominent role of inflammation. For this reason, we suggest

that future studies on airway microbiome include healthy controls living in similar environmental conditions to limit unintended bias.

Pulmonary Microbiome Is Altered in Equine Asthma

On the basis of β -diversity analysis at the family level, the pulmonary microbiome of asthma-affected horses seems distinct from that of healthy horses in terms of their relative abundance of bacterial families, whereas oral and nasal microbiomes are not. This is consistent with the differences observed in patients with asthma (5, 10, 12), but because corticosteroids can influence the diversity of lung communities (10), at least some of the differences observed in other studies could be secondary to corticosteroid exposure. In the present study, we show that the altered microbiome observed in chronic pulmonary inflammatory conditions can be present even in animals receiving no inhaled or oral corticosteroids and living in the same environment as their controls. Consistent with previous findings, we found differences in relative abundance (5, 10, 12), but unlike others, we did not observe differences in overall diversity (5, 10, 12) or bacterial burden (12). Despite the variations between groups and environments, the most commonly represented phylum in healthy and asthmatic lungs was Proteobacteria, followed by Bacteroidetes and Firmicutes, three phyla commonly found in previous studies of humans and animals (4, 5, 10, 27).

As mentioned above, it is difficult to separate the effect of the environment and the effect of inflammation induced by inhaled antigens, because both could have an effect on the local environment of the lungs. Because the difference between groups is driven mainly by the moderate antigen exposure environment, where inflammation is present in asthmatic horses only, and because the lower dispersion of healthy BALF is lost when inflammation is present (Figure 7B), it is possible that the induced inflammation changes the microenvironment of the bronchoalveolar space, creating new niches for bacterial growth (28). This could explain similarities in the lung microbiome of healthy and asthma-affected horses in the high antigen exposure environment (whereas healthy horses also have some degree of pulmonary inflammation) and suggests that the role of the immune system is important

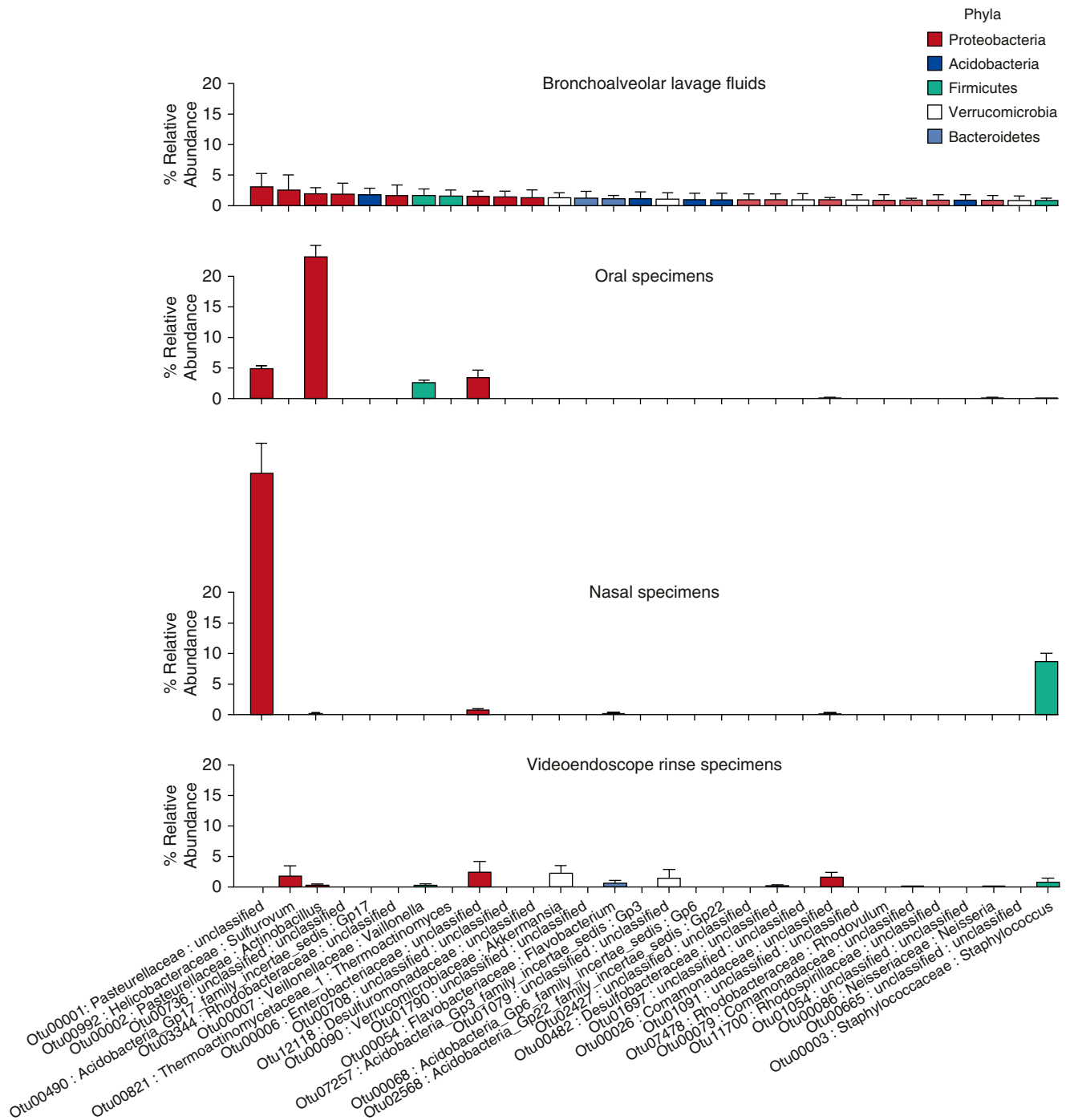


Figure 6. Comparison of the 30 most common OTUs found in BALF.

in regulating the lung microbiome. Bacterial dysbiosis could then induce a disproportionate reaction of the immune system, leading to an inflammatory loop in which primed inflammatory cells of the lungs disturb the bacterial population by secreting inflammatory cytokines (28), to which bacteria may have the potential to adapt (29). Alternatively, the communities

inhaled in this environment could themselves drive, in part, the inflammation. An inherently altered microbiome cannot be ruled out on the basis of our data, owing to the low power to detect differences when environments are analyzed separately. Also, the relatively low resolution of the MiSeq platform could have played a role in the lack of difference observed at the OTU

level. Finally, it is also possible that the variation observed in healthy horses reflects the normal adaptability of a healthy lung to adverse conditions.

Pulmonary Microbiome Is Distinct from Oral and Nasal Microbiomes

Microbiomes of the mouth, nose, and lungs are distinct in horses. The dissimilarity between

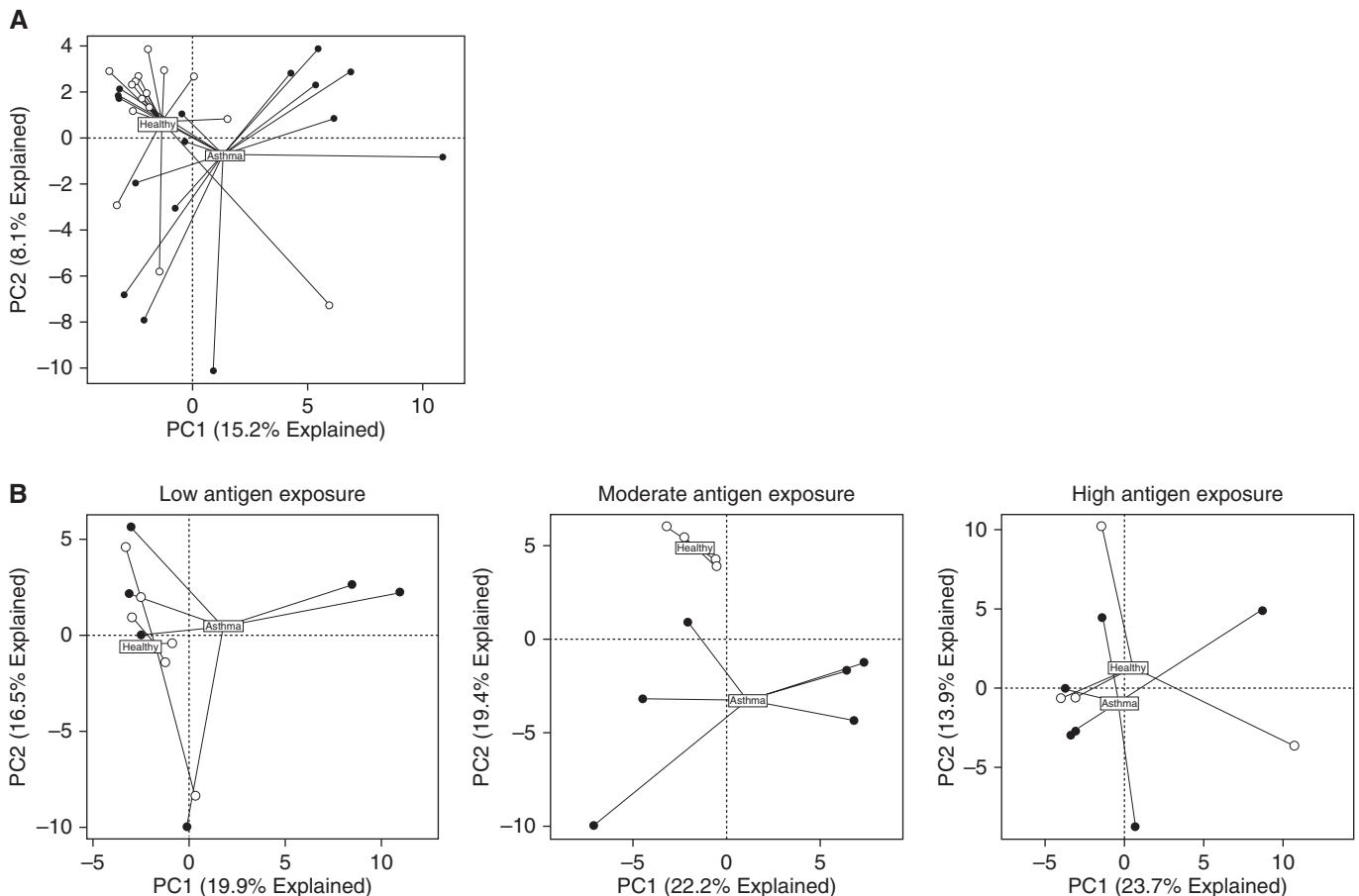


Figure 7. PC analysis of the pulmonary microbiome of healthy and asthmatic horses. Specimens are represented at the family level of taxonomic designation. (A) Pooled data. (B) Horses separated by environments.

nasal and lung communities indicates that the method of sampling effectively limited contamination by the nasal passages and avoided significant carryover from the upper airways, a concern raised previously (30). Nevertheless, a greater similarity could have been expected for biological reasons, such as the large minute ventilation (60 L/min at rest) of these obligate nasal breathers (31). It also supports the concept that the lung microbiome is its own ecological niche with distinct ecologic pressures (2) and not merely a diluted version of the upper airways' bacterial content (7). The oral and pulmonary communities appear to cluster more in humans than in horses (5, 7, 30, 32), possibly because microaspirations played a lesser role in populating the lower airways of horses owing to their long velum separating the oropharynx from the nasopharynx, their nonupright position, and their eating and sleeping behaviors.

Potential Pitfalls

Contamination of the samples with irrelevant bacterial DNA is a major concern

in studies sequencing low-biomass microbiome. As expected, numerous bacterial DNA sequences were found in all reagents and scope rinse samples, but because they were different from the biological samples and did not cluster by environment, they played a minor role in the overall biological sample analysis. Also, the sequencing depth did not allow for all OTUs to be identified at the species level, and for some OTUs, the identification could have been limited by the fact that databases are based in large part on bacteria found in humans. There is also a relatively low macrophages-to-lymphocytes ratio in equine BALF (in our data and in the literature [33]) that could influence the host interactions with the bacterial microbiome and limit interspecies extrapolations. Finally, the low number of horses in each group could have precluded identifying statistically significant differences between groups in the low and high antigen exposure environments.

Conclusions

This study shows that in untreated animals housed together, pulmonary, oral, and nasal microbiomes are influenced by environmental conditions, but only the pulmonary microbiome differs between horses with and without asthma. The difference between asthmatic and healthy horses was present mainly when airway inflammation was present in horses with asthma but not in control horses, suggesting that the altered lung microbiome observed in asthma might not be inherent but coincident with inflammation. Further studies will determine if this altered lung microbiome contributes to persistent inflammation in equine and human asthma. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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LUNG MICROBIOME IS INFLUENCED BY THE ENVIRONMENT AND ASTHMATIC STATUS IN AN EQUINE MODEL OF ASTHMA

Gabrielle Fillion-Bertrand, Robert P Dickson, Roxane Boivin, Jean-Pierre Lavoie, Gary B Huffnagle, Mathilde Leclere

Online data supplement

Methods

Lung function

Lung function was measured on standing, unsedated horses, using an impulse oscillometry system (IOS), as previously described (E1). Pulsated electrical waves from 0.5 Hz to 10 Hz were applied for 30 seconds to the respiratory system with a loud speaker. A mask was sealed on the nose of horses and they breathed through a heated pneumotachograph (E2). Pressure and flow signals measured were processed by fast Fourier transform and used to calculate resistance, reactance and impedance. Lung function was reported as the resistance at 5 Hz over the resistance at 10 Hz (R5/R10 ratio), used as a simple proxy for the positive frequency dependence observed in healthy horses over 5 Hz, which is lost as airway resistance increases in exacerbated equine asthma (E1, E3).

Bronchoalveolar lavages

Bronchoalveolar lavage fluid was collected on standing, sedated horses using a 2.5 m videoendoscope (Olympus Medical System Corp., Tokyo, Japan). To avoid upper airway contamination, the videoendoscope was first advanced in a protective sheath through the nasal passages and down to the trachea. Then the videoendoscope was further advanced out of the sheath

and into the right main bronchi until it lodged in a 10 mm diameter bronchus. Two 250 ml boluses of warm isotonic saline were instilled and aspirated as described previously (E4). Between each horse, the videoendoscope and the protective sheath were disinfected with multi-enzymatic solution, immersed for 10 minutes in a 1 % concentrated 21.4 % W/W Potassium monopersulfate solution (Virkon®, 02125021, Vetoquinol inc. Lavaltrie, QC, Canada), and rinsed with ultra-pure injectable pharmaceutical grade water. BALF was immediately placed on ice and processed within 2 hours of collection. Thin-layer cells were prepared with a cytocentrifuge (Rotorfix, Hettich, Berlin, Germany, 1000 rpm, 5 minutes) and stained with a modified Wright-Giemsa (DiffQuick, Fisher Scientific, Waltham, MA, USA). Differential cell counts were obtained from a count of approximately 400 cells. 5 mL aliquots were used for immediate DNA extraction, and 15 mL was stored at -80°C. Remaining BALF cells were centrifuge at 1600 rpm at 4 °C, washed twice in PBS 1X and total cell count was determined (ADAM automatic Cell Counter, Montreal-Biotech Inc., Montreal, QC, CA).

Negative and positive sequencing control specimens

Prior to each BALF collection, 50 mL of sterile saline was instilled through the main channel of the videoendoscope, collected in sterile tubes, and processed as the other samples above (scope rinse specimens). Twenty reagent control specimens (sterile water, buffers used in DNA extraction) were also sequenced. A mock community of known bacterial DNA composition was used to evaluate run-to-run variation.

DNA extraction

Total DNA was extracted from 5 mL of each sample using QIAGEN DNeasy® Blood & Tissue Kit (Qiagen, Toronto, ON, CA) following manufacturer's instructions with minor modifications.

Briefly, samples were centrifuged at $16,000 \times g$ for 30 min and 360 μL of lysis buffer was added to cell pellets and transferred to 0.7 mm garnet bead tubes (VWR, Radnor, PA, USA). Samples were bead beaten for 1 min at the homogenize setting of a Mini-BeadBeater-8 (Biospec, Bartlesville, OK, USA) and centrifuged for 1 min at $16,000 \times g$. Samples were incubated for 1h at 55°C with proteinase K and 30 min at 70°C with a guanidine hydrochloride buffer provided with the kit. We then continued with the DNeasy® Blood & Tissue Kit protocol (Qiagen) starting with step 3 (DNA precipitation with ethanol).

High throughput sequencing

Sequencing was performed using the Illumina MiSeq IEMFileVersion 4 platform, using a MiSeq Reagent Kit V2 (500 cycles), according to the manufacturer's instructions with modifications for low biomass samples. The V4 region of the bacterial 16S rRNA gene was amplified using published primers (E5) and the dual-indexing sequencing strategy developed and described by Kozich *et al.* (E6). Briefly, Accuprime High Fidelity Taq was used in place of Accuprime Pfx SuperMix. Primary PCR cycling conditions were 95°C for two minutes, followed by 20 cycles of touchdown PCR (95°C 20 seconds, 60°C 20 seconds and decreasing 0.3 degrees each cycle, 72°C 5 minutes), then 20 cycles of standard PCR (95°C for 20 seconds, 55°C for 15 seconds, and 72°C for 5 minutes), and finished with 72°C for 10 minutes.

Quantitative PCR (mixed bacterial DNA)

Relative bacterial load was estimated by quantifying bacterial DNA using qPCR. QuantiTect SYBR Green PCR Kit (Qiagen, Toronto, ON, CA) was used with modified universal primers for bacterial DNA region (Forward: 5'-ACACGGCCCAGACTCCTAC-3' and Reverse: 5'-TATTACCGCGGCTGCTGGC-3'). 2 μL of DNA was added to obtain a final reaction volume of

20 µL containing 0.5 µM of each primer. Amplification of the DNA was performed in a Rotor-Gene RG3000 (Corbett Research, Sydney, AS). The parameters used for quantifying the mixed 16S rRNA gene were as follows: 95°C for 10 min, followed by 50 cycles at 95°C for 10 s, 59°C for 15 s and 72°C for 20 s. Copy number of 16S in each sample was calculated relative to a standard curve.

Data analysis

Sequence data was processed and analyzed using the software mothur v.1.27.0 according to the Standard Operating Procedure for MiSeq sequence data using a minimum sequence length of 250 base pairs (E7). For each experiment and sequencing run, a shared community file and a phylotyped (genus-level grouping) file was generated using operational taxonomic units (OTUs) binned at 97 % identity generated using the `dist.seqs`, `cluster`, `make.shared` and `classify.otu` commands in mothur. OTU numbers were arbitrarily assigned in the binning process and are referred to throughout the manuscript in association with their most specified level of taxonomy. Classification of OTUs was carried out using the mothur implementation of the Ribosomal Database Project (RDP) Classifier and the RDP taxonomy training set 9 (fasta reference = `trainset9_032012.pds.fasta`, taxonomy reference = `trainset9_032012.pds.tax`), available on the mothur website. Sequences are available via the NCBI Sequence Read Archive (accession number PRJNA361129).

We performed microbial ecology analysis using the vegan package 2.0-4 (E8) in R (version 3.2.3 (2015-12-10) -- "Wooden Christmas-Tree". Copyright (C) 2015 The R Foundation for Statistical Computing. Platform: x86_64- w64-mingw32/x64 (64-bit)) (E9). For relative abundance and ordination analysis, samples were normalized to the percent of total reads and we restricted analysis to OTUs that were present at greater than 1 % of the sample population; all OTUs were included

in diversity analysis. Direct community similarity comparisons were performed using the Bray-Curtis similarity index. We performed ordinations using Principal Component Analysis on Hellinger-transformed normalized OTU tables generated using Euclidean distances (E10). We determined significance of differences in community composition using PERMANOVA (*adonis*) with 10 000 permutations using a Euclidean distance matrix.

Weights and ages were analyzed by unpaired two-tailed t-tests. Lung function, BALF differentials, 16S quantification by qPCR, and community diversity and richness data was analyzed using a repeated measures linear model. Normality was assessed by visual inspection of the data and Kolmogorov-Smirnov tests. Data was log-transformed where indicated. Spearman correlations were done between sequencing and qPCR quantification. Unless mentioned otherwise, standard deviation ($\pm$ SD) is reported.

Identification of procedural contaminants

Reagents used in DNA isolation and library preparation contain bacterial DNA that can contaminate sequence-based studies of microbial communities (E11). As detailed above, we collected and sequenced 36 scope rinse specimens and 20 reagent control specimens to identify the influence of procedural contamination. Specimens were processed in a randomized order to minimize the risk of false pattern formation due to reagent contamination. We compared the bacterial taxa detected in control specimens using PERMANOVA (*adonis*).

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Figure Legends

Figure E1. Principal component (PC) analysis of bronchoalveolar lavage fluid (BALF) in low antigen exposure conditions (LOW, blue circles), moderate antigen exposure (MOD, green circles) and high antigen exposure (HIGH, red circles). Specimens are represented at the OTU level of taxonomic designation. Lung communities clustered by environmental conditions (PERMANOVA, $P = 0.004$).

Table E1. Bronchoalveolar lavage cell counts and percentage of volume return. *: Different from low antigen exposure, †: Difference between groups in the same environment.

	Low antigen exposure		Moderate antigen exposure		High antigen exposure	
	Mean ± SD Healthy	Equine asthma	Mean ± SD Healthy	Equine asthma	Mean ± SD Healthy	Equine asthma
Neutrophils (x10⁴ cells/mL)	0.10±0.11	0.71±1.36	0.32±0.35 †	5.30±5.5 *†	1.59±2.19 *†	15.81±15.22 *†
Lymphocytes (x10⁴ cells/mL)	12.71±6.46	8.84±4.30	10.27±2.39	13.60±16.39	10.98±2.60	19.88±23.59
Macrophages (x10⁴ cells/mL)	6.02±2.52	4.18±2.05	4.95±0.44	6.64±7.92	7.90±2.28	11.02±19.59
Mastocytes (x10³ cells/mL)	1.18±1.05	1.14±1.22	1.82±0.74	1.53±2.13	1.96±1.08	4.18±7.47
Eosinophils (x10³ cells/mL)	1.67±2.03	0.50±0.52	1.78±0.93	3.21±3.14 *	1.36±0.86 *	3.20±1.96 *
Total cell count (x10⁵ cells/mL)	1.91±0.76	1.39±0.48	1.59±0.29	2.60±2.85	2.08±0.46	4.74±5.63
Volume return (%)	45 ± 13 †	30 ± 8 †	48 ± 12	36 ± 12	46 ± 6 †	32 ± 7 †

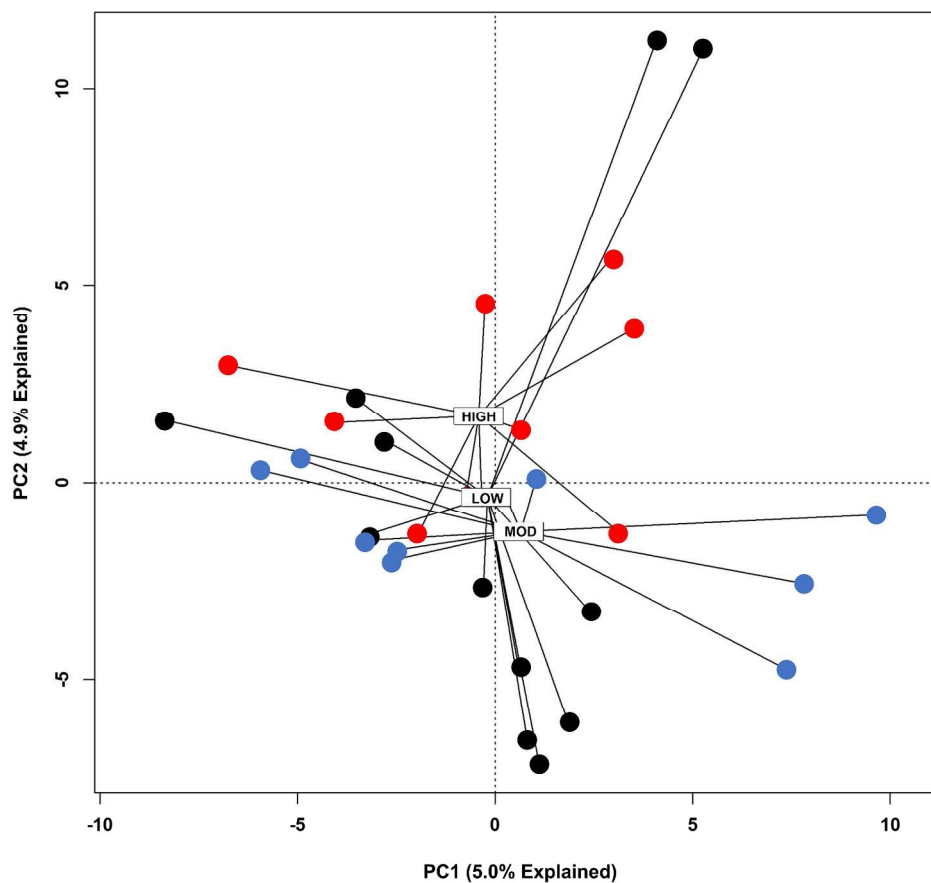


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