THE EFFECT OF LOW-DUST FORAGES AND THE ROLE OF PRO-RESOLVING LIPID MEDIATORS IN MILD-MODERATE EQUINE ASTHMA

by

Carla Josefa Olave Olivares

A Dissertation

Submitted to the Faculty of Purdue University

In Partial Fulfillment of the Requirements for the degree of

Doctor of Philosophy

Department of Veterinary Clinical Sciences

West Lafayette, Indiana

August 2020

THE PURDUE UNIVERSITY GRADUATE SCHOOL
STATEMENT OF COMMITTEE APPROVAL

Dr. Laurent L. Couetil, Chair
College of Veterinary Medicine

Dr. Harm HogenEsch
College of Veterinary Medicine

Dr. Jae H. Park
School of Health Sciences

Dr. John Burgess
Department of Nutrition Science

Approved by:
Dr. J.C. Scott-Moncrieff

ACKNOWLEDGMENTS

First, I am grateful for the financial support that made this dissertation possible; my thanks goes to the Grayson-Jockey Club Research Foundation, USDA National Institute of Food and Agriculture, Animal Health project (Grant/Award Number: IND020767AH), Boehringer Ingelheim VetMedica (Advancement in Equine Research Award), Equine Guelph, State of Indiana, Purdue University College of Veterinary Medicine research account funded by the total wager tax, Haygain, and C&M Forage.

I would like to express my sincere gratitude to my advisor, Dr. Couetil, for his continuous support, knowledge, advice, patience, motivation, and guidance during my PhD. I would like to thank Dr. Ivester for her time, patient, and support, but especially for her invaluable friendship. Additionally, I would like to thank the rest of my advisory committee, Dr. HogenEsch and Dr. Park, and Dr. Burgess for their important advice and guidance.

My sincere thanks goes to Donna Griffey, RVT, Laura Murray, RVT, Theresa Jeffrey, and Dr. Mukhopadhyay, for their priceless technical support, and to numerous undergraduate students for all their hard work. A special thanks to Emily Hess, Kylie Zehner, and Mary Kate Grubbs for all the help and long days at the racetrack.

This dissertation would not have been possible without the support of trainers, track personnel, and horses, especially to Mr. Joe Davis, Mr. Miguel Cazarez, and Mr. Jose Cazarez, for their enormous support and availability.

I would also like to acknowledge Dr. Uberti and Dr. Moran for their encouragement and letters of recommendation; without them, I wouldn't be here.

Finally, I want to recognize my support system. I'm grateful to my parents, brother, family, and friends in Chile for their continuous encouragement and advice, and also to those friends that I feel lucky to call my Purdue family; thank you all for being there whenever I needed and for making this race a better one.

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LIST OF ABBREVIATIONS

EA	Equine asthma
ALX/FPR2	Formyl peptide receptor 2
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
COPD	Chronic obstructive pulmonary diseas
DHA	Docosahexaenoic acid
DRV1	D-series resolvin ligands-1
DRV2	D-series resolvin ligands-2
EHV	Equine herpesvirus
EPA	Eicosapentaenoic acid
ERV	E- series resolvin ligand
FAs	Fatty acids
IAD	Inflammatory airway disease
IgE	Immunoglobulin E
IL	Interleukin
LAL	Limulus amebocyte lysate
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
Lx	Lipoxins
NFκB	Nuclear Factor-κB
PG	Prostaglandin
PM1	Particles less than 1 μm
PM10	Particles less than 10 μm
PM2.5	Particles less than 2.5 μm
PRLM	Pro-resolving lipid mediators
PUFAs	Polyunsaturated fatty acids
RAO	Recurrent airway obstruction
RvD	D-series resolvins
RvE	E-series resolvins
SD	Standard deviation
Th-1	T-helper 1
Th-17	T-helper 17
Th-2	T helper 2
TNF-α	Tumor necrosis factor-α
TW	Tracheal wash
Ω-3	Omega-3 polyunsaturated fatty acids
Ω-6	Omega-6 polyunsaturated fatty acids

ABSTRACT

Mild-moderate equine asthma (EA) is a commonly encountered disease of racehorses that affects performance. Decreasing dust exposure is crucial in the treatment of equine asthma. Dry hay, because of its high dust content, is known to increase the risk of airway inflammation. Feeding haylage, steamed hay, or hay pellets instead can help to decrease dust exposure. Haylage may also contribute to the resolution of airway inflammation by providing higher levels of omega-3 polyunsaturated fatty acids (Ω-3). Higher levels of Ω-3 are associated with an increase in pro-resolving lipid mediators (PRLM), essential molecules in the resolution of airway inflammation.

The studies presented in this dissertation were designed to test the hypothesis that feeding low-dust forages would decrease airway inflammation in racehorses, and that haylage would provide superior resolution of airway inflammation compared to other low dust forages due to changes in systemic Ω-3 and PRLM concentrations associated with increased dietary Ω-3 intake.

Three clinical trials were conducted to determine the effect of low-dust forages on airway inflammation. Environmental exposures were measured at the breathing zone, and bronchoalveolar lavage (BAL), and differential cytology counts were performed as measure of airway inflammation.

The first clinical trial was a pilot study performed with 7 Standardbred racehorses. Horses were randomly assigned to eat hay (n=3) or haylage (n=4) for 6 weeks while in training. Measurements were performed at baseline and after 2, 4, and 6 weeks. Results showed a decrease in respirable dust, and β-glucan exposure in the horses fed haylage when compared to those fed hay. BAL neutrophil proportion was significantly lower at weeks 2, 4, and 6 when compared to baseline and at week 6 when compared to horses fed hay.

The second clinical trial was performed on 19 mild asthmatic horses from the teaching herd. The diet of these horses was changed from dry hay to haylage (n=9) or hay pellets (n=10) for 6 weeks. Measurements were performed at baseline, week 3, and week 6. Results indicated that horses eating haylage and hay pellets were exposed to similar dust levels that were significantly lower than when they were eating dry hay. BAL neutrophil proportion was significantly lower in horses eating haylage when compared to baseline and to horses eating hay pellets at week 6. Horses eating haylage exhibited a significant decrease in stearic acid

concentration at week 6. Pro-resolving lipid mediators (Resolvin D1, Resolvin E1, and Lipoxin A_4) did not affect neutrophil apoptosis or efferocytosis *in vitro*.

The third clinical trial was performed on 73 thoroughbred racehorses actively racing and training. Horses were randomly assigned to eat dry hay or to change the forage to steamed hay or haylage. No other change in the management of the horses was allowed. Measurements were performed at baseline (n=73), week 3 (n=69), and week 6 (n=53). Results indicated that respirable dust exposure was significantly reduced when racehorses were fed steamed hay or haylage in place of dry hay. Respirable dust exposure was positively associated with BAL neutrophil proportions. Feeding haylage also significantly decreased exposure to respirable endotoxins. Horses eating haylage for 3 weeks had significantly lower BALF neutrophil proportion when compared with baseline. Also, at week 3, horses eating haylage showed a significant decrease in mast cell proportion, and horses eating steamed hay had a significant decrease in eosinophil proportion. At week 6, horses eating haylage maintained significantly lower BALF neutrophil proportions compared to baseline, and horses eating hay for 6 weeks. Concentration of Ω-3 and PRLM were not increased in horses eating haylage when compared to horses fed hay. Eicosapentaenoic acid was significantly higher on the horses eating haylage when compared to horses eating steamed hay.

In conclusion, feeding low-dust forages is sufficient to decrease breathing zone exposure of horses to respirable dust. Despite similar dust exposure, haylage was the only low-dust forage that resulted in resolution of neutrophilic airway inflammation; however, the mechanism remains unclear.

CHAPTER 1. INTRODUCTION

Mild-moderate equine asthma (EA), previously referred to as inflammatory airway disease is an inflammatory disease of the equine respiratory system. It is more commonly encountered in racehorses but can occur in horses of any breed and at any age. Airway inflammation observed in racehorses has been associated with coughing and poor performance.

Exposure to organic dust is central to the development of this disease. Evidence suggests that decreasing dust exposure can improve respiratory health by decreasing airway inflammation and improving clinical signs in severely asthmatic horses. Airway inflammation in racehorses has been linked to increased exposure to small size dust particles (respirable dust) and pro-inflammatory components of the organic dust. The effect of low-dust diets for the prevention and treatment of mild-moderate EA in horses has not been demonstrated.

The high incidence of mild-moderate EA in racehorses may be explained by some of the management practices used at the racetracks in the United States. Racehorses are generally fed dry hay with no access to pasture and maintained in stalls for extended periods of time. Dry hay has higher dust content and lower nutritional value when compared to grass pasture. It has been demonstrated that dust exposure can be reduced just by changing forage. Feeding haylage, hay pellets, or steamed hay can all decrease dust exposure compared to dry hay. However, it is unknown if feeding low-dust forages instead of dry hay is sufficient to resolve airway inflammation in horses with mild-moderate EA and how quickly the effect might be expected.

Airway inflammation in human asthma persists due to an ineffective return to airway homeostasis. Dietary fatty acid composition impacts the resolution of inflammation. Essential polyunsaturated fatty acids (PUFAs) that include omega-3 PUFAs (Ω-3) and omega-6 PUFAs (Ω-6) cannot be synthesized by mammals; consequently, their availability depends on dietary intake. Higher levels of Ω-3 are associated with an increase in PRLM, essential molecules in the resolution of airway inflammation and restoration of homeostasis. Ω-6 are precursors to various pro-inflammatory fatty acids (eicosanoids). Eicosanoids are crucial in the acute response of inflammation, and their principal role is initiating the influx of neutrophils to the inflammation site. After the initial damage is controlled, a class switch from eicosanoids to PRLM needs to occur to avoid further tissue damage by inflammatory cells. PRLM promote resolution by stopping the influx of inflammatory cell to the inflammation site and by increasing neutrophils apoptosis,

efferocytosis, and macrophage phagocytosis of bacteria and debris. The role PRLM in equine airway inflammation and plasma concentrations has not been reported yet.

Both Ω-6 and Ω-3 metabolic pathways are competing with each other because of shared enzymatic steps, and a higher intake of Ω-3 could play a role in the mitigation of airway inflammation. On the other hand, diets with high Ω-6 content, such as dry hay, would favor chronic inflammation. Diet supplementation with Ω-3 has been shown to improve clinical signs and airway inflammation in horses with severe EA when combined with low-dust diet. Similarly, increased Ω-3 intake appears beneficial in people with asthma. Haylage, a forage widely used in Europe to manage horses with severe EA, has not been investigated as a forage option for racehorses in the United States. This forage presents several advantages compared to dry hay: first, it is low-dust and second, it contains higher levels of Ω-3.

The primary purpose of the study was to determine the effect of low-dust forages on dust exposure and airway cytology of racehorses actively training, and to investigate the role of pro-resolving lipid mediators in the resolution of airway inflammation in horses. The specifics aims were:

1. Determine if feeding haylage or steamed hay can reduce dust exposure and airway inflammation in racehorses when compared to dry hay.
2. Compare respirable dust, β-glucan, and endotoxin exposures in the breathing zone between thoroughbred racehorses fed hay, haylage, and steamed hay over 6 weeks.
3. Compare neutrophil, mast cell, and eosinophil proportions in bronchoalveolar lavage fluid between thoroughbred racehorses fed hay, haylage, and steamed hay over 6 weeks.
4. Compare BAL fluid neutrophil proportions and PRLM (Lipoxin A_4, Resolvin E1, and Resolvin D1) in healthy horses fed hay for at least 2 months and after 6 weeks on haylage or hay pellets.
5. Determine the effect of PRLM on apoptosis of equine neutrophils and efferocytosis of equine neutrophils by alveolar macrophages *in vitro*.

CHAPTER 2. LITERATURE REVIEW

2.1 Equine Asthma

Equine asthma (EA) has been recommended as the preferred terminology to describe both inflammatory airway disease (IAD) and recurrent airway obstruction (RAO).[1] This new terminology paradigm further defines IAD as mild-moderate EA and RAO as severe EA.[2–5]

2.1.1 Similarities between equine and human asthma

In the past decades, there has been a constant debate about the link and similarity among chronic airway inflammation diseases in horses and chronic obstructive pulmonary disease (COPD) and asthma in people. The term COPD was used in the 1980s to describe horses with clinical signs of severe EA.[6,7] Nowadays, this terminology and the link with human COPD has been rejected since the diseases are fundamentally different in etiology, clinical presentation, and pathophysiology.[8] In the 2007 American College of Veterinary Internal Medicine consensus statement,[7] the term RAO or heaves was recommended to describe this disease. But recently a new terminology was proposed, where RAO is referred to as severe EA and IAD as mild-moderate EA, because of the similarities that these diseases share with human asthma.[1–3,5] Furthermore, the role of this new terminology is to help increase awareness of horse-owners and veterinary professionals.[1,5]

Asthma affects 7.7% of the human population in the USA.[9] This disease has been described as a heterogeneous condition, usually characterized by chronic airway inflammation.[9] Asthma is defined by a history of respiratory symptoms such as wheeze, shortness of breath, cough, chest tightness, and airflow limitation that varies over time and in intensity. [9] The different asthma phenotypes are determined by clusters of clinical, pathophysiological, and demographic characteristics.[10,11] The most common phenotypes are allergic asthma, non-allergic asthma, adult-onset asthma with persistent airflow limitations, and asthma associated with obesity.[9] Also, asthma can be differentiated based on severity (mild, moderate, and severe) and the severity is determined by the level of therapy required for the control of symptoms.[9]

In general, human asthma and EA are chronic inflammatory lung diseases that present cellular and structural changes in the airways.[5,9] Equine asthma shares similarities with some but not all the phenotypes of human asthma; for example, the horse is an appropriate animal model for allergic asthma, non-allergic asthma, and adult-onset asthma.[5]

Both diseases are triggered by the inhalation of environmental antigens.[9,12] Equine asthma usually occurs during exposure to dust or allergens in the stable while some horses with severe EA develop seasonal signs while at pasture.[1,13] The factors that influence the development of human asthma have been divided into host factors (genetic predisposition, obesity, and sex) and environmental factors (indoor and outdoor allergens such as pollen, dust mites, and animal dander).[9,14]

The patient's age when clinical signs develop varies between different human asthma phenotypes.[9,15] Allergic human asthma is mainly observed during early childhood and gradually decreases with age,[15] similarly mild-moderate EA is frequently observed in racehorses between 2 and 4 years old.[1] The presentation in racehorses has been described to be as high as 90% in standardbreds racehorses, and 80% in thoroughbred racehorses.[16,17] Non-allergic asthma and late-onset asthma in people are more common during adulthood (> 40 years old),[9,15] comparable with the presentation of severe EA that is most commonly observed on adult horses (older than 7 years).[18,19]

Clinical signs of EA are variable; horses with mild-moderate EA have chronic, often subtle signs such us occasional cough, poor performance but no increase of respiratory effort at rest.[1,2] The classic signs during severe EA exacerbation are regular to frequent coughing, exercise intolerance, abnormal breath sounds, increased airway secretions, and increased respiratory efforts at rest,[13] and the presentation of clinical signs can change with the severity of the disease.[1] Similarly, the clinical symptoms in human asthma are coughing, wheezing, shortness of breath, and tightness of the chest, which vary in intensity and over time, combined with airway hyperresponsiveness and expiratory airflow limitation of fluctuating severity. Only 27% of asthmatic people show daily symptoms.[9]

Asthma in people and EA are diseases that can be controlled in most cases. In general, the prognosis for well-managed asthma patients is good, and they can maintain normal activities into old age.[20] In severe EA, when offending antigens are removed from the horses' environment, affected horses improve progressively, and eventually are clinically indistinguishable from healthy

animals, and their airway function and bronchial cytology tend to normalize. This disease cannot be cured, but it can be controlled.[2,21] On the other hand, mild-moderate EA has been described to improve spontaneously with or without treatment,[1] thereby resembling allergic asthma in people where the incidence and signs decrease with advancing age.[9,15]

Human asthma has a predominant T helper 2 (Th-2) response, with the secretion of interleukin (IL)-4, IL-5, and IL-13.[17] Eosinophils, mast cells, and CD4+ T-lymphocytes are the principal cells involved.[22] Secretion of IL-5 by CD4+ T cells plays an essential role in eosinophil recruitment, especially in allergic-asthma. The accumulation of eosinophils in the airways has been described to occur after the early asthmatic response in antigen-challenged asthmatics, and it is the most common cell observed in the allergic phenotype of asthma.[23] This response is similar to the immune response observed in mild-moderate EA were the presence of mast cells, and eosinophils in bronchoalveolar lavage fluid (BALF) is common.[1,17,24] There is some evidence that the increase in Th-2 response seen in horses with mild-moderate EA is associated with the increase of IL-4, and IL-5 concentrations that makes mast cell inflammation more common in these horses when compared with horses with severe EA. Eosinophilic inflammation has been observed in young racehorses; similar to allergic asthma in people, that is more common during childhood. Eosinophils in BALF are rare in older horses with mild-moderate EA and are not observed in horses with severe EA.[17,25]

Airway neutrophilia is commonly observed in EA, especially during an acute severe asthma exacerbation. The presence of airway neutrophilia supports the role of a T-helper 17 (Th-17) immune response and its ability to evoke cell-mediated immunity and phagocyte-dependent inflammation.[5] During asthma exacerbation in people, IL-17 concentration is elevated and correlated with the presence of neutrophils and loss of lung function.[26] Neutrophils, T-lymphocytes , and macrophages are the primary cells present in the airways of severe asthmatic horses during exacerbation.[3] Airway neutrophilia and higher concentrations of IL-5 and IL-9 in BALF lymphocytes or neutrophils of horses affected with severe EA compared to controls support the link between Th-2-type immune response in horses affected with severe EA.[27,28] The presence of high levels of IFN-γ in BALF and constant levels of mRNA expression of IL-4 and IL-13 in BALF-derived cells and neutrophilic inflammation suggests the role of a T-helper 1 (Th-1) response.[29,30] Comparable to asthma in people, it has been observed that horses have an increase in IL-17 expression during severe EA exacerbation.[31] In the non-allergic human asthma phenotype,

neutrophils in sputum are more commonly observed, and a Th-1 immune response, with cell-mediated immunity and phagocyte dependent inflammation, is observed.[9]

In EA and human asthma, similar structural changes are observed in airway remodeling.[9,32] Both diseased have thickening of the airway as a consequence of the deposit of submucosal extracellular matrix and airway smooth muscle hyperplasia. Mucous gland hyperplasia and hypersecretion produce an accumulation of mucopurulent exudate that physically reduces the airway lumen.[8,33] Some differences between EA and human asthma are that in the horse, the lamina propria increases in thickness instead of thinning, and that the larger airways rather than smaller airways are more affected in human asthma.[32] Airway remodeling in EA has been mainly described in severe cases,[32] but recent studies have reported the presence of airway remodeling in mild-moderate cases of the disease.[34]

Immunoglobulin E (IgE) serum concentrations are elevated and associated with some of the symptoms observed during human asthma exacerbation.[35] IgE is responsible for the activation of allergic reactions. In asthma, the production of aeroallergen-specific IgE has been connected with the severity of the disease and bronchial hyperresponsiveness.[35] The role of IgE in severe EA is still controversial; some studies have demonstrated an increased serum IgE concentration in horses with severe EA particularly against recombinant allergens,[36] but others have failed to identify any significant differences between horses with EA and controls.[37] Recently, the detection of serum IgE was determined to have a good correlation with IgE concentration in BALF of horses with severe EA and controls,[38] while other authors were only able to detect antigen-specific IgE in BALF of horses with mast cell inflammation.[39]

In human asthma and severe EA, bronchoconstriction is one of the central causes of airway narrowing and interference with airflow. In asthma, bronchial smooth muscle contraction occurs as the result of the IgE dependent release of mediators from mast cells that include histamine, leukotrienes, and prostaglandins.[40] In severe EA, airway smooth muscle tone is controlled by the autonomic nervous system and circulating levels of receptor agonist and antagonist.[8] In severe EA and human asthma, increased mucus production, airway hyperresponsiveness, and pulmonary remodeling also contribute to the airflow obstruction. In severe EA and human asthma the airflow obstruction can be reversible,[2,9] but in human asthma, airflow limitations may later become persistent.[9]

Airway hyperresponsiveness is used to describe an increased tendency of airways to constrict in an exaggerated response to irritant stimuli.[41] This phenomenon is observed in asthma when the airways are exposed to an aerosolized chemical mediator such as histamine, and a similar response is observed in horses affected with EA. The mechanisms influencing airway hyperresponsiveness are multiple and include airway wall thickening, airway smooth muscle contractile properties, airway inflammation, dysfunctional neuroregulation, and structural changes.[1,9,41]

Asthma and EA share many characteristics, and the horse appears to be a suitable model for the study of asthma. Nonetheless, it is crucial to consider that both human and equine asthma are heterogeneous diseases with different clinical presentations, and some of the critical factors to consider when we talk about the similarities are the stage of the disease, chronicity, and possibly differences in the pathogenic pathways.[3]

2.1.2 Mild-moderate equine asthma

The main presentation of mild-moderate EA is in racehorses between 2-4 years, but horses of any age and discipline can develop this syndrome.[1,42,43] This disease is the second most common reason for veterinary care in young racehorses, and the most common chronic lung inflammation in this population of horses.[44] The main clinical signs of mild-moderate EA are poor performance, occasional chronic intermittent cough and affected horses exhibit normal breathing at rest.[1] Since the clinical signs are nonspecific, the diagnostic confirmation is achieved by the detection of excessive tracheal mucus and mild increase of inflammatory cells in BALF.[1] The recommended threshold values for airway cytology are >5% for neutrophils, >2% for mast cells and >1% for eosinophils.[1] Furthermore, the use of a tracheal wash (TW) to determine the presence of airway inflammation is not recommended, because of the absence of association between tracheal wash cytology and performance,[45,46] and also because the agreement between cytological findings of TW and BALF is controversial. In general, the agreement between BALF and TW cytology has been described as weak ,[46–48] and only one study that included horses with mild-moderate and severe EA has been able to demonstrate a correlation between BALF and TW neutrophilia.[49] The limited pulmonary dysfunction generated by the mild inflammation can only be detected with sensitive methods.[50–52]

Recently, the diagnosis by BALF cytology of mild-moderate EA has been described to be as high as 80-90% in racehorses.[16,17] The prevalence of excess tracheal mucus has been reported to range from 13-22% in racehorses in the United States,[53,54] and 0.5 – 45% around the world.[55–57] Poor performance in racehorses and reduced willingness to perform in other disciplines have been associated with the accumulation of excess tracheal mucus.[53,58] Also, airway mastocytic and neutrophilic inflammation have been related to reduce performance in racehorses.[17]

In racehorses, even mild airway inflammation can affect performance.[17] The real impact of mild-moderate EA in performance depends not only on the severity of the disease [17] but also the equestrian discipline performed.[59] Airway inflammation may affect athletic performance because of impaired gas exchange produced by problems in the diffusion or limitations of ventilation.[59]

Mild-moderate equine asthma: Pathogenesis

Little is known about the molecular pathways, and the role of the immune system in the pathogenesis of mild-moderate EA.[60] Cytokine profiles associated with mild-moderate EA cytological phenotypes are controversial.[61–63] Different cytokine patterns between cytological phenotypes further support differing pathophysiologic mechanisms. During mast cell inflammation, the expression of Th2 cytokines such as IL-4 and IL-5 in BALF is enhanced.[61,62] Also, there is an association of higher BALF mast cell proportion with airway hyperresponsiveness and exposure to respirable β-glucan.[17,64] These responses have been associated with the role of aeroallergen in mild-moderate EA.[17,24,64] Mast cells can release different types of proteases, cytokines, and pro-inflammatory mediators; they play a central role in the pathophysiology of allergic diseases in people.[65] The Th2 polarization of the immune response during mast cell inflammation also suggests a role of an adaptive immune response in mild-moderate EA.[61,62]

Neutrophilic inflammation has been related to the activation of the innate immune system, and with an increase in BALF tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and IL-1β concentrations.[61,62] In severe EA, IL-8, IL-4, IL-17, and IL-23 contribute to the influx and accumulation of neutrophils in the horse's airways.[30,66,67]

The role of eosinophils in the pathogenesis of mild-moderate EA is not entirely understood.[1] The presence of eosinophils in BAL has been described at any age and with different severities,[46,48] but the association between environmental exposure and eosinophils in BAL has

only been demonstrated for respirable dust exposure in the horses' breathing zone of young racehorses entering training.[24]

Mild-moderate equine asthma: Risk factors

There are various risk factors that contribute to the presentation of mild-moderate EA. It is essential to consider that the presentation is usually not related to only one factor but the combination of several potential factors.

Genetic predisposition has not been thoroughly investigated, but in horses with severe EA, family history has been related to the presentation of the disease in some breeds, such as warmblood horses.[68,69] Also, age has been associated with the incidence of the disease. Mild-moderate EA is more common in horses between 2 and 4 years old and is less common in older horses.[1] The risk decreases with age, suggesting the development of resistance or immunity to infectious agents.[70,71] In general, older horses downregulate the secretion of inflammatory cytokines, but horses with mild-moderate EA do the opposite.[72] Mild-moderate EA cytological phenotypes are influenced by age with BALF eosinophilia being more often observed in young racehorses entering training,[24] while BALF neutrophilia is more common in older horses.[17,50] On the other hand, the presentation of mast cell inflammations is observed in both age groups.[17,24] Some studies have described that the real risk factor is not the age, but the time spent in training.[55] Horses entering training at four years old are significantly more likely to be affected with mild-moderate EA than horses of the same age that had been in training for years. Horses entering training have a significantly higher amount of mucus in trachea compared with horses that have been in training for a more extended period.

However, the primary risk factors are closely associated with the etiology of this disease. mild-moderate EA etiology is poorly understood but has been described as multifactorial[73], including environmental,[71,74–77] bacterial,[55,78]and viral components.[71,79] Non-infectious agents, especially organic dust, appear to play a central role in the development of the disease, [80] while the contribution of infectious agents is currently unclear.[1]

Risk factors associated with bacteria exposure

The role of bacterial infection is controversial, but an infectious component in mild-moderate EA is highly suspected.[57,71,78] Specific bacteria species have been associated with increased risk of airway inflammation, and the isolation of these bacteria in tracheal wash (TW) has provided evidence of their potential role in the presentation of mild-moderate EA.[55,57,71,78,81,82]Airway inflammation has been closely associated with the isolation of *Streptococcus zooepidemicus, Streptococcus pneumonia, Mycoplasma equihinis,* and *Pasteurella/Actinobacillus*-like species.[57,70,71]

Furthermore, the isolation of *Streptococcus zooepidemicus* or *nonhaemolytic Streptococci* increased the odds of a higher mucus score, with an increased effect when both were isolated together.[10] Some studies in racehorses have associated the presence of bacteria in tracheal wash with tracheal mucus,[81] coughing,[57] and increase tracheal neutrophil proportion.[70,78] More recently a study in an older population of horses with mild-moderate EA did not find an association between any specific bacteria detected by qPCR in TW and clinical signs, tracheal mucus or BALF inflammation.[83]

The microbiome present in oral, nasal and lung communities has been related to disease status and appears to be clustered by environmental condition (low antigen exposure, moderate antigen exposure, and high antigen exposure).[82] The lung communities are the only ones affected by disease status, and the difference was only observed when horses with severe EA had lung inflammation. This finding suggested that the altered lung microbiome in asthma might not be a risk factor for the presentation but concomitant with lung inflammation.[82]

In a recent study of horses with mild-moderate EA a specific overgrowth in tracheal aspirates was not observed, but rather a lower bacterial load was detected using qPCR.[83] The dysbiosis noted in these horses could be a consequence of chronic inflammation, previous treatments or a perpetuating factor of inflammation.

Risk factors associated with viral exposure

In human asthma, viral infection plays an important role in disease induction, disease exacerbation, and airway neutrophilic inflammation.[9] The role of viral infection in mild-moderate EA is still debated, but there is some evidence of the increased risk of EA with viral infections.[84,85]

In a case-control study with client-owned pleasure or low-level sport horses, viral exposure was associated with an increase in the risk of mild-moderate EA;[85] horses with neutrophilic or mast cell inflammation and clinical signs (cough, exercise intolerance, poor performance, or nasal discharge) were significantly more likely to have positive titers to equine rhinitis virus A than horses without inflammation. On the contrary, a longitudinal study performed on young Standardbred racehorses failed to find any association between rhinitis B virus, and rhinitis A virus PCR or seroconversion and poor performance.[86]

Both alphaherpesviruses (equine herpesvirus type 1 (EHV-1) and EHV-4) and gammaherpesvirus (equine herpesvirus 2 (EHV-2)) have been associated with long-lasting airway inflammation.[87] EHV-1, EHV-2, and EHV-5 have been associated with airway inflammation and poor performance in some studies,[47,88] but the association between poor performance and EHV-1, EHV-2, and EHV-4 was not observed in Standardbred trotters.[86] Also, no association between the detection of EHV-1 and EHV-4 in trachea or nasopharynx with the clinical presentation of mild-moderate EA was observed in young racehorses.[79]

Equine herpesvirus 2 was described to be the more prevalent virus in TW of horses with airway inflammation and poor performance.[89,90] Moreover, EHV-2 DNA detection was associated with prolonged airway inflammation and an increase in airway neutrophilia. Morphological abnormalities of exfoliated epithelial cells and ciliocytophthoria were associated with EHV-2 and EHV-5 in BALF by PCR.[87,89] Positive nasal PCR for EHV-2 has been related to the diagnosis of mild-moderate EA.[85]

Risk factors associated with environmental exposure

Exposure to different non-infectious agents present in organic dust can increase the risk of airway inflammation, even in healthy horses.[74,91] Organic dust usually has a heterogeneous composition including materials from microbial, plant, and animal sources. Organic dust may contain pathogenic or non-pathogenic living or dead bacteria and fungi, allergens, bacterial endotoxins, β-glucans, pollen, and plant fibers.[92] The two major pro-inflammatory components of organic dust are mold β-glucans and bacterial endotoxins.[93] Endotoxins are part of the cell wall of gram-negative bacteria, and β-glucans are constituents of most fungi, some bacteria, and numerous plant cell walls.[92] Some inorganic particulates are of less importance, but still contribute to the

disease presentation, such as silicates from dusty arenas or oil fly ash from diesel machinery being used inside large barns.[94]

Stable air quality is influenced by particulates originating from the environment outside and inside the stable, and both should be considered to identify horses at the most significant risk.[95] Stabling is associated with a higher risk of inflammation in both the upper and lower airway of young horses,[74] and a higher number and percentage of neutrophils in BALF has been observed in stabled horses.[74,96–98] Introducing otherwise healthy horses to stall confinement is sufficient to trigger the neutrophilic reaction in the airway.[74]

Indoor air quality in stables can be affected by building design (number and location of windows and doors), quality of feed and bedding, and stable management practices that include methods of stall cleaning, raking and sweeping, type of ventilation, and contamination from road dust, vehicle emission, and pollen.[95] Respirable dust exposure is up to 13 times higher in horses in common stable conditions (hay with straw) compared with pasture.[75] Similarly, endotoxin exposure is significantly higher (8 fold) in stables than on pasture.[99]

Increased exposure to particulate matter with diameter less than 1 μm (PM10) is associated with an increase in tracheal mucus, and the odds of having visible mucus are increased when horses are in a stall with high PM 10 concentrations.[100] Increased neutrophil proportions in TW are associated with PM2.5 (particulate matter with diameter less than 2.5 μm) and PM10 concentration.[100,101] Airway inflammation, especially neutrophilic inflammation, occurs during environmental challenge, not only in severely asthmatic horses but also in clinically healthy animals.[74,91] In healthy horses, despite the influx of neutrophils into the airways, no increase of mucus accumulation after exposure to hay dust is observed.[2,28]

Airway inflammation has been related to respirable dust but not inhalable dust in the horses' breathing zone.[29] Inhalable dust are particles that can be inhaled from the surrounding air, with a 50% cut-point at <100 μm, and respirable dust are particles that can penetrate deep into the lungs, with a 50% cut-point at <4 μm.[102] In young Thoroughbred horses entering training, breathing zone respirable dust exposures are correlated with BALF eosinophilic inflammation,[24] and in older racehorses, the correlation is with neutrophil proportions in BAL.[16,17]

Horses housed in stalls have a greater risk of developing mild-moderate EA, because they are continually exposed to potential triggering factors present in the forage and bedding, as well as poor ventilation.[103] Enclosed stables have lower ventilation and higher particulate exposure than

open-sided barns.[95] The type of forage has a more significant influence than the bedding on respirable dust concentrations in the breathing zone of the horses.[104,105] Horses with severe asthma kept in a stall for two months under low-dust conditions (silage and cardboard bedding) did not have any changes in lung function or BAL cytology, compared to the same horses in the pasture.[106] However, lung function and BAL cytology data were significantly different after only 1 to 3 days in poor hygiene conditions (straw and dusty hay). Forages such as pelleted feed, steamed hay, and haylage have demonstrated a decreased exposure to dust in the horses' breathing zone.[104,107–110] Another critical factor is the system of feeding; feeding hay from a haynet increases exposure to respirable dust and endotoxins in the horses breathing zone as compared to feeding hay on the ground.[24]

They are many variables that affect the amount of dust generated, and microbiological contamination in forages, for example, climatic factors, and agricultural practices are important to the hygienic quality of the hay.[111] Fungal and dust contamination are higher if the moisture of the hay remains high during or after the hay is baled. Feeding hay from round bales results in higher risk of neutrophilic inflammation in TW[43] and BALF.[91]

Endotoxin is a potent inflammatory agent, and much of its toxicity is associated with the lipid-A components.[112] Endotoxin inhalation induces a dose-dependent neutrophilic airway inflammation response in healthy and asthmatic horses.[113] Comparison of the effect of acute endotoxin inhalation and hay/straw challenge suggest that inhaled endotoxin is not the sole cause of severe equine asthma. Recently, a protective effect of low doses of endotoxin in respirable dust upon BALF neutrophil proportion has been described.[17]

High levels of β-glucans are associated with respiratory symptoms in people.[112] In racehorses, β-glucan exposure, as measured at the horses' breathing zones, has been associated with mast cell proportions in BALF.[17] IL-6 is upregulated in stabled horses and is associated with high concentrations of respirable dust, ß-glucan, cold weather, and lower endotoxin.[114]

Pirie et al. described the effect of nebulizing a hay dust suspension (HDS) on the airways,[12,113] and reported a dose-dependent airway neutrophilic response both in healthy and asthmatic horses.[113] Further experiments were conducted with HDS that was separated into specific fractions to describe the effect of endotoxins and β-glucan in airway inflammation.[12] In both cases, nebulization with the specific components generated neutrophilic inflammation, but HDS generated a higher response. Endotoxin and β-glucan are important for the induction of

neutrophilia in the airway. Still, it is the synergic actions of the different compounds present in organic dust that generates the hallmark response in the airway.

Mitigation of dust exposure associated with equine asthma

For the treatment, prevention, and control of airway inflammation in mild-moderate EA, environmental control of dust exposure has been proposed as the primary strategy[115] however, until our studies, no clinical trial has demonstrated the effectiveness of this strategy. The pharmacological treatment of mild-moderate EA is widely used, despite weak evidence of the efficacy of corticosteroids and bronchodilators in racehorses with the disease.[116] The control of inflammation without the use of drugs is especially crucial in racehorses, where the use of many drugs is restricted.

Keeping horses on pasture without access to hay is often not possible or practical; therefore, many studies have focused on different options to decrease dust exposure of stabled horses. Studies have provided strong evidence that the reduction of dust exposure is a treatment option for horses with severe EA,[117] but there is only circumstantial evidence regarding the effect on mild-moderate EA.

Concentration and number of respirable particles vary with stable design, management, time of the day, the season, and location of the stall within the stable.[95] In the USA, one study observed higher PM10 concentration and total particles but lower respirable dust concentrations during the winter when compared with summer.[118] Another study observed that average particle concentration was lower in July and higher between September and November, but number of particle between 2-5 μm were higher in July and lowest in November.[95] On the other hand, in Norway, lower outdoor temperatures were associated with higher respirable dust in stabled horses, the cause of this increment was mainly related to the type of ventilation system in the barn, and if the doors and windows were kept open or closed.[119] It is important to consider that stall management significantly influences the background exposure in the next stall, and the same influence is expected with storage areas.[104,120] The two main strategies to decrease dust exposure are to increase ventilation in the barn and to change management to low-dust feed and bedding.[1,117]

The effect of bedding on dust exposure has been extensively studied. There is evidence that bedding can impact the concentration of endotoxins and particulate exposure.[103] Area generation of PM10 is higher in horses bedded with wheat straw when compared to wood shavings

and straw pellets.[121] Bedding type can also influence measurements of fungal air contamination,[122] horses eating hay and bedded with pellets had lower fungal area air contamination when compared with peat-shavings and straw as bedding. In the same study, peat-shavings bedding generated less dust when compared with wood pellet and crushed wood; however, the change in bedding material did not influence the horses' clinical evaluation, blood gases or tracheal mucus.[122]

When horses are fed steamed hay and bedded on wood shavings, respirable dust concentrations measured in the stall and at the breathing zone of the horse are lower when compared to horses fed hay and bedded on straw or shaving, and that horses fed haylage and bedded on straw in two different barn types.[107] Also, the change from poor quality hay and straw to complete pelleted feed and wood shavings significantly reduces the respirable dust burden in the horse's breathing zone and the exposure to aeroallergens, such as *Aspergillus fumigatus*.[110] The respirable endotoxin concentration and respirable dust exposure can also be affected by the change from hay and straw in a barn to haylage with shavings by a 5- to 10-fold decrease, respectively.[75]

As mentioned, forage has a more significant impact on horse exposure to dust.[104] It has been demonstrated using area measurements that the highest dust exposure in a barn occurs while the horses are eating.[121] Also, this conclusion was corroborated by studies comparing hay and haylage in low and high dust exposure types of bedding.[105] Horses fed haylage were exposed to lower total dust than horses fed hay independent of the bedding type.

Hay, the most common forage fed to horses, is the primary source of dust exposure.[103] Indirect evidence suggest that exposure to dust of horses eating hay is associated with the presentation of lung inflammation.[91,123]

Horses fed hay in a stall can have an 8-fold increase in exposure to endotoxin concentration in the breathing zone when compared with horses at pasture.[99] The deleterious effect of endotoxin on airway inflammation has previously been described.[12] In addition, feeding horses from a haynet can increase the exposure to endotoxins 3-fold and respirable dust 5-fold when compared to horses fed on the ground.[24]

Alternative feed options, such as soaked hay, steamed hay, pelleted feed, and haylage, have been studied in the management of equine asthma. Soaking the hay immediately before feeding can decrease respirable dust exposure in the horses breathing zone by 60%,[115] but soaking hay for 24 hours has been related to an increased in yeast count, enterobacteria, and lactic acid bacteria.[124]

Watering and steaming hay may decrease PM10 and PM2.5 released *in vitro* by nearly 90% compared to hay,[108] and any significant reduction in PM10 and PM2.5, requires the moisture content of the forage to be higher than 25%.

The use of complete pelleted feed in place of poor quality hay can decrease the total particle concentration by 70% and respirable particle concentration by 99% in the stall.[125]

Finally, feeding haylage has shown to decrease respirable dust exposure on the horses breathing by 60-70% when compared to hay.[104] Furthermore, the use of haylage as forage with shavings as bedding has been demonstrated to decrease the endotoxin in inhalable dust by five-fold when measured in the breathing zone of the horse when compared with horses eating hay with straw bedding.[105]

The beneficial effect of reduced dust exposure on clinical outcomes, such as airway inflammation of horses with severe asthma, has been well documented.[126–130] Although, several strategies are available to decrease dust exposure in the breathing zone of horses, no studies have yet reported the efficacy of such strategies in horses with mild-moderate EA.

2.1.3 Conclusions

Mild-moderate EA is commonly encountered in racehorses and affects their performance. The main risk factor for the development of airway inflammation is exposure to organic dust and pro-inflammatory mediators present in dust. Exposure to dust, and pro-inflammatory mediators is determined by ventilation, activity in the barn, external contaminants, but especially by the type of forage. There are different strategies to mitigate dust exposure, such as feeding horses with low dust forages and avoiding the use of dry hay. Still, no evidence of the impact of low-dust forages on airway inflammation of racehorses with mild-moderate EA has been reported.

2.2 Role of omega-3 polyunsaturated fatty acids in airway inflammation

2.2.1 Omega-3 polyunsaturated fatty acids structure and metabolism

Fatty acids (FAs) and their metabolites play a decisive role in regulating persistence and the resolution of airway inflammation.[131] They have a structural function as constituents of cell membrane phospholipids; also their derivatives are involved in cell signaling, and neutral lipid FAs are important for energy storage.[132]

Dietary FAs are long-chain hydrocarbons that can be separated into four categories: saturated, mono-unsaturated, PUFAs, and trans fats.[133] The differences between these FAs are their biochemical structure. FAs are differentiated by the length of the carbon chain and the presence or absence of double bonds between carbon atoms. Saturated FAs, such as palmitoleic and oleic acids, have the maximum number of hydrogen atoms attached to every carbon, and if a pair of hydrogen atoms are missing because of a double bond between two carbon atoms, these FAs are called PUFAs.[134]

Polyunsaturated FAs can be further classified as Ω-3 and Ω-6 depending on the carbon chain length and the position of the first double bond in the fatty acid chain.[135] The first double bond of Ω-3 is located between the third and fourth carbon atoms from the terminal methyl group, while on Ω-6, the first double bond is located between the sixth and seven carbon atoms from the terminal methyl group (Figure 2.1).[134] Mammals are not able to convert some FAs to PUFAs, and that is why these FAs are essential PUFAs. Specifically, mammals cannot convert oleic acid into linoleic acid (Ω-6), and α-linolenic acid (Ω-3).[134,136] The relative availability of these molecules is determined by dietary intake. Plants contain an enzyme capable of inserting a double bond at positions 3 and 6 from the terminal methyl that mammals don't have, allowing plants to synthesize linoleic acid and α-linolenic acid.[134] Therefore, horses like other herbivores, obtain those compounds by grazing or eating other plant products like hay or grain. In the horse's diet, dry hay is considered higher in Ω-6 content when compared with pasture that has a higher Ω-3 content.[137]

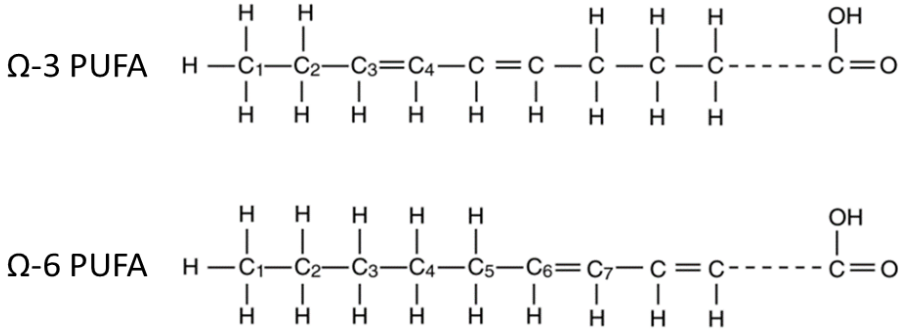


Figure 2.1. Structure of omega-3 and omega-6 polyunsaturated fatty acids. Adapted from Lunn and Theobald 2006

Polyunsaturated FAs are an important constituent of phospholipids of all cell membranes, and the FA composition of different cell membranes is determined by metabolic properties and tissue, but also depends on the type of PUFA that is available.[138] Also, synthesis of the various Ω-3 and Ω-6 compete for the same enzymatic pathways involved in the fatty acid molecule elongation and denaturation.[134] Once Ω-3 and Ω-6 are ingested, they can be metabolized into physiologically active compounds.[135] The nature of lipid mediators produced from both pathways of PUFAs results in different biological activities.[138,139] Linoleic acid is the precursor of arachidonic acid that can be further transformed into pro-inflammatory lipid mediators or eicosanoids, such as prostaglandins, thromboxane, and leukotrienes (Figure 2.2).[139,140] On the other hand, α-linoleic acid is the precursor of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Both DHA and EPA migrate with inflammatory exudate and are release from cell walls to sites of inflammation where they are the precursors of PRLM.[136] Pro-resolving lipid mediators may be derived from eicosanoids (lipoxins (Lx)), DHA (D-series resolvins (RvD), protectins, and maresins) or EPA (E-series resolvins (RvE)) (Figure 2.2).[136,141]

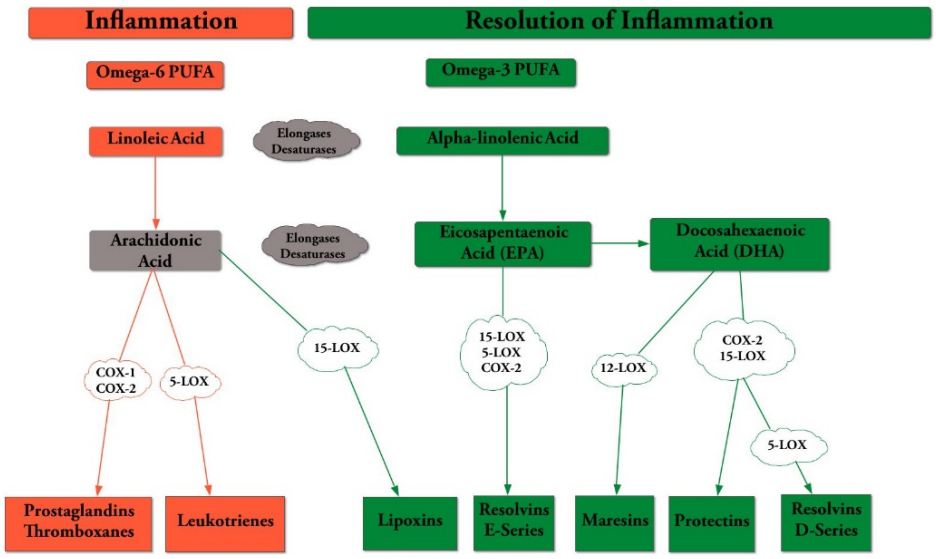


Figure 2.2. Overview of Ω-3 and Ω-6 pathways into eicosanoids (red boxes) or pro-resolving lipid mediators (green boxes). COX: cyclooxygenase; LOX: lipoxygenase. Adapted from Wendell and Holguin 2014.

Excessive intake of Ω-6 has been associated with increased inflammatory diseases in people, because of the upregulation of arachidonic acid-derived eicosanoids.[142] These molecules are essential modulators of the immune response through complex interactions with neutrophils

and, as such, play a crucial role in the acute phase of inflammation.[135,143] Because of the competition between Ω-6 and Ω-3 metabolic pathways, higher intake of Ω-3 may prevent airway inflammation by partially replacing Ω-6 in the cell membranes of erythrocytes, neutrophils, and monocytes, making Ω-3 more available to be converted into PRLM and suppressing in part the production of eicosanoids.[135,144-145] Multiple studies suggest a beneficial role of Ω-3 in many diseases including asthma in people and horses.[129,146]

2.2.2 Pro-resolving lipid mediators and their effect during inflammation resolution

Inflammation is a dynamic process that starts with an acute inflammatory response to eliminate the insult resulting from tissue exposure to a pathogen or an allergen.[147] Acute inflammation is often characterized by the coordinated action of chemokines, cytokines, and other pro-inflammatory mediators, such as eicosanoids.[147] Eicosanoids are central in the initiation of the acute inflammation and are necessary for the activation of immune cells and the syntheses of pro-inflammatory signals.[131] Prostaglandin E_2 and I_2 are responsible for the transmigration of neutrophils from post-capillary venules to the inflammation site.[140] Leukotriene-B_4 generates the chemoattractant gradient for the migration.[148,149] Also, eicosanoids are going to generate a switch from monocytes to pro-inflammatory macrophages that proliferate and affect the function of resident tissue macrophages to pro-inflammatory resident macrophages.[148] There is evidence that chronic and uncontrolled inflammation occurs in the airways when the resolution of an acute inflammatory response is incomplete.[150] For this reason, and to prevent further tissue damage, the acute inflammation process needs to resolve.[147] Resolution of inflammation is an active process that takes place in overlapping phases,[147] and the initial pro-inflammatory events are central in programming resolution by promoting the synthesis and release of pro-resolving mediators.[151,152] The class switch from eicosanoids to PRLM occurs when the neutrophils congregate in inflammation site.[140,153] If the class switch does not occur, it can lead to enhanced production of eicosanoids, tissue damage by neutrophils, and chronic inflammation.[144]

Resolution of inflammation and return to tissue homeostasis can only occur if neutrophils are eliminated, and resident macrophages and lymphocytes return to pre-inflammatory numbers and phenotypes.[148] Pro-resolving lipid mediators are produced in the inflammation site to limit and stop the infiltration of new neutrophils and stimulate the efferocytosis and phagocytosis by

macrophages of cellular debris.[140] In response to an inflammatory event, PRLM stimulate molecular and cellular events to resolve inflammation and restore tissue homeostasis. [154–157]

At a cellular level, different *in vitro* studies in human cells, and *in vivo* models of inflammatory diseases (mice and rabbits) have described that the main resolution mechanisms activated by PRLM are the limitation of leukocyte recruitment, induction of neutrophil apoptosis, enhancement of efferocytosis by alveolar macrophages, and phagocytosis, microbe killing and containment at the site of inflammation.[140,148,158,159] In murine models of airway inflammation, PRLM appear to play a role also in the regulation of the adaptive immune response by selectively downregulating the production of Th-2 pro-inflammatory cytokines by natural killer, T, and B cells.[158,160]

In humans, PRLM are ligands of G-protein-coupled receptors. Formyl peptide receptor 2 (ALX/FPR2), chemR23 or E- series resolvin E ligand (ERV), D-series resolvin ligands-1 (DRV1), and D-series resolvin ligands-2 (DRV2) are G-protein-coupled receptors, and their primary ligands are LxA_4, RvE1, RvD1, and RvD2, respectively, but they are capable of interacting with other PRLM.[161] For example, neutrophils during inflammation mobilize ALX/FPR2 receptor to the cell surface where RvD1 is going to interact with it to produce a pro-resolutive signal in the cells. RvD1 interacts with the specific receptor DRV1 during tissue homeostasis phase.[162,163] The ALX/FRP2 receptor is present on the surface of T-cells, macrophages, neutrophils, eosinophils, and airway epithelial cells.[161] ERV is expressed by brain cells, dendritic cells, epithelial cells, and airway cells, and the interaction with its ligand RvE1, increases macrophage phagocytosis and reduces pro-inflammatory cytokines production.[161,164] DRV1 is expressed in neutrophils, lymphocytes, macrophages, and monocytes.[161] DRV2 is expressed in neutrophils, monocytes, and macrophages. The interaction with RvD2 can increase efferocytosis, macrophages phagocytosis, and decrease the influx of neutrophils. [165] The receptors for protectins and maresins have not been described.[161]

Pro-resolving lipid mediators can also exert antagonism on phlogistic receptors *in vitro*. RvE1 and Maresin-1 are capable of inhibiting the receptor BLT1 for Leukotriene-B_4,[166,167] potentially generating a local decrease of BLT1 signals reducing neutrophil influx, inhibiting Nuclear Factor-κB (NFκB) signaling, inducing neutrophil apoptosis and macrophages efferocytosis.[167,168]

In addition, some PRLM are capable of interacting with their receptors to promote the expression of other PRLM that can interact with a specific receptor to further promote resolution of inflammation. For example, when RvE1 interacts with ERV1, it promotes the synthesis of LxA_4 for ALX/FPR2-mediated resolution of allergic airway inflammation.[169]

The effect of each one of the PRLM on receptor activation and intracellular signaling is dependent on the cell type and the organ where the cells are located.[170]

Resolvin E1 plays a role in the resolution of inflammation by stopping neutrophil migration to tissues, stimulating neutrophil apoptosis, and activating phagocytosis.[140,161,168] Resolvin E1 and RvE2 also decrease the synthesis of cytokines, and adhesion molecules, and inhibits NFκB signaling.[171] Resolvin E1 has been shown to have a protective and pro-resolution role in human neutrophils *in vitro* and in mice models of airway inflammation.[168,169] Resolvin D series are produced by neutrophils and macrophages,[154,172,173] and are able to promote apoptosis of neutrophils, block signaling of NFκB, and stimulate polarization of macrophages to an anti-inflammatory phenotype.[161,174] RvD1 has demonstrated anti-inflammatory and pro-resolution effects in mice models of lung inflammation.[175,176]

Lipoxins are produced from arachidonic acid, but they produce an anti-inflammatory effect by reducing reactive oxygen species by neutrophils, decreasing trans-endothelial migration of cells and pro-inflammatory cytokines production, increasing apoptosis of neutrophils, and increasing efferocytosis.[146,177,178] LxA_4 modulates innate and adaptive immune response regulating leucocyte migration, T-lymphocytes, and dendritic cells.[140,177] The effect of LxA_4 on efferocytosis has been demonstrated in mice models of asthma and with human cells *in vitro*.[179,180] A decreased LxA_4 has been detected in the sputum of children and adults with severe asthma.[178,181]

Maresins are produced by macrophages.[182] They limit neutrophil infiltration, enhance macrophage phagocytosis, and conversion from pro-inflammatory to anti-inflammatory macrophages.[160,182,183] Also, Maresin-1 has been described as broncho-protective in murine models of airway inflammation.[160]

Protectins are produced by monocytes, neutrophils, eosinophils, and T-cell.[172,184–186] Protectin-1 has anti-inflammatory and neuroprotective properties by blocking intracellular signals of NFκB, and by inhibiting TNF-α, IFN-γ, and prostaglandin synthesis.[147,184] A decrease in protectin-1 synthesis from eosinophils has been reported in severe asthmatics, as well as a decrease concentration in exhaled breath condensate during asthma exacerbation.[187,188]

2.2.3 Omega-3 polyunsaturated fatty acids supplementation in asthma

In the past few decades, several clinical trials have been performed to determine the effect of Ω-3 supplementation in human asthma. Results have been inconclusive and in some cases contradictory.[189–194] Some of the inconsistencies between studies can be explained by differences in dose, length of treatment, type of supplement and study population.[188]

A placebo-controlled study showed no effect of 6 months of supplementation with 3.2 g of EPA and 3.2 g of DHA per day on lung functions or symptoms in asthmatic adults.[189] However, newer studies have been able to shown a beneficial effect of Ω-3 supplementation in asthmatic patients. In a crossover study of asthmatic athletes with bronchoconstriction, consumption of 3.2 g of EPA and 2.0 g of DHA or a placebo for 3 weeks, did not have an effect on pre-exercise lung function, but supplementation did improve the post-exercise pulmonary function when compared with the placebo diet.[190] Also, a decrease of *in vitro* production of eicosanoids and plasma pro-inflammatory cytokines was observed when compared with baseline and placebo diet. Another placebo-controlled study performed in mild-moderate asthmatic young adults supplemented with 3.2 g of EPA and 2.0 g of DHA for 3 weeks showed that patients presented an improvement in pulmonary function, decrease in bronchodilator usage, and decreased sputum differential cell count when compared with the placebo group.[191] A study of asthmatic adults after low-dose allergen challenge showed a reduction in bronchial inflammation after 5 weeks of supplementation with 450 mg of EPA and 180 mg of DHA per day when compared with placebo group.[192]

Furthermore, an epidemiological study using a validated survey of young adults in the United States reported that Ω-3 intake was inversely associated with asthma incidence after adjusting for dietary and socioeconomic confounders.[195] There was a more significant association observed with DHA supplementation when compared to EPA. Similarly, in other studies, DHA was found to be more beneficial on lung function.[193,195] In children, higher consumption of Ω-3 was associated with a reduction of the effect of indoor exposure to PM2.5, while higher consumption of Ω-6 presented the opposite effect.[194]Also, a higher intake of Ω-6 was associated with higher odds of asthma worsening.

The benefit of Ω-3 supplementation has also been described in other species. In a study of feline asthma, cats with experimentally-induced asthma supplemented with Ω-3 (20 mg of BioVex lipids and 10 mg of luteolin) for 4 weeks[196] demonstrated that Ω-3 was integrated into the red

blood cell membrane after the supplementation, and resulted in decreased airway hyperresponsiveness however, no beneficial effect was observed on airway cytology.[196]

In horses with severe EA, a crossover feed trial was performed to determine the effect of supplementation with seal blubber oil (rich in Ω-3) or sunflower oil (rich in Ω-6) for 10 weeks.[197] After the Ω-3 supplementation period, total cell count in BALF was significantly lower, plasma EPA and DHA were significantly higher, and EPA was successfully incorporated into leukocyte membranes.

Also, in horses with severe EA, supplemented with two doses of Ω-3 rich in DHA (1.5 and 3g) for 8 weeks and fed a low-dust diet experienced rapid clinical improvement and a marked decreased in BAL neutrophil proportion independent of the dose when compared to horses fed only a low-dust diet and placebo.[129]

2.2.4 Omega-3 polyunsaturated fatty acids in equine diet

In human studies, the incorporation of EPA and DHA in inflammatory cells occurs in a dose-response fashion,[198], and the recommended intake ratio of Ω-6:Ω-3 is 4:1.[135] The ratio of Ω-6:Ω-3 has been described to be 1:3 in orchard grass and 1:2 alfalfa.[199] The recommended intake ratio of Ω-6:Ω-3 has not been established in horses, but considering that pasture is the horse primary diet an intake ratio of Ω-6:Ω-3 of 1:2 may be recommended.[199]

It is essential to consider that α-linolenic acid conversion to EPA and DHA is limited (less than 15% in humans);[200] consequently the levels of EPA and DHA that are consumed directly are crucial to increase the levels of blood and tissue PUFAs. In ruminants, intake of pasture has been related to higher levels of α-linolenic acid in milk and meat,[201] and higher levels of EPA and DHA in meat when compared to dry hay.[202] Similarly, in a study of horse-meat, yearling horses fed pastures presented a higher meat Ω-3 content when compared with hay.[203]

The effect of supplementation of Ω-3 in blood levels of horses has been described. A study fed horses two different Ω-3 supplements for 3 months, the first supplement had α-linolenic acid (2 g), EPA (7.6 g) and DHA (26.6 g), and a second had only α-linolenic acid (38 g).[204] The first supplement showed an increase of EPA and DHA blood levels, but the effect was not observed with the second supplement, demonstrating the importance of the direct supplementation of DHA and EPA. Another study demonstrated that horses supplemented with 3 and 6 g of DHA for 4

weeks exhibited increased blood levels of DHA and that the levels were similar between the two dosages.[129]

Racehorses are mainly fed conserved forages with no access to pasture. The content of Ω-3 in forages depends on the vegetative stage.[199,201] The progression of the vegetative stage generates an increase in Ω-6 concentration and a decrease in Ω-3. Also, haymaking conditions have an impact in Ω-3 content, the most important are drying conditions.[199] Poor drying conditions have been related to a decrease in Ω-3 content in hay. Haylage and silage are not affected by the drying process and have similar content of α-linolenic as pasture grass, and higher content than dry hay.[137,199]

2.2.5 Conclusions

In summary, PUFAs are essential constituents of all cell membranes. The concentration of Ω-6 and Ω-3 will determine the nature of lipid mediators produced with pro-inflammatory or pro-resolution activities, respectively. Pro-resolving lipid mediators are essential for the resolution of inflammation, while an increase in eicosanoids has been related to chronic inflammation. Ω-3 and Ω–6 are essential FA that must be supplied in the diet. Therefore, nutritional quality and especially PUFA content of horses' diet need to be considered for the prevention and management of inflammatory diseases, such as EA.

CHAPTER 3. DUST EXPOSURE AND PULMONARY INFLAMMATION IN STANDARDBRED RACEHORSES FED DRY HAY OR HAYLAGE: A PILOT STUDY

3.1 Abstract

Respirable dust exposure is linked to airway inflammation in racehorses. Feeding haylage may reduce dust exposure by 60-70%. The objective of this study was to compare dust exposure, airway cytology, and inflammatory cytokine concentrations between horses fed haylage or hay over 6 weeks while in training.

Seven healthy Standardbred horses were randomly assigned to be fed hay (n =3) or haylage (n =4) for six weeks while training on a treadmill. Dust exposure was measured gravimetrically at the breathing zone. Endotoxin and β-glucan concentrations in respirable dust were measured. Bronchoalveolar lavage fluid (BALF) cytology was determined at baseline and after 2, 4, and 6 weeks. Cytokine concentrations (interferon-γ, tumor necrosis factor-α and interleukin-4) were measured in BALF at baseline and week 6. The effect of forage on exposure, airway cytology and cytokines was evaluated using generalized linear mixed models. Adjusted p-value <0.05 was considered significant.

The results showed that respirable dust exposure was lower in horses fed haylage than hay (0.02±0.001 mg/m^3 vs. 0.06±0.01 mg/m^3; p=0.03). Beta-glucan exposure was lower in horses fed haylage than hay (69±18 pg/m^3 vs. 160±21 pg/m^3; p=0.02). By week 6, horses fed haylage had lower BALF neutrophilia than horses fed hay (0.7±0.2% vs. 4.0±0.7 %; p=0.0004). In horses eating haylage, BALF neutrophil proportion decreased between baseline (2.2±0.5%), week 2 (0.8±0.3%; p=0.01) and week 6 (0.7±0.2%; p=0.03). Interleukin-4 concentration in BALF was higher at week 6 (14.4±4.6 pg/ml) in horses fed hay compared to baseline (2.9±4.6 pg/ml; p=0.007).

In conclusion, feeding haylage instead of hay to horses in training can reduce exposure to respirable irritants and mitigate airway neutrophilia.

3.2 Introduction

Mild-moderate equine asthma (EA), previously known as inflammatory airway disease,[1] is a non-septic inflammatory disease of the equine respiratory system.[17] Clinical signs may include

decreased performance and chronic, intermittent cough.[50,53] Diagnosis is confirmed by detecting excessive tracheal mucus with endoscopy or increased proportion of inflammatory cells on bronchoalveolar lavage fluid (BALF) cytology.[1] Mild EA may be diagnosed in up to 80% of actively racing Thoroughbreds based on BALF cytology.[17]

Horses are exposed to high concentrations of aerosolized particles inside barns.[103] Exposure to barn environment is associated with airway disease both in horses[17,205] and people working in horse barns.[206] Barn dust contains a variety of organic particles such as fungi, molds, endotoxin, β-glucan, debris, and bacteria.[103,104] Dust composition depends on husbandry practices such as feed and bedding used.[103] Two major pro-inflammatory components of organic dust are β-glucans, originating mainly from fungi and plant cell walls, and bacterial endotoxins.[92] The main source of respirable dust in stabled horses is dry hay.[103] Feeding hay has been shown to be associated with up to a 10-fold increase in exposure to airborne dust in the horses' breathing zone when compared to pasture.[75] Other forage options with lower dust exposure are available. Haylage, for example, results in a 60-70% reduction in breathing zone measures of dust exposure compared to hay. [104] Haylage is typically grown and cut at similar stages as hay, except that it is harvested when the moisture is still high (30-50 %).[207] Feeding silage to severely asthmatic horses during stabling can maintain them in clinical remission,[208] but whether feeding haylage to racehorses in training benefits airway health is currently unknown.

Neutrophilic airway inflammation in racing Thoroughbreds is correlated with respirable dust exposure, and mast cell inflammation is associated with β-glucan exposure.[17] Both types of inflammation are negatively related to performance.[17] Exposure data also suggest a positive interaction between endotoxin and dust particulates.[17,61] Feeding horses with moderate to severe EA a low dust diet helps reduce airway inflammation and improves clinical signs such as performance and cough.[129] However, it is unknown if reducing exposure to respirable dust and associated irritants in healthy horses in training decreases airway inflammation.

Cytokine profiles associated with cytological phenotypes of mild-moderate EA are controversial.[61–63] Neutrophilic inflammation in horses may lead to an activation of the innate immune system with an increase in tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) concentration in BALF[63] but others reported only increased expression of interleukin (IL)-1β.[61]

An increase in helper T-cell type 2 (Th-2) cytokine expression, such as IL-4 in BALF, has also been reported in horses with mast cell inflammation.[61,62]

Therefore, we hypothesized that horses in training fed haylage for 6 weeks will be exposed to lower respirable irritants (dust, endotoxin, β-glucan) and exhibit significantly lower BALF neutrophil and mast cell proportions, and pro-inflammatory cytokine concentrations (IL-4, TNF-α, IFN-γ) than horses fed dry hay.

3.3 Material and methods

3.3.1 Experimental Design

A prospective clinical trial was designed as a pilot study to compare the effect of forage on dust exposure and airway inflammation in horses during training. Seven healthy Standardbred racehorses (3 mares and 4 geldings) between 4 and 9 years old and body weight of 454 ± 45 kg were used in this study. Prior to enrollment, horses had been housed at a single private facility on pasture during the day and stabled during the night. For the study, horses were housed in the same climate-controlled barn sharing air space in individual stalls bedded with wood shavings and managed identically with the exception of the assigned forage. Horses were trained 5 days a week on a high-speed treadmill over a 6-week period to establish uniform fitness using a protocol similar to a previous study.[209]

After enrollment, horses were allocated into two groups, one fed good quality alfalfa hay (n=3) and the other fed haylage (n=4) using simple randomization through a random number table. Both forages were fed on the ground. Those horses assigned to be fed haylage were gradually transitioned from hay to haylage over a period of 7 days. Consumption of forages increased progressively from 1.8% body weight/day during week 1 to 2.2% body weight/day by week 6. Horses were also fed increasing amounts of concentrate (Omolene 200®, Purina Animal Nutrition, St Louis, Missouri) in accordance to the training intensity and consumption increased from 0.2% body weight/day at the start of training up to 1% body weight by the end of week 6. At baseline, physical examination, hematology, endoscopy of the respiratory tract and BAL were performed on each horse. Horses were enrolled if they showed no evidence of respiratory or systemic disease by physical examination and complete blood count. Endoscopy was repeated at week 6. Physical examination and BAL were repeated at weeks 2, 4 and 6. Dust exposure was measured at the

breathing zone of each horse on two occasions between week 4 and week 6. Horses achieved peak fitness by the end of week 6 as indicated by the plateau reached by the horses' speed for a blood lactate of 4 mmol/L.

3.3.2 Breathing zone respirable particulate measurements

Gravimetric filter sampling was conducted as previously described.[80] Respirable and inhalable particulate samples were collected in the horse's breathing zone over the course of 6 hours using a personal sampler secured to the noseband of the halter. The respirable fraction (particles with 50% cutoff of 4 μm that can penetrate deep into the lungs) was collected onto glass fiber filters (type A/E, diameter of 37 mm) using an aluminum cyclone (P225-01-02, SKC, Inc., Eighty-Four, Pennsylvania) and the inhalable fraction (particles with 50% cutoff of 100μm that can be inhaled from surrounding air) onto 25 mm PVC filters using an IOM sampler (SKC, Inc., Eighty-Four, Pennsylvania). Both samplers were connected by flexible tubing (Tygon, Saint Gobain, France) to sampling pumps (AirChek 2000, SKC, Inc., Eighty-Four, Pennsylvania) which were secured to a surcingle on the girth of the horse. The horse was free to eat, drink, and move around the stall as usual. The change in weight of each filter was divided by the volume of air sampled to determine dust exposure in mg/m^3.

3.3.3 Beta-glucan and endotoxin analysis

Respirable dust samples were stored at -20°C until measurement of β-glucan and endotoxin. The content of β-glucan and endotoxin in the respirable dust was measured using a kinetic chromogenic Limulus amebocyte lysate (LAL) technique (NexGen PTS0, Charles River Laboratories, Wilmington, Massachusetts) as described by Ivester et al.[17]

3.3.4 Endoscopic Examination

A 7.9 mm OD flexible video endoscope was passed through the ventral meatus to the level of the larynx, while the horses were restrained with a lip twitch. Laryngeal function was recorded, and any upper airway abnormalities were noted. Then the endoscope was advanced down the trachea, until the carina was visible, to assess the mucus score. A score between 0-4 was assigned

to tracheal mucus.[97] A dilute lidocaine solution (0.2%, 30-60 mL) was sprayed into the airway (larynx and carina) as the endoscope was removed to prevent coughing during the BALF procedure.

3.3.5 Bronchoalveolar lavage

Horses were sedated with butorphanol (0.02-0.04 mg/kg IV; Torbugesic, Zoetis, Parsippany-Troy Hills, New Jersey) and with xylazine hydrochloride (0.2-0.5 mg/kg IV; AnaSed, Akorn Animal Health, Lake Forest, Illinois). A sterile BALF tube (300 cm long; 10 mm outer diameter; Bivona Medical Technologies, Gary, Indiana) was passed through the nose and wedged into a distal bronchus. Two hundred fifty (250) mL of sterile 0.9% sodium chloride were instilled and recovered using 60 ml syringes. BALF was filtered through sterile gauze and immediately placed on ice and processed within one hour of collection. Cytospin preparations were performed and slides processed with modified Wright stain. Differential cell count was determined by enumerating 600 cells per horse by a single individual (CO); epithelial cells were not included in the cells counted.

3.3.6 Cytokine measurements

In order to determine cytokine concentrations, ELISA tests were performed on BALF supernatant collected at baseline and week 6. Samples were stored at -80˚C until analyses. BALF samples were diluted as needed. TNF-α, IL-4 and IFN-γ were measured using equine-specific ELISA kits (R&D Systems, Minneapolis, Minnesota) according to the manufacturer's instructions. The readings were measured using a plate reader (BioTek, Winooski, Vermont). Measures were performed in duplicate and average recorded.

3.3.7 Data analysis

Generalized linear mixed models were constructed to determine the effect of forage on dust, endotoxin, and β-glucan exposures and to evaluate the effect of forage over time on BALF inflammatory cell proportions and cytokine concentrations.[17] Model assumptions and residual distributions were checked graphically. Significance of post hoc pairwise comparisons was controlled by Tukey's post hoc method, and an adjusted p value of <0.05 was considered significant. Data analyses were performed using ProcGLIMMIX SAS v.9.4 (SAS Institute, Cary,

North Carolina), and graphs were made with GraphPad Prism 8 (GraphPad Software, San Diego, California).

3.4 Results

None of the horses developed any signs of respiratory disease. Both feeding protocols were well tolerated, and no adverse effects were observed during the duration of the study.

3.4.1 Exposure to organic dust

Respirable dust exposure was significantly lower in the horses eating haylage when compared with horses eating hay (p=0.03, Figure 3.1). Exposure to inhalable dust was not different between groups (p=0.3, Figure 3.2). Respirable β-glucan exposure was also lower in horses eating haylage when compared with horses eating hay (p=0.02, Figure 3.3). Respirable endotoxin concentration was not different between groups (Hay=1.6±0.3 EU/m^3; Haylage=1.6±0.3 EU/m^3; p=0.8).

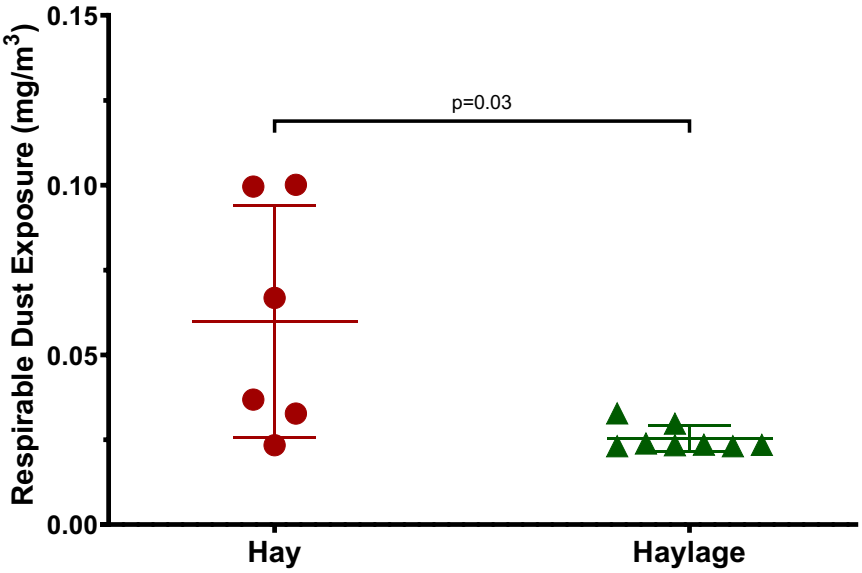


Figure 3.1. Average and standard deviation of respirable dust exposure in the breathing zone of horses fed hay or haylage. Hay n=6, haylage n=8 (2 measurements per horse).

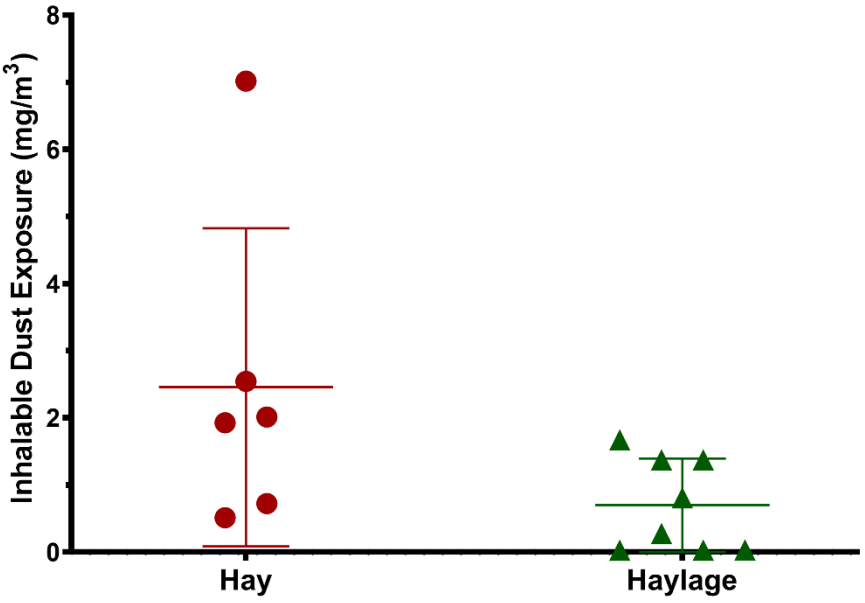


Figure 3.2. Average and standard deviation of inhalable dust exposure in the breathing zone of horses fed hay or haylage.

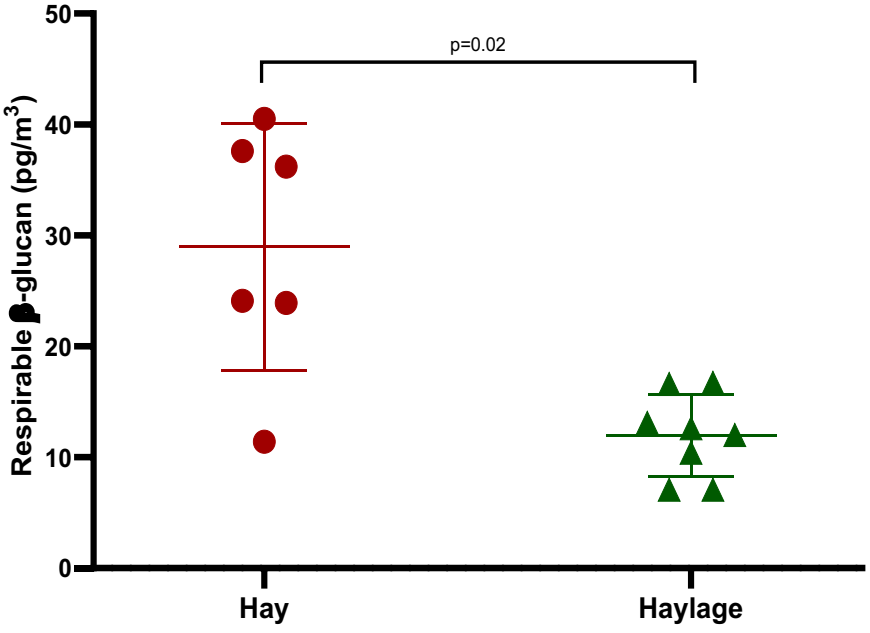


Figure 3.3. Average and standard deviation of respirable β-glucan exposure in the breathing zone of horses fed hay or haylage.

No laryngeal dysfunction was identified. Mucus scores were not different between groups at baseline (hay=0.3 ± 0.3; haylage=0.5 ± 0.3; p=0.7) or week 6 (hay=0.3 ± 0.3; haylage=0.2 ± 0.3; p=0.8).

3.4.2 Airway cytology

The mean BALF recovered was 190 ± 17 ml. At baseline three of the horses (1 in the haylage group and 2 in the hay group) demonstrated BALF mast cell inflammation (>2%), but all had normal neutrophil proportions (<5%). BALF neutrophil proportions were affected by forage assignment over time (p=0.002). The proportion of neutrophils was significantly lower by week 2 in horses eating haylage when compared with baseline (p=0.01, Figure 3.4). By week 6, horses fed haylage had a significantly lower proportion of BALF neutrophils compared to baseline (p=0.03) and compared to horses fed hay at week 6 (p=0.0004). The proportion of neutrophils in the horses fed hay was significantly higher at week 6 when compared with baseline and week 2 (p=0.01 and p=0.001, respectively).

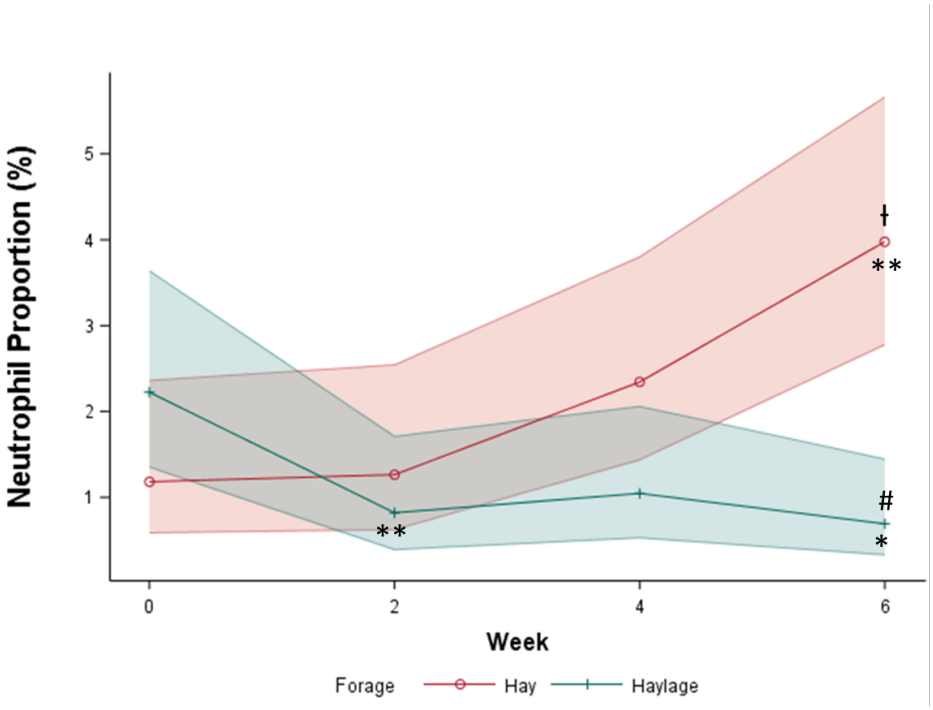


Figure 3.4. Bronchoalveolar lavage neutrophil proportion over time in horses fed hay or haylage. Lines represent the mean and bands represent 95% confidence interval. * denotes differences compared to baseline *= 0.03**=0.01. † denotes differences with week 2 p=0.001. # denotes differences between groups p=0.0004.

Other BALF cell proportions were not different between horses fed hay or haylage at any time point (Table 3.1).

Table 3.1. Summary of BALF mast cells, eosinophils, macrophages, and lymphocytes proportion. Data presented as mean ± SD.

		Hay	Haylage
BALF Mast Cell proportion (%)	Baseline	2.7 ± 0.7	1.7 ± 0.6
	Week 2	3.5 ± 0.9	1.7 ± 0.5
	Week 4	3.0 ± 0.8	2.1 ± 0.6
	Week 6	2.4 ± 0.7	2.3 ± 0.5
BALF Eosinophils proportion (%)	Baseline	2.7 ± 0.7	1.7 ± 0.6
	Week 2	3.5 ± 0.9	1.7 ± 0.5
	Week 4	3.0 ± 0.8	2.1 ± 0.6
	Week 6	2.4 ± 0.7	2.3 ± 0.5
BALF Macrophages proportion (%)	Baseline	57 ± 6.1	59 ± 3.1
	Week 2	60 ± 6.0	68 ± 4.6
	Week 4	57 ± 5.0	64 ± 1.0
	Week 6	53 ± 1.8	65 ± 4.0
BALF Lymphocytes proportion (%)	Baseline	39 ± 6.4	37 ± 2.5
	Week 2	35 ± 5.7	30 ± 4.5
	Week 4	38 ± 1.5	33 ± 1.5
	Week 6	40 ± 1.8	40 ± 1.8

3.4.3 Cytokine concentrations

The BALF concentration of IFN-γ, and TNF-α did not vary between horses eating hay or haylage over time ($p=0.8$ and $p=0.7$, respectively; Figure 3.5). The BALF IL-4 concentration differed between the groups over time ($p=0.008$; Figure 3.5), with a significant increase in IL-4 concentrations measured after 6 weeks in horses eating hay ($p=0.007$; Figure 3.5) when compared to baseline.

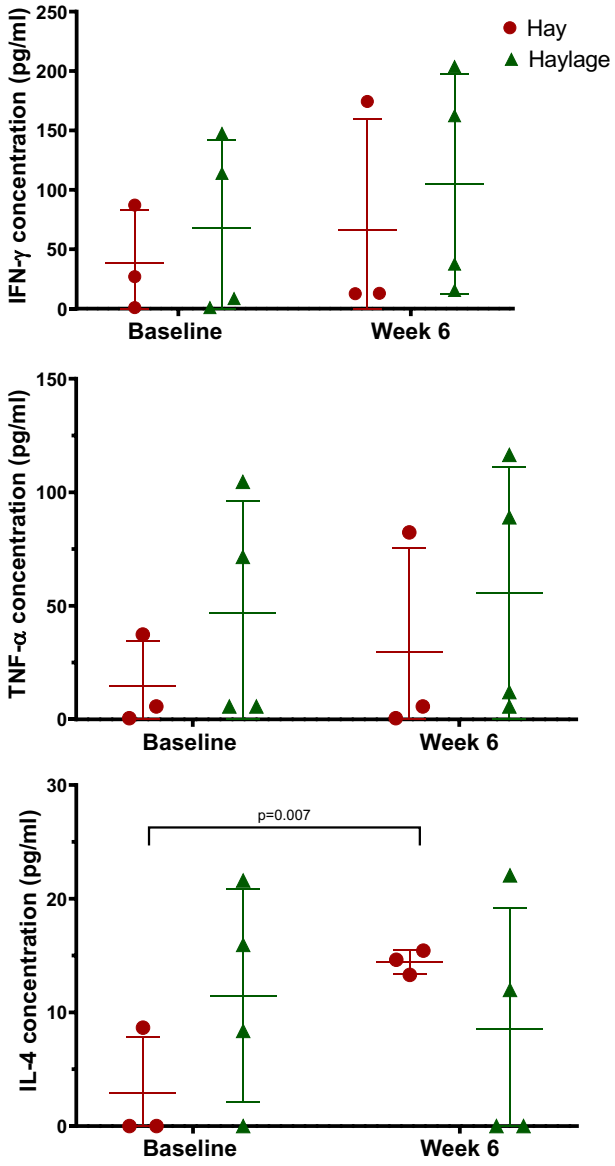


Figure 3.5. Average and standard deviation of BALF IFN-γ, TNF-α, and IL-4 concentrations over time in horses fed hay or haylage.

3.5 Discussion

Feeding haylage to horses during training resulted in a lower exposure to respirable dust and β-glucan concentrations when compared to horses fed hay but did not affect exposure to inhalable dust or respirable endotoxin. Pulmonary neutrophilia was reduced as early as 2 weeks after feeding haylage, while horses eating hay demonstrated increased BALF neutrophil

proportions between weeks 2-6. BALF IL-4 concentrations increased over the course of 6 weeks in horses fed hay but did not change in those fed haylage. Neither TNF-α nor IFN-γ concentrations varied with forage over time. Results from this pilot study suggest that feeding low dust forage such as haylage without any other management changes is sufficient to improve airway health in racehorses in training.

Haylage is a conserved grass that is considered a cross between hay and silage.[210] This forage is cut and allowed to dry but it is wrapped airtight in plastic when the moisture is about 65%.[207] One of the main concerns about feeding haylage is the potential risk of Clostridium botulinum toxin ingestion if the forage is not preserved properly (e.g. puncture of plastic cover). To minimize these risks, it is recommended to carefully examine the bales for the presence of molds, and for bales to be fed within a 3-7 days of opening.[211,212] A killed vaccine against *Clostridium botulinum* type B is available and should be considered before introducing haylage to the diet however, botulism may be caused by other serotypes and there is no cross-protection between serotypes. None of the horses in this study were vaccinated and no side effects developed while horses were eating haylage. Care was taken to discard any part of the haylage bale that appeared grossly molded before feeding the horses.

Exposure to respirable dust was 3 times lower in horses eating haylage when compared to horses eating hay, despite the fact that horses were housed in the same air space with the same bedding. The exposures measured in this study were similar to a previous study reporting mean breathing zone respirable dust exposures of 0.064 ± 0.04 mg/m^3 and 0.026 ± 0.01 mg/m^3 when a pony was housed on shavings and fed either hay or haylage, respectively.[104] Respirable dust breathing zone exposures in the horses eating hay were also similar to those reported in Thoroughbred racehorses fed dry hay and bedded on sawdust (0.055 ± 0.09 mg/m^3).[17,24] In contrast, breathing zone measures of exposure reported in the current study are markedly lower than those reported for a single pony housed under the low dust conditions of shavings and silage (0.22 mg/m^3) and instead comparable to the measures obtained when the same horse was at pasture (0.08 mg/m^3).[75] Dust exposure is also affected by individual horse eating behavior with some horses burying their noses while eating hay or shaking it, therefore, resulting in higher exposure in the breathing zone.[24] The eating behavior of the pony used in this study may have contributed to the higher dust exposure. Additionally, differences in sampling techniques and equipment or quality of the forage may have contributed to the variability. Furthermore, those studies did not report the

effect of varying exposure levels on airway cytology making management recommendations difficult to issue.

Exposure to inhalable dust was not different between groups and did not appear to impact BALF neutrophil proportions. These findings are in agreement with previous studies that found no association between exposure to inhalable dust in the breathing zone and airway inflammation in racehorses.[17,24]

Respirable β-glucan exposure was significantly lower with haylage compared to hay. Horses eating hay were exposed to almost 3 times higher β-glucan levels (160 ± 21.2 pg/m^3) than in a previous study of racehorses eating hay while bedded on sawdust bedding (55.5 ± 66.2 pg/m^3).[17] In that study, β-glucan concentration was positively correlated with BALF mast cell proportion[17], but this association was not present in the current study. Some horses presented airway mast cell inflammation but the average BALF proportion did not change over time. The β-glucan exposure of horses eating haylage (69 ± 0.57 pg/m^3) in the present study was closer to the concentration previously described for horses eating hay.[17] The hay used in the present study appeared grossly of good quality, but these differences in β-glucan levels may reflect differing mold content in hay between studies.

Endotoxin exposure was not different between horses fed hay or haylage in the present study. The endotoxin exposure measured was lower than in a report of Thoroughbred racehorses fed dry hay while housed on sawdust bedding (7.35 ± 12.8 EU/m^3).[17] The concentrations were considerably lower than those reported by Berndt et al,[99] where endotoxin exposure of horses eating hay while bedded on straw was 7080 EU/m^3 and that of horses on pasture was 850 EU/m^3. The median endotoxin exposures of both the horses eating hay (1.48 EU/m^3) and haylage (1.71 EU/m^3) were lower than those described for young Thoroughbreds in training eating dry hay from the ground bedded with sawdust (59.2 EU/m^3).[24] Endotoxin measurements can be highly variable based upon methods and sample handling.[92] The lower endotoxin exposures measured in the current study may relate to the air-conditioned space with a high ventilation rate in which the horses were housed or may reflect differences in the hay quality between studies.

Horses eating haylage had a reduction in BALF neutrophil proportions after 2 weeks in the study, and this reduction was still present at week 6, while horses eating hay experienced an increase in BALF neutrophil proportions after 2 weeks. Considering that the horses were kept in

the same air space and were all bedded on wood shavings, the only differences between the groups was the respirable dust and the β-glucan exposures associated with forage assignment. A previous study described a positive association between respirable dust and BALF neutrophil proportions such that each 0.1 mg/m^3 increase in respirable dust is predicted to increase BALF neutrophil proportion by 30%.[17] Horses presented relatively low BALF proportions at baseline (1.9 ± 1.3%), presumably due to being housed mainly at pasture prior to enrollment. The BALF neutrophil proportions of horses eating hay at week 6 (4.0±0.66%) was consistent with results from Thoroughbred racehorses eating hay while on sawdust bedding (4.8 ± 4.0%).[17] Though the mean neutrophil proportion at all time points was considered within the reference range, it is important to consider that even a small increase in the neutrophil proportion can have a negative effect on racing performance.[17]

Of the cytokines measured in BALF, only IL-4 concentration was different between groups, increasing by week 6 in the horses fed hay, but not in those fed haylage. IL-4 production is a hallmark of a Th2-type response, suggesting the role of aeroallergens in the development of mild equine asthma, as also suggested by the presence of mast cells or eosinophils in the airways of some horses with mild asthma.[61,62] Mast cell inflammation was observed in some of the horses from both groups during the study, but the mean mast cell proportion did not change over time. IL-4 is also a potent activator of neutrophils at the site of inflammation and this activation produces the release of other pro-inflammatory cytokines such as IL-8 that are involved in the influx of more neutrophils to the site of inflammation.[66] Thus, the increase in IL-4 concentrations may be related to the increase of BALF neutrophil proportions in the horses fed hay. The finding that TNF-α and IFN-γ were not different between groups after 6 weeks was consistent with a previous report,[61] although these cytokines have been related to Th-1 polarization and neutrophilic airway inflammation in racehorses in another study.[63] It is important to note that for the current study no correction for BALF dilution was made although, this potential confounding factor was likely minimum considering the small variation in BALF volume return compared to the volume instilled (76.0 ± 6.8 %). The main limitation of this study was its small sample size. The small number of horses used in the current study may have limited our ability to detect any effect of forage on TNF-α and IFN-γ concentrations. However, the data suggest that there was no effect of forage on BALF cytokine concentrations overtime rather than a lack of power to detect an effect. In fact, a sample size calculation showed that in order to detect a difference between baseline and week 6 in TNF-

α concentrations, we would need a sample size of 102 horses per group, suggesting minimal effect of forage upon this cytokine.

3.6 Conclusions

Feeding haylage to healthy racehorses in training reduced exposure to respirable dust and β-glucan, resulting in attenuation of airway neutrophilia when compared to horses eating hay. These preliminary findings will require further studies to determine the effect of low dust forages on lung inflammation in racehorses in natural conditions at the track and determine if this management change can prevent or mitigate mild equine asthma.

CHAPTER 4. EFFECTS OF FORAGE AND PRO-RESOLVING LIPIDS ON AIRWAY INFLAMMATION IN HORSES

4.1 Abstract

Objective: This study compared resolution of airway inflammation when horses transitioned from high-dust to two low-dust forages that presumably differed in omega-3 polyunsaturated fatty acids (Ω-3) content.

Animals: 20 horses with mild airway inflammation

Procedures: Horses previously eating hay were fed pellets (low-Ω-3, n=10) or haylage (high-Ω-3, n=9) for 6 weeks. Dust exposure was measured in the breathing zone using a real-time particulate monitor. Bronchoalveolar lavage (BAL) was performed at baseline, week 3, and week 6. Apoptosis of circulating neutrophils and efferocytosis displayed by alveolar macrophages was quantified by flow cytometry. Plasma lipid concentrations were measured by liquid chromatography–tandem mass spectrophotometry. Mixed models were constructed to examine the effect of forage upon BAL cytology, lipid concentrations and pro-resolving lipid mediator (PRLM) treatments upon neutrophil apoptosis and efferocytosis

Results: Dust exposure was highest with hay feeding ($p<0.01$) and equivalent between haylage and pellets ($p=0.9$). BAL neutrophil proportions decreased significantly in horses fed haylage (baseline: 11.8±2.4%; week 6: 2.5±1.1%; $p=0.0017$) but not pellets (baseline: 12.1±2.3%; week 6: 8.5%±1.7%; $p=0.28$). At week 6, horses eating haylage had lower BAL neutrophil proportions than those eating pellets ($p=0.014$), and a lower concentration of stearic acid than at baseline ($p=0.048$). PRLM treatments did not affect neutrophil apoptosis or efferocytosis ($p>0.9$).

Conclusions and clinical relevance: Horses fed haylage displayed resolution of airway inflammation despite a similar reduction in dust exposure as those fed pellets. The clinical improvement was not associated with an increase in Ω-3 or an effect of PRLM on neutrophil apoptosis or efferocytosis. Feeding haylage improves airway inflammation beyond that due to reduced dust exposure, though the mechanism remains unclear.

4.2 Introduction

Mild equine asthma is frequently encountered in performance and pleasure horses,[16,17,43] and the disease has been related to respirable organic dust exposure.[17,80] Current treatments rely principally on a combination of pharmacological and environmental management. Drugs used to control airway inflammation are mainly glucocorticoids; however, treatment may not be effective,[116] and their use in competing horses is complicated by potential side effects and drug residues. Therefore, control and prevention of equine asthma without pharmacologic intervention is key.[1]

The main source of dust exposure to horses is hay, the most commonly used forage in horses.[80] Feeding horses low-dust forages, like hay pellets or haylage, can decrease dust exposure in the horse's breathing zone by 60-70% compared to hay and by 90% when compared to poor quality hay, respectively.[104,110] But the resolution of neutrophilic inflammation may require months of low-dust conditions.[127,213] Asthmatic airway inflammation is thought to reflect a failure to resolve inflammation, preventing a return to homeostasis after an inflammatory trigger.[214]

The fatty-acid composition of an individual's diet influences the formation of inflammatory mediators related to many chronic diseases, such as asthma in humans.[141,215] Omega-3 polyunsaturated fatty acids (Ω-3) and Ω-6 intake determines cell membrane composition and differences in the dietary intake of these fatty acids can modify downstream production of pro- and anti-inflammatory mediators.[141,216] Pro-resolving lipid mediators (PRLM) derived from Ω3 are central to the resolution of inflammation,[141] at least in part due to increased apoptosis and clearance of inflammatory neutrophils by efferocytosis.[214] In humans, lipoxin A_4 (LxA4) and resolvin E1 (RvE1) increase neutrophil apoptosis *in vitro*.[158,168] In murine models of airway inflammation, resolvin D1 (RvD1) decreases neutrophilic inflammation and enhances efferocytosis.[175]

Haylage is more abundant in Ω-3 compared to hay;[137] therefore, it may provide additional benefit to equine airway health beyond that of reducing dust exposure. The purpose of this study was to compare resolution of airway inflammation, and plasma PRLM in horses transitioned from a high dust-low Ω-3 diet (hay) to a low dust-high Ω-3 diet (haylage) or low dust-low Ω-3 (hay pellets), and to determine the effect of PRLM on apoptosis of equine neutrophils and efferocytosis of equine neutrophils.

Consequently, we hypothesized that horses transitioning from a high dust-low Ω-3 diet to a low dust-high Ω-3 diet will exhibit a faster resolution of bronchoalveolar lavage fluid (BALF) neutrophilia than horses transitioned to a low dust-low Ω-3 diet (hay pellets), and that horses fed with the high Ω-3 diet will have higher plasma concentrations of PRLM. Also, we hypothesized that PRLM (LxA4, RvD1, and RvE1) would increase the apoptosis of equine neutrophils and their efferocytosis by alveolar macrophages *in vitro*.

4.3 Material and methods

4.3.1 Study design

The study was designed as a prospective trial with 20 clinically healthy horses from the University teaching herd. Fifteen mares and 5 geldings with an average age of 16 ± 6 years, and an average weight of 485 ± 41 kg were kept in dry lots and had free access to round bales of hay from covered feeders for at least 6 weeks prior to the start of the study (Figure 4.1) . Horses were not randomized to groups but rather allocated by housing requirements. One group of horses was fed haylage, and the other one fed hay pellets for six weeks. The horses were housed in two separate dry lots at the same facility but did not share a fenceline. The study was performed during the winter (February and March 2019), so the dry lots remained free of grass. The horses fed haylage were introduced to the new forage gradually over 7 days. Horses on the hay pellet group were fed alfalfa timothy pellets (DuMOR®[a]). Horses were fed forage based on an estimated intake of approximately 2% of body weight per day. They had free access to clean water. At baseline, physical examination, bronchoalveolar lavage (BAL), and blood were collected on each horse. Horses were deemed healthy based on a normal physical examination. Sample collection was repeated at week 3 and week 6. The Purdue University Animal Care and Use Committee approved all procedures.

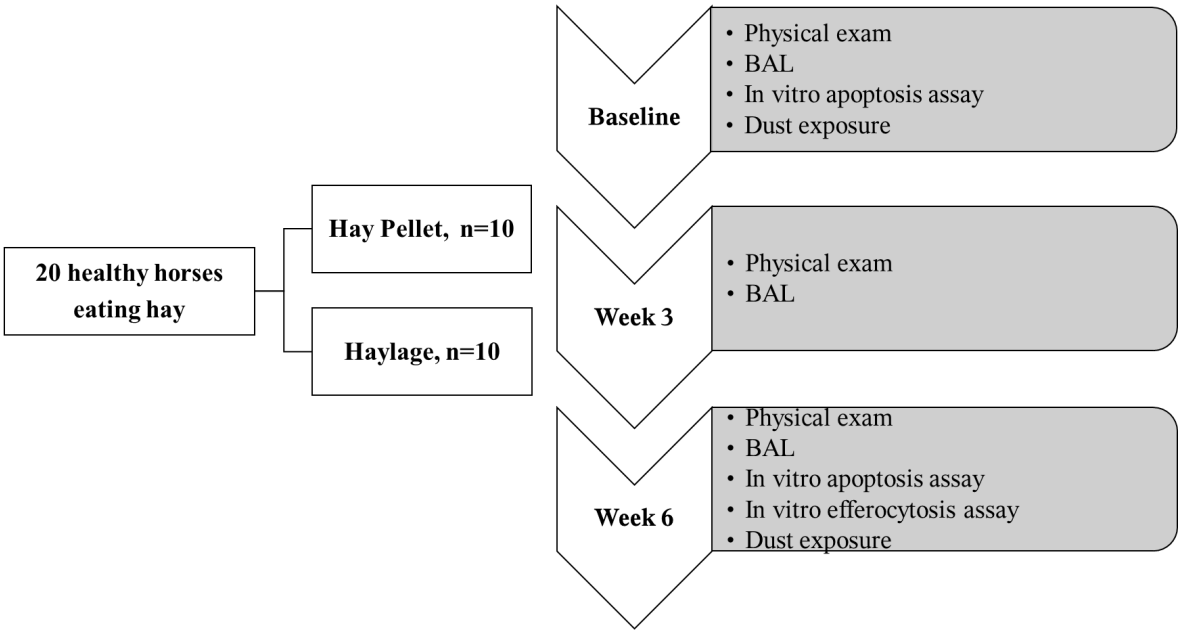


Figure 4.1 Study design.

4.3.2 Clinical score

Horses were trailered to the laboratory located 2 miles from the farm and allowed at least 30 minutes to acclimate before examination. Upon physical examination, a clinical score (range: 0-21) based on cough, nasal discharge, respiratory efforts, and auscultation was determined as previously described.[129]

4.3.3 Blood collection and processing

Blood was collected by jugular venipuncture with a vacutainer into evacuated tubes containing EDTA. Within one hour of blood collection, samples were centrifuged at 1500 g at room temperature for 10 minutes. Plasma was aspirated carefully and placed into plastic tubes with no additives. Plasma was stored at −80°C until lipid quantification.

4.3.4 Bronchoalveolar lavage

Horses were sedated intravenously with butorphanol[b] (0.02-0.04 mg/kg IV) and xylazine hydrochloride[c] (0.2-0.5 mg/kg IV). A sterile BAL tube[d] (300 cm long; 10 mm outer diameter) was passed through the nose and wedged into a distal bronchus. Two hundred fifty (250) mL of sterile 0.9% sodium chloride were instilled and recovered using 60 ml syringes. The BALF was

immediately placed on ice and processed within one hour of collection. Cytospin preparations were performed and slides processed with modified Wright stain. Differential cell counts were determined by enumerating 600 cells per horse by a single individual unaware of forage assignment (CO). Horses were returned to the farm once recovered from sedation.

4.3.5 Particulate exposure measurements

Exposures to particulate matter with diameter less than 1 μm (PM1), particulate matter with diameter less than 2.5 μm (PM2.5) and particulate matter with diameter less than10 μm (PM10) in the horse's breathing zone were measured with a real-time particulate monitor (OPCN2), for 20 minutes on two occasions: when the horses ate dry hay from round bales (four horses per group) and while the horses ate the assigned low-dust forage (10 horses on the pellet group, and 9 horses on the haylage group). The monitor was secured to the crown piece of a break-away halter, and the sampling tube tip was secured to the noseband of the halter in order to sample dust at the breathing zone of the horse. The horse was free to move around, eat, and drink as usual.

4.3.6 Plasma lipids quantification

Lipids mediators were analyzed using targeted liquid chromatography-tandem mass spectrometry (LC-MS/MS) from plasma samples at the Metabolite Profiling Facility, Discovery Park, Purdue University. Five hundred (500) μl of plasma were transferred to a 5 ml vials and spiked with 500 pg of LxA4-d5 standard[f], 500 pg RvD1-d5[f], 2500 pg of PGE_2-d4[f] and 250 pg of RvE1-d4[f]. Methanol (2 ml) was added to the spiked samples. Samples were vortexed for 1 minute and centrifuged at 14,000 rpm for 10 minutes to precipitate the proteins. The supernatant was collected and transferred to a new vial to be evaporated and stored at -80°C until analysis. The dried lipid extracts were reconstituted with 50 μl of methanol/water at 1:1 volume ratio and submitted for targeted quantification by LC-MS/MS.[217,218] Ten (10) μl of the reconstituted sample was delivered to a column (Acquity UPLC BEH C18 (1.7 μm 2.1x100)[g]) through a multisampler (G7167B) into a QQQ6470A triple quadrupole mass spectrometer[h] equipped with ESI Jet Stream ion source. The binary pump flow rate was set at 0.3 ml/min in an Agilent UPLC (G7120A) using water and 0.1% formic acid as mobile phase A and acetonitrile and 0.1% formic acid as mobile phase B. The LC column was pre-equilibrated for one minute with 20% B and a linear gradient to

100% B was set in 28 minutes. Then returned to 20% B in 2 minutes and re-equilibrated for 3 minutes. Concentrations in ng/ml of plasma were obtained by calculating by the ratio of the areas of the endogenous and the deuterated internal standard (IS), then multiplied by the concentration of the IS. For molecules without deuterated internal standard, calibration curves were done with 5 serial dilutions of the stock solution starting at 100 µg/ml as the highest concentration and limit of quantification of quantification of 5 µg/ml and limit of detection of 1 µg/ml. The dynamic range and linear ion intensity response (R^2=0.99) of the calibration curves were observed for over four orders of magnitude. Data processing was carried out by using a dedicated software (MassHunter B.06.00[h]).

4.3.7 *In vitro* assessment of neutrophilic apoptosis

Neutrophils were collected from peripheral blood obtained by jugular venipuncture and isolated with a discontinuous density gradient with Percoll®[i] and centrifugation within two hours of collection.[219] Equine neutrophils were incubated for 30 min with no treatment, PRLM (LXA4 at 100 nM, RvE1 at 10 nM, or RvD1 at 10 nM)[f], or PMA at 100 nM[i] (positive control) at 37°C in 24-well cell culture plates. PRLM concentrations were obtained from similar studies in other species.[158,168,220] After incubation, the neutrophils were washed and resuspended in PBS. Apoptosis was quantified by flow cytometry[i] with FITC-AnnexinV[k], following the manufacturer's instructions, and propidium iodide[k] was used to exclude dead cells. Gating was done manually using a dedicated software (PlateAnalyzer™). [l]

4.3.8 *In vitro* assessment of efferocytosis

Alveolar macrophages were isolated from bronchoalveolar lavage fluid (BALF) by cell culture in complete RPMI-1640 medium[m] for 4 h at 37°C in a humid chamber with 5% CO_2.[221] Neutrophils isolated the day before and incubated in complete RPMI-1640 medium for 16 h at 37°C in a humid chamber with 5% CO_2 to allow the neutrophils to age and become naturally apoptotic as previously described.[222] After the incubation period, neutrophils were stained with Celltracker™ green[m] and alveolar macrophages with Tag-it violet™.[n] Stained neutrophils were co-incubated with stained alveolar macrophages from the same animal, at an approximate ratio of 3:1. Cells were co-incubated and treated with PRLM (LXA4 100 nM, RvE1 10 nM, RvD1 10 nM),

for 30 min at 37°C, 5% CO_2. Following incubation, cells were washed to eliminate all the neutrophils that were not phagocytized. Samples were analyzed by flow cytometry[j], and data analyses were performed using a dedicated software (PlateAnalyzer™). [1]

4.3.9 Statistical analysis

An exploratory analysis of correlations between the various lipid mediators, age, BALF neutrophil proportion, and BALF total nucleated cell counts at baseline was performed by calculating Spearman rank correlations with un-adjusted p-values. Generalized linear mixed models were constructed to examine the effect of forage assignment upon BALF cytology over time, and to compare *in vitro* neutrophil apoptosis and efferocytosis between forage groups and *in vitro* treatments. All models were controlled for age. Tukey-adjusted p-values < 0.05 were considered significant. An a priori sample size calculation indicated that a sample size of 20 horses (10 horses per forage group) would provide 80% power to detect a clinically relevant difference of 5% in BALF neutrophil proportions between groups at a significance level of $\alpha=0.05$. Data analyses were performed using ProcGLIMMIX SAS v.9.4o, graphs were made with SAS v.9.4o ,GraphPad Prism 8p, and MetaboAnalyst 3.0q.

4.4 Results

4.4.1 Horses

One horse from the haylage group was removed from the study due to development of pneumonia during the study day 42. Both feeding protocols were well tolerated by the horses, and no adverse effects were observed during the study. The mean ages of the horses from the pellet and haylage groups were 14.9 ± 6.0 years and 14.4 ± 6.0 years, respectively. Clinical score was not different between groups at baseline (pellet= 3.9 ± 0.8; haylage= 4.9 ± 0.9, p=0.4) or week 3 (pellet= 3.9 ± 0.8; haylage= 5.3 ± 0.9, p=0.2). At week 6, clinical score was significantly higher in the horses fed haylage (4.5 ± 0.8) than those fed pellets (1.9 ± 0.6; p=0.01).

4.4.2 Dust exposure

Breathing zone measure of PM1, PM2.5, and PM10 were available for 8 horses when eating from hay round bales. Four horses were assigned to hay pellet group and 4 horses were assigned to haylage group. Breathing zone measures of exposure were obtained for 10 horses while eating pellets, and 9 horses eating haylage. Dust exposures to PM1, PM 2.5 and PM 10 in the horses' breathing zone were significantly higher when horses were eating round bales of hay (baseline measurement) than when consuming hay pellets (week 6 measurement; Figure 4.2; Appendix A) or haylage (week 6 measurement; n=9; Figure 4.2; Appendix A). Exposures to PM1, PM2.5, and PM10 were not different between horses eating pellets and haylage (Figure 4.2; Appendix A).

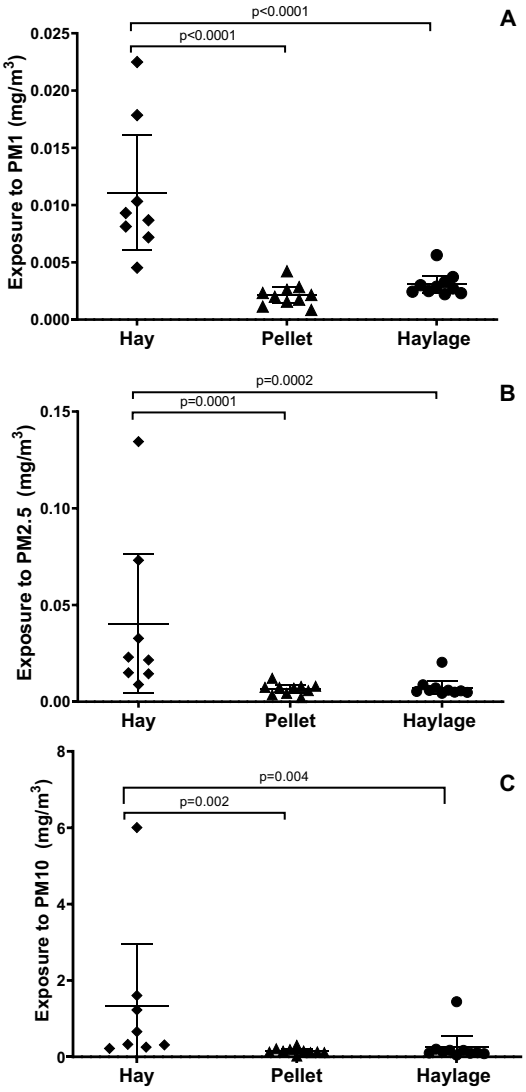


Figure 4.2. Scatter plot of dust exposure in horses breathing zone while eating hay, pelleted hay and haylage. A) Particulate matter with diameter less than 1 µm (PM1) concentration. B) PM2.5 concentration. C) PM10 concentration. Horizontal bars indicate mean and 95% confident interval.

4.4.3 Airway cytology

Horses fed haylage experienced a marked decrease in BALF neutrophil proportions between baseline and week 6 (p=0.0017), while those fed pellets experienced a mild but non-significant decrease (p=0.28; Figure 4.3; Appendix B). At week 6, horses eating haylage had significantly lower BALF neutrophil proportions than those eating pellets (p=0.014; Figure 4.3, Appendix B). BALF cytology data for macrophages, lymphocytes, mast cells, and eosinophils showed no effect of time or forage (Table 4.1).

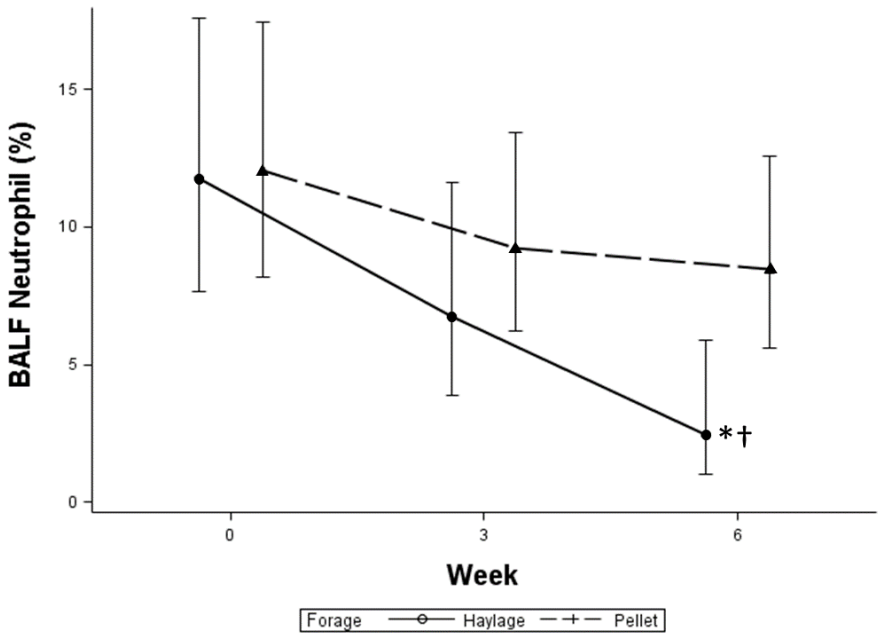


Figure 4.3. Generalized linear mixed model of the effect of forage assignment on bronchoalveolar lavage fluid neutrophil proportions overtime. Horizontal bars indicate 95% confident interval. †Significantly different from baseline (p=0.0017). * Significantly different from pellet group at week 6 (p=0.014).

Table 4.1. Summary of BALF cytology. Data presented as mean ± SD.

	Time	Pellets	Haylage
BALF Mast Cells proportion (%)	Baseline	2.0 ± 0.4	1.9 ± 0.4
	Week 3	1.9 ± 0.4	2.1 ± 0.3
	Week 6	2.0 ± 0.3	1.5 ± 0.3
BALF Eosinophils proportion (%)	Baseline	0.5 ± 0.2	0.2 ± 0.2
	Week 3	0.1 ± 0.1	0.3 ± 0.2
	Week 6	0.2 ± 0.2	0.1 ± 0.1
BALF Macrophages proportion (%)	Baseline	37 ± 3.4	43 ± 3.8
	Week 3	38 ± 2.9	41 ± 3.7
	Week 6	39 ± 3.0	53 ± 3.5
BALF Lymphocytes proportion (%)	Baseline	49 ± 2.6	42 ± 2.7
	Week 3	51 ± 2.3	50 ± 2.8
	Week 6	50 ± 2.3	42 ± 2.6

4.4.4 Plasma lipids quantification

The only quantifiable PRLM was RvD1 and the other PRLM (RvE1, LxA4, Maresin 1, and Protectin 1) were not detected. No effect of forage or time was observed on RvD1, arachidonic acid, EPA, DHA, oleic acid, linoleic acid, palmitoleic acid, palmitic acid, or PGE_2 ($p>0.25$; Appendix C). Stearic acid concentration in plasma was affected by forage over time ($p=0.048$), with a significant decrease at week 6 in horses eating haylage when compared with baseline ($p=0.048$, Figure 4.4). Linolenic acid concentration decreased over time regardless of forage assignment ($p=0.01$).

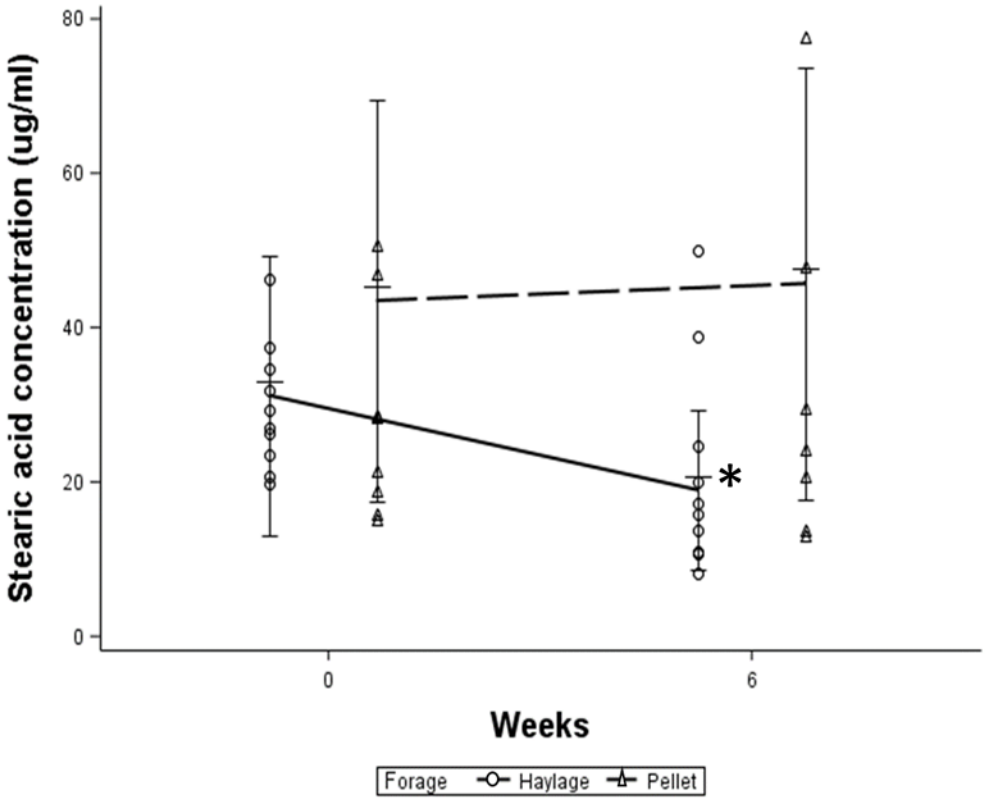


Figure 4.4. Mean and 95% confidence interval of plasma stearic acid concentration per group over time. *Significantly different from baseline (p= 0.04).

There was a negative correlation between PGE_2 and the age of the horses (Rs=-0.5, p=0.04). DHA and EPA were positively correlated, and DHA was negatively correlated with BALF total nucleated cell count (Table 4.2). Concentrations of plasma palmitoleic acid, palmitic acid, oleic acid, linoleic acid, arachidonic acid and stearic acid were significantly correlated (Appendix D).

Table 4.2 Spearman rank correlation of DHA, EPA, RvD1, age, and neutrophil proportion, and total nucleated cell count (TNCC) in BALF. Correlation coefficient (unadjusted p-value). Statistically significant correlations are in bold.

	Age	RvD1	EPA	DHA	TNCC	Neutrophil (%)
Age	1	-0.1 (0.7)	0.02 (0.9)	0.19 (0.4)	-0.38 (0.1)	0.30 (0.2)
RvD1		1	0.37 (0.1)	0.40 (0.1)	-0.07 (0.8)	-0.10 (0.7)
EPA			1	**0.84 (<0.0001)**	-0.35 (0.1)	-0.06 (0.8)
DHA				1	**-0.49 (0.03)**	-0.13 (0.6)
TNCC					1	0.15 (0.5)
Neutrophil (%)						1

4.4.5 *In vitro* determination of apoptosis and efferocytosis

Apoptosis did not differ between forage groups or time points. The only effect of treatment was observed with an increase in apoptosis with PMA (positive control) compared to no treatment in both groups (Figure 4.5; Appendix E) as expected.

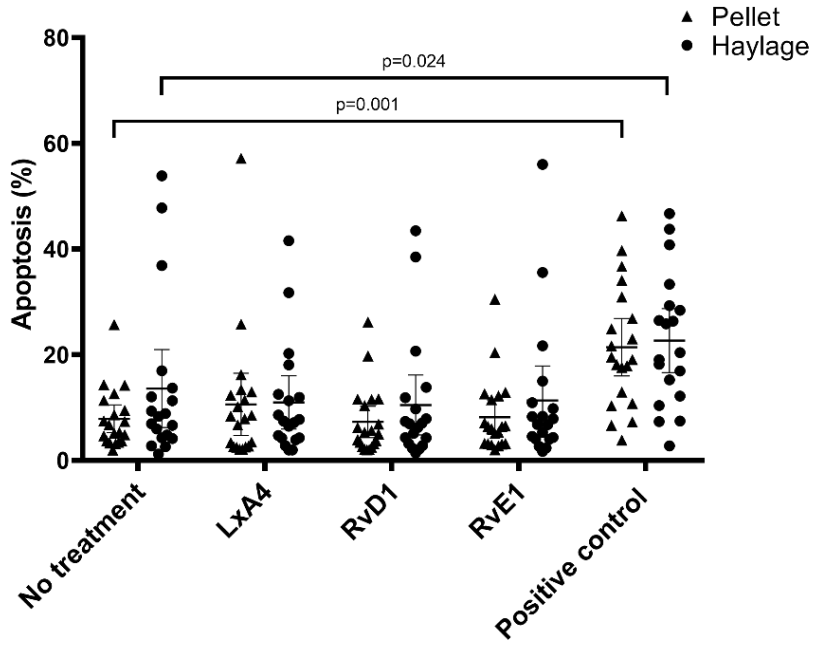


Figure 4.5. Scatter plot of neutrophil apoptosis (%) with baseline and week 6 data pooled. Horizontal bars indicate mean and 95% confident interval.

Overnight incubation in culture media resulted in apoptosis in roughly 50% of isolated neutrophils (FITC-AnnexinVpositive) at the moment time of the efferocytosis assay. Efferocytosis was significantly higher in the horses eating pellets when compared with the horses eating haylage ($p=0.005$; Figure 4.6; Appendix F). There was no difference in efferocytosis between PRLM treatments ($p>0.33$).

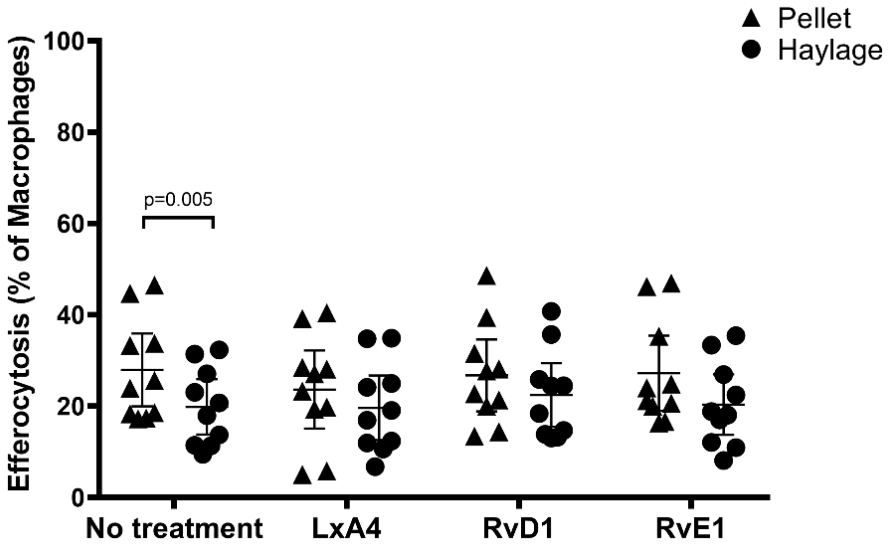


Figure 4.6. Scatter plot of efferocytosis (%) at week 6. Horizontal bars indicate mean and 95% confident interval.

4.5 Discussion

The purpose of this study was to study the role of dust exposure and lipid mediators in the resolution of neutrophilic airway inflammation when horses transitioned from high-dust to low-dust forages. Another objective was to gain insight into the mechanism of resolution of airway neutrophilia by studying the effect of PRLM on apoptosis of equine neutrophils and efferocytosis of neutrophils by alveolar macrophages *in vitro*. We found that horses fed hay from round bales were exposed to high levels of small dust particles (PM1, PM2.5, and PM10) in the breathing zone, and this exposure was associated with mild neutrophilic airway inflammation. Transitioning horses' diet from hay to haylage resulted in a marked decrease in airway neutrophilia over 6 weeks. Only a mild decrease in airway neutrophilia, albeit not statistically significant, was observed in horses fed hay pellets for 6 weeks, despite a similar reduction in dust exposure as horses fed haylage. Stearic acid was the only lipid mediator measured that differed between horses fed haylage and

pellets. Contrary to our hypothesis, efferocytosis was higher in horses eating hay pellets when compared to those eating haylage, and we found no evidence of any direct effect of pro-resolving treatments on apoptosis or efferocytosis *in vitro*.

As anticipated, breathing zone measures of PM1, PM2.5, and PM10 were not different between low-dust forages, and both pelleted hay and haylage resulted in significantly lower dust exposure when compared to round bale hay. Exposure to PM2.5 and PM10 has been related to visible tracheal mucus score in racehorses.[100] Similarly, PM10 has been associated with tracheal mucus score and also neutrophil counts in tracheal wash cytology in racehorses.[101] In humans, PM2.5 has been associated with an increase in the prevalence and morbidity of respiratory diseases such as lung cancer and asthma.[223] As expected the PM10 measured at the breathing zone of horses eating round bales (0.12 mg/m^3) was higher than those reported for stabled Thoroughbreds eating hay (0.073 mg/m^3).[101]

All horses displayed mildly increased neutrophil proportions at baseline. These horses were kept on dry lots during the winter and fed round bales of hay for at least 6 weeks prior to the study. Similar results had been described in a population of horses eating round bales of hay during winter, where 80% of the horses presented with a mild to moderate increase in BAL neutrophil proportions however, dust exposure was not measured.[91] After 6 weeks on low-dust diets, both groups of horses in the current study showed a decrease in BAL neutrophil proportion, but this decrease was only statistically and clinically significant in horses eating haylage. In previous studies, horses with severe equine asthma exacerbation placed on a low-dust diet (pasture and pelleted feed) demonstrated an improvement in BAL neutrophilia, but only after 2 to 6 months.[127,213] BAL neutrophil proportions fell below 10% in severely asthmatic horses eating pelleted feed and kept on pasture, but only after 6 months on this regimen.[127] Horses with severe equine asthma kept on grass pasture, a diet naturally higher in Ω-3 than dried forages,[137] may exhibit resolution of BALF neutrophilia within 2 months.[130] Similarly, horses with severe asthma eating a low-dust diet (complete pelleted diet) and an Ω-3 supplement for 2 months displayed significantly greater improvement in clinical signs and BAL neutrophilia when compared to a group that received the low-dust diet with a placebo supplement.[129] Taken together, these results suggest that the presumed higher content of Ω-3 of the haylage fed in the current study contributed to the rapid resolution of BALF neutrophilia, similar to that seen with Ω-3 supplementation and access to pasture.

We were unable to detect most of the targeted PRLM using LC-MS/MS. This technique has been used to measure plasma PRLM in humans and rodents,[224,225] but the detection of these molecules remains challenging.[226] These mediators display bioactivity at concentrations in the picomolar and lower nanomolar ranges.[227] PRLM such as LXA4, RvD1, RvE1, maresin1, and protecting 1 may have been present at concentrations and activity that differed between forage groups but were below our limit of detection. We were only able to measure plasma RvD1 from the horses in this study, and 20 samples were under the limit of detection (50 pg/ml). The average plasma concentration at baseline in the horses was 142 ± 169 pg/ml; this concentration is higher than those previously described in humans (24.4 ± 2.5 pg/ml).[224,228] RvD1 concentration did not change with the haylage, despite the presumed higher content of Ω-3. The only plasma lipid affected by forage was stearic acid, which significantly decreased with the consumption of haylage. Stearic acid is a saturated fatty acid present in forages[229] such as hay, and has been reported to increase as forages become more mature.[230] A possibility is that pellets were made from more mature alfalfa grass as compared to haylage. Stearic acid has been related to inflammatory processes such as osteoarthritis and obesity in humans. *In vitro* studies with stearic acid indicate a pro-apoptotic effect on macrophages[231] and the capacity to enhance the production of oxygen radicals by neutrophils.[232] Stearic acid was correlated with oleic acid and arachidonic acid, lipid mediators that are associated with inflammatory processes in humans.[233] Thus, the effect of haylage upon stearic acid concentrations may reflect decreased dietary intake compared to pellets or a potential role of this molecule in airway inflammation, or both.

Prostaglandin E_2, a fatty acid that is derived from arachidonic acid, was negatively correlated with age at baseline. The opposite has been reported in humans and mice models with an increase in PGE_2 production seen with aging. This increase in synthesis has been related to various chronic diseases in elderly humans, such as arthritis, and cancer.[234] On the other hand, PGE_2 has been described to prevent allergen–induced bronchoconstriction and to reduce airway hyper-responsiveness and inflammation in bronchial asthma in humans.[235] In horses with severe asthma, diminished production of PGE_2 by airway mucosa has been reported,[236] while others found increased BALF PGE_2 concentration.[237] In the current study, plasma PGE_2 concentrations decreased after 6 weeks in the horses fed haylage, but the change did not reach statistical significance. This could be related to the small sample size and the high variability of the measurements. Power calculations were performed, and to detect the effect of forage on RvD1

between forage groups at week 6, we calculated that a sample size of 52 horses per group would be necessary.

Decreased neutrophilic airway inflammation over the 6 weeks course of the study as a result of low-dust exposure was expected to be secondary to enhanced neutrophil apoptosis and efferocytosis mediated by PRLM. Horses fed haylage did indeed exhibit improved resolution of BAL neutrophilia, but we did not find increased neutrophil apoptosis nor increased efferocytosis in this group. Instead, horses on the low-Ω-3 pellet diet demonstrated greater alveolar macrophage efferocytosis. The increased efferocytosis observed in the pellet group may be an indication that alveolar macrophages maintained an activated state due to continued inflammation, while resolution of inflammation in the haylage group resulted in a more quiescent population of alveolar macrophages.[238] Neither apoptosis of neutrophils harvested from the systemic circulation nor efferocytosis of apoptotic neutrophils by alveolar macrophages was affected by pro-resolvins treatments at the concentrations and incubation times used in this study. PRLM have been reported to affect both apoptosis and efferocytosis *in vitro* in other animal models and humans.[155,158,168,175,214] The concentrations of the PRLM and incubation times used in the current study were reportedly effective in previous studies with rodent or human cells,[158,168] but may not have been sufficient for equine cells. Another potential explanation is that multiple PRLM are needed to work in concert. Alternatively, other PRLM such as maresins[239] may be more important in resolving inflammation in the horse. It is also possible that the accelerated resolution of inflammation observed in the horses fed haylage in this study was mainly due to inhibition of transendothelial migration of neutrophils to the lung by PRLM.[153] Such an effect would not be apparent with the type of *in vitro* studies performed in this study.

One limitation of this study was that the groups were made by convenience and not randomised. Other unobserved differences between the groups may have obscured the effect of forage in this study. This latter possibility is unlikely since horses were studied at the same time and were kept in paddocks in close proximity. However, a randomised, cross over study design would have been best to reduce confounding factors. Also, as previously noted, the detection method may not have provided the necessary sensitivity to detect differences in PRLM between the groups, as evidenced by our inability to detect most of the targeted molecules. A larger sample size may have enabled us to detect an effect of forage on those molecules we were able to measure. Finally, Ω-3 and Ω-6 are important components of cell walls.[240] In other species, such as felines[196]

and humans,[241] DHA and EPA have been detected in red blood cells with higher concentrations than plasma. Therefore, measuring the concentration of these molecules in red blood cells could be considered for future studies.

4.6 Conclusion

In conclusion, horses transitioning from round bale hay feeding to haylage and pelleted hay had a comparable reduction in dust exposure to PM1, PM2.5, and PM10, but only those fed haylage for 6 weeks experienced a significant reduction in BALF neutrophilia. This clinical effect was not accompanied by an *in vitro* effect of PRLM treatments on neutrophil apoptosis or efferocytosis. The reduction in neutrophilic airway inflammation exhibited by horses fed haylage is greater than expected by the decreased dust exposure alone however, the mechanism remains unclear.

4.7 Footnotes

[a] TSC, Terre Haute, Indiana 47802

[b] Torbugesic, Zoetis, Parsippany-Troy Hills, New Jersey, 07054

[c] AnaSed, Akorn Animal Health, Lake Forest, Illinois, 60045

[d] Bivona Medical Technologies, Gary, Indiana, 46406

[e] Alphasense, Essex, United Kingdom

[f] Cayman Chemical, Ann Arbor, Michigan 48108

[g] Waters, Milford, Massachusetts, 02451

[h] Agilent Technologies, Santa Clara, California, 95051

[i] Sigma-Aldrich, Saint Louis, Missouri, 63103

[j] CytoFLEX, Beckman Coulter, Indianapolis, Indiana, 46268

[k] BD Biosciences, San Jose, California, 95131

[l] Purdue University Cytometry Laboratories, West Lafayette, Indiana, 47907

[m] Thermo Fisher Scientific, Waltham, Massachusetts, 02451

[n] Biolegend, San Diego, California, 92121

[o] SAS Institute, Cary, North Carolina, 27513

[p] GraphPad Software, San Diego, California, 92108

[q] MetaboAnalyst 3.0 software (http://www. metaboanalyst.ca)

CHAPTER 5. EFFECTS OF LOW-DUST FORAGES ON RACEHORSES' DUST EXPOSURE, AIRWAY CYTOLOGY, AND PLASMA OMEGA-3 CONCENTRATIONS: A RANDOMIZED CLINICAL TRIAL

5.1 Abstract

Mild equine asthma is commonly encountered in racehorses and has been associated with dust exposure. Dust exposure and dietary intake of polyunsaturated fatty acids can be changed by altering forage. The purpose of the study was to compare dust exposure, bronchoalveolar lavage fluid (BALF) cytology, plasma omega-3 levels, and plasma pro-resolving lipid mediators (PRLM) concentration between racehorses fed dry hay, steamed hay, and haylage for 6 weeks.

In this prospective clinical trial, Thoroughbred racehorses actively racing and training were randomly assigned to dry hay, steamed hay, or haylage. Bronchoalveolar lavage (BAL) and differential cell count on cytospin preparations were performed at baseline and after three and six weeks. Exposure to dust was measured on two occasions. Measurements of plasma polyunsaturated fatty acids (PUFAs) and PRLM were measured at baseline and week 6. Mixed models were constructed to examine the effect of forage assignment upon BAL fluid cytology, dust exposure, PUFAs levels, and PRLM concentration.

Data was obtained from 69 horses at week 3 (hay=24, steamed hay=21, haylage=24), and 53 horses at week 6 (hay=17, steamed hay=18, haylage=18). Respirable dust exposure was significantly higher on horses fed hay when compared to steamed hay ($p=0.01$) or haylage ($p=0.005$). Exposure to PM10 was significantly lower on horses fed haylage when compared to hay ($p<0.001$) and steamed hay ($p=0.01$), and significantly lower on horses fed steamed hay when compared to hay ($p=0.004$). Horses eating haylage had a lower proportion of BALF neutrophils at week 3 ($p=0.025$) and 6 ($p=0.003$) compared to horses eating hay and compared to baseline ($p=0.04$). Horses eating haylage exhibited a decrease in mast cell proportions only at week 3 ($p=0.003$) when compared to horses eating hay. Horses eating steamed hay had a decrease in eosinophil proportion at week 3 when compared to baseline ($p=0.03$), and to horses eating hay ($p=0.04$). Eicosapentaenoic acid (EPA) was significantly higher on the horses eating haylage when compared to pooled data from horses eating steamed hay and hay.

In conclusion, feeding low-dust forages reduce dust exposure. Haylage was the only forage that achieved resolution of neutrophilic inflammation within the time frame of the study; this effect could be related to the observed increase of plasma EPA.

5.2 Introduction

Mild-moderate equine asthma (EA) is a commonly encountered respiratory disease in racehorses.[1,17,53] The prevalence of mild-moderate EA in racehorses when determined by endoscopic evidence of excess tracheal mucus has been estimated to be 13-22% in the United States[53,54] and 0.5 – 45% around the world.[55,56,242] However, based on bronchoalveolar lavage fluid (BALF) cytology, mild-moderate EA was diagnosed in 80% of examined Thoroughbreds racing in the US [17] and over 90% of trotters racing in France.[16] While airway inflammation in mild-moderate EA is frequently subclinical, the associated accumulations of tracheal mucus and increased proportions of inflammatory cells recovered in BALF have been related to impaired racing performance,[17,53] presumably due in part to impaired gas exchange.[59,243]

Exposure to organic dust is central in the development of mild-moderate EA.[17,24] Horses that live inside stables are exposed to high concentrations of aerosolized particles and gases cumulatively.[24] Dust composition in horse barns depends on the source and conditions under which bedding and feed materials are grown, harvested, and stored.[103] Endotoxin and β-glucan are two well-characterized pro-inflammatory mediators present in varying concentrations in organic dust.[92] Endotoxins are part of the cell wall of gram-negative bacteria, and as ligands of toll like receptor (TLR)-4 receptors play a major role in innate immune signaling.[244] Beta-glucans are constituents of most fungi, some bacteria, and numerous plants cell walls.[92] Beta-glucans activates TLR-2 in the airway epithelia further influencing the nature of the innate immune response in the airway.[245] In racehorses, respirable β-glucan exposure has been related to BALF mast cell proportions, while respirable dust exposure has been associated with BALF neutrophil proportions.[17] Furthermore, respirable endotoxin exposure appears to modulate the neutrophilic inflammatory response to organic dust.[17]

Environmental management is key in the control of dust exposure and potentially the prevention of mild-moderate EA, especially in racehorses were drugs commonly used to control inflammation may not be effective [116] and should be avoided due to the legal implication of drug

residue detection during racing.[1] Racehorses remain in their stall about 23 hours per day, where they are constantly exposed to higher concentrations of dust than when at pasture.[121] Feeding dry hay is associated with a 4-fold increase in exposure to dust in the horses' breathing zone compared with exposure at pasture.[80] Other forages with higher moisture and lower dust production are available options. Haylage, a conserved forage that is grown and cut at similar stage as hay but is harvested when moisture is about 65%,[246] has been reported to reduce respirable dust exposure in the horses breathing zone between 60-70 percent when compared to day hay.[120] Steaming hay is another method that results in 95% lower respirable dust release *in vitro* when compared to dry hay.[247] The effect of low dust forages on racehorse's dust exposure and associated airway inflammation has not been reported yet.

While dust exposure appears key to the development of airway inflammation in asthmatic horses, resolution of inflammation is often prolonged and incomplete once low-dust management has been instituted.[127,129,130] There is evidence that the chronic and uncontrolled inflammation present in the airways of human asthmatics is the result not only of the increased or repetitive exposure to the triggering stimulus, but also of impaired pro-resolving pathways.[158,248] Pro-resolving lipid mediators (PRLM) are enzymatically derived from polyunsaturated fatty acids (PUFAs). In response to an inflammatory event, PRLM stimulate molecular and cellular events responsible for resolving inflammation and restoring tissue homeostasis,[154,155] such as limitation of leukocyte recruitment, induction of neutrophil apoptosis, and enhancement of efferocytosis by alveolar macrophages at the site of inflammation.[148,158]

Pro-resolving lipid mediators are derived from essential omega-6 (Ω-6) and omega-3 PUFAs (Ω-3).[136] Mammals are not capable of synthesizing essential PUFAs, such as α-linolenic acid ((Ω-3) and linoleic acid (Ω-6),[216] so the relative availability of these molecules is determined by dietary intake. Excessive intake of Ω-6 has been associated with increased inflammatory diseases in people, because of the upregulation of arachidonic acid-derived inflammatory mediators, such as prostaglandins, thromboxane, and leukotrienes.[142] There is competition between Ω-6 and Ω-3 metabolic pathways, and a higher intake of Ω-3 may help mitigate airway inflammation in humans[145] and horses with severe asthma.[129] Racehorses are typically fed dry hay without access to pasture. Dry hay contains lower levels of α-linolenic acid when compared to pasture and haylage.[137] There is evidence that increased Ω-3 consumption associated with pasture

grazing can change horse-meat PUFAs composition,[203] and that supplementation with Ω-3 can increase blood levels of Ω-3.[129] Therefore, in addition to lowering dust exposure, feeding haylage to racehorses may provide additional benefits due to higher Ω-3 content.

Consequently, we hypothesized that changing the forage fed to racing Thoroughbreds from dry hay to haylage or steamed hay will significantly reduce breathing zone exposure measures of respirable dust, β-glucan, and endotoxin and this will result in significantly lower BALF proportions of neutrophils and mast cells. In addition, we expect horses fed haylage will display an enhanced resolution of inflammation compared to horses fed steamed hay that will be associated with higher concentration of plasma PUFAs and PRLMs.

5.3 Material and methods

5.3.1 Experimental Design

A prospective randomized feed trial was designed to compare respirable dust exposure and markers of airway inflammation between horses fed dry hay, haylage, or steamed hay. The study was performed during two racing meets between May 2018 and October 2019. Trainers enrolled horses that resided at the racetrack barns for a minimum of 6 weeks by providing informed consent (Appendix G) and completing a short questionnaire (Appendix H) detailing length of ownership, vaccination history, and any history of respiratory illness. Horses with signs of respiratory infection or systemic illness (fever, abnormal hematology, decreased appetite) were excluded. Upon enrollment, horses were allocated to receive dry hay, haylage, or steamed hay as their sole source of forage using simple randomization through computer-generated random number. Trainers were offered complementary vaccination of their horses against *Clostridium botulinum* type B before assignment to haylage. No other change was made to the horses' management. Horses continued to be fed the same amount of grain, mineral and vitamin supplements according to trainer's preference. All horses were bedded on saw dust. Horses fed hay and steamed hay were eligible for re-enrollment.

At baseline, physical examination, blood collection, endoscopy of the respiratory tract, and bronchoalveolar lavage (BAL) were performed. For those horses assigned to receive haylage, the forage was gradually introduced while hay was gradually excluded from the diet over the course of 7 days. Those horses assigned to dry and steamed hay groups continued to be fed the hay they

received prior to enrollment. Trainers were provided with haylage (C&M Forage) and with a commercially available hay steamer (Haygain™) and instructed on its use. Haylage with visible mold growth was discarded, and trainers were instructed to feed haylage bales within 3 days once opened. Horses remained on the assigned forage for 6 weeks. At 3 and 6 weeks, breathing zone measures of respirable dust exposure, physical and endoscopic examinations, and BAL were performed. During the study, training and racing schedules were continued as usual. The Purdue University Animal Care and Use Committee and the Indiana Horse Racing Commission approved all procedures.

5.3.2 Dust exposure measurements

Gravimetric sampling

Dust exposure measurements were performed at the breathing zone for 3 hours using gravimetric filter sampling as previously described [80]. Briefly, the respirable fraction (50% cutoff of 4 μm) was collected onto polyvinyl chloride (PVC) filters (GLA5000, diameter of 37 mm) using an aluminum cyclone (P225-01-02, SKC, Inc., Eighty Four, PA, USA). The cyclone was secured to the noseband of the halter in order to sample dust at the breathing zone of the horse. A sampling pump (AirChek 2000, SKC, Inc., Eighty Four, PA, USA) was secured to a surcingle placed around the girth of the horse. The pump was connected to the cyclone with flexible tubing (Tygon, Saint Gobain, France) secured to the mane and forelock of the horse. Particulate measurements were determined gravimetrically by subtracting the average of three weights taken before sampling from the average of three weights obtained after sampling. Subsequently, PVC filters were stored at -20°C until elution for β-glucan and endotoxin analyses. Differences between pre and post exposure weight less than 0.02 μg was consider below limit of detection.[118] The detection limit for respirable dust concentration was 0.047 mg/m^3.

Real time sampling

Exposure to particulate number, to particulate matter with diameter less than 1 μm (PM1), particulate matter with diameter less than 2.5 μm (PM2.5), particulate matter with diameter less than 10 μm (PM10) (diameter ≤ 10μm), and particulate matter with diameter between 2.5 and 10

μm ($PM_{10-2.5}$) were measured in the horse's breathing zone in real-time using a particulate monitor (OPCN2, Alphasense, Essex, UK). The monitor was secured to the crown piece of a break-away halter, and sample tubing extending from the inlet was secured to the noseband of the halter in order to sample dust at the breathing zone of the horse. The horse was free to move around the stall, eat, and drink as usual.

Beta-glucan and endotoxin analysis

Respirable dust samples from week 6 were stored at -20°C until analysis. The content of β-glucan and endotoxin in respirable dust was measured using a kinetic chromogenic Limulus amebocyte lysate technique (NexGen PTS0, Charles River Laboratories, Wilmington, MA, USA) as previously described.[17] The limits of detection of the measurements were 43.7 pg/m^3 and 0.02 EU/m^3 for β-glucan and endotoxin, respectively. The precision of the assay was consider to be equal to the lower limit of detection multiplied by the square root of 2 (10 pg/ml β-glucan and 0.005 EU/ml for endotoxin) and this value was multiplied by the dilution factor and divided by the volume of air sampled.[118]

5.3.3 Clinical Score

Upon physical examination, a clinical score (range: 0-21; Appendix I) based on cough, nasal discharge, respiratory efforts, and auscultation was determined as previously described.[129]

5.3.4 Endoscopic examination

Horses were restrained with a nasal twitch and a 7.9 mm OD flexible endoscope was passed through the ventral meatus to the level of the pharynx. A score was assigned to the degree of pharyngeal lymphoid hyperplasia present (range: 0-4).[24] Any upper respiratory tract abnormality was noted. The endoscope was advanced into the trachea and tracheal mucus scored (range: 0-4).[97] To facilitate BAL, the carina and larynx were sprayed with a 0.4% lidocaine solution as the endoscope was removed (20–30 ml at each site).

5.3.5 Bronchoalveolar lavage

Horses were sedated by intravenous injection of xylazine hydrochloride (0.2-0.5 mg/kg; AnaSed, Akorn Animal Health, Lake Forest, ILL, USA) and butorphanol (0.02-0.04 mg/kg; Torbugesic, Zoetis, Parsippany-Troy Hills, NJ, USA). A sterile BAL tube (300 cm long; 10 mm OD; Bivona Medical Technologies, Gary, IN, USA) with inflatable cuff was passed through the nose and wedged into a peripheral bronchus. Two hundred fifty mL of sterile 0.9% NaCl were infused and recovered manually using 60 ml syringes. The BALF was filtered with gauze and immediately placed on ice. Samples were processed within one hour of collection. Cytological specimens were prepared by cytospin centrifugation and processed with modified Wright's stain. Differential cell counts were performed on 600 cells by a single observer (CO); epithelial cells were not included in the cells counted. The following threshold values were used to determine the presence of airway inflammation in BALF (i.e. mild-moderate EA): neutrophils % >5%, mast cells % >2%, or eosinophils >1%.[1]

5.3.6 Fatty acid analysis

Venous blood was collected from the jugular vein into evacuated tubes containing EDTA for CBC and plasma separation at baseline, week 3 and week 6. Plasma samples were stored at -80°C until analysis. Fatty acid analysis of plasma was performed on freshly thawed samples after extraction of lipids by the Folch method,[249] isolation of phospholipids by solid phase extraction using silica cartridges, and methylation utilizing boron trifluoride and gas chromatography.[250] Fatty acid measured included linoleic acid, α-linolenic acid, arachidonic acid, EPA, and DHA.

5.3.7 Pro-resolving lipid mediators analysis

Plasma samples from baseline and week 6 were stored at -80˚C until analyses. PRLM were measured using equine-specific ELISA kits for resolvin D1 (RvD1) and resolvin E1 (RvE1) (MyBiosource, San Diego, CA, USA) according to the manufacturer's instructions. The readings were measured using a plate reader (BioTek, Winooski, VT, USA). Measures were performed in duplicate and the average was recorded.

5.3.8 Data Analysis

Statistical analysis:

Generalized linear mixed models were constructed to examine the effect of forage assignment upon dust exposure, BALF cytology, PRLM concentration, and PUFAs concentration, controlling for age, trainer, and repeated measures. Model assumptions of residual distributions were checked graphically. Significance of post hoc pairwise comparisons was controlled by Tukey's post hoc method, and an adjusted p-value of < 0.05 was considered significant. Data analyses were performed using ProcGLIMMIX SAS v.9.4 (SAS Institute, Cary, NC, USA), and graphs were made with GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA), MetaboAnalyst 3.0q (http://www. metaboanalyst.ca), and SAS.

Sample size calculations:

Preliminary data demonstrated a mean BALF neutrophil proportion of 4.75% (standard deviation= 3.95%) in horses racing at the racetrack and a mean BALF neutrophil proportion of 0.75% (a 4-fold reduction from baseline) in horses fed haylage for 6 weeks. A sample size of 15 horses per group, or 45 total horses, would provide a 90% power to detect a statistically significant difference between groups with $p \leq 0.05$. To allow for subject dropout over the 6-week enrollment period, a target enrollment of 20 per group for a total of 60 horses was planned.

5.4 Results

5.4.1 Horses

Forty-three horses were enrolled in the study. Twenty-one of them were re-enrolled, 12 horses were re-enrolled once, and 9 horses twice. Seventy-three measurements were performed at baseline, 69 at week 3 and 53 at week 6 (Figure 5.1). The drop-out rate between baseline and week 3 was a 7%, and between week 3 and week 6 was 30%; 13 horses left the barn for causes not related with the study, 4 couldn't be sampled because of conflicts with the barn's schedule, and 4 horses couldn't continue on the forage assigned due to an operating error. Five stallions (12%), 20 geldings (47%), and 18 mares (42%) were enrolled, and the age was 4.0 ± 1.7 years (mean ±

standard deviation). Four trainers participated, one with 33 horses (77%), one with 6 horses (14%), and two with 2 horses each (4.5%). Clinical and BAL parameters measured at baseline for horses enrolled in the study did not differ between forage groups (Table 5.1). At baseline, mild-moderate EA was observed in 66 out of 73 measurements (90%). Mast cell inflammation was the most commonly observed (36%), followed by mixed inflammation (33%), neutrophilic inflammation (18%) and eosinophilic inflammation (4%).

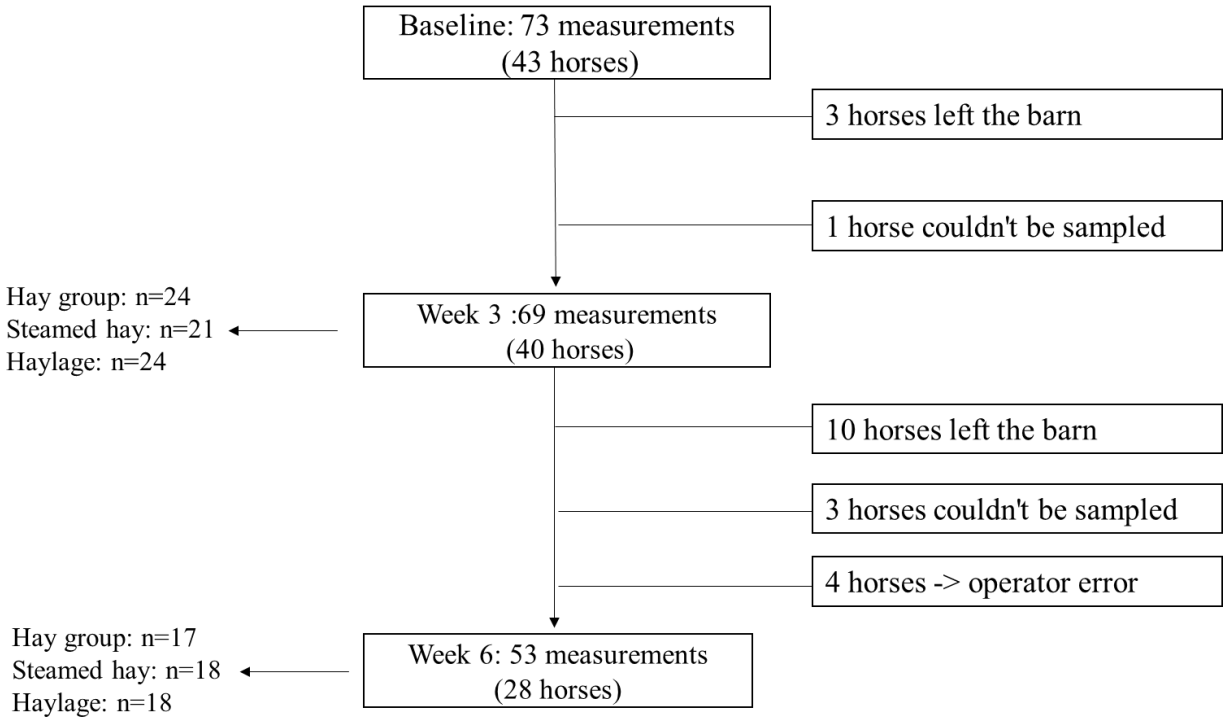


Figure 5.1. Flow diagram of horses that participated in the trial and forage assignment.

Table 5.1. Clinical and airway cytology variables of horses assigned to each forage group. Data presented as mean ± SD.

	Hay	Steamed Hay	Haylage	p-value
Clinical Variables				
N	28	22	23	
Age	3.6 ± 1.2	3.9 ± 1.2	4.2 ± 2.0	p = 0.40
Sex	16 geldings, 8 mares, 4 stallions	12 geldings, 10 mares	7 geldings, 15 mares, 1 stallion	
Respiratory rate (breaths per min)	20 ± 2	21 ± 5	19 ± 4	p = 0.30
Clinical Score	2 ± 1	2 ± 1	2 ± 1	p = 0.54
Mucus Score	1 ± 1	1 ± 1	1 ± 1	p = 0.77
Bronchoalveolar lavage fluid cytology				
Total nucleated cell count (cells/μl)	303 ± 112	291 ± 64	273 ± 104	p = 0.50
Macrophages (%)	50.3 ± 9.7	49.1 ± 9.6	50.0 ± 9.0	p= 0.87
Lymphocytes (%)	40.0 ± 8.2	41.0 ± 7.0	42.1 ± 8.7	p= 0.65
Neutrophils (%)	4.8 ± 2.9	5.2 ± 4.0	5.4 ± 3.6	p= 0.84
Mast cells (%)	2.9 ± 1.5	2.9 ± 1.3	2.5 ± 1.0	p= 0.86
Eosinophils (%)	2.6 ± 8.1	2.1 ± 7.7	1.0 ± 1.0	p= 0.60

5.4.2 Effect of forage on dust exposure

Gravimetric respirable dust data collected during the second year of sampling was not included in the analyses due to a technical error with the scale. During the first year, 72 measurements of respirable dust were performed, 48 of those measurements were under the limit of detection, 8 on the horses fed hay, 19 on the horses fed steamed hay and 21 on the horses eating haylage. Horses' exposure levels to respirable dust while fed steamed hay (n=28) or haylage (n=27) were significantly reduced compared to horses fed dry hay (n=17; p=0.01 and p=0.005, respectively; Figure 5.2).

Real-time exposure data was collected in all the horses at week 3 and week 6. Horses' average particle number exposures over the 3-hour sampling period did not vary significantly with forage (Hay= 2.8± 0.17 particles/m^3 n=41; Steamed hay= 2.8±0.16 particles/m^3 n=39; Haylage=2.7±0.16 particles/m^3 n=42). Average PM1 and PM2.5 did not change with forage assignment (Figure 5.3 A-B). Average PM10 and $PM_{10-2.5}$ were significantly higher on horses eating hay (p=0.01) and steamed hay (p<0.001; Figure 5.3 C-D) when compared with horses eating

haylage. PM10 was also significantly higher on the hoses eating hay when compared with the horses eating steamed hay (p=0.04; Figure 5.3 C).

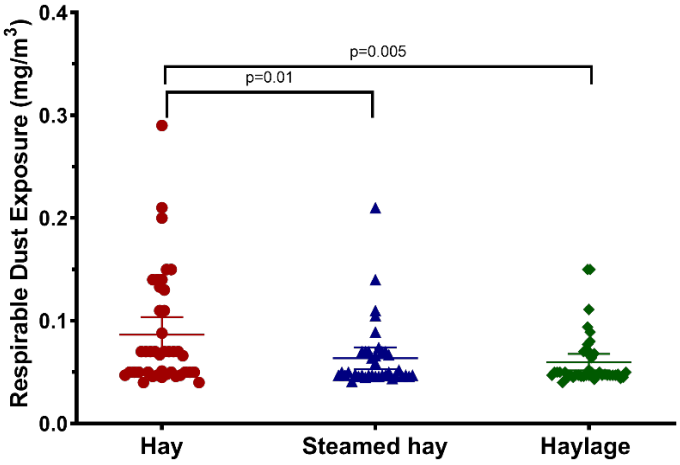


Figure 5.2. Scatter plot of breathing zone respirable dust concentration in horses fed dry hay, steamed hay or haylage. Horizontal bars indicate mean and 95% confidence interval.

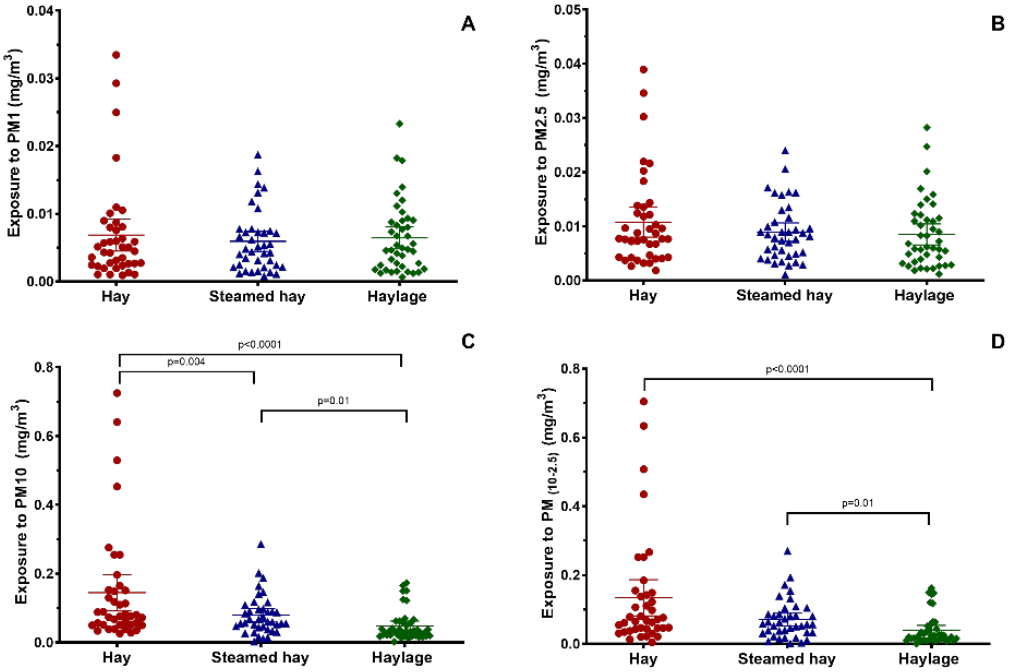


Figure 5.3. Scatter plot of breathing zone particle matter (PM) concentrations in horses fed dry hay, steamed hay or haylage. A) Particulate matter with diameter less than 1 µm (PM1) concentration. B) PM2.5 concentration. C) PM10 concentration D) $PM_{10-2.5}$ concentration. Horizontal bars indicate mean and 95% confidence interval.

5.4.3 Effect of forage on β-glucan and endotoxin concentration

Fifty-one filters from week 6 were measured for respirable β-glucan concentration. Forty-five filters were under limit of detection in the β-glucan assay. The limit of detection for respirable β-glucan concentration was 43.7 pg/m^3. Respirable β-glucan concentration was not different between groups (Hay=48.3.2±4.21 pg/m^3; Steamed hay=46.43±6.3 pg/m^3; haylage= 44.5±2.2pg/m^3; p=0.7).

Respirable endotoxin concentration was obtained from 35 filters. Respirable endotoxin concentration of horses eating hay was significantly higher than those eating haylage (p=0.003) but not different from horses eating steamed hay (p=0.2; Figure 5.4).

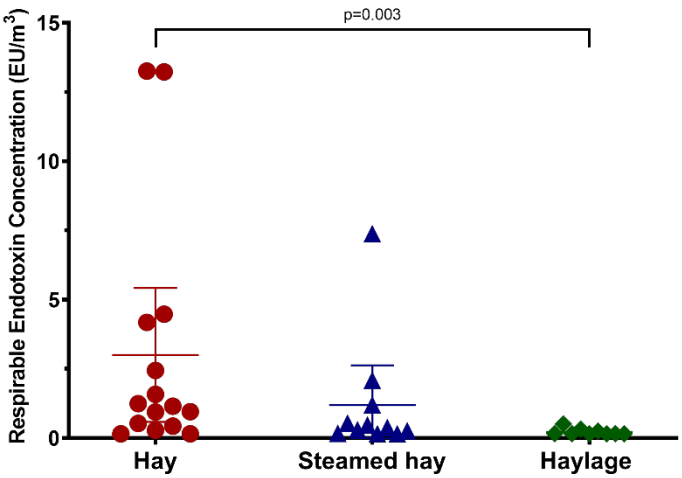


Figure 5.4. Scatter plot of respirable endotoxin concentration in respirable dust collected from horses eating dry hay, steamed hay and haylage. Horizontal bars indicate mean and 95% confidence interval.

5.4.4 Effect of forage on clinical score and tracheal mucus

Clinical score increased over time regardless of forage assignment (p=0.03; Figure 5.5). Respiratory rate was not affected by forage or time (p=0.66).

Mucus score was significantly higher in the horses eating hay at week 6 when compared with baseline (p=0.042) and week 3 (p=0.035; Figure 5.6). In addition, mucus score was significantly higher at week 6 in the horses eating hay than those eating haylage (p=0.048; Figure 5.6).

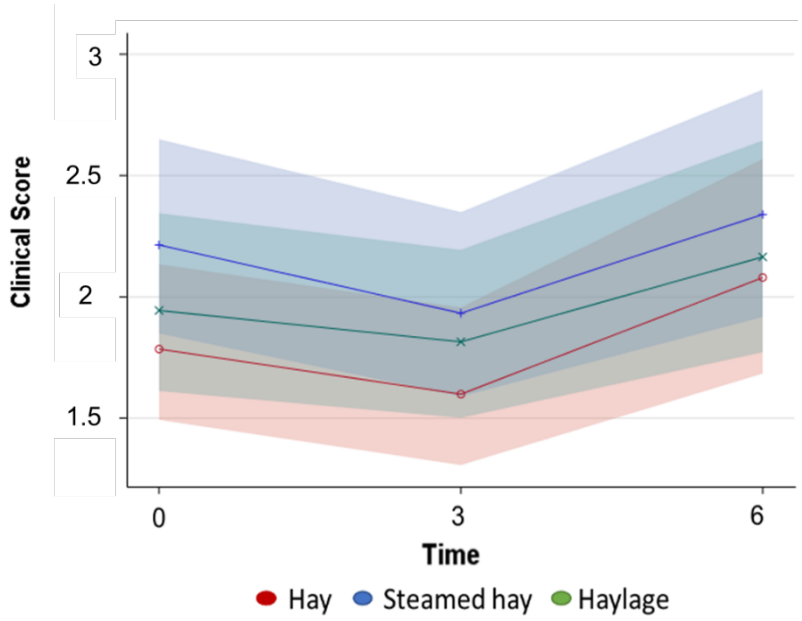


Figure 5.5. Clinical score per group over time. Generalized linear mixed model of clinical score per forage group over time. Solid line= mean response, and band= 95% confidence interval.

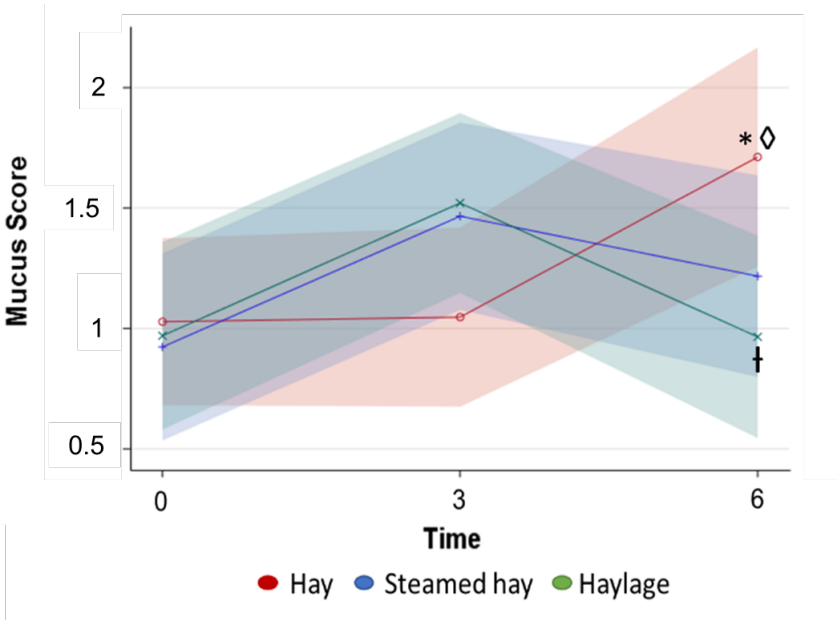


Figure 5.6. Mucus score per group over time. Generalized linear mixed model of mucus score per forage group over time. Solid line= mean response, and band= 95% confidence interval. *Different from baseline p=0.042. ◊ Different from week 3 p=0.035 ǂ Different from hay p=0.048

5.4.5 Effect of forage on airway cytology

Horses eating haylage had a lower BALF neutrophil proportion at week 3 (p=0.024) and week 6 (p=0.003) compared to horses eating hay (Figure 5.7). After 6 weeks, horses fed haylage had a lower proportion of BALF neutrophils compared to baseline (p=0.037). Horses consuming steamed hay experienced a decrease in BALF neutrophil proportions after 3 and 6 weeks compared to baseline however, the drop was not statistically significant (p>0.2). Neutrophil proportions in BALF were positively associated with increasing respirable dust exposure (p<0.0001; Figure 5.8).

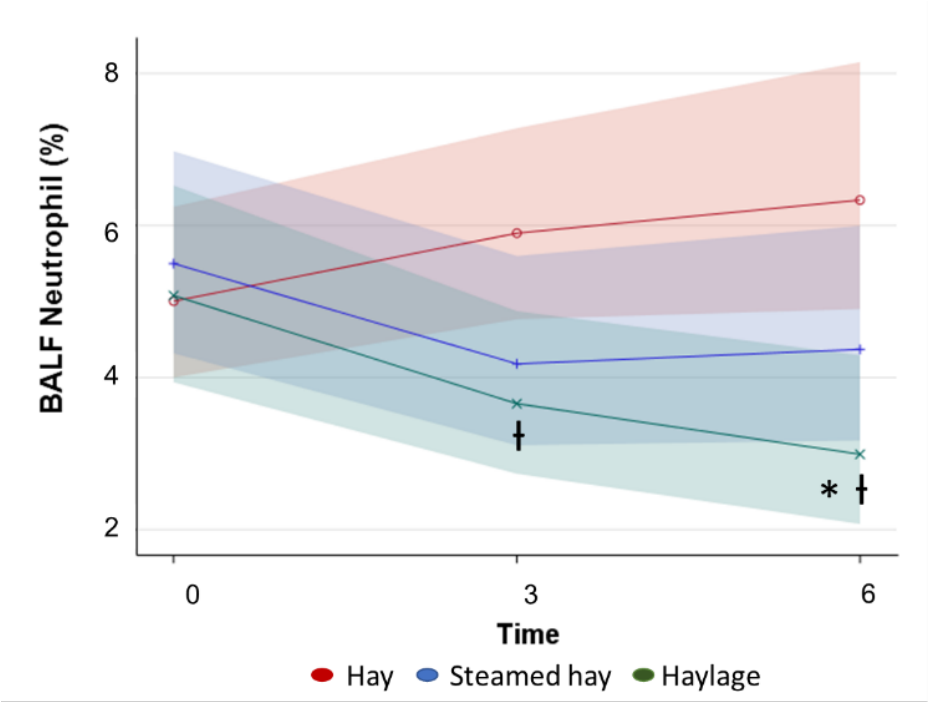


Figure 5.7. BALF neutrophil proportion per group over time. Generalized linear mixed model of BALF neutrophil (%) per forage group over time. Solid line= mean response, and band= 95% confidence interval. *Different from baseline p=0.037. † Different from hay p<0.024

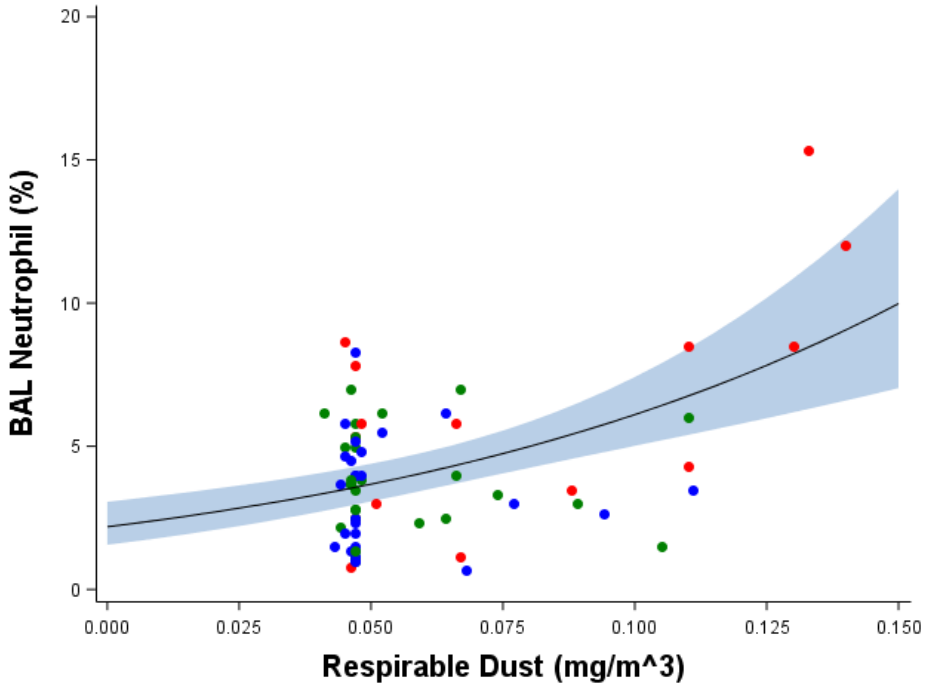


Figure 5.8. Association between BALF neutrophil (%) and breathing zone respirable dust exposure. Generalized linear mixed model of BALF neutrophil percentage versus respirable dust exposure. Solid line = predicted mean response fit at age = 4.47 years. Band = 95% confidence interval of the mean response. Red dots=hay; blue dots= steamed hay; green dots=haylage.

Horses eating haylage exhibited a significantly lower BALF mast cell proportions at 3 weeks when compared horses eating hay (p=0.003) and steamed hay (p=0.02), however mast cells were back to baseline levels by week 6 (Figure 5.9). No significant change in BALF mast cell proportion was detected in horses eating hay or steamed hay during the 6-week period.

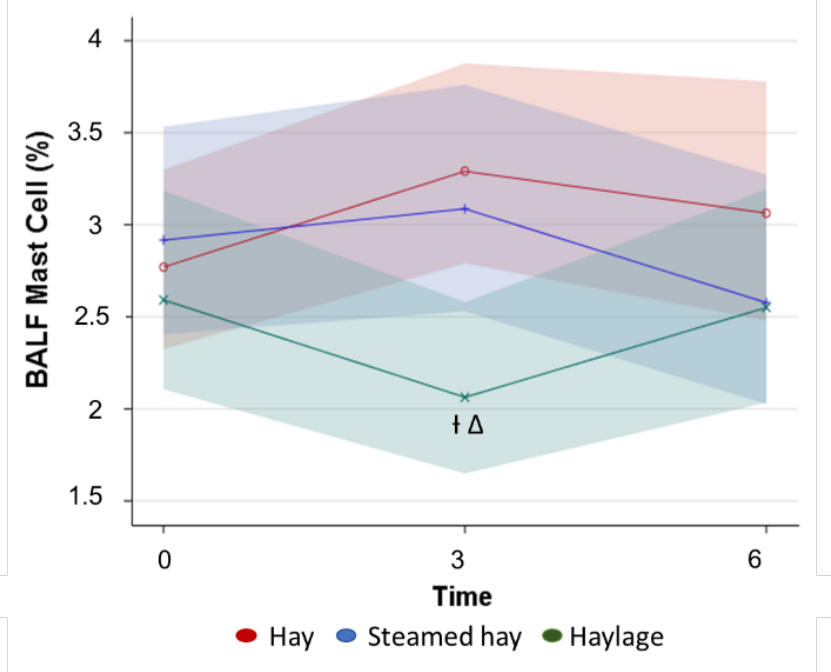


Figure 5.9. BALF mast cell proportion per group over time. Generalized linear mixed model of BALF mast cell (%) per forage group over time. Solid line= mean response, and band= 95% confidence interval. ǂ Different from hay (p=0.003) Δ Different from steamed hay p=0.02.

Horses eating steamed hay exhibited a decrease in BALF eosinophil proportions after 3 weeks compared to baseline (p=0.03) and to horses eating hay (p=0.04; Figure 5.10). However, by week 6 BALF eosinophil proportions in horses fed steamed hay were not significantly different from horses fed hay. No significant change in BALF eosinophil proportions was detected in horses eating dry or haylage during the 6-week period.

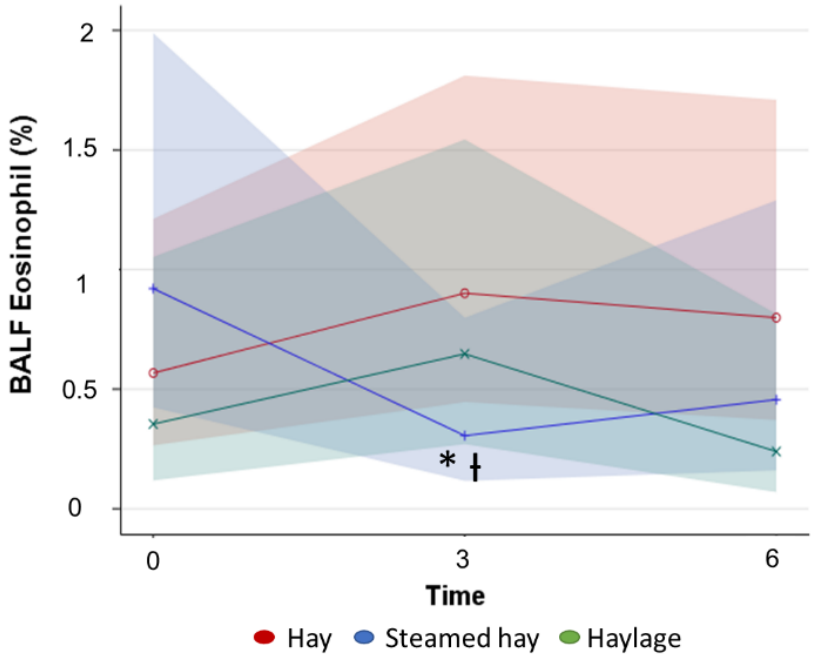


Figure 5.10. BALF eosinophil proportion per group over time. Generalized linear mixed model of BALF eosinophil (%) per forage group over time. Solid line= mean response, and band= 95% confidence interval. *Different from baseline p=0.02. ŧ Different from hay p=0.03.

5.4.6 Effect of forage on blood polyunsaturated fatty acids and pro-resolving lipid mediators

No effect of forage or time was observed on linoleic acid, γ-linolenic acid, α-linoleic acid, steridonic acid, eicosadienoic acid, dihome-γ-linolenic acid, arachidonic acid, docosadienoic acid, and DHA (p>0.1; Appendix J). Effect of forage was observed on EPA (p=0.014). EPA relative concentrations were highest after 6 weeks of eating haylage; after controlling for multiple comparisons, EPA concentrations at week 6 were significantly higher when compared to pooled data from horses eating steamed hay and hay (p=0.04; Figure 5.11). The ratio of EPA to arachidonic acid was significantly higher in the horses eating haylage for 6 weeks when compared with baseline (p=0.005; Figure 5.12), and when compared with pooled data from horses fed hay and steamed hay at week 6 (p=0.0002; Figure 5.12). No effect of time or forage was observed on RvD1 (p=1.0) or RvE1 (p=0.4) plasma concentrations.

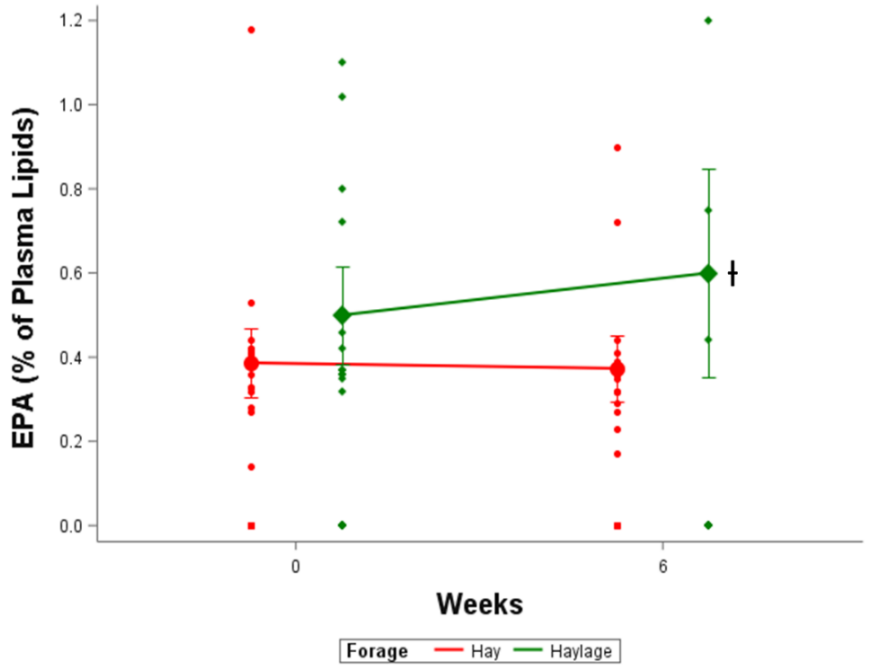


Figure 5.11. Mean and 95% confidence interval of EPA relative concentration per group over time. Hay= Pooled data from horses eating hay and steamed hay. ǂ Different from hay p=0.04. Large circle= mean relative EPA concentration in horses eating hay or steamed hay; small circles= individual measurements of relative EPA concentrations in horses eating hay or steamed hay; large diamond= mean relative EPA concentration in horses eating haylage; small circles= individual measurements of relative EPA concentrations in horses eating haylage; whiskers=95% confidence interval for the mean.

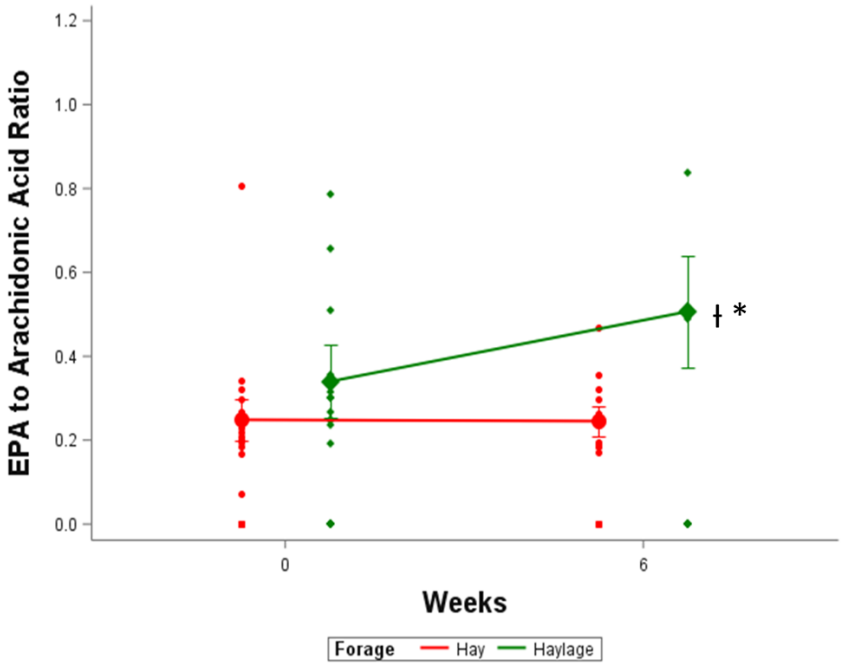


Figure 5.12. Mean and 95% confidence interval of EPA to arachidonic acid ratio per group over time. Hay= Pooled data from horses eating hay and steamed hay. *Different from baseline p=0.05. ǂ Different from hay p=0.0002. Large circle= mean relative EPA to arachidonic acid ratio in horses eating hay or steamed hay; small circles= individual measurements of relative EPA to arachidonic acid ratio in horses eating hay or steamed hay; large diamond= mean relative EPA to arachidonic acid ratio in horses eating haylage; small circles= individual measurements of relative EPA to arachidonic acid ratio in horses eating haylage; whiskers=95% confidence interval for the mean.

5.5 Discussion

In the present study, we investigated the effect of feeding dry hay, and low-dust forages (steamed hay and haylage) to racehorses on dust exposure, BALF cytology, plasma PUFAs, and PRLM concentrations. This prospective trial demonstrates that breathing zone measurements of respirable dust exposure can be significantly reduced by feeding racehorses steamed hay or haylage in place of dry hay as the only management change. Feeding haylage also significantly decreased exposure to respirable endotoxins compared to dry hay. Most importantly, after 3 weeks of eating haylage, horses already had significantly lower BALF neutrophil and mast cell proportions than horses eating dry hay. In addition, horses eating steamed hay showed a significant decrease in eosinophil proportions. After 6 weeks on haylage, horses maintained decreased BALF neutrophil proportions compared to baseline and to horses eating hay, but BAL mast cell

proportions were no longer significantly reduced. Plasma concentration of EPA was higher in horses eating haylage but all other PUFAs and PRLM concentrations were not different between groups.

At baseline, the forage groups did not differ in overall clinical parameters or BALF cytology. Under the criteria from the ACVIM consensus statement on equine asthma,[1] 90% of the horses met the cytological criteria for mild-moderate EA at enrollment, similar to the results previously described in standardbred racehorses (>90%),[16] and a similar population of thoroughbred racehorses (80%).[17] Mast cell inflammation was the most commonly observed BALF cytological profile, comparable to previous results in thoroughbred racehorses after racing.[17] In that study eosinophilic inflammation was observed in only 4% of BALF samples [17] but in the present study eosinophilic inflammations was observed in 19% of baseline measurements. This difference could be explained by the higher number of 2 years old horses sampled in the present study, since eosinophilic inflammation appears to be encountered more frequently in younger horses.[24]

It is important to note that none of the horses that dropped out of the study experienced any adverse effect from either the feeding or sampling protocol. Haylage and steamed hay were well tolerated, and no adverse effects or change in feces appearance were observed during the study. Botulism has been previously associated with feeding haylage to horses but it is thought to be due to feeding moldy or poorly preserved forage.[251] In this study, as has been recommended,[211,212] horsemen were advised to discard any haylage with visible mold growth and haylage bales were fed within 3 days once opened.

As hypothesized, exposure to respirable dust in breathing zone was significantly lower in horses eating steamed hay or haylage when compared to eating dry hay, with approximately 30% reduction. The mean respirable dust exposure measured in the breathing zone of horses eating hay (0.080 mg/m^3) was comparable with previous studies of thoroughbred racehorses eating hay and bedded on sawdust (0.055-0.090 mg/m^3),[17,24] and another study of a pony fed hay and bedded on shavings (0.064±0.04 mg/m^3).[104] In the latter study, the exposure when the pony was fed haylage (0.026 ± 0.01 mg/m^3) was lower than those measured in the current study (0.053 ± 0.016 mg/m^3) however, it is difficult to draw conclusions based on measurements taken in a single animals because breathing zone exposure levels vary markedly between horses.[24] The reported exposure of horses eating steamed hay on pasture (0.0015 mg/m^3)[252] were lower than those observed in

this study (0.056 ± 0.018 mg/m^3). The lower exposure in horses fed steamed hay on pasture would be expected because of a likely higher ventilation outdoors compared to stalls. Another possibility may be the high number of filters under the limit of detection (0.047 mg/m^3) in this study, especially considering the high number of filters that were under the limit in horses eating haylage (78%), and steamed hay (68%). Future studies measuring respirable dust exposure by gravimetric methods in horses fed low dust forages should consider extending sampling time to 6 hours in order to yield measurements above the detection level.

Average PM 10 and $PM_{10-2.5}$ were affected by forage and significantly lower for the horses eating haylage when compared with steamed hay and hay. Also, PM10 was significantly lower in horses eating steamed hay when compared with horses eating hay. In racehorses higher exposure to breathing zone PM10 has been associated with increased tracheal mucus accumulation, and neutrophil proportion in tracheal wash.[101] In humans, increased PM2.5 and PM10 exposures have been associated with asthma exacerbation.[253] In addition, $PM_{10-2.5}$ has been related with the presentation of asthma in children,[254] for each 0.001 mg/m^3 increase in the average coarse particles, the prevalence of asthma increased by 0.6%. The current study demonstrates that substituting dry hay for a low dust forage such as steamed hay or haylage may reduce horse's exposure to PM10 by an average of 44% and 66%, respectively. Average particle number concentration was not affected by forage. The majority of the particles measured were under 1 μm; these particles were not affected by forage, and are probably environmental particles that the horse is inhaling while standing inside the stall and not eating and may be influenced by other environmental pollutants such as vehicular traffic.[95]

Respirable β-glucan concentration did not change with forage in the current study. In a previous study of Thoroughbred racehorses that used the same methodology, the mean β-glucan concentration was 55.5±66.2 pg/m^3 [17]compared to 48.3±4.2 pg/m^3 in horses fed hay in the present study. The main difference between the studies was the sampling time, which was 3 hours longer in the previous study.[17] In the current study, sampling time was limited by the need to perform dust sampling and clinical measurements in the morning after feeding horses and to complete all procedures prior to the barn personnel leaving the premises.

Respirable endotoxin concentrations were significantly lower in horses eating haylage when compared with horses eating hay. Airborne endotoxin concentration in horses eating haylage was reported to be similar to horses on pasture, and significantly lower than horses fed hay.[75] This

is similar to the results observed in this study in respirable endotoxin concentration. A study on a horse eating steamed hay was described to have a lower respirable endotoxin activity (15 times) when compared with horses eating hay.[252] In the current study no difference in respirable endotoxin concentration was observed between horses fed hay or steamed hay.[252] The median endotoxin exposure of horses fed hay was 1.2 EU/m^3 in the current study, while median exposures reported for horses eating hay range from 2.2 EU/m^3 to 7080 EU/m^3.[1,2] The variability between endotoxin exposures in these studies can be explained by the high variability that endotoxin measurements can have upon methods of sampling and handling.[92]

Surprisingly, clinical score changed over time independent of forage. Though this change was significant, the increase was small, roughly 1 point on a 21-point scale, and would not be expected to be clinically relevant in this population of horses. Clinical scoring is not sufficiently sensitive to differentiate horses with mild-moderate EA from healthy horses, and further diagnostic test such as endoscopy and BAL should be performed.[117] Mucus score increased from baseline (score=1) to week 6 (score=2) when horses were eating hay, and this was significantly greater than mucus scores of horses fed haylage (score=1). It is important to consider that mucus scores can be affected by the time elapsed between exercise and endoscopy,[73] and that timing was not standardized in the present study. However, those results are consistent with a median mucus score of 2 that was reported in a study of Thoroughbreds examined approximately one hour after racing and all these horses were fed hay.[17] These findings are clinically relevant because mucus scores of grade 2 or higher have been associated with poor performance in thoroughbred racehorses.[53]

A significant reduction in BALF neutrophil proportions at week 3 and week 6 was only observed in horses fed haylage. This effect was not observed when horses ate steamed hay, despite similar respirable dust exposure. This difference may be explained in part by higher exposure to PM10 and $PM_{10-2.5}$ when horses were fed steamed hay compared to haylage. The only change to horses' management was the forage fed to the horses; training and racing schedules were not affected by our study. The mean BALF neutrophil proportion at baseline (4.8 ± 3.95%) was similar to that previously described in Thoroughbreds after racing (4.75 ± 3.95%).[17] In this study, the median BALF neutrophil proportion at baseline (4.12%) and at week 6 on horses fed hay (6.2%) was lower than the one observed in Standardbred racehorses (10%).[16] In horses with severe asthma, feeding haylage and using low dust bedding can maintain horses in clinical remission and minimal

BAL neutrophilia (<10%) for months.[106] A similar beneficial effect was observed in horses with mild asthma in the current study: BALF neutrophil proportion in horses eating haylage were significantly lower by week 3 (3.66 ± 0.53%) with further improvement after 6 weeks (2.99 ± 0.5%). Even though the average BALF neutrophil proportion at baseline was within the normal range; it is important to recognize that even small changes in BALF neutrophil proportion can have an effect in racehorse performance,[17] therefore the decrease observed in the haylage group should be considered as clinically relevant. In the current study, for each 0.1 mg/m^3 increase in respirable dust, a 2.7 fold increase in neutrophil proportions was observed as compared to 1.3 fold increase in a previous study.[17] Consequently, changing the forage from dry hay to haylage is sufficient to control neutrophilic airway inflammation in racehorses. The effect of endotoxin exposure in neutrophilic inflammation is complex, since the relationship between these two variables may be parabolic[17] and not be linear as previously suggested.[12] Horses exposed to high doses of endotoxin (48,000 EU) and to high concentrations of dust exposure (0.2 mg/m^3) present and increase in neutrophilic inflammation demonstrating a synergic effect between respirable dust and endotoxin concentration.[12] On the other hand, horses exposed to naturally occurring levels of respirable endotoxin, increasing endotoxin exposure may mitigate the neutrophilic response to respirable dust exposure.[12,17]

The apparent effect of haylage on mast cell inflammation was transient. Mast cell inflammation has been positively associated with respirable β-glucan.[17] However, β-glucan measurements were below the assay detection limit in 88% of the samples. A more sensitive measure of respirable β-glucan in this study may have helped to explain the changes in mast cell proportions. In horses eating steamed hay, a significant but transient decrease was observed in BALF eosinophil proportions at week 3 when compared to baseline and to horses eating hay. The difference was no longer significant at week 6. The clinical relevance of this change in eosinophil proportions is unclear because mean BALF eosinophil proportions were under the 1% threshold for eosinophilic inflammation in each group.[1] However, future studies are needed to understand the clinical effect of BALF eosinophilia on racehorses' performance.

In the USA, racehorses are frequently fed hay as their sole forage with no access to pasture. Dry hay has lower Ω-3 content when compared to haylage and fresh grass.[137] Ω-3 are the main precursors for the production of PRLM;[136] molecules that are crucial in the resolution of inflammation.[214] Plasma levels of PUFAs and PRLM did not vary with forage over or time. Except

for EPA that was significantly higher in horses eating haylage when compared with horses eating steamed hay, but not with horses eating hay. In horses with severe asthma, the addition of a Ω-3 supplement to a low dust diet demonstrated a faster improvement in clinical signs and BALF cytology when compared to horses fed a low-dust diet with a placebo,[129] and plasma DHA concentrations were significantly higher in the treated horses. In humans, some studies have shown that diets with higher content of DHA and EPA may be protective against inflammatory diseases like asthma while others studies have not found this association.[188] EPA can compete with arachidonic acid as a substrate for enzymes and be converted to PRLM,[139] and *in vitro* studies of alveolar macrophages from humans with asthma have shown EPA to be a more potent inhibitor of pro-inflammatory mediators than DHA.[255] Furthermore, EPA is an important precursor of the resolvin E-series of PRLM;[214] the differences in EPA between horses eating haylage and steamed hay may explain the differences in BALF neutrophils proportions observed in this study. Human athletes supplemented with Ω-3 (3.2 g EPA and 2.2 g DHA) show a suppression in exercise-induced bronchoconstriction associated with a decrease in circulating inflammatory cytokines in vitro and production of leukotrienes from neutrophils *in vitro*.[190] The measurement of Ω-3 was performed by measuring the fatty acid composition of neutrophil phospholipids rather than in blood, and an increase in the treatment group was observed;[190] perhaps this technique would have allowed detection of a change associated with feeding haylage, as the techniques in the current study failed to demonstrate a significant change in the blood concentrations of these molecules. In previous studies when a DHA supplement (3-6 g) was administered to horses an increase in DHA blood levels was observed.[129]

The lack of effect of forage upon RvD1 and RvE1 concentrations may indicate that other, unmeasured PRLM may have been involved in the enhanced resolution of airway inflammation when horses were fed haylage.[131] For example, resolvin E2 is also derived from EPA and regulates neutrophil chemotaxis, activates phagocytosis, and enhances the synthesis of anti-inflammatory cytokines.[171]

A limitation of our study was that most of the horses in the study were provided by a single trainer. Compliance with the study design required cooperation and extra man hours that some trainers found burdensome, which limited their participation.

5.6 Conclusions

The current study demonstrated that feeding steamed hay or haylage in place of dry hay can decrease respirable dust exposure in horse's breathing zone. Feeding haylage can decrease neutrophilic inflammation as early as 3 weeks after switching forage. There is an apparent benefit of feeding haylage over steamed hay despite the similar reduction in respirable dust that may be explained by the higher plasma EPA concentration achieved when fed haylage. Future studies are needed to determine the effect of Ω-3 in horses with mild airway inflammation.

CHAPTER 6. CONCLUSIONS

This dissertation was able to corroborate dust exposure in the horses' breathing zone can be reduced by changing the forage from dry hay to low dust forages, such as haylage, steamed hay, and hay pellets. As expected, gravimetric measurement of respirable dust exposure in the horses' breathing zone was reduced when horses were fed haylage and steamed hay. We were able to measure exposure to different particle size fractions of particle matter (PM) using portable particle counters attached to the halter and connected to the breathing zone of the horse by flexible tubing. The use of a real-time sampler decreased the time required to evaluate particulate exposure of horses. On the other hand, the calculated respirable dust exposure performed with the data collected from the real-time device was poorly correlated with the gravimetric respirable dust measurements that are considered as the gold standard. For this reason, we were not able to compare respirable fractions on the horses that only real-time sample was performed. Respirable dust measurements are important since an association between those particles and airway inflammation has been described in the past and was corroborated on one of our studies. In the future, real-time devices could be used to determine the activities where the horse is exposed to higher particle concentration, particle numbers, and the size of the particles involved.

An increase in respirable dust exposure was positively associated with increase in BALF neutrophil proportion. The change in forages from dry hay to low dust forages has an impact on airway inflammation of horses with mild EA. As hypothesized, despite similar dust exposure between low-dust forages, haylage was the only forage that resulted in significantly lower neutrophil proportion after 6 weeks.

Concentrations of plasma Ω-3 or PRLM were not higher on the horses fed haylage when compared to hay pellets or baseline measurements when horses were fed hay. Consequently, the role of these molecules in the decrease in neutrophilic inflammation observed on the horses fed haylage cannot be confirmed in this dissertation. Still, when levels of Ω-3 were compared between horses fed dry hay, steamed hay, and haylage. The only difference observed was on plasma EPA levels, which was higher on horses eating haylage when compared to those eating steamed hay. Measurements of essential fatty acids and PRLM in cell membranes from neutrophil or red blood cells could help detect the changes in essential fatty acids after supplementation. To determine if

Ω-3 mediate resolution of airway inflammation, a clinical trial would be needed to investigate the effect of Ω-3 supplementation in racehorses with mild EA.

Further *in vitro* work would be necessary to better understand the role of PRLM in equine neutrophils and macrophages because no effect was observed of the individual PRLM used on apoptosis or efferocytosis at the concentrations and incubation times used. The determination of PRLM by LC-MS/MS was more complicated than expected. Most of the concentrations were under the limit of detection. Although, we were able to detect RvD1 and RvE1 by ELISA, the gold standard is considered to be LC-MS/MS since it is supposed to be a more sensitive and specific method for the determination of PRLM. Future studies determining the concentration of these molecules in tissues or cell walls could help us better comprehend the dynamics of these molecules in equine airway inflammation.

We were able to modify the diet of racehorses successfully and performed multiple BALs without interrupting training or racing schedules. We can conclude that feeding haylage resulted in lower respirable dust exposure and decreased airway neutrophilia within 3-6 weeks and that haylage presents additional benefits beyond reducing dust exposure. We also confirmed that mild EA in common in Thoroughbreds racing in Indiana, as previously described. An estimation of the prevalence of mild EA in Thoroughbreds racing in different parts of the country and its effect on performance would give us a better understanding of the true impact of the disease in horses racing in the United States, and it would help to understand the influence of factors such as climate or pollution on lung health.

APPENDIX A. SUPPLEMENTARY S 4.1

Summary of dust exposure. Data presented as mean mean ± SD.

	Hay	Pellet	Haylage
PM 1 (mg/m^3)	0.008± 0.0008	0.002± 0.0003	0.003±0.0003
PM 2.5 (mg/m^3)	0.01 ±0.002	0.003 ±0.001	0.005 ±0.001
PM 10 (mg/m^3)	0.15 ±0.06	0.03±0.01	0.06 ±0.02

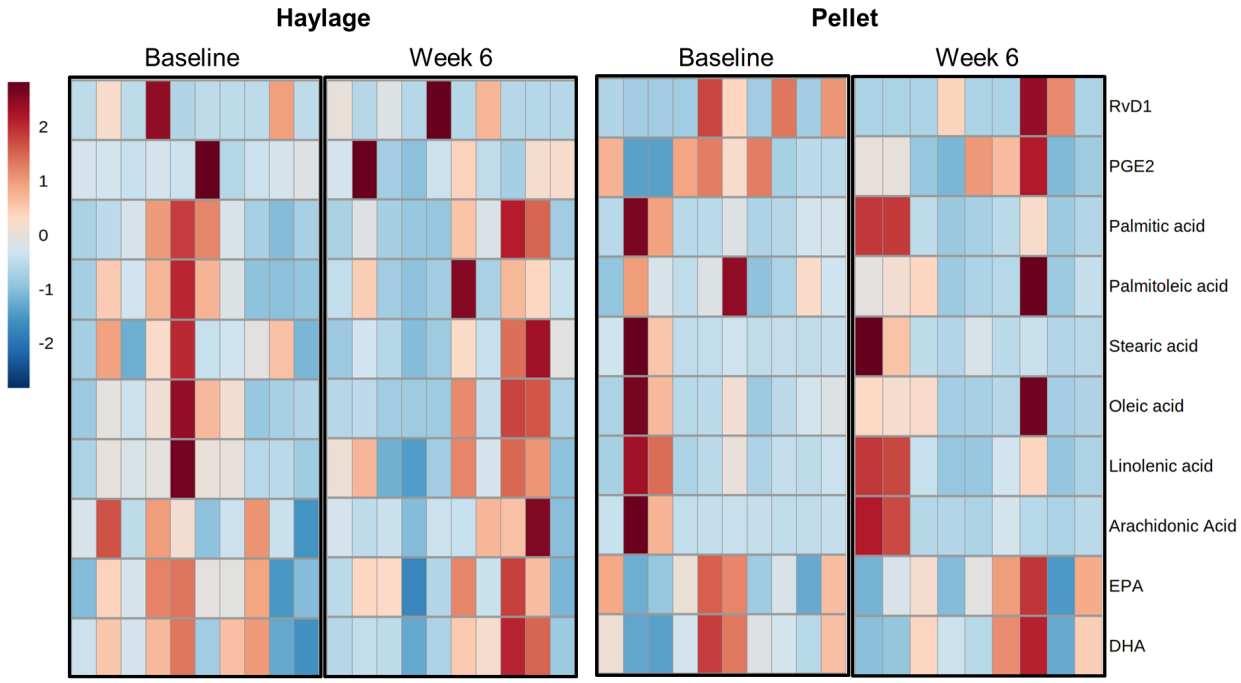
APPENDIX B. SUPPLEMENTARY S 4.2

Summary of BALF neutrophil proportion. Data presented as mean ± SD.

	Time	Pellets	Haylage
BALF Neutrophil (%)	Baseline	12.1 ± 2.3	11.8 ± 2.4
	Week 3	9.2 ± 1.7	6.8 ± 1.8
	Week 6	8.5±1.7	2.5 ± 1.1

APPENDIX C. SUPPLEMENTARY S 4.3

Heatmap displaying the concentration of plasma lipids at baseline and week 6 by forage group. Each column represents a horse.



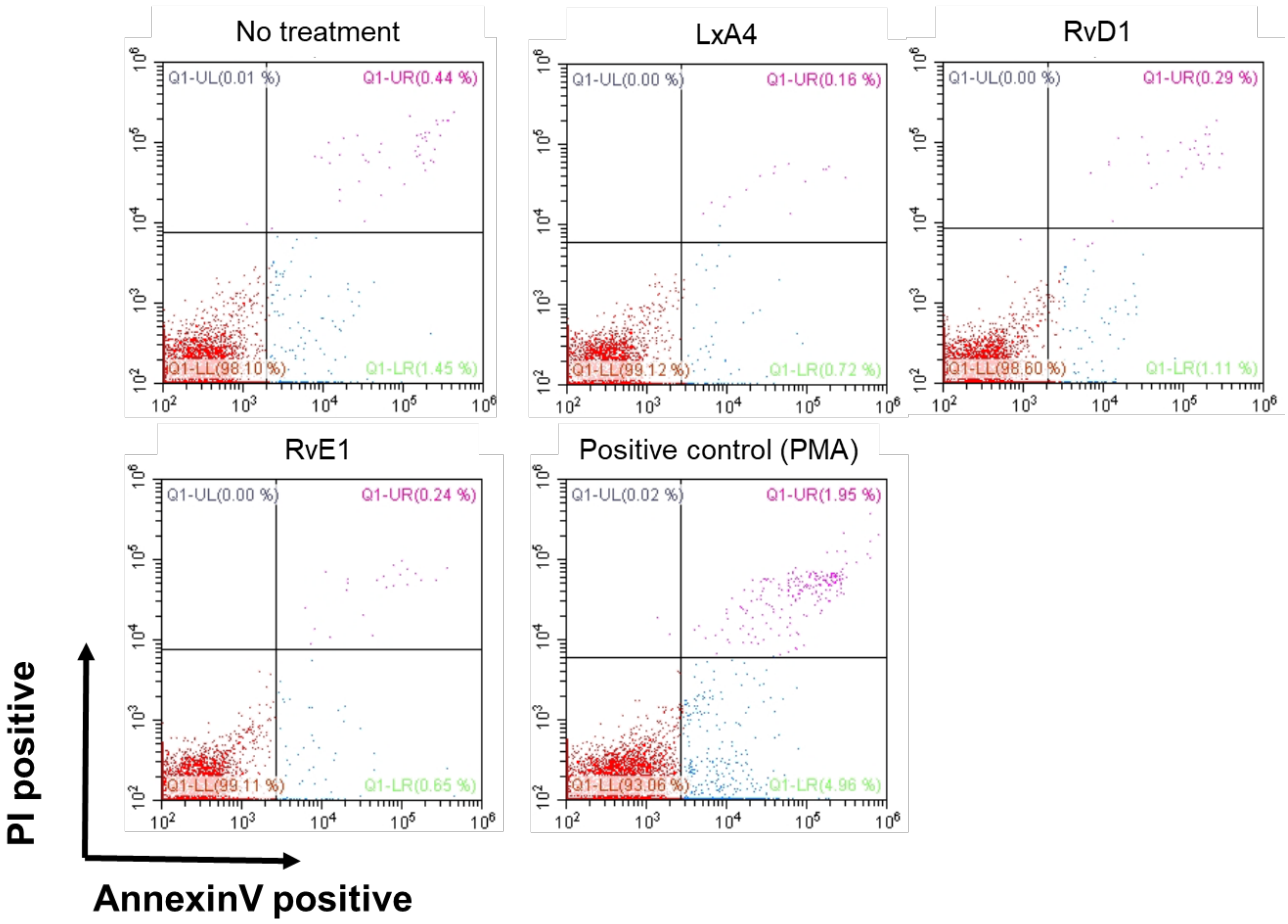
APPENDIX D. SUPPLEMENTARY S 4.4

Spearman rank correlation coefficient between plasma lipid concentrations. Correlation coefficient (unadjusted p-value). Statistically significant correlations are in bold.

	Palmitic acid	Palmitoleic acid	Oleic acid	Linolenic acid	Stearic Acid	Arachidonic acid
Palmitic acid	1	**0.89 (<.0001)**	**0.91 (<.0001)**	**0.68 (0.001)**	0.43 (0.06)	0.36 (0.1)
Palmitoleic acid		1	**0.89 (<.0001)**	**0.67 (0.001)**	0.31 (0.2)	0.30 (0.2)
Oleic acid			1	**0.82 (<.0001)**	**0.47 (0.04)**	0.31 (0.2)
Linolenic acid				1	**0.57 (0.01)**	0.40 (0.08)
Stearic Acid					1	**0.74 (0.0002)**
Arachidonic acid						**1**

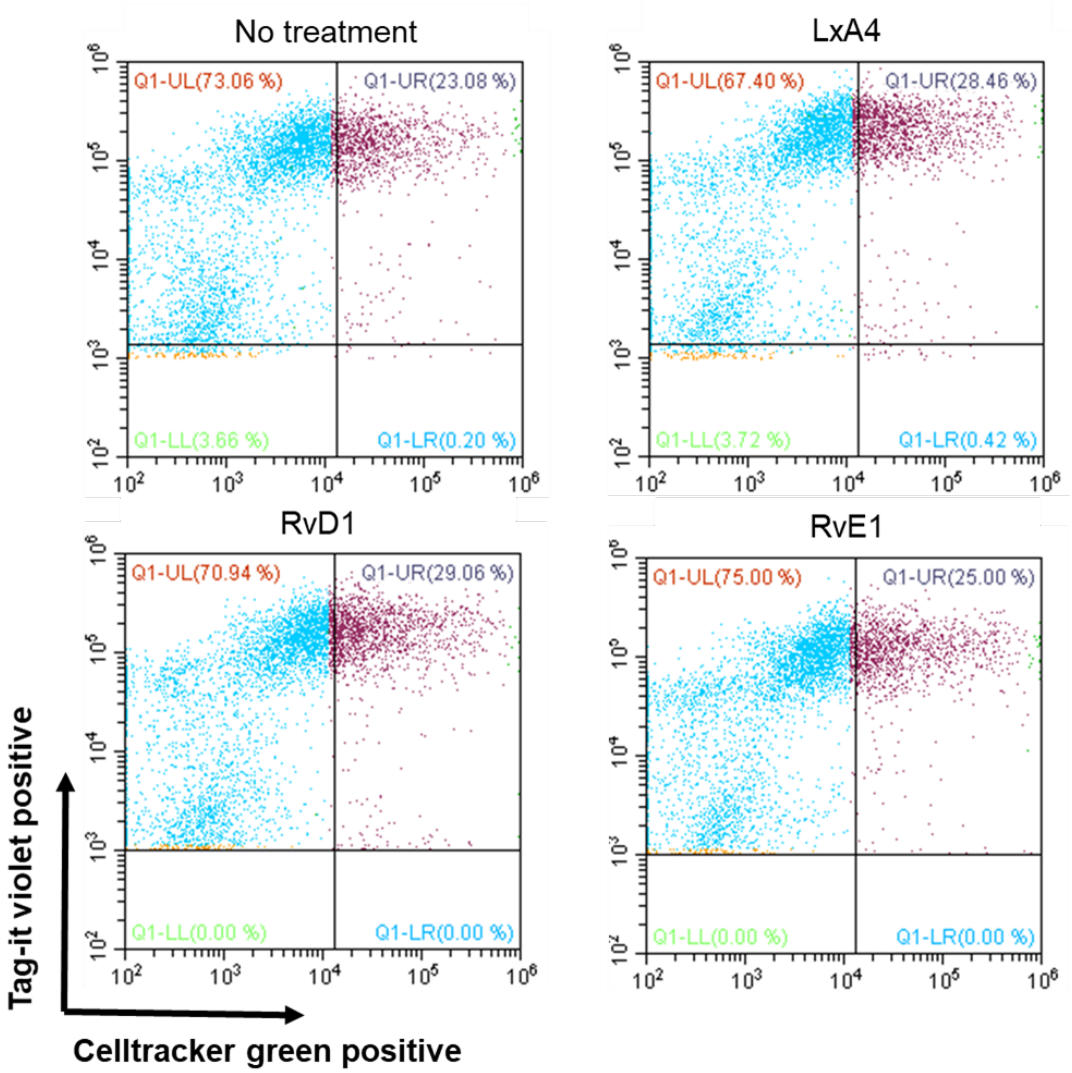
APPENDIX E. SUPPLEMENTARY S 4.5

In vitro determination of apoptosis. Unstained cells (red) are considered as viable cells. AnnexinV positive and propidium Iodide (PI) negative cells (blue) were considered as cells undergoing apoptosis. AnnexinV negative and PI positive cells were considered as dead cells.



APPENDIX F. SUPPLEMENTARY S 4.6

In vitro determination of efferocytosis. Tag-it violet positive and celltracker green negative cells are alveolar macrophages. Tag-it violet positive and celltracker green positive cells were considered as efferocytosis.



APPENDIX G. SUPPLEMENTARY S 5.1

PURDUE UNIVERSITY COLLEGE OF VETERINARY MEDICINE **Research Consent Form**	**Horse Name:**_____ **Study #:** LDF **Owner:**_____ Stallion / Gelding / Mare

Role of Stable exposure in airway inflammation.

(Title of study or trial)

Clinical Investigators: Dr. Laurent Couëtil, Dr. Kathleen Ivester, Dr. Carla Olave

Purpose of Study:

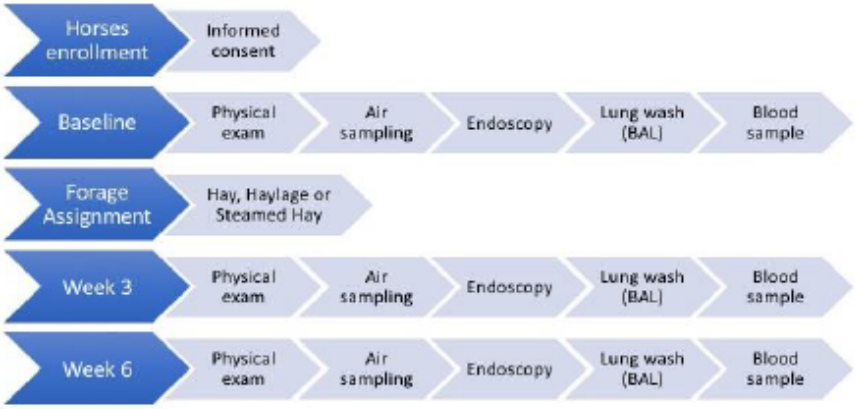
Equine asthma, or inflammatory airway disease (IAD) is a common cause of poor performance in athletic horses. Inflammation and accumulation of mucus in the airways interferes with the movement of oxygen from the lung into the blood stream, limiting the performance of horses during strenuous exercise. Affected horses typically train well and may only cough occasionally. The best way to detect equine asthma is by visualization of excess mucus by endoscopy of the airways after exercise or by identification of airway inflammation by lung wash. Asthmatic horses can be treated with anti-inflammatory drugs (corticosteroids); however, treatment may not be effective, and the risk of residue resulting in a positive drug test is always a concern.

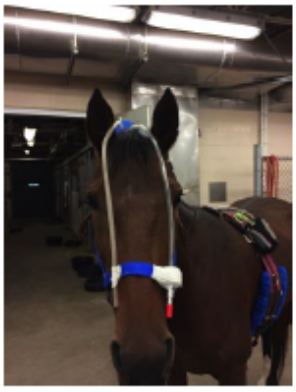
Exposure to dust in the stall, particularly from hay, is likely an important contributor to the onset of equine asthma. Feeding horses steamed hay or grass silage (haylage) is an effective way to decrease dust levels; however, it is unknown if it would be sufficient to control airway inflammation in asthmatic racehorses. The body has natural ways to resolve inflammation by using compounds present in the diet, such as omega-3 fatty acids, to decrease inflammation and heal tissue. Fresh grass and haylage have high levels of omega-3 fatty acids compared to dry hay. Racehorses typically are fed dry hay and do not have access to fresh grass. Consequently, in addition to lowering dust levels, feeding haylage to racehorses may provide additional benefits compared to steamed hay due to higher omega-3 content.

The goal of the study is to compare the effect of feeding dry hay, steamed hay, or haylage on dust level, airway inflammation and blood omega-3 levels in Thoroughbreds actively racing in Indiana. We hope to find that horses fed haylage or steamed hay will be exposed to lower levels of dust, resulting in decreased airway inflammation compared to horses fed dry hay. In addition, we believe that haylage will result in the most significant improvement, and this will be associated with higher blood levels of omega-3 fatty acids.

Eligibility: To be eligible for participation in this study, horses must be Thoroughbreds eligible to race at Indiana Grand Racing and Casino free from clinical respiratory disease.

Procedures:





At baseline, 3 weeks, and 6 weeks, we will perform a physical examination including body temperature and chest auscultation. **A blood sample (20 cc) will be taken by jugular venipuncture to look for any signs of subclinical infection and measure biomarkers of airway disease.** Any horses that have signs of infection will be excluded from the study.

1. The horse will be restrained with halter and nose twitch (or other method recommended by owner/trainer) and a small endoscope (OD = 6.5 mm) will be passed through the nose. The laryngeal mechanics and degree of nasopharyngeal inflammation will be recorded. Then the scope will be advanced down the trachea to assess the degree of exercise-induced hemorrhage, inflammation, and secretions. Through the endoscope biopsy channel, a sterile guarded cytology brush will be introduced and used to collect a sample of airway secretions.
2. Before removal of the endoscope, dilute lidocaine solution (0.2%, 30-60 mL) will be sprayed onto the airway to decrease coughing
3. Horses will be sedated by i.v. injection of xylazine hydrochloride (0.2-0.5 mg/kg) and butorphanol (0.01-0.03 mg/kg).
4. A small, sterile tube (10 mm in diameter; 3-meter long) will be passed through the nose, down the trachea, and into the lungs.
5. Sterile fluid (250 ml saline solution) will be infused through the tube and recovered immediately by aspiration with a syringe. This fluid contains the lung secretions (bronchoalveolar lavage fluid or BALF) and will be used for the laboratory tests.
6. The tube will be removed and the horse will be monitored as it wakes up from sedation.

Dust collection:

1. The horse will be fitted with a surcingle and allowed to adjust to the surcingle before proceeding with the remaining steps.
2. A small air sampling pump will be secured to the surcingle and attached to a collection cassette with soft, clear plastic tubing.
3. The collection cassette will be secured to the halter of the horse, and the tubing will be attached to the horse's mane in order to allow free movement of the head and neck. The pump will draw air into the cassette where the dust particles will be caught on a filter.
4. A small, real-time particle monitor, roughly 105 grams in weight (OPC-N2, Alphasense LTD) will be attached to the crown of the halter.
5. The samples will be collected over the course of several hours during daytime hours while personnel in the barn are available to monitor the horse. Following sampling, all equipment will be removed from the horse. The filter will be weighed to determine the amount of dust collected. Further laboratory testing will examine different components within the dust. While equipped for air sampling, the horse will be free to move about the stall with normal access to feed and water.

Associated Risks: *Need to clearly state the likely risks associated with procedures and treatments so that the client understands the risk they are assuming.*

Airway endoscopy and BAL procedures have been performed on thousands of horses and are well tolerated. No harmful effects on the lungs or lung secretions are expected. All air sampling is minimally invasive. Horses will be closely monitored to ensure that they do not exhibit signs of stress or anxiety during sampling. In case a horse would exhibit signs of stress deemed excessive by the trainer or clinician (e.g. rearing up), the procedure would be aborted and horse removed from the study.

As with any forage, feeding haylage carries a risk of botulism intoxication. Care has been taken during the production of the haylage product to minimize this risk.

Compensation:

Testing will be free of charge to the owner. Results of the study will be communicated in writing to the trainer upon completion of the study.

Incentives:

Results of physical examination, complete blood count, endoscopic examination of the airway, and cytological evaluation of BALF will be communicated to the trainer.

Questions about this project may be directed to Dr. Laurent Couëtil, at 765 494-8548.

I understand that my decision to allow my animal to participate in this study is entirely voluntary. I am free to withdraw my animal from this study at any time without compromising the quality of care provided to my animal. I understand that if I chose to withdraw my animal from the study, that there may or may not be the opportunity to re-enroll my animal.

I also understand that there may be other reasons why my animal could be withdrawn from the study. If my animal's health worsens, it may not be safe for him/her to remain in the study. For example, some treatments are only safe when the overall health of an animal is good and major organs are functioning normally. I have been informed that the veterinarians attending to my animal will discuss with me any concerns that could arise regarding my animal's continued participation. I also understand that if I am unable to follow the study protocol in regards to giving medications, returning my animal for evaluation, or other items in the protocol, that my animal may need to be withdrawn from the study. Furthermore, I am aware that some studies may be stopped earlier than planned. I have also been informed that the information gained from the study, regardless of how long my animal or other animals participate, will be used in an attempt to make progress to improve the outlook for other animals, as well as my animal. I recognize it is very important, and I have provided all information which is relevant and which is requested regarding my animal's medical history.

I acknowledge that I have read and understand this consent form, and all my questions have been answered to my satisfaction. I have been assured that all personal identifying information will be kept confidential. I understand that results of this study may be shared, published, or used for educational instruction. This is important in improving veterinary care for animals. I also authorize the release of all data, including, but not limited to, medical data, photographs and videotapes.

I am aware that this study has been reviewed and approved by the Purdue Animal Care and Use Committee of Purdue University.

As a volunteer, I give my informed consent to the Purdue University Veterinary Teaching Hospital to enroll my animal in this study, according to the explanations and conditions presented in this document. I agree to hold harmless the Board of Trustees of The Trustees of Purdue University, the Purdue University Veterinary Teaching Hospital, and its officers, employees, agents and assigns from any and all liability, claims and actions that may arise from participation in this study.

☐ I have received a copy of this Consent form.

_____
Printed Name: Owner (or authorized agent)

_____ _____
Signature Owner (or authorized agent) Date

_____
Printed Name: Witness

_____ _____
Witness Signature Date

(Original to Medical Records; PACUC Approval #1111000181)

APPENDIX H. SUPPLEMENTARY S 5.2

Purdue University Inflammatory Airway Disease Research: Low Dust Forage Study **Baseline**

Baseline History Questionnaire

Horse Name: _____ Study #: LDF

All information will remain confidential.

Date: _____ Trainer: _____

How long have you trained/owned the horse? _____

How would you rate the horse's performance

Above expectations	As expected	Below expectations

_____

_____

Describe the horse's training schedule:

Distance: _____ Surface: _____

_____

_____

Please list any medications that the horse receives:

Does the horse requiere treatment with?:

Corticosteroids (i.e Dexamethasone)	Yes	No
Phenylbutazone (bute) or banamine	Yes	No
Antibiotics	Yes	No

Drug	Dose/Route	When the drug was given?	Schedule (how often)

Does the horse cough?

No	Less than once/day	1-4 times/day	5 or more times/day

When/where is your horse ussually coughing?

In stall	During or after exercise	When fed hay	During grooming	While cleaning the stall

Other (specify):

How long have you noticed the cough? _____

Does the horse have nasal discharge?

No	Watery	White mucus	Yellow mucus

Other (specify):

When is the discharge usually visible ?

In stall	During or after exercise	When fed hay	During grooming	While cleaning the stall

Other (specify):

Please describe frequency/severity of discharge: _____

_____

Has the horse bled in the past?

Yes	No

When and how did you diagnose bleeding? _____

Does the horse make abnormal respiratory noise when working?

Yes	No

If yes, please describe the noise: _____

Please describe any other past respiratory problems: _____

_____

Did this require treatment

Yes	No

If yes, what treatment was used?

Drug	Dose/Route	When the drug was given?	Schedule (how often)

Vaccination and deworming history

Vaccination	Date	Frequency	Deworming	Date	Frequency

Please describe any other illnesses (i.e neurologic or lameness): _____

_____

Management:

Does the horse get turned out?

Yes	No

Please describe diet:

Grain/Hay/Supplements	Amount	Number of times/day	Time

Is hay fed in?

Ground	Hay net in stall	Hay net outside of stall	Varies

What type of bedding is used?

Shaving	Sawdust	Straw	Pellets	Paper	Peat Moss

Other:

APPENDIX I. SUPPLEMENTARY S 5.3

Purdue University Inflammatory Airway Disease Research: Low Dust Forage Study

Horse Name: _____ Study #: _____

Date: _____ Trainer: _____

Barn: _____ Stall: _____

Time: _____ Temperature: _____ Pulse: _____

Temperature:					
Humidity:					

Respiration:	<15 0	15-20 1	21-25 2	26-29 3	>30 4
Nasal Discharge:	None 0	Serous 1	Mucopurulent 2		
Abdominal Lift:	None 0	Mild 1	Pronounced 2		
Nostril Flare:	None 0	Present 1			
Tracheal Sounds:	Normal 0	Increased Intensity 1	Mucus Movement 3		
Crackles	None 0	Present 2			
Wheezes	None 0	Present 2			
Cough	None 0	Intermittent 1	Paroxysmal 3		
Total Clinical Score:	/19				

Laryngeal Score: Right/Left	Movement Abduction Grade	Synchronous Full I	Asynchronous Full II	Asynchronous Partial III	Paralysis None IV
Palate Function:					

Pharyngeal Lymphoid Hyperplasia	None 0	Few, small white follicles dorsal pharynx 1	Many small white follicles/few pink follicles dorsal and lateral walls 2	Many pink/edematous and white follicles extending below GP openings 3	Coalescing edematous follicles entire nasopharynx 4

Sedation Time: Xylazine: _____ Butorphenol: _____

Tracheal Mucus	None 0	Singular small blobs 1	Larger singular blobs, not confluent 2	Mucus ventrally confluent (forming stream) 3	Large ventral pool 4	Profuse mucus covering > 25% of lumen 5

Exercise Induced Pulmonary Hemorrhage	No blood seen 0	One or more flecks of blood, up to twoshort streams (<25% length of trachea) 1	Long stream of blood (>50% length of trachea) or more than 2 short streams, less than 1/3 circumference 2	Multiple streams of blood covering more than 1/3 circumference of trachea; no blood pooling at thoracic inlet 3	Blood covering more than 90% of tracheal surface with blood pooling at thoracic inlet 4

Lidocaine infused: Time: _____

Prime BAL tube ☐

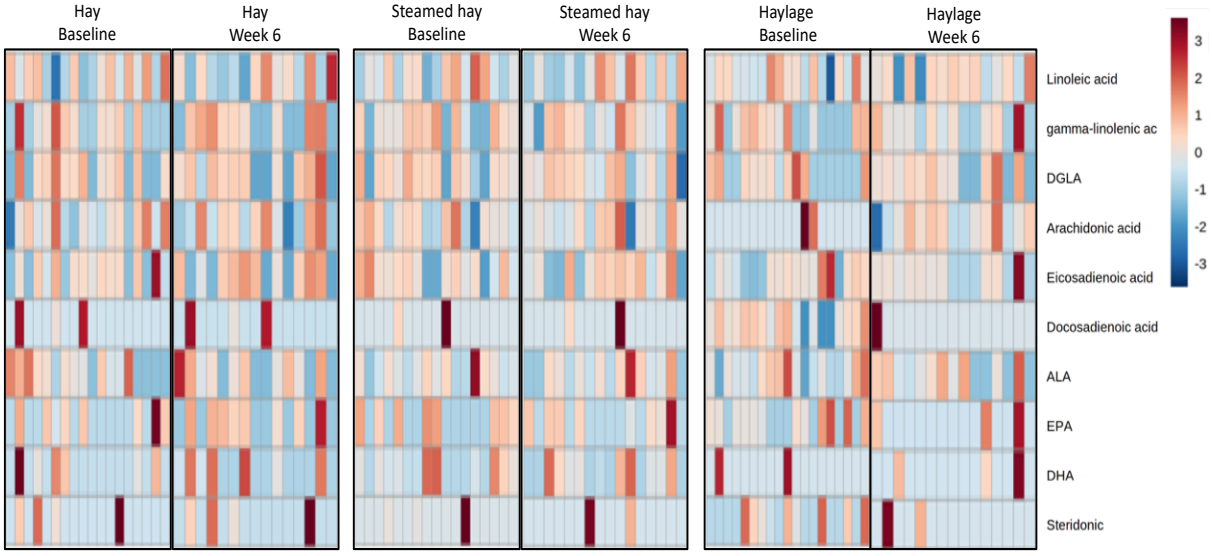
Bronchoalveolar Lavage Infused: _____ Recovered: _____

Time: _____ Appearance: _____

Signature: _____

APPENDIX J. SUPPLEMENTARY S 5.4

Heatmap displaying relative concentration of plasma PUFAs at baseline and week 6 by forage. Each column represents a horse. Data was mean-centered and divided by the standard deviation of each PUFA to scale the features for visualization purposes.



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VITA

Carla J Olave Olivares

Academic Record

2017-present	PhD degree in Veterinary Science, Purdue University – Graduation expected June 2020.
2016	M.S in Veterinary Science, Universidad Austral de Chile.
2014	Specialization in Clinical Veterinary Sciences, mention Equine Medicine and Surgery, Universidad Austral de Chile.
2013	D.V.M., Universidad Austral de Chile.
2007	Deutsche Schule, Puerto Montt, Chile.

Language Skills

Spanish	Native language.
English	Advance knowledge.
German	Intermediate knowledge (Vordiplum approved).

Employment

Other Professional Appointments

January 2017-Current	Research assistant, Department of Veterinary Clinical Sciences, Purdue University College of Veterinary Medicine.
March 2015-November 2016	Research assistant, Department of Pharmacology, Faculty of Veterinary Science, Universidad Austral de Chile.
December 2012-March 2013	Summer Internship, Large Animal Medicine and Surgery; Institute of Veterinary Clinical Sciences, Universidad Austral de Chile, Valdivia, Chile.

Awards and Honors

2015	Chilean national scholarship for Masters in Chile - CONICYT (Chile's National Commission for Scientific and Technological Research).

Memberships in Academic, Professional, and Scholarly Societies

2019-Present	American Association of Equine Practitioners
2020	American College of Veterinary Medicine

DISCOVERY

Publications

Peer Reviewed Publications

Published

1. **Olave C**, Morales N, Uberti B, Henriquez C, Sarmiento J, Ortloff A, Folch H, Moran G. Tamoxifen induces apoptotic neutrophil efferocytosis in horses. Veterinary Research Communication. 2018.
2. **Olave C,** Morales N, Uberti B, Henriquez C, Sarmiento J, Ortloff A, Folch H, Moran G. Tamoxifen induces apoptotic neutrophil efferocytosis in horses. Veterinary Research Communication. 2018.
3. Borlone C, Morales N, Henriquez C, Folch H, **Olave C**, Sarmiento J, Uberti B, Moran G. *In vitro* Effects of Tamoxifen on Equine Neutrophils. Research in Veterinary Science. 2016.

In Preparation

4. **Olave C,** Ivester K, Couetil L, Mukhopadhyay A. Dust exposure and pulmonary inflammation in Standardbred racehorses fed dry hay or haylage. The Veterinary Journal.

Doctoral Thesis Title

- *The effect of low-dust forages and the role of pro-resolving lipid mediators in mild-moderate equine asthma.* Purdue University. 2020 (expected)

M.S. Thesis Title

- *Effect of tamoxifen in neutrophils of healthy horses and the role of estrogen receptors in this effect. Universidad Austral de Chile. 2016.*

D.V.M Thesis Title

- *Comparison of the sedative effects of acepromazine-tramadol and acepromazine-tramadol-midazolam association in dogs. Universidad Austral de Chile. 2013.*

Abstracts and Proceedings – Research/Clinical Investigations

Abstract Presentations

1. Olave C, Ivester K, Couetil L. Effect of hay and haylage on particulate exposure and bronchoalveolar lavage cytology in Standardbred racehorses. 35th symposium of the Veterinary Comparative Respiratory Society, October 2017, Champaign, Ill.
2. Olave C, Ivester K, Couetil L, Park JH. Effect of forage type on particulate exposure in the breathing zone of racehorses. 36th symposium of the Veterinary Comparative Respiratory Society, October 2018, Auburn, AL.
3. Olave C, Ivester K, Park JH, Couetil L. Effect of forage type on respirable dust exposure and airway cytology in Thoroughbred racehorses. Havemeyer Equine Asthma Workshop, May 2019, Custer, SD.

Poster Presentations

1. Olave C, Ivester K, Couetil L, Robinson J, Park J. Role of dietary pro-resolving lipid mediators in equine asthma. Health and disease poster session, Purdue University, West Lafayette, IN. 2020.
2. Olave C, Ivester K, Park J, Couetil L. Effect of forage type on respirable dust exposure and airway cytology in Thoroughbred racehorses. PVM Research Day, Purdue University, West Lafayette, IN. 2019.
3. Olave C, Ivester K, Couetil C, Mukhopadhyay A. Effect of hay and haylage on lung health in Standarbred racehorses. PVM Research Day, Purdue University, West Lafayette, IN. 2018.
4. Olave C, Uberti B, Folch H, Morales N, Henriquez C, Moran G. *In vitro* effects of tamoxifen on equine neutrophil respiratory burst and phosphatidylserine expression. ACVIM forum, National Harbor, MD. 2017.
5. Olave C., Folch H. *In vitro* effect of tamoxifen on phagocytosis of neutrophils and efferocytosis in equines. 13th Annual meeting of the Immunology Society of Chile, Puerto Varas, Chile. 2015.

6. Cordero, F., Olave, C., Bustamante, H., Gajardo, G., Diaz, J., Meneses, C., Uberti, B., Menarim, B. Horner syndrome in two Chilean horses after intravenous administration of Selenium. 18th Chilean Congress of Veterinary Medicine, Santiago, Chile. 2014.
7. Olave, C., Uberti, B., Bustamante, H., Cordero, F., Diaz, J., Gajardo, G., Samudio, O., Meneses, C., Menarim, B. Congenital fibroblastic epidermal cysts in a 4-month-old foal. 18th Chilean Congress of Veterinary Medicine, Santiago, Chile. 2014.
8. Olave C, Bustamante H, Minder P, Alfaro A. Comparison of the sedative effects of acepromazine-lodamart and acepromazine-lodamart-midazolam association in dogs. 18th Chilean Congress of Veterinary Medicine, Santiago, Chile. 2014.
9. Olave C, Cordero F, Diaz J, Gajardo G, Uberti B, Menarim B. Description of front limb neurectomy of the deep branch of the lateral palmar nerve in Chilean Mare. 18th Chilean Congress of Veterinary Medicine, Santiago, Chile. 2014.
10. Uberti B, Diaz J, Gajardo G, Olave C, Menarim B. Exploration of causes of Selenium deficiency in a pre-mountainous range establishment in the Region of Los Ríos, Chile. 18th Chilean Congress of Veterinary Medicine, Santiago, Chile. 2014.

Professional Meetings and Courses Attended

1. "Havemeyer Equine Asthma Workshop"; May 2019, Custer, South Dakota.
2. "36th symposium of the Veterinary Comparative Respiratory Society, Advance in Respiratory Diagnostic Imaging"; October 2018, Auburn, Alabama.
3. "35th symposium of the Veterinary Comparative Respiratory Society, Advance in Respiratory Diagnostic Imaging"; October 2017, Champaign, Illinois.
4. "Care and use of experimental animals, bioethical issues"; January 2016, Valdivia, Chile
5. "13th Annual meeting of the Immunology Society of Chile"; October 2015, Puerto Varas, Chile.
6. "58th Annual meeting of the Biology Society of Chile"; October 2015, Puerto Varas, Chile.
7. "18th Chilean Congress of Veterinary Medicine"; December 2014, Santiago, Chile.
8. "17th Chilean Congress of Veterinary Medicine"; November 2012, Valdivia, Chile.
9. "Equine distal lameness"; November 2012, Valdivia, Chile.
10. "ANEVET congress of actualization in Veterinary Medicine"; September 2012, Valdivia Chile.

Research Grants

From External Sources

1. Agency/Title of Grant: **Grayson-Jockey Club Res. Fdn. / Effects of low-dust forage on racehorses lung health**
2. Duration of Funding (Dates): **Two (2) years (4/2018-3/2020**
3. Total amount of award: **$126,457**
4. Your role: **Co-I**
5. If Co-PI, for how much of the total funding are you directly responsible: **NA**
6. Co-Investigators (Roles): **Couetil L (PI), Burgess J (Co-I), Ivester K (Co-I), Park J (Co-I), Moore G (Co-I)**

1. Agency/Title of Grant: **Boehringer Ingelheim/ Role of dietary pro-resolving lipid mediators in equine asthma**
2. Duration of Funding (Dates): **One (1) year (1/19-1/20**
3. Total amount of award: **$15,000**
4. Your role: **Co-I**
5. If Co-PI, for how much of the total funding are you directly responsible: **NA**
6. Co-Investigators (Roles): **Couetil L (I), Burgess J (Co-I), Ivester K (Co-I), Parl J (Co-I), Moore G (Co-I)**

1. Agency/Title of Grant: **CONICYT (Chile)/ Effects of tamoxifen on pro-resolutive mechanisms of inflammation in horses with recurrent airway obstruction**
2. Duration of Funding (Dates): **Three (3) years (1/2016-1/2019**
3. Total amount of award: **$241,000**
4. Your role: **Co-I**
5. If Co-PI, for how much of the total funding are you directly responsible: **NA**
6. Co-Investigators (Roles): **Grabiel Moran-Ruz (PI), Uberti B (Co-I), Folch H (Co-I), Morales M (Co-I), Herniquez C (Co-I)**

Research Proposals Submitted and Pending or not funded

From External Sources

Not Founded

1. Agency/Title of Grant: **American Association of Equine Pract. Foundation/ Is Tracheal Wash a Useful Tool to Diagnose Equine Asthma in the Field?**
2. Duration of Funding (Dates): **One (1) year (8/19-8/20**
3. Total amount of award: **$19,965**
4. Your role: **PI**
5. If Co-PI, for how much of the total funding are you directly responsible: **NA**
6. Co-Investigators (Roles): **Couetil L (Co-I), Ivester K (Co-I),**

ENGAGEMENT

Committee Service (Including Appointed or Elected Offices)

Purdue University

2019-Present Purdue Chilean Association (Treasurer)