Impact of stressors on immune system parameters in yearling horses and fermentation characteristics and aerobic stability of inoculated corn silages

by

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Abstract

A study was conducted in a Latin rectangle to determine the impact of stressors on immune function in yearling horses. After 51 d of supplementation with either block 1, a molasses block containing 14.30% fat or block 2, a molasses block containing 7.58% fat, horses were subjected to three forms of stress. Blood samples were collected 6 h pre-stress and at h 1, 6, 12, 24, 48, 72, 96, and 168 post stress. Nasopharyngeal flush (NPF) was conducted 6 h pre-stressor and h 12 and 72 post-stressor. Blood was analyzed for fatty acids, various hematological parameters, cytokines, PGE_2, and cortisol. Nasopharyngeal flush samples were analyzed for IgA. Composition of molasses blocks were found to be inconsistent with documented formulation and n-6:n-3 ratios were found to be similar between blocks. Therefore, main effect of hour was evaluated. White blood cells were elevated ($P < 0.01$) above baseline at subsequent sampling times and platelet count was elevated ($P = 0.01$) at h 1, 6, 24, 48, and 96 h post-stressor compared to baseline. Lymphocyte concentrations were reduced ($P < 0.01$) at h 1 compared to baseline but were elevated above baseline by h 6 ($P < 0.01$). Neutrophil concentrations were elevated ($P < 0.01$) at h 1 and 6 compared to baseline. Nasal mucosal IgA concentrations had a tendency ($P < 0.07$) to be greater at h 12 relative to baseline. Serum cortisol dropped to concentrations below baseline ($P < 0.01$) at h 1 and 6 but increased above baseline by h 12 ($P < 0.01$). It appears that stressors applied were sufficient to induce mild changes in several of the hematological parameters evaluated but an overall more profound effect may have been displayed if stressors were more profound or prolonged.

A second experiment was conducted to evaluate fermentation characteristics and aerobic stability of whole-plant corn silage after inoculation with three different bacterial inoculants. Treatments consisted of *Lactobacillus plantarum* and *Lactobacillus buchneri* (LPB), *L.*

plantarum, *L. buchneri*, and *Lactobacillus diolivorans* (LPBD), and *L. diolivorans (*LD*)*. Silages were sampled on d 0, 14, 39, and 90. Propionate was elevated in silages inoculated with LPBD on d 39 and d 90 compared to other treatments ($P < 0.03$). 1, 2-propanediol was elevated ($P < 0.01$) in silages inoculated with LPB and LPBD on d 39 and d 90 compared to CON and LD silages. An effect of sampling day ($P < 0.05$) was detected for DM, aNDF, ADF, ash, starch, pH, total VFA, acetate, propionate, lactate, ethanol, and 1,2-propanediol. When silages sampled on d 14, 39, and 90 were subjected to an aerobic stability test, only treatment differences were detected ($P < 0.05$) for lactate: acetate and 1-propanol. Sampling day differences were detected ($P < 0.05$) for pH, total VFA, acetate, propionate, butyrate, lactate:acetate, 1-propanol, and 1, 2-propanediol. Overall, results suggest that epiphytic microflora populations largely dominated the ensiling process as indicated by the relative similarities in silages throughout the trial.

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"For I know the plans I have for you," declares the Lord, "plans to prosper you and not to harm you, plans to give you hope and a future." ~ Jeremiah 29:11.

Dedication

To my mother, Suzanne, and late father, Rock. Thank you for instilling in me a love of learning and a love of life.

I am incredibly blessed to be your daughter.

Chapter 1 - A Review of the Literature-Equine

1.1 Introduction

Dietary supplementation of omega-3 fatty acids (n-3 FA) in equine diets has become increasingly popular, due in part, to their immunomodulatory effects in humans and domestic animals (Calder and Grimble, 2002). Because of their ability to be incorporated into the phospholipid cellular membrane of cells involved in the immune response, n-3 FA have the potential to attenuate stress-induced immune responses that are generally considered to have anti-inflammatory effects (Calder, 2002). Alteration of the immune response is advantageous in all species but especially in the performance horse.

Performance horses are frequently exposed to stressors such as extended trailering, contact with pathogens, and exercise. This stress, if chronic, may result in immune system dysregulation. If the physiological responses to stress can be altered by supplementation with n-3 FA, such as by decreasing secretion of pro-inflammatory cytokines, the overall health status and performance of the equine could be improved.

Omega-3 FA supplementation can be delivered via top-dressing oils or incorporation into a concentrate. An alternative delivery method includes *ad libitum* consumption of a block or tub containing n-3 FA. However, efficacy of such a product is largely dependent on its level of intake and is relatively unexplored.

1.2 Stress

Horses, as a species, are exposed to a litany of stressors throughout their lives including weaning, training, and competition. Stress can be experienced psychologically, physiologically, or physically and has been shown to decrease performance, increase susceptibility to disease, and induce behavioral issues (Art and Lekeux, 2005). While the stress response depends greatly on

the training, handling, and disposition of the animal, horses are a reactive species sensitive to changes in their environment, predisposing them to elevated stress responses. While repeated exposure to stressors, such as long hauls in the trailer or performance in different environments, may lead to adaptation and thus decrease physiological response to stress, further mitigation of the stress response at a cellular level may help decrease the negative effects of stress.

Stress generally begins with a stimulus, which causes the brain to react, ultimately inducing an action by the body (Dhabhar, 2014). Chemicals, such as epinephrine and cortisol, are secreted due to the sympathetic nervous system and communication between the pituitary and adrenal glands and hypothalamus. Cortisol, a glucocorticoid hormone, concentration increases in the blood in response to a stressor, initially increases the availability of glucose from muscles, and induces gluconeogenesis in the liver, thus increasing the amount of energy available for fighting off or fleeing a stressor. A stress response depends on many factors, such as previous exposure to the stimulus and concentration of stress hormones in the body, making the response highly variable between individuals. Stress responses also depend on the length of the stimulus. Acute stress is considered to be momentary to several hours long, whereas chronic stress extends past hours and continues into days. Acute stress leads to upregulation of the innate and adaptive immune system, whereas chronic stress can down-regulate the actions of the same systems. This prolonged but down-regulated response predisposes the body to low-grade inflammation (Dhabhar, 2014).

Exogenous and Endogenous Stress

Stress is the result of outside factors affecting the internal homeostatic state and can be broken down into 2 categories: endogenous and exogenous stress. Stressors experienced outside the body but that produce an internal reaction are classified as exogenous stressors. Generally,

these are a product of the environment (i.e. pathogenic exposure and long distance trailering; Art and Lekeux, 2005). Situations such as these compromise the immune system and can result in disease. Endogenous stressors, such as oxygen availability, result from exogenous stressors, such as work during a training regime. When a horse is at work, its body is in a state of stress, and homeostatic balance is altered. Water and electrolyte balances change, as well as the rate in which oxygen is utilized and carbon dioxide is produced in order to meet new demands on the body (Art and Lekeux, 2005).

Both forms of stress can have potent and lasting impacts on health status of the horse. Whether there is endogenous stress in the form of pathogenic exposure and subsequent infection or changes in red blood cell (RBC) concentration, the body must manage the stress response to fight infection and return to a homeostatic state.

1.3 The Immune System

The immune system protects the body from foreign attack. Whether the challenge is pathogenic or situational, proper immune function is vital to the maintenance of homeostasis. Main purposes of the immune system are to contain and remove the immune challenge (i.e. pathogen), reduce further injury to the body, and begin tissue repair (Frellstedt et al., 2014). In order to accomplish these tasks, the immune system activates its two-component structure: the innate immune system and acquired immune system.

The Innate Immune System

The innate immune system is the first and immediate line of defense to pathogenic invasion and consists of physical tissue barriers, such as skin and mucosal membranes, that produce antimicrobial chemicals and cellular components (Abbas and Lichtman, 2005). These components include white blood cells (WBC; i.e. neutrophils, monocytes, eosinophils,

basophils, macrophages, and natural killer cells) and cytokines derived from macrophages (Calder, 2001).

Cells of the Innate Immune System

White blood cells, or leukocytes, are the first line of defense for the body and are produced in the bone marrow. White blood cells are classified by the presence or absence of granulocytes that contain enzymes that assist in killing pathogens. While WBC as a whole are vital to immune function, the various WBC isotypes have specific functions within innate immunity.

Neutrophils

Neutrophils are polymorphonuclear cells that make up 40 to 80% of the differential cell count (DCC) in humans (Calder, 2001). Neutrophils increase the body's inflammatory response to injury by secretion of pro-inflammatory cytokines such as TNF-α. Neutrophils also function as the main phagocyte of innate immunity and possess cytoplasmic granules that aid in pathogen neutralization (Calder, 2001).

Monocytes

Monocytes are mononuclear cells that originate in bone marrow and increase in concentration in response to chronic inflammation and stress (Tizard, 2013). A primary role of monocytes is maturation and differentiation into macrophages and dendricytes.

Macrophages

Macrophages are an integral part of the innate immune system that originate from monocytes. Functioning similarly to phagocytotic neutrophils, the macrophage response time to stimuli is much slower. Once macrophages are active, they signal for initiation of the acquired immune response. These antimicrobial cells also produce pro-inflammatory cytokines and

eicosanoids when stimulated. These molecules, like tumor necrosis factor-α (TNF-α) and prostaglandin E_2 (PGE_2), are essential to the proliferation of immune responses and tie the innate and acquired immune systems together (Abbas and Lichtman, 2005).

Eosinophils and Basophils

Eosinophils and basophils are both polymorphonuclear cells. Eosinophils are involved in the production of various cytokines, such as IL-4 and TNF-α, and are associated with immune responses to parasites, allergies, and asthma. Basophils, the least abundant WBC, release histamine and are closely associated with eosinophils in that they are important to allergic reactions and asthma. Upon infection, basophils travel from the bone marrow to the site of infection (Abbas and Lichtman, 2005).

Natural Killer Cells

Natural killer cells (NK cells), a class of lymphocytes, are activated by interferons, a class of cytokines. Natural killer cells respond to a threat in a non-specific manner by releasing cytotoxic granules, helping neutralize pathogens (Pier et al., 2004).

Molecules of the Innate Immune System

Cytokines are proteins that transmit cellular signals, help regulate cellular activity, and can induce both pro-inflammatory and anti-inflammatory responses. While cytokines can also originate from lymphocytes as part of the acquired immune system, their involvement in innate immunity mediates local and systemic inflammation and helps with pathogen elimination. Common cytokines involved in innate immunity include TNF-α, IL-6, and IFN-α. Tumor necrosis factor-α, released by activated macrophages, neutrophils, monocytes, NK cells, and B and T cells, aids in removal and repair of damaged tissue by activating the vascular endothelium, increasing vascular permeability (Hines et al., 1996). This allows recruited cells of the immune

system to enter the damaged tissue to aid in its protection and repair. Tumor necrosis factor-α also increases B and T lymphocyte production by the acquired immune system, and it increases antibody production.

While normal, pro-inflammatory cytokine activity is beneficial, excessive cytokine activity is undesirable (Calder, 2001). In order to prevent overproduction and amplified inflammation, anti-inflammatory cytokines are produced as built-in inflammatory mediators. The most common forms include interleukin-4 (IL-4), interleukin-10 (IL-10) and tumor growth factor-β (TGF-β).

Interferon-α is one of the first molecules involved in an innate immune response to a pathogen and primarily works to inhibit further pathogenic replication by releasing enzymes that prevent RNA and DNA replication. This cytokine also encourages the development of cells in the acquired immune system, such as T helper cells, to recognize and rapidly eliminate pathogens (Abbas and Lichtman, 2005).

Prostaglandin E_2 (PGE_2), another chemical normally secreted by pro-inflammatory cells, induces fever, vasodilation, swelling, and pain. Produced by macrophages and stimulated neutrophils, PGE_2 is an eicosanoid, which are 20 C immunologic mediators, similar to leukotrienes (LT). Leukotrienes are produced mainly by inflammatory cells and are acted upon by 5-lipooxygenase to produce LTB_4, which along with PGE_2, have the largest impact on the intensity of the inflammatory reaction. Prostaglandin E_2 (PGE_2) can also have immunosuppressive effects by reducing proliferation of lymphocytes and decreasing production of pro-inflammatory cytokines, though this typically occurs during allergic reactions and asthma (Calder and Grimble, 2002).

These main cellular components of innate immunity aid in preventing invasion and subsequent damage to the host, but when the innate immune system cannot adequately eliminate the challenge, the adaptive immune system is stimulated.

Acquired Immunity

Acquired, or adaptive immunity is dependent on the action of lymphocytes and the antibodies and cytokines they release. Classified as either B or T cells, these lymphocytes are able to recognize, respond, and eliminate antigens in a specific manner, which is dependent on previous exposure to the foreign invader. These "memories" of previous pathogen exposure are retained due to antigen specific receptors on T and B lymphocytes. When activated, they produce antibodies that remain in circulation and help prevent re-infection of the host, allowing for a quicker and more efficient protective response in the future. Acquired immunity is made up of 2 different immune responses; humoral immunity and cell-mediated immunity (Abbas and Lichtman, 2005).

Humoral Immunity

Originating in bone marrow, B cells initiate the humoral immune response by secreting antibodies after sensing an antigen. These proteins, also known as immunoglobulins, aid in neutralization of antigens by directly preventing binding to cellular surfaces by opsonization, when a pathogen is identified by an antibody and eliminated by a phagocyte.

Immunoglobulins are the primary protein component of blood and are the most common protein found on the surface of mucosal tissue. In general, antibodies cause an inflammatory response as well as stimulation of phagocyte activity. This allows serum antibodies to proliferate the site of infection, ultimately assisting in pathogen neutralization (Abbas and Lichtman, 2005).

Immunoglobulins can be classified into 1 of 5 major isotypes: immunoglobulin G (IgG), immunoglobulin M (IgM), immunoglobulin A (IgA), immunoglobulin E (IgE), and immunoglobulin D (IgD). Each type differs in its function, structure, and antigen specificity. Measurement of these immunoglobulins can elucidate the behavior of the acquired immune system.

The most common immunoglobulins are IgG, IgM, and IgA, though immunoglobulin type and concentration differ between species. Immunoglobulin G is found throughout the body and its concentration increases following stress (Cruse et al., 2004). An increase in IgG is seen when an antigen is re-introduced to the immune system. Immunoglobulin M is bound to the membrane of B cells, which, when stimulated during acute infection, activates the activity of B cells. When presented with a novel stressor, IgM is associated with the initial immune response and increases in concentration. Immunoglobulin A is heavily concentrated and predominately located in mucosa, tissues, and secretions, such as saliva and intestinal and bronchial secretions (Cruse et al., 2004). Commonly associated with disorders of the mucous membranes and resulting secretions, such as sinus infections, IgA helps block pathogenic cells from attaching to the mucosal surface (Pier et al., 2004).

Cell-Mediated Immunity

T lymphocytes mediate the activity of cell-mediated immunity and eliminate pathogens that escaped destruction by the humoral immune system (Abbas and Lichtman, 2005). The parent T cells are used for formation of 2 types of T cells: α T cells and β T cells. β T cells have cytotoxic action and remove pathogens by recognition of exogenous protein antigens. α T cells remove pathogens that have endogenous protein antigens and further differentiate into 1 of 2 types of T helper cells (Th cells).

During an immune challenge, an antigen is presented to a naïve T cell and IL-2 is produced, inducing an increase in T cell production. If the naïve T cell senses IL-12, it will differentiate into a T_H1 cell and if IL-4 is present, differentiation into T_H2 cell will occur (Calder, 2002).

Helper T1 cells function mainly to kill intracellular pathogens and are essential to cell mediated immunity. Helper T1 cells increase interleukin-12 (IL-12) production, which stimulates further T cell production and IFN- γ that increases proliferation of macrophages. These cytokines activate macrophages, natural killer T (NKT) cells and cytotoxic β T cells (Mosmann and Sad, 1996). Helper T1 cell responses are generally considered pro-inflammatory, largely due to their production of the pro-inflammatory cytokines IL-12 and TNF-α.

An integral part of humoral immunity, T_H2 cells stimulated by IL-4 help rid the body of pathogens. They produce IL-4 and IL-10 and when combined with IL-4, produce IgE, which makes up a large part of the immune response to allergens. Interleukin-10 and IL-4 work together to decrease the activity of cell mediated immune response and immunity (Abbas and Lichtman, 2005).

Helper T cells are an essential part of the immune system because different types reduce the activity of other T_H cells due to signals from other cytokines that originate from macrophages and B cells. This opposing action is what gives the lymphocytes their potency and influence over the immune response. When exposed to a stressor or a pathogen, one of the helper T cell types increases and proliferates. If T_H2 cells produce IL-4, the activity of the generally pro-inflammatory T_H1 cells as well as the secretion of the associated pro-inflammatory cytokines is inhibited. The opposite is true when T_H1 cells produce IFN- γ, which decreases T_H2 cell activity (Morel and Oriss, 1998).

Proper cooperation between immune and inflammatory cells is made possible by the presence and activity of interleukins (IL). These proteins are essential for cellular growth and function and can be pro-inflammatory or anti-inflammatory (Simopoulos, 2002a).

Interleukin-4 (IL-4), a lymphocyte derived T helper cell, is a nonspecific effector molecule that stimulates the change of B cells to IgE (Cruse et al., 2004). This cytokine has potent anti-inflammatory effects due to its antagonistic action on the activity of interferon- γ (IFN- γ), resulting in decreased macrophage activity. Interleukin-4 is decreases the activity of cell-mediated immune reactions, and thereby decrease inflammation (Abbas and Lichtman, 2005).

Interleukin-10 (IL-10) regulates the amount of B cells and IgA involved in an immune response. Interleukin-10 also decreases macrophage activity (Cruse et al., 2004). Like IL-4, IL-10 has anti-inflammatory effects and functions to decrease IFN- γ, thereby decreasing pro-inflammatory reactions (Abbas and Lichtman, 2005).

Interferon- γ and IL-2 are also involved in T-cell function and development. Interferon- γ works to activate macrophages and is closely involved in both innate and adaptive immunity. Classified as "anti-viral," IFN- γ is known for its ability to potentiate macrophage and NK cell activity and function (Cruse et al., 2004), thus increasing inflammation as a result of pathogen exposure (Abbas and Lichtman, 2005).

Interleukin-2 is largely involved in the acquired immune response, as it is produced due to recognition of antigens on T cells. This interleukin increases lymphocyte proliferation while also regulating lymphocyte activity by T cell production. Thus, an increase in IL-2 will decrease pro-inflammatory reactions due to its oversight of T cell activity (Abbas and Lichtman, 2005).

Eicosanoids are also involved in acquired immunity. Prostaglandin E_2 alters IgE production by B lymphocytes (Calder and Grimble, 2002) and leukotrienes can increase TNF-α, IL-1, and IL-6 through stimulation of neutrophil adhesion to endothelium of the vascular system (Calder and Grimble, 2002; Calder, 2003); thus increasing the pro-inflammatory reaction.

Together, innate and acquired immunity work together to prevent infection of the host. Immune system activity can be altered by a multitude of factors, such as previous pathogen exposure, nutritional plane, physiological status, or stress. Therefore, strengthening the immune system is advantageous in many circumstances, especially in those where novel environments and pathogens are encountered.

1.4 Immune Response to Stress

Immune responses to stress are generally considered protective, pathological, or regulatory (Dhabhar, 2014). These responses result from communication between the immune system, damaged tissues, and inflammatory mediators released from immune and non-immune cells. Processes involved with wound healing and pathogen elimination are considered immunoprotective and initially utilize the innate immune system before stimulating the adaptive immune system. Pathological immune responses are seen in instances of autoimmune disorders, where the body is generally working to eliminate what it perceives as an endogenous threat. Regulatory immune responses are classified as reactions between different types of immune cells, such as regulation of pro-inflammatory cytokines by production of anti-inflammatory cytokines (Dhabhar, 2014).

The stress response and subsequent release of immunologic chemicals is greatly affected by duration and severity of stress. In periods of acute stress, the innate and adaptive immune systems are upregulated; whereas wth chronic stress, the constant load on the immune system

results in dysregulation of immune function. Controlled responses to acute stress typically induce positive changes, whereas chronic stress results in deleterious effects (Dhabhar, 2014).

While outward signs of stress in the equine may present as behavioral changes, such as flared nostrils, an unwillingness to stand still, or vocalization, the impact of stress at the cellular level alters production and proliferation of immunological chemicals and cells.

Acute stress

Acute stress can last from several minutes to hours and can be physiological or psychological. Physiological stressors, such as exercise, can result in an inflammatory response due to damage to tissues caused by mechanical movement (Hines et al., 1996; Frellstedt et al., 2014). Psychological stress can be experienced when animals are restrained, exposed to something new, or handled by people (Grandin, 1997).

When involved in a "fight or flight" response due to acute stress, defense mechanisms are activated to help promote survival. Many physiological changes take place at this time, which are initialized by leukocytosis and subsequent increase in leukocyte concentration in the blood. This increase in WBCs potentiates the production of pro-inflammatory cytokines such as IL-6 and TNF-α (Hines et al., 1996) and activation of NK cells, B cells, T cells and Th cells (Dhabhar et al., 1995). After an initial acute increase, leukocyte concentration in the blood quickly decreases and returns to baseline concentration after removal of the stressor (Dhabhar et al., 1995). Neutrophils also increase due to stress, but they generally remain elevated after the body has returned to rest (Pedersen, 2000). Under stress, pro-inflammatory cytokine production is also up-regulated resulting in inflammation, swelling, and heat at the site of assault. An increase in the WBC count and its subcategories would be expected during stress or disease as these cells are essential for removing the pathogens from the host. Raidal et al. (1997) measured neutrophil

activity in equines after trailering and found decreased neutrophil concentrations 36 h after transportation ended. Lymphocyte populations initially increase in the blood and are recruited to areas where they are needed to fight an assault, but after prolonged exertion, a decrease in lymphocyte subpopulations are observed (Dhabhar, 2014).

Tumor necrosis factor-α increases during bodily injury, fever, septic shock, acute exercise, and social stressors (Slavish et al., 2015), causing a pro-inflammatory response (Cruse et al., 2004). Tumor necrosis factor-α also causes accumulation of coagulants at a site of injury, thereby decreasing entrance of more pathogens into the body (Abbas and Lichtman, 2005). Elevated levels of TNF-α are considered one of the most indicative signs of sickness or infection. Demonstrated in horses by Barton and Collatos (1999), blood and peritoneal fluid from horses suffering from colic contained elevated TNF-α concentrations. Increases in pro-inflammatory cytokines are also found in humans in a disease state, such as coronary heart disease and cancer (Simopoulos, 2002a).

Stress also alters activity of anti-inflammatory cytokines. Stressors, such as test anxiety, caused the anti-inflammatory cytokines IL-4 and IL-10 to decrease in the saliva of anxious humans (Maes et al., 1998). But, in humans who were not easily stressed, salivary IL-10 increased after exposure to a social stressor (Maes et al., 1998).

Immunoglobulin concentration is also impacted by stress. Salivary IgA was reported to decrease in humans after prolonged running (Nieman et al., 2002). Similar results were observed in horses after 12 h of head elevation but returned to normal 24 h post-stressor (Bobel et al., 2016). A decrease in IgA levels may reduce mucosal protection from infection and predispose the body to infection.

Stress can be quantified by the measurement of serum cortisol. Cortisol generally increases as a result of stress. In the equine, an increase in plasma cortisol can be 2 to 3 times greater than baseline and maximal levels are typically found approximately 30 min post-exercise before returning to baseline levels within 2 hr after cessation of the stressor. Cortisol secretion causes increased deposition of glycogen and an increase in utilization of fat stores in order to reserve glycogen that may be needed if stress is prolonged (Marlin and Nankervis, 2002). King et al. (2009) introduced horses to trailering and subsequent stall tying and found greater cortisol levels 30 min after trailering compared to the horses' non-stressed counterparts. Unfortunately, concentration of serum cortisol can be confounded by sampling protocols (Cook and Bond, 2010) and is greatly impacted by circadian rhythm (Irvine and Alexander, 1994). Because of this, interpretation can be difficult, but nevertheless, it is a useful evaluation parameter for quantification of stress levels.

Dysregulation of Immune Function by Stress

Stress induces a litany of negative physiological responses, such as inhibition of key immune cells, namely neutrophils, leukocytes, macrophages, natural killer cells, and T and B lymphocytes. Stress also alters pro-inflammatory cytokine production and inhibits cytokines that are needed to allow the immune system to adequately adapt to immune challenges (Padgett and Glaser, 2003). The interaction between the immune system and the stress response is a delicate balance, but one that provides for substantial protection for the body.

1.5 Fat Supplementation in the Equine

Increasing dietary fat by supplementation is common practice in the equine industry. Providing energy in the form of fat rather than starch or sugar helps manage or prevent metabolic health problems, reduce the heat of metabolism, lessen gastrointestinal bulk, and decrease the

risk of cecal acidosis. While there are many benefits to increased dietary fat, fat source may impact a horse's physiology.

Fat may be administered via top-dress, an extruded high-fat pellet, or incorporated into a commercial feed. A less widely used method for fat delivery includes *ad libitum* access to lick tubs or blocks.

Commercial feed companies use fat sources that are affordable, accessible, and easy to store and incorporate into the ration. These commonly include soybean oil, corn oil, or sunflower oil. While providing concentrated energy to the diet, most supplement fat sources are predominately composed of the polyunsaturated omega-6 fatty acid (n-6 FA) linoleic acid: (C18:2n-6: LA). Linoleic acid constitutes 65% of fat found in sunflower oil and more than 50% of fat content in corn and soybean oil (Bézard et al., 1994; Calder and Grimble, 2002). While n-6 FA are necessary for proper health, omega-3 FA (n-3 FA) are also essential and have been shown to improve immune response and function (Calder and Grimble, 2002).

Fatty Acids

Initial classification of fatty acids depends on the presence or absence of double bonds. Saturated FA do not have a double bond while unsaturated FA have a single (monounsaturated) or multiple (polyunsaturated; PUFA) double bonds. Molecules with less than 6 C are considered short chain fatty acids (SCFA), medium chain fatty acids (MCFA) possess 6 to 16 C, and long chain fatty acids (LCFA) have more than 16 C (Friedberg, 1992).

Further classification of FA depends on whether they are essential in the diet. Plants, possessing the necessary enzymes for desaturation and elongation of any FA, readily convert 18 C fatty acid molecules into LA and alpha linolenic acid (18:3n-3; ALA ; Calder, 2013). Animals, on the other hand, cannot synthesize these long chain PUFA *de novo* as they lack Δ12 and Δ15

desaturases, key enzymes used to change shorter chained FA, like oleic acid (C18:1n-9) into LA or LA into ALA (Calder and Grimble, 2002). As a result, the n-3 and n-6 PUFA are considered essential fatty acids (EFA) and must be added to the diet.

Supplementation of Essential Fatty Acids

Fatty acids are essential for many cellular processes including maintenance of cell membrane integrity and fluidity, transmission of cellular signals, regulation of gene expression, and retinal and neural development (Yaqoob et al., 1995; Warren and Vineyard, 2013). The physiological impact of FA depends initially on its ability to be incorporated into cells which depends on the amount provided in the diet.

On average, humans consume approximately 14 g/d of LA and 2 g/d of ALA (Calder and Grimble, 2002). For the equine, the NRC (2007) recommends a LA intake of 0.5% DM/d, or approximately 50 g/d for a 1000 lb mature horse consuming 2% of BW in DM/d. Currently, there is no formal recommendation regarding dietary intake of ALA (Warren and Vineyard, 2013). Despite the LA recommendation, there is not a well-defined requirement in the horse for total EFA in the equine diet, and even in horses fed low-fat diets, a deficiency in EFA has not been identified. In other species, deficiencies are manifested by hair loss, poor skin quality, and reduced reproductive efficiency (Warren and Vineyard, 2013).

Forages typically contain more ALA than LA, whereas concentrates generally have the opposite ratio. General consensus is that daily consumption of LA and ALA should be sufficient in a horse consuming good quality forage and a concentrate (Warren and Vineyard, 2013). In cases where diet quality is in question or horses are maintained solely on pasture, supplementation with LA or ALA may be helpful to provide these EFA if deficiencies are suspected.

Omega-6 Fatty Acids

Omega-6 FA are the most common long chain PUFA found in the majority of human and equine diets. Made up of at least 18 C and possessing more than 1 double bond, the n-6 designation comes from the location of the first double bond found 6 C away from the methyl end of the C chain. Sources of n-6 FA are generally affordable, easily accessible, and stable at room temperature under various storage conditions. Omega-6 fatty acids are consumed in 1 of 2 forms: as the parent form, LA, or as the longer chain derivative of LA, aracadonic acid (ARA, C20:4n-6). If consumed as LA, the FA can be converted by various enzymes into ARA, though this conversion pathway is not extremely effective. When consumed as ARA, this n-6 FA is readily used to produce pro-inflammatory eicosanoids

Omega-3 Fatty Acids

Omega-3 FA have their first double bond located 3 C away from the methyl end of the C chain and originate from either plant or marine sources. The most common plant based n-3 FA is alpha-linoleic acid (C18:3n-3; ALA), which is the parent n-3 FA of eicopentaenoic acid (C20:5n-3; EPA) and docosahexaenoic acid (C22:6n-3; DHA; Calder, 2001). Fresh and preserved forages typically contain shorter chain n-3 FA and are generally less concentrated, whereas longer chain and more concentrated sources can be found in flaxseed and fish oil.

Flaxseed is marketed as whole flaxseed, ground flaxseed, flaxseed meal, or flaxseed oil. Flaxseed is the richest plant source of n-3 FA and predominately comprised of ALA (Ander et al., 2004). Flaxseed and flaxseed oil have been fed to horses for decades because of their apparent benefits to hair coat and they are credited with making horses “shiny,” though this has not been quantified in controlled research trials. Although a common ingredient, there is disagreement regarding the most effective manner of supplementation for the horse. Because of

its hard-outer seed coat, some argue that flaxseed should be further processed, by grinding and stabilization to prevent oxidation or fed as an oil to maximize its incorporation into the tissues, whereas others contend that further processing is unnecessary. Supporting this, Aymond and Van Elswyk (1995) found that laying hens fed a diet containing 15% ground flaxseed had greater n-3 FA concentration in their egg yolks as compared to their counterparts fed whole flaxseed. However, at 5% inclusion there were no differences between treatment groups. Thus, the form and inclusion rate of flaxseed in the diet appears to impact the incorporation of n-3 FA into tissues in chickens.

Omega-3 fatty acids are also found in marine sources and are most commonly consumed in the form of fish oil. Although ALA is present in marine sources, these n-3 FA sources predominately consist of the longer chain n-3 FAs EPA aid and DHA.

Biosynthesis Between Essential Fatty Acids

Alpha-linolenic acid and LA can be immediately utilized, or they may undergo alteration within the body to form longer-chained derivatives. Both EFA are subjected to the same pathway, which begins with the rate limiting action of Δ6- desaturase and adds a double bond to the C chain. The resulting derivatives, gamma linolenic acid (C18:3n-6; GLA) from LA and stearadonic acid (C18:4n-3, SA) from ALA, are further changed by elongase enzymes to dihomo-gamma-linolenic acid (C20:3n-6; DGLA) and eicosatetraenoic acid (C20: 4n-3), respectively, which are acted upon by Δ5- desaturase to form arachidonic acid (C20:4n-6; ARA) from the LA parent and eicosapentaenoic aid (C20: 5n-3; EPA) from ALA. EPA can be further elongated to docosapentaenoic acid (C22:5n-3; DPA) before undergoing further elongation, desaturation by Δ6- desaturase, and beta-oxidation to docosahexaenoic acid (C22:6n-3; DHA).

Shortcomings of Reliance on PUFA to EFA Bioconversion for EFA Supply

Because both n-3 FA and n-6 FA acids are formed from the same enzymatic pathways, there is constant competition for desaturation and elongation (Calder and Grimble, 2002). When n-3 FA are present, Δ6-desaturase preferentially converts ALA rather than LA, resulting in greater conversion of ALA to EPA and thereby decreasing the conversion of LA to ARA (Sprecher et al., 1995; Hussein et al., 2005).

Due to the rate limiting nature of initial Δ6 desaturation (Hess et al., 2013) and the rapid oxidation rate of ALA compared to other FA (Nettleton, 1991), reliance on this conversion process is not sufficient to produce all necessary EPA and DHA. Williams and Burdge (2006) estimated that humans bioconvert less than 10% of ALA to EPA and less than 0.10% of ALA to DHA. In horses, ALA supplementation results in increased EPA and DHA deposition in cellular membrane phospholipids (Vineyard et al., 2010). But, because of unknown and assumed low conversion rates of ALA to these longer chain PUFA in the equine, the most efficient supplementation method of EPA and DHA is through fish oil rather than flaxseed oil. Unlike plant sources of n-3 FA which must be elongated and desaturated into longer chain FA EPA and DHA can be readily incorporated into cellular membranes (Calder, 2001).

Differences in incorporation rates have been demonstrated in several studies. Hess et al. (2012) compared the effects of marine and flaxseed-based n-3 FA on serum FA composition in horses. When supplemented for 90 d, marine-and flaxseed-sourced n-3 FA led to decreased circulating levels of LA, while EPA and DHA concentrations increased after supplementation with marine sourced n-3 FA only. Additionally, Vineyard et al. (2010) found supplementing encapsulated fish oil to horses for 70 d increased ($P < 0.05$) EPA and DHA in plasma while flaxseed meal had no effect on circulating FA. Ross-Jones et al. (2014) supplemented horses

with either 38 g of ALA via flaxseed or 38 g of ALA+ 7.6 g EPA + 26.6 g of DHA for 90 d and measured the amount of FA in synovial fluid. Synovial fluid from horses fed the combination of n-3 FA had greater concentrations of EPA and DHA when compared to horses fed flaxseed. Therefore, direct supplementation of EPA and DHA appears to be an effective method to alter FA concentration within the body.

Balance between Essential Fatty Acids

Elevated amounts of n-6 FA compared to n-3 FA may negatively impact health of the equine. Specifically referring to the immune system, excessive dietary n-6 FA can result in overproduction of pro-inflammatory molecules, which predisposes the body to increased swelling, heat, and chronic low-grade inflammation (Simopoulous, 2002a; Dhabhar et al., 1995). Greater intake levels of n-6 FA are typically found in humans who consume diets rich in starch and meat, typical of a traditional "Western diet" and often have n-6 to n-3 ratios around 16 to 20:1 (Simopoulos, 2002b), whereas a diet of lean meat, fish, and vegetables provides more n-3 FA. Humans consuming a diet with elevated n-6 FA are predisposed to health problems resulting from inflammation, heart disease, metabolic imbalances, and arthritis (Calder, 2003).

Equine diets typically contain more n-6 FA:n-3 FA when compared to humans, but further decreasing this ratio may offer additional benefits, as evidenced by improvements in locomotion in osteoarthritic horses (Woodward and Nielsen, 2005). Therefore, altering the type of FA fed to the equine can potentially benefit overall well-being, especially in horses already in a state of compromised health.

1.6 EFA and Immune System

Essential FA found in cells of the immune system are predominately n-6 FA and typically consist of approximately 25% ARA, 8% LA and 2% DGLA. The n-3 FA of ALA, EPA, and

DHA are found in much lesser concentrations, not exceeding 4% of the total fat composition of the cell (Gibney and Hunter, 1993; Yaqoob et al., 2000). After incorporation of n-3 and n-6 LCFA into phospholipid membranes, fluidity of cellular membranes, cell signaling molecules, and synthesis of key bioactive inflammatory mediators are altered (Calder and Grimble, 2002).

Eicosanoids

In order to impact immune function, FA must be incorporated into cell membrane phospholipids in their long chain forms. During an immune challenge, these LCFA can be oxidized immediately and utilized to form eicosanoids, such as PGE_2. The synthesis of such compounds is a vital regulatory process, as eicosanoid biosynthesis links innate and acquired immunity (Simopoulos, 2002a). Eicosanoids are classified into 1 of 4 categories: leukotrienes, prostacyclins, thromboxanes, and prostaglandins (PG). Prostaglandins, prostacyclins and thromboxanes are considered prostanoids and are produced via the cyclooxygenase (COX) pathway, whereas leukotrienes are produced via the 5-lipooxygenase (LOX) pathway (Calder and Grimble, 2002).

Eicosanoids are not synthesized continuously in the body; production is induced when cells receive signals due to trauma or sense the action of other immune cells (Funk, 2001). Initial formation of eicosanoids from ARA via the COX pathway begins when phospholipase A_2 acts on cellular membranes to induce release of ARA. Cyclooxygenase activity on free ARA produces the intermediate prostaglandin PGH_2. Depending on enzyme affinity, PGH_2 can be further metabolized to 2-series thromboxanes (TX) and PG, such as PGE_2 and TXA_2 (Funk, 2001; Calder and Grimble, 2002). These eicosanoids, while important to the immune system, are pro-inflammatory, which can be detrimental in excess.

Eicosanoids are derived from ARA, DGLA, or EPA and their role in promoting or preventing inflammation is dependent on their parent FA (Mori and Beilin, 2004). If derived from ARA, eicosanoids are pro-inflammatory, but eicosanoids synthesized from EPA and DGLA are anti-inflammatory. The anti-inflammatory eicosanoids aid in pathogen exclusion, tissue repair, and vasodilation (Mori and Beilin, 2004).Thus, altering the source of FA and their subsequent eicosanoids, can effectively modulate immune response.

Because ARA is the commonly the predominant FA found in modern human diets, immune cells typically reflect its plentiful concentration. Therefore, ARA is the most common precursor for eicosanoid synthesis, largely resulting in pro-inflammatory eicosanoid production within the body (Funk, 2001; Tsatsanis et al., 2006). If dietary n-6 FA acids are replaced by n-3 FA, ALA and more specifically EPA, will be present in larger amounts, thus increasing anti-inflammatory molecules due to an increase in competition between n-3 FA and n-6 FA for enzyme activity (Simopoulos, 2002b). Competition between EPA, ARA, and DGLA occurs because EPA directly inhibits activity of phospholipase A_2, preventing release of ARA from phospholipids while also preventing COX from oxidizing ARA, thereby increasing anti-inflammatory eicosanoid production (Calder and Grimble, 2002). Eicosanoid synthesis from EPA produces 3- and 5-series inflammatory mediators, like PGE_3, which are considered to be low- or anti-inflammatory. While less potent than 2- and 4-series eicosanoids, such as PGE_2, these eicosanoids fill binding sites, reducing the ability of pro-inflammatory eicosanoids to bind cells and elicit pro-inflammatory effects (Mickleborough et al., 2008).

Cellular membranes have a direct impact on immune function. Thus, increased dietary n-3 FA would alter membrane FA composition and subsequent physiological and immune responses (Simopoulos, 2002a). Supporting this, Jangale et al. (2013) fed diabetic rats 10%

flaxseed oil or 10% fish oil. They observed both diets reduced blood glucose and hepatic inflammatory gene expression for TNF-α and IFN- γ. Conversely, horses supplemented with 3% added corn oil had increased PGE_2 levels when their bronchoalveolar lavage fluid (BALF) cells were isolated and subjected to an endotoxin challenge, indicative of a pro-inflammatory response (Hall et al., 2004).

Benefits of Supplementation with Fish Oil

Omega-3 FA can increase the flexibility of cellular membranes, thereby increasing circulation and vasodilation, while decreasing inflammation (Monteverde et al., 2016). Increased vasodilation improves supply of oxygen, nutrients, and phagocytes to injured tissues, thus quickening the repair process. Additionally, EPA is capable of forming anti-inflammatory eicosanoids and resolvins that reduce inflammation (Calder, 2001). Omega-3 FA also have been shown to decrease the activity of cells that present antigens to immune cells thereby decreasing the magnitude of an immune response (Simopoulos, 2002a).

Eicosapentaenoic acid and DHA are rapidly incorporated into cellular membranes without need for enzymatic alteration. Thus, fish oil is considered to be more effective than flaxseed oil as an anti-inflammatory and immunomodulating supplement (Wallace et al., 2003; Zhao et al., 2007), and fish oil has positive impacts on humans suffering from heart disease and cancer (Calder et al., 2002; Gurr et al., 2002). Rats supplemented with fish oil and subjected to an exercise regimen had lower heart rates than control animals (Lortet and Verger, 1995). O'Connor et al. (2004) reported similar outcomes where serum cholesterol and heart rates were decreased in exercising horses supplemented with 342 mg of fish oil/kg BW/d. Though fish oil appears to be effective at modulating changes, dosage, overall conditioning of experimental subjects, and saturation of cellular membranes seem to have a large impact on magnitude of

response. Raastad et al. (1997) found that fish oil supplementation had no impact on highly trained soccer players.

While fish oil appears to be an effective health promoter, its odor and concern with long-term stability have limited its use in equines. Fish oil is also generally more expensive than to flaxseed oil.

Benefits of Supplementation with ALA

Alpha-linolenic acid can mitigate inflammation and alleviate symptoms of rheumatoid arthritis, among other ailments in humans, due to its ability to reduce pro-inflammatory cytokine production (Caughey et al., 1996; Wallace et al., 2003; Zhao et al., 2007). The amount of ALA and the ratio of ALA to other PUFA in the diet appear to be major factors in determining its impact on immune response. Data collected on humans indicate that 15 g/d ALA decreases pro-inflammatory cytokine production, but the determination of whether this effect is due to ALA itself or the subsequent conversion to EPA and DHA is not clearly defined (Simopoulos, 2002a). Additionally, isolated hearts from rabbits fed a 10% flaxseed diet displayed reduced arrytthmatic activity when subjected to blood loss and subsequent rehydration (Ander et al., 2004), indicating that flaxseed supplementation may be beneficial for heart health.

When horses were fed a diet containing 8% flaxseed oil for 8 wk and macrophages were collected and subjected to an endotoxin challenge, flaxseed oil supplemented horses' macrophages produced less TNF-α compared to their non-supplemented counterparts (Morris et al., 1990). These results suggest that ALA was readily incorporated in the cell membrane as indicated by reduction in pro-inflammatory cytokine production.

Vineyard et al. (2010) postulated that the impact of ALA supplementation may not be quantifiable in horses consuming good quality forage, because of the plentiful amount of ALA in

fresh roughage. Because membranes were likely already saturated with ALA, dietary supplemental ALA likely does not change membrane composition. Because of this, benefits of ALA supplementation may be more likely when administered to horses consuming poorer quality hay or diets that are heavily concentrate based.

Impact of ALA Supplementation on Skeletal Muscle

While incorporation of ALA into cellular membranes is less efficient than EPA and DHA assimilation, ALA supplementation may still have beneficial effects. After supplementation of ALA via flaxseed, greater levels of the n-3 FA docosapentaenoic acid (DPA; C20:5n-3) were present in skeletal muscle in horses compared to those who were supplemented with marine-sourced n-3 FA (Hess et al., 2012). Though DPA has not been extensively researched, its incorporation into skeletal muscle indicates that ALA supplementation may have an impact on health, though this influence has yet to be fully elucidated.

Degenerative muscle diseases are characterized by an overactive inflammatory response typified by large increases in TNF-α. Carotenuto et al. (2016) supplemented flaxseed to hamsters with muscle myogenesis and re-evaluated muscle condition post-supplementation. Upon re-assessment, muscles of ALA supplemented hamsters had increased indicators of regeneration, as well as little to no further muscle damage. Thus, flaxseed supplementation may help muscular dystrophy patients by decreasing TNF-α concentration by exerting a protective effect on the tissue. It is plausible that persistent administration of flaxseed would change the muscle microenvironment, alleviating or lessening muscle damage. Supplementary ALA may additionally be involved with expression of caveolin-3, a protein only found in muscle that maintains cardiac and skeletal muscle function. However, any benefits from ALA supplementation have yet to be quantified (Carotenuto et al., 2016).

Impact of PUFA Supplementation on Immune Function and Stress

Eicosanoids, cytokines, and immunoglobulins can be quantified to evaluate immune response to stress as well as for measurement of the effect of PUFA supplementation on immune function (Pier et al., 2004). Prostaglandin E_2 decreases in rats and humans in response to n-3 FA supplementation in healthy and diseased states (Sargi et al., 2013), though this effect depends heavily on the source of n-3 FA. In equines, supplementary n-3 FA reduced PGE_2 concentration in some studies and were unchanged in others, most likely due to horse to horse variation. Hall et al. (2004) observed an increase in PGE_2 when lipopolysaccharide (LPS) challenged equine bronchoalveolar lavage fluid (BALF) cells were isolated from horses supplemented with corn oil compared to horses supplemented with fish oil.

When humans were supplemented with increasing amounts of n-3 FA over a 12-wk period with a final dose of 2 g of EPA+DHA/d, concentrations of TNF-α and IL-6 were decreased in stimulated peripheral blood mononuclear cells (PBMC; Trebble et al., 2003). Additionally, Meydani et al. (1991) found reduced TNF-α concentration in plasma from people when supplementation was greater than 2.4 g EPA+ DHA/d. Dosage of n-3 FA appears to have significant impact on potency of the immunomodulatory effect, which is demonstrated in multiple studies that administered EPA and DHA at rate of < 2g/d and saw no differences in cytokine production (Calder, 2001; Thies et al., 2001).

Fish oil can decrease cortisol in humans subjected to mental stress. When men were provided 7.2 of supplementary fish oil per day for 3 wk, their cortisol levels were decreased after undergoing a mathematical examination, though this effect is considered to be highly dose dependent (Bhathena et al., 1991; Delarue et al., 2003) and influenced by stressor perception.

1.7 Delivery of Supplement by Molasses Blocks

Providing livestock with supplements via a block or tub is common practice in animal agriculture. Supplement blocks can provide an array of nutrients. Blocks are generally classified as either salt, mineral, or protein. Salt and trace mineral blocks are typically provided free-choice and should be provided to all horses (NRC, 2007). Protein tubs, although more commonly fed to cattle, can help meet the nutritional needs of horses consuming fresh or dried forages or that require supplemental nutrients in addition to a forage and concentrate diet. These blocks typically contain a mixture of protein, fat, fiber, vitamins, and minerals, and frequently contain molasses as a carrier.

Fat can be administered in several ways but providing it alongside other nutrients via a block is a low-management and cost-effective method to meet a horse's nutritional requirements. To our knowledge, the efficacy of immunomodulation by supplemental FA delivered by a molasses block has not been evaluated.

1.8 The Paradox of Immunology

The immune system is made up of a delicate conglomeration of reactions that work in concert to protect the body from harm. Alterations of this system can occur due to stress but the magnitude of effect that these challenges can have may be ameliorated by supplementation of n-3 FA. While reduction in cellular reactions to stress can be beneficial, there is still a need for these reactions to take place to prevent invasion by foreign substances. Therefore, the purpose of altering immune response to stress by application of anti-inflammatory FA is not to eliminate a reaction, but to reduce its magnitude.

When evaluating the immune status of the horse, care must be taken to consider many pertinent factors. Omega-3 FA supplementation has the potential to benefit health status of the

horse, but, these effects have been clearly shown to be transient and depend heavily on fat source, delivery method, and dose.

1.9 Summary

Methods to increase the productivity and longevity of performance horses continue to be researched. Due to constant exposure to new environments and pathogens, optimizing function of the immune system by n-3 FA supplementation has the potential to greatly impact the equine. Though research on human and animal supplementation with these long chain PUFA have displayed positive effects, no studies to our knowledge have been conducted that evaluate the efficacy of daily n-3 FA supplementation by use of molasses blocks. Therefore, the following study was conducted to evaluate the impact of *ad libitum* molasses block consumption containing n-3 FA on immune markers following a stressor.

Chapter 2 - Impact of stressors on immune system parameters in yearling horses

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Abstract

An experiment was conducted to examine the immunomodulatory effects of stress on various hematological and immunological parameters in yearling horses. Horses had *ad libitum* access to Smooth Bromegrass hay and poured molasses bocks and fed a concentrate formulated for growing horses provided at 0.5% of BW/d. Horses were individually stalled for d 1-11 and group housed in dry-lots for d 12-59. After 51 d, horses were subjected to a 60-min trailer ride, 15-min of forced exercise, and 2-h elevated head tie. Blood samples were collected 6 h before initiation of the stressors and at h 1, 6, 12, 24, 48, 72, 96, and 168 post-stressor. Nasopharyngeal flushes (NPF) were collected 6 h before stress and h 12 and 72 post-stress. At the end of the sampling week, d 59, treatments were removed, and horses were maintained on their grain and forage diet for 41 d to serve as a wash-out period. At the end of the 41-d, horses were placed back onto the forage, block, and grain diet and protocols were repeated. Blood was analyzed for fatty acids, hematological parameters, cytokines, prostaglandin E_2, and cortisol. NPF samples were analyzed for IgA. White blood cells were elevated ($P < 0.03$) above baseline at subsequent sampling times and platelet count was elevated at h 1, 6, 24, 48, and 96 h post-stressor compared to baseline. Lymphocyte concentrations were reduced ($P < 0.01$) at h 1 compared to baseline but were elevated above baseline by h 6 ($P < 0.01$). Neutrophil concentrations were elevated ($P < 0.01$) at h 1 and 6 compared to baseline. Nasal mucosal IgA concentrations had a tendency ($P < 0.07$) to be greater at h 12 relative to baseline. Serum cortisol dropped to concentrations below baseline ($P < 0.01$) at h 1 and 6 but increased above baseline by h 12 ($P < 0.01$). It appears that stressors applied were sufficient to induce mild changes in several hematological parameters. A more profound effect may have been displayed if stressors were more intense or prolonged.

Introduction

Stress is an unavoidable part of an equine's life and is commonly experienced as a consequence of stressful events and environment (i.e. long-distance trailering and pathogenic exposure). While stress can initiate the important fight-or-flight reaction and enhance wound healing, stress can cause overproduction of immune molecules that can be damaging if chronic (Dhabhar, 2014) and can result in compromised immune function and ultimately disease (Art and Lekeux, 2005). The potential for compromised health due to stress is a result of many factors. But, relationships between pro-inflammatory cytokine and lipid mediator production in the stress response have been ascertained. The magnitude of a response to stress depends heavily on the perception of the stressor by the host (Koolhaas et al., 1999) and ability of the host to manage the stressor. If the body is unable to effectively cope with the stressor, physiological function and behavior can be altered (Tsigos et al., 2016). Therefore, investigation into the hematological alterations caused by stress warrant thorough examination and understanding.

Materials and Methods

Animals, Treatments and Diets

Animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee at Kansas State University prior to implementation. Ten yearling American Quarter Horses (mean age = 14 mo; SEM = 2 mo), 7 geldings and 3 mares (average BW = 407.5 kg; SEM = 8.9 kg), were used in a 2 x 10 (treatment x horse) Latin rectangle replicated over 2 periods. Horses were randomly assigned to their initial treatment which consisted of *ad libitum* consumption of either a commercially available molasses-based block formulated to contain greater levels of n-3 FA via flaxseed and flaxseed oil (block A; HorsLic Omega Elite; New Generation Supplements, Belle Fourche, SD) or a molasses-based block containing sunflower oil

(block B). Nutrient composition of molasses-based blocks were quantified by Midwest Laboratory (Omaha, NE) and are shown in Table 2.1. Variation between molasses-based blocks are shown in Table 2.2 and were quantified at the Ruminant Nutrition Laboratory at Kansas State University (Manhattan, KS). Block composition was determined after trial period was complete. Due to an error at manufacturing, FA profiles of the blocks were quite different from intended formulation. Treatment blocks were not iso-lipid (14.3% and 7.58% total fat for n-3 FA and n-6 blocks, respectively). Additionally, the n-3 FA: n-6 FA between treatments were similar (Table 2.1) and inconsistent in composition (Table 2.2). As a result, block affect was removed from analysis (see "Statistical Analyses").

Prior to initiation of the trial, all horses underwent a 7-d acclimation to *ad libitum* Smooth Bromegrass hay and 0.5% BW/d AF of an oat- and corn-based concentrate (Tables 2.3 and 2.4). The same diet was fed throughout the study, including the washout period.

Timeline, Housing, and Data Collection

For the first 10 d of period 1, horses were housed in individual box stalls (3.66 x 3.05 m) with rubber mats covered with pine shaving and equipped with automatic waterers. From 0700 on d 8 to 0700 on d 11, horses were continuously videotaped to determine frequency of block consumption (consumption events/d) while in confinement. Meals were defined as separate events if there was a break in consumption of 5 min or longer. Blocks were weighed at 0700 and 1900 each day. At 0700 on d 11, horses were relocated to 1 of 2 dry lots sharing a common center fence and group housed in their respective treatment groups with *ad libitum* access to treatment blocks. From 0700 on d 18 to 0700 on d 21, consumption data were collected by continuous videotaping to determine time determine frequency of block consumption

(consumption events/d) while group housed. Horses were maintained in their respective dry lots for the remainder of period 1 which concluded on d 59.

On d 52 at 0900, baseline blood and nasal flush samples were collected. Jugular venipunctures were performed utilizing an 18-gauge x 25.4-mm needle (BD; 305195; Franklin Lakes, NJ). Blood was collected into three 10-mL non-heparinized vacutainer blood tubes (BD; 366430; Franklin Lakes, NJ) and one 4-mL EDTA coated vacutainer (BD; 367844; Franklin Lakes, NJ). Nasopharyngeal flushes (NPF) were performed according to procedures utilized by Bobel et al. (2016). In brief, a 3.33 mm x 55.88 cm polypropylene catheter (Covidien LTD; 8890703518; Dublin, Ireland) was inserted into the pharyngeal region of the nostril and flushed with 120 mL of 0.9% sterile saline via two 60-mL sterile syringes. Proper catheter position was confirmed by simultaneous flow of fluid out of both nostrils. Fluid was collected at the base of each nostril through sterile urinary funnels (Parter Medical; 244917 Carson, CA) and into 90-mL sterile specimen cups (Parter Medical; 244917 Carson, CA).

At 1500 h, horses were subjected to a series of events that simulated common stressors encountered by performance horses. First, horses were randomized between treatment groups, loaded into 1 of 2 trailers and hauled for 1 hr. Upon return, they underwent 15 min of forced exercise via an automated panel walker (Priefert, Mount Pleasant, TX). Exercise regimen consisted of 2-min walk (6.4 km/h), 3-min trot (13 km/h), and 10-min canter (19 km/h). Finally, horses were subjected to a prolonged, elevated head tie during which horses could not drop their head below the point of their withers for 2 h. The completion of the elevated head tie was considered time post-stressor. Following conclusion of the elevated head tie, blood samples were collected after h 1, 6, 12, 24, 48, 72, 96, and 168 h. Nasopharyngeal flushes were conducted at h 12 and 72 post-stressor. Horses were allowed continuous access to their supplement blocks

throughout the remainder of the collection period that ended on d 59. At 0700 on d 60, treatments were removed, treatment groups were combined, and horses underwent a 42-d washout period whereby they only consumed only their basal diet of hay and concentrate. Period 2 began at 0700 on d 104, whereby horses were separated back into their original treatment groups, assigned to the opposite treatment, and procedures from d 1 to d 59 as described above were repeated. An experimental schematic is provided in Fig. 2.1.

Collection of Consumption Data

Video was recorded onto compact flash memory cards (SanDisk, 92889, Milpitas, CA) using cameras (Panasonic, WV-CP310, Kadoma, Japan) secured to pipe fencing in barns and dry lots and data were recorded onto utilizing digital video recording devices (Stack LTD, DVR3-130, Sycamore, IL). Video was recorded in 48-min intervals. Video was observed using a desktop media player (VLC Media Player). Consumption event duration and frequency were recorded by observers while playing video at maximum 2x speed. A consumption event was defined as a bout of consumption lasting more than 15 sec and meal conclusion was recorded when a break from consumption lasted more than 5 min. Mean consumption event size was determined using the following equation:

$$\frac{Number\ of\ consumption\ events\ in\ 24\ h}{(Beginning\ block\ weight - Ending\ block\ weight)}$$

Consumption event size was estimated when horses were housed in dry lots by calculating the weight of treatment blocks for 24-h periods and dividing by the number of horses in the treatment groups (n = 5).

Fatty Acid Composition of Treatment Blocks

Molasses block composition was evaluated utilizing methods described by Sukhija and Palmquist (1988) through derivitizing to methyl-esters by interesterification. Gas

chromatography (GC) was performed using a Hewlett Packard 5890 GC equipped with a flame ionization detector and a Supelco SP-2560 capillary column (100 m x 0.25 mm x 0.20 μM; Supelco, St. Louis, MO). Injector and detector temperatures were both 250°C and H flow rate was 1-ml/min with a split ratio of 1:100. Oven temperature began at 140°C and increased at a rate of 2°C/min to 200° C and then increased at a rate of 4°C/min to 245°C where it was held for 17 min. Fatty acid quantification was performed using a commercially available FA methyl ester standard mix (Supelco 37 FAME Mix; 47885-U, Supelco, St. Louis, MO).

Serum Parameters

Whole blood samples were centrifuged at 2,000 x g for 25 min. Serum was removed and sixteen 0.5-mL aliquots were transferred to 2-mL polypropylene tubes (Thermo Fischer Scientific; S06514; Waltham, MA) and frozen at -20°C until analysis. Ethylenediaminetetraacetic acid evacuated tubes were refrigerated and used for complete blood count (CBC) analysis within 4 hours of blood collection.

Complete blood count

Complete blood cell counts were performed using an IDEXX Procyte Dx Haematology Analyzer (IDEXX, Westbrook, Maine) within 4 h of blood collection. 4-mL EDTA evacuated tubes were inverted and individually placed into hematology machine to run CBC on each horse at each time point. White blood cell (WBC), red blood cell (RBC), hematocrit (HCT), platelet count, and differential cell count consisting of lymphocytes (LYM), neutrophils (NEU), monocytes (MON), eosinophils (EOS), and basophils (BAS) were recorded. Complete blood count were collected at h 1, 6, 12, 24, 48, 72, and 96 post-stressor.

Cortisol and PGE_2

Cortisol concentration was measured at baseline and h 1, 6, 12, 24, 48 using a commercially available ELISA kit (Neogen, 402710, Lexington, KY). Prostaglandin E_2 was measured in serum at baseline and h 1 and 6 post-stressor. Prostaglandin E_2 was quantified by the Gastrointestinal Physiology Laboratory at the Kansas State University College of Veterinary Medicine using a commercially available monoclonal ELISA kit (Cayman Chemical, 514010; Ann Arbor, MI).

Cytokines

Tumor necrosis factor-α was measured in samples obtained at baseline and h 1 and h 6 post-stressor via ELISA by the Gastrointestinal Physiology Laboratory at the Kansas State University College of Veterinary Medicine using a commercially available TNF-α equine ELISA kit (ThermoFisher Scientific, ESS0017; Waltham, MA). Interleukin-4 (IL-4), interleukin-10 (IL-10), interleukin-17 (IL-17), interferon-α (IFN- α), interferon- γ (IFN- γ) concentrations were determined at baseline and h 1, 6, 12, 24, 48, 72, and 96 post-stressor. Analyses were performed as described in Wagner and Freer (2009) by sandwich of 2 ELISA assays at the Animal Health Diagnostic Laboratory at Cornell University (Ithaca, NY).

Immunoglobulins

Immunoglobulin M (IgM) concentration was determined in samples collected at baseline and 96 h post-stressor via a commercially available radial immunodiffusion assay (Triple J Farms, 803411; Bellingham, WA) at the Clinical Programs Center, College of Veterinary Medicine, Cornell University (Ithaca, NY). Total immunoglobulin G (IgG) concentration was measured in samples obtained at baseline and 168 h post-stressor. Quantification was obtained according to manufacturer's instructions using a commercially available ELISA kit (Immunology Consultants Laboratory, Inc, E-70G, Portland, OR).

Nasopharyngeal Flush Samples

Fluid obtained via NPF were transported on ice to the laboratory for filtration before freezing. Chilled samples were vortexed, filtered through sterile 74-micron nylon mesh (ELKO Filtering Co. NCO36680, Miami, FL) and subsequently filtered through a 40 μm cell strainer (Corning Inc., 352340; Corning, NY) into 50-mL polypropylene tubes (Corning Inc., 352070; Corning, NY). Tubes were then centrifuged at 400 x g for 5 min. Equal subsamples were collected from each polypropylene tube, combined into twelve 1-mL aliquots and frozen at -80°C until analysis.

Immunoglobulin A

Samples obtained at baseline, h 12, and h 72 via NPF were analyzed using an equine specific ELISA (Immunology Consultants Laboratory, Inc. E70-A; Portland, OR) following the manufacturer's protocol at The University of Florida (Gainesville, FL).

Statistical Analyses

As stated above, effect of treatment (block) will be ignored in the results due to manufacturing errors. Still, treatment remained a fixed effect in the statistical model. Log transformed values for consumption data were analyzed in SAS (Version 9.4) using Proc MIXED in a linear mixed model with treatment as the fixed effect. Horse and treatment period were random effects. Denominator degrees of freedom adjusted using the DDFM= KR option. Pairwise tests for differences in least-squares means were conducted to compare the levels of significant fixed effects with no other adjustment for multiplicity. Statistical significance was defined as a p-value of $P < 0.05$, and $0.05 < P < 0.10$ was considered a tendency.

Log transformed values for each equine blood or nasal fluid parameter were analyzed in SAS (Version 9.4) using Proc MIXED in a linear mixed model. Treatment, sampling hour, and

interaction between treatment and sampling hour were fixed effects while horse and period were random effects. Sampling hour was used as a repeated measure and denominator degrees of freedom adjusted using the DDFM= KR option. Since experimental unit was horse within period, horse by period was the subject of the REPEATED statement. Covariance structure for the repeated measures was selected from among compound symmetry, heterogeneous compound symmetry and unstructured based on the Akaike information criterion. Pairwise tests for differences in least-squares means were conducted to compare the levels of significant fixed effects with no other adjustment for multiplicity. Statistical significance was defined as a p-value of $P < 0.05$, and $0.05 < P < 0.10$ was considered a tendency.

Results

As previously stated, treatment blocks were not iso-lipid (14.3% and 7.58% total fat for n-3 FA and n-6 blocks, respectively) and contained similar n-3 FA: n-6 FA as a result of a manufacturing error. Therefore, block effect will not be discussed when examining serum and NPF data. The following results and discussion, with exception to consumption data, will only analyze response to stress over time.

Horses gained an average of 0.22 kg/d throughout the trial which resulted in an average weight gain of 35.83 kg (SEM = 3.29 kg). There was no effect of treatment on body weight ($P > 0.05$).

Consumption of Blocks

There was no interaction between block and housing on block consumption events nor differences between blocks ($P > 0.89$; Table 2.5). Horses had more consumption events while stalled (39.45 meals/d; $P < 0.01$) compared to when group housed (13.59 meals/d). Horses consuming block A ate approximately 39.92 meals/d while stalled and 13.54 meals/d while

group housed. Horses consuming block B ate approximately 38.98 meals/d while in confinement and 13.64 meals/d while group housed. Horses in stalls tended ($P = 0.051$) to consume more of block A (601.66 g/d) compared to block B (450.52 g/d; Table 2.6). When group housed, horses consumed more ($P = 0.04$) of block B (321.28 g/d) than block A (301.63 g/d).

Blood Parameters

Hematology

The hematological parameters of WBC, RBC, HCT, platelet count, lymphocytes (LYM), neutrophils (NEU), monocytes (MON), and eosinophils (EOS) varied between sampling hours ($P < 0.02$; Table 2.7). Compared to baseline, WBC concentrations were elevated ($P < 0.03$) at all subsequent sampling hours. White blood cell concentrations at h 1 and h 6 were elevated ($P < 0.01$) compared to h 24, 48, 72 and 96. Baseline RBC and HCT concentrations were similar to all subsequent sampling hour concentrations ($P > 0.05$) but samples collected at h 6 were elevated compared to h 1, 24, 48, 72, and 96 ($P < 0.05$).

Platelet counts were elevated ($P < 0.01$) at h 1, 6, 24, 48, and 96 h compared to baseline but were lowest ($P < 0.05$) and similar to baseline at h 12 ($P > 0$ .05). Compared to baseline, lymphocytes decreased ($P < 0.01$) at h 1 but elevated ($P < 0.01$) at h 6, 24, 48, 72, and 96. Compared to h 1, whole blood lymphocytes were elevated ($P < 0.01$) at h 6, 24, 48, 72, and 96.

Neutrophils were elevated ($P < 0.01$) at h 1 and 6 compared to baseline but were similar to baseline at h 24, 48, 72, and 96. Monocytes were elevated ($P < 0.01$) compared to baseline at all subsequent sampling hours. Monocytes measured at h 1 were lesser ($P < 0.05$) than h 6, 24, 48, and 96. There was no impact on BAO ($P > 0.05$). Eosinophils were reduced ($P < 0.01$) at h 1 and 6 compared to baseline, similar to baseline at h 24 and 72, and greater ($P < 0.01$) than baseline at h 48 and 96.

Immunoglobulins

Immunoglobulin M and G were not different between sampling hours ($P > 0.05$; Table 2.8). Nasal mucosal IgA at h 12 tended to be elevated ($P < 0.07$; Table 2.9) compared to baseline but were similar ($P > 0.05$) to baseline at h 72. Compared to h 12, h 72 IgA was decreased ($P < 0.02$).

Cytokines, Eicosanoids, and Hormones

Approximately 75% of serum samples analyzed for IFN-α and IFN-γ were below the detectible limit of the assay and those parameters are not reported. There were no differences detected between sampling hours for serum IL-4, IL-10, IL-17, nor TNF-α ($P > 0.05$; Tables 2.10 and 2.11). Cortisol decreased ($P < 0.01$; Table 2.12) in serum at h 1 and 6 compared to baseline but was elevated above baseline by h 12 ($P < 0.01$). Compared to h 1 and h 6, serum cortisol was elevated ($P < 0.01$) at h 12, 24, and 48. Cortisol had a tendency ($P < 0.06$) to be less at h 24 compared to baseline and less ($P < 0.01$) at h 48 compared to baseline. Prostaglandin E_2 was similar between hours ($P > 0.05$; Table 2.13).

Discussion

While stress associated with a fight or flight response is essential for survival in the wild, situational stress, such as exposure to novel environments or a training regime, can potentially alter immune system functionality (Dhabhar, 2009). Altering immune activity can increase susceptibility to pathogens and disease (Calder, 2007) as decreases in immunological cell activity, such as T-helper cells, can impact activity of the acquired immune response (Padgett and Glaser, 2003). Therefore, the impact of stressors should continue to be investigated in order to further understand physiological changes that result. This can allow for development of strategies to improve immune function can enhance total body health status.

Hematological Parameters

Horses were exposed to 3 different stressors including a 1-hr trailer haul, 10-min canter, and 2-h elevated head tie. Therefore, hematological alterations were expected due to physiological changes that occur during acute stress, including increased respiration, increased fluid lost through sweat, and splenic contraction (Snow et al., 1983; Iversen et al., 1994).

During acute stress, WBC are released from the spleen and enter circulation. Once mobilized, they travel to tissues such as nasal and oral mucosa to combat pathogenic invasion. An increase in WBC due to leukocytosis is a common effect of exercise (Korhonen et al., 2000). The increase in WBC demonstrated at h 1 and h 6 post-stressor indicates that the stressors applied did increase the concentration of WBC in circulation. While detection of a return to baseline levels of WBC were not seen, this could have been due to distribution of sampling hours or unknown physiological effects.

Under stress, RBC and HCT (packed cell volume) increase as a result of splenic contraction. Additionally, stressors that prevent the *ad libitum* consumption of water, such as lengthy trailering, acute exercise, and standing tied result in an increase in RBC and HCT concentration in the blood (Smith et al., 1996). In the present study, measurements of RBC and HCT indicated that concentrations were similar to baseline but were different between samples collected post-stressor. Both RBC and HCT were most concentrated at 6 h post-stressor. An increase in HCT due to exercise agrees with findings from Monteverde et al. (2016) and Di Filippo et al. (2016) who measured HCT after horses were subjected to periods of stress and found elevated concentrations in circulation after the stressor had ended. Conversely, Smith et al. (1996) saw elevated post-stressor RBC and HCT concentrations compared to baseline, but

samples were collected from horses in that study after being hauled for 24 h. Therefore, the differences in stressor length between this study and the present study are quite different.

Neutrophils increase following acute inflammation and stress, such as with repeated exercise (Korhonen et al., 2000) because they migrate from endothelium of blood vessels and enter circulation (Rossdale et al., 1982). In the present study, the completion of the stressor period appears to have induced neutrophilia by h 1. While concentration increases, their phagocytotic capacity has been shown to be reduced in horses up to 36 h post-transport, which may increase the susceptibility of the body to disease (Raidial et al., 1997). Lymphocytes are also negatively impacted by stress (Padgett and Glaser, 2003). Results from the present study compliment this data whereby lymphocytes concentration was decreased below baseline 1 h post-stressor.

Immunoglobulins

In humans, a positive correlation between job stress and serum IgG and IgM has been noted (Nakata et al. 2000). Authors also reported decreased T and NK cells due to high-stress employment. However, B cell activity of horses in the current study was unaffected by stress.

Immunoglobulin A is the primary antibody found in nasal mucosa and the first line of defense against pathogenic invasion of mucous membranes (Pier et al., 2004). In the current study, mucosal IgA increased over baseline at h 12 post-stressor. In contrast, Bobel et al. (2016) found diminished mucosal IgA 12 h post-stressor. These discordant results are likely due to differences in the magnitude and length of stress that the animals were exposed to.

Cortisol

Cortisol is largely involved in a stress response due to its effect on glucose utilization as it shunts glucose away from muscles and towards the brain. Cortisol also increases the action of

catecholamines like epinephrine, and mitigates immune response by preventing overproduction anti-inflammatory molecules (Ganong, 1988). Cortisol secretion varies throughout the day, following a circadian rhythm with greatest concentrations in the morning followed by a subsequent decrease throughout the day (McIntosh et al., 1981).

Bobel et al. (2016) subjected horses to an elevated head tie for 12 h and then quantified serum cortisol after stress. Concentrations were elevated post-stressor and returned to baseline within 12 h following the elevated head tie. Similarly, Fazio et al. (2008) reported increased cortisol in horses subjected to trailer hauling between 100 to 300 km and argued that transportation could be considered psychological stress if less than 3 h in length. Cortisol was also elevated in blood sampled from horses immediately following a 24 h trailer haul (Smith et al., 1996). In the present study, serum cortisol concentrations were less than baseline at h 1 and 6 post-stressor, were greater than baseline at 12 h and declined below baseline after 24 h. Therefore, it appears that the magnitude of stress in the present study was responsible for discordant effects compared to other research. Also, the sampling times occurring in the evening at h 1, 6, 24, and 48, in the present study, could have impacted the accurate measurement of this serum parameter.

Prostaglandin E_2 and Cytokines

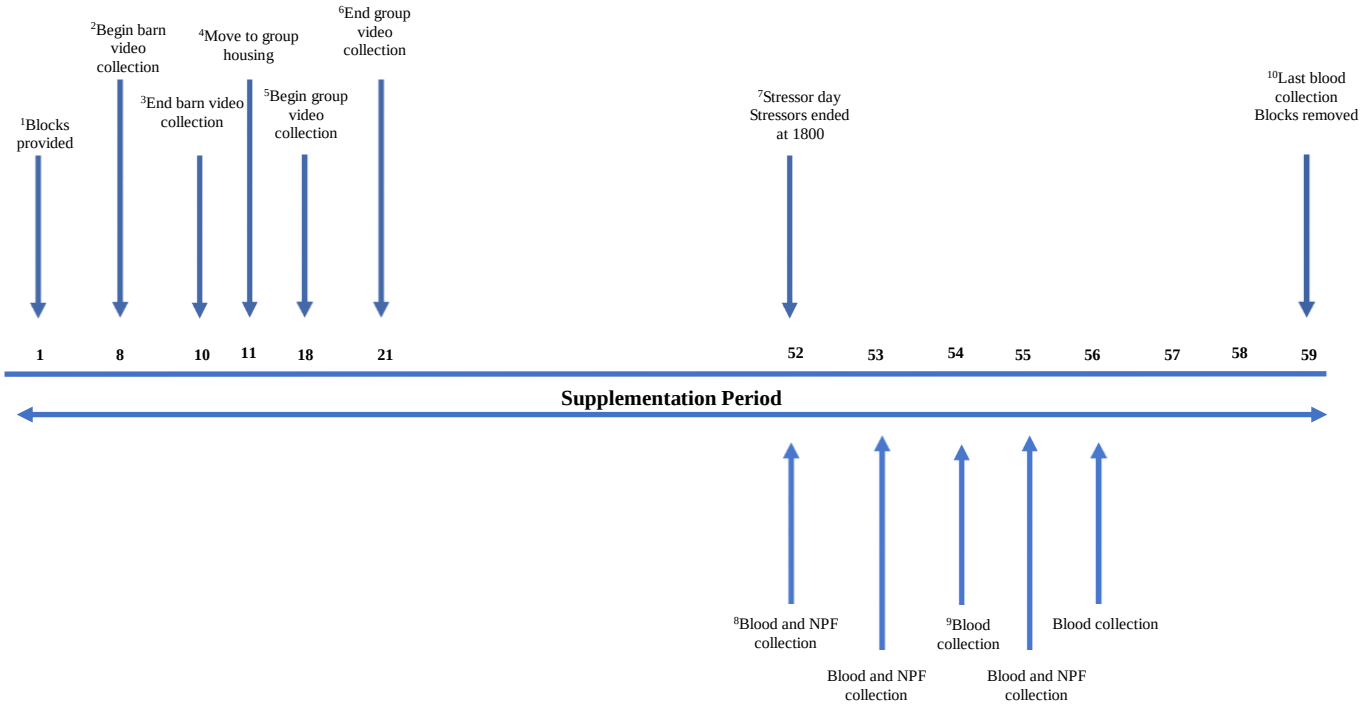
Activation of the stress response is regulated in part by prostaglandins. These lipid mediators are produced from cyclooxygenase and play a role in regulating the inflammatory response to an immune challenge (Elander, 2010). In the present study, PGE_2 concentrations were no different between sampling hours. Tumor necrosis factor-α is a useful marker of inflammation as it increases early in a homeostatic challenged state, such as disease (Barton and Collatos, 1999). Closely associated with fever and general signs of lethargy, TNF-α is secreted

by macrophages recruited to the site of infection (Morris et al., 1990). Exercise also can increase production of TNF-α, which remains elevated for at least 2 h following exercise (Donovan et al., 2007). There were no differences detected in serum TNF-α concentration. Interleukin-4 and IL-17 are generally considered to be pro-inflammatory cytokines, whereas IL-10 is generally considered anti-inflammatory. Himmerich et al. (2013) subjected rats to a forced swim or restraint and found that IL-4, IL-10 and TNF-α concentrations were all elevated compared to controls. In the present study, no differences between treatment or sampling hour were observed for IL-4, IL-10, and IL-17. The lack of measureable differences in PGE_2, TNF-α, and IL-4, IL-10, and IL-17 concentration in the current study may be attributed to improper sampling times or inadequate stress level.

Summary

Stress and the stress response are commonly characterized as being immunosuppressive but it is more appropriate to summarize the stress system as being immunomodulatory (Tsigos et al., 2016). The stress response in horses is an important consideration of equine management as some stress can be considered beneficial but experiencing prolonged stress can cause dysregulation of behavior and immune function. In the present study, while hematological parameters, such as WBC, NEU, and MON appeared to be altered due to application of stressors, stress susceptibility due to intra-animal variation may be a contributing factor to the lack of differences displayed between immunological parameters such as cytokines and PGE_2. It is possible that stressors were not great enough to elicit a corresponding physiological response or serum samples were collected at inappropriate time to detect differences in immunological parameters. In the future, magnitude and length of stressors should be altered in order to better identify the point at which immunological parameters are altered.

Figure 2.1 Experimental schematic during 59-d supplementation period



[1]Blocks provided to horses for *ad libitum* consumption while individually stalled
[2]Initiation of barn consumption data collection to quantify number of consumption events and length of consumption events while stalled
[3]Conclusion of barn consumption data collection to quantify number of consumption events and length of consumption events while stalled
[4]Horses were relocated from stalled housing to a drylot and allowed *ad libitum* consumption of blocks while group housed
[5]Initiation of barn consumption data collection to quantify number of consumption events and length of consumption events while group housed.
[6]Conclusion of barn consumption data collection to quantify number of consumption events and length of consumption events while group housed.
[7]Baseline samples were collected at 0900 h, stressors applied at 1500 h and ceased at 1800 h
[8]Collection of blood and nasopharyngeal flush samples (NPF)
[9]Collection of blood samples only
[10]Samples were collected at 168 h post-stressor and blocks were removed

Table 2.1 Fatty acid composition of molasses blocks[1] (AF Basis)

Fatty acid, g/100 g[2]	Block A, g/100 g	Block B, g/100 g
LA[3]	2.47	1.21
ALA[4]	6.98	3.58
EPA[5]	< 0.01	< 0.01
DHA[6]	< 0.01	< 0.01
Total n-3 FA[7]	6.99	3.58
Total n-6 FA	2.48	1.21
Total Fat	14.30	7.58
n-6:n-3	0.35	0.34

[1]Provided to yearling horses on an *ad libitum* basis
[2]Of sample
[3]Linoleic acid
[4]Alpha-linolenic acid
[5]Eicosapentanoic Acid
[6]Docohexanoic acid
[7]Fatty acid

Table 2.2 Variability in fatty acid composition of treatment blocks

	Block A[A], g/100g				Block B[B], g/100g			
Fatty acid, g/100 g[1]	**Min**	**Mean**	**Max**	**STD DEV**	**Min**	**Mean**	**Max**	**STD DEV**
Sum FA[2]	7.14	10.96	14.02	0.39	4.50	7.07	9.24	0.28
Sum n-3	3.50	6.29	8.23	0.29	0.91	1.65	3.31	0.18
Sum n-6	1.30	1.45	1.65	0.02	0.52	1.20	1.58	0.08
n-6:n-3	0.18	0.24	0.45	0.02	0.00	0.92	1.36	0.09

[A]Block contained 14.30% fat
[B]Block contained 7.58% fat
[1]Of sample
[2]Fatty acid

Table 2.3 Dietary nutrient analysis (DM basis)

Item	Bromegrass hay[1]	Concentrate[2]
Dry matter, %	87.70	86.27
Crude protein, %	9.46	15.4
Crude fat, %	1.48	5.40
ADF, %	38.6	9.0
NDF, %	65.2	8.0
DE, Mcal/lb	1.29	1.69
Calcium, %	0.76	1.04
Phosphorous, %	0.19	0.78
Magnesium, %	0.16	0.17
Potassium, %	1.92	0.97
LA[3], g/100 g	0.20	2.12
ALA[4], g/100 g	0.34	0.12
EPA[5], g/100 g	0.01	< 0.01
DHA[6], g/100 g	0.01	< 0.01
Total n-3 FA[7], g/100 g	0.34	0.12
Total n-6 FA, g/100 g	0.20	2.12

[1]Fed *ad libitum* to all horses
[2]Fed 0.5% of BW (as-fed) to all horses
[3]Linoleic acid
[4]Alpha-linoleic acid
[5]Eicosapentanoic Acid
[6]Docohexanoic acid
[7]Fatty acid

Table 2.4 Composition of concentrate

Ingredient	% of Concentrate[1]
Corn	30.00
Oats	40.10
Molasses	10.00
Soybean meal, 48%	12.69
L-Lysine	0.40
Limestone	1.30
Salt	0.50
Monocalcium phosphate	1.44
Vitamin A 30,000	0.03
Vitamin D 30,000	0.00
Vitamin E 20,000	0.50
Copper sulfate	0.02
Zinc oxide	0.02
Sodium selenite	0.08
DL-methonine	0.12

[1]Fed 0.5% of BW/d (as-fed) to all horses

Table 2.5 Effect of block, housing, and interaction between block and housing on consumption events for yearling horses

	Housing							
	Barn		Group			*P*-value		
Treatment*	Block A[A]	Block B[B]	Block A	Block B	SEM	Block	Housing	Block × Housing[2]
Consumption events/d[1]	39.92	38.98	13.54	13.64	1.141	0.95	< 0.01	0.90

*Treatments provided *ad libitum* to yearling horses
Means and SEM were log transformed, analyzed, and back-transformed for reporting
[1]Average number of separate times block was consumed per 24 h period calculated by individual horse
[2]Interaction between block and housing
[A]Block contained 14.30% fat
[B]Block contained 7.58% fat

Table 2.6 Effect of stall or group housing on *ad libitum* block intake by yearling horses

	Treatment*			
Housing	**Block 1[2]**	**Block 2[3]**	**SEM**	***P*-value**
Stalled, intake g/d	601.61[a]	450.52[b]	1.534	0.051
Group, intake g/d	301.63[c]	321.28[d]	1.123	0.04

*Treatments provided *ad libitum* to yearling horses
[1]All means and SEM were log transformed, analyzed, and back-transformed for reporting
[a]Block 1: Molasses block containing 14.30% fat
[b]Block 2: Molasses block containing 7.58% fat
[a,b]Denotes a tendency for a treatment difference ($P < 0.10$)
[c,d]Denotes a treatment difference ($P < 0.05$)

Table 2.7 Effect of sampling hour on whole blood characteristics of yearling horses before and after three types of stressors[1]

		Sampling hour							*P*-value
Item	**Baseline[ψ]**	**1**	**6**	**24**	**48**	**72**	**96**	**SEM**	**Hour**
WBC[2], K/µL[3]	9.3	11.8[a]	11.7[a]	10.0[b]	10.0[b]	9.9[b]	9.8[b]	1.06	< 0.01
RBC[4], M/µL[5]	8.46	8.28*[ac]	8.89*[b]	8.41*[adf]	8.51*[de]	8.50*[d]	8.28*[cef]	1.05	< 0.01
HCT[6], %	33.10	32.26*[a]	34.88*[b]	32.83*[acd]	33.29*[ce]	33.31*[c]	32.26*[ade]	1.05	< 0.01
Platelets, K/µL	134.02	159.30[ae]	165.31[ac]	164.15[ad]	160.08[a]	139.03*[b]	169.25[cde]	1.09	0.01
Lymphocytes, K/µL	4.84	4.46[a]	5.60[b]	5.44[b]	5.49[b]	5.41[b]	5.48[b]	1.09	< 0.01
Neutrophils, K/µL	3.68	6.63[a]	5.33[b]	3.69*[c]	3.66*[c]	3.68*[c]	3.54*[c]	1.08	< 0.01
Monocytes, K/µL	1.43	1.47[a]	1.55[b]	1.52[c]	1.52[c]	1.48[ad]	1.50[cd]	1.01	< 0.01
Basophils, K/µL	1.04	1.03	1.03	1.03	1.03	1.03	1.03	1.01	0.20
Eosinophils, K/µL	1.19	1.13[a]	1.15[a]	1.20*[b]	1.23[c]	1.20*[b]	1.23[c]	1.07	< 0.01

Means were log transformed, analyzed, and back-transformed for reporting
[abcdefg]Means with different superscripts within serum parameter are different ($P > 0.05$)
*Denotes that means are similar to baseline within a serum parameter ($P > 0.05$)
ψBaseline is defined as serum collected prior to application of stressors
[1]Stressors included 60-min trailer haul, 15-min forced exercise, and 2-h elevated head tie
[2]White blood cells
[3]Kilo per microliter
[4]Red blood cells
[5]Million per microliter
[6]Hematocrit

Table 2.8 Effect of sampling hour on serum antibodies of yearling horses before and after three types of stressors[1]

	Sampling hour			*P*-value
Antibody	**Baseline$^{\psi}$**	**168**	**SEM**	**Hour**
IgG, ng/mL	69.13^{a}	69.07^{b}	1.13	0.09
	Baseline$^{\psi}$	**96**		
IgM, mg/dL	56.87	58.65	1.11	0.49

Means were log transformed, analyzed, and back-transformed for reporting
^{a,b}Means with different superscripts tend to be different ($P < 0.10$)
ψBaseline is defined as serum collected prior to application of stressors
[1]Stressors included 60-min trailer haul, 15-min forced exercise, and 2-h elevated head tie

Table 2.9 Effect of sampling hour on antibody concentration in NPF[1] fluid of yearling horses before and after three types of stressors[2]

	Sampling hour				*P*-value
Antibody	**Baseline$^{\psi}$**	**12**	**72**	**SEM**	**Hour**
IgA, µg/mL	41.06	54.53^{*a}	37.43^{*b}	1.38	0.04

Means were log transformed, analyzed, and back-transformed for reporting
^{a,b}Means with different superscripts are different ($P > 0.05$)
*Denotes that means are similar to baseline within a serum parameter ($P > 0.05$)
ψBaseline is defined as serum collected prior to application of stressors
[1]Nasopharyngeal flush fluid
[2]Stressors included 60-min trailer haul, 15-min forced exercise, and 2-h elevated head tie

Table 2.10 Effect of sampling hour on serum interleukins of yearling horses before and after three types of stressors[1]

	Sampling hour									*P*-value
Cytokine	**Baseline$^{\psi}$**	**1**	**6**	**12**	**24**	**48**	**72**	**96**	**SEM**	**Hour**
IL-4, pg/mL	79.45	86.69	70.37	47.24	85.58	82.34	95.62	49.67	1.80	0.29
IL-10, pg/mL	7.40	10.11	8.41	7.12	7.64	8.04	10.94	8.57	1.94	0.75
IL-17, U/mL	25.33	27.26	24.14	24.24	21.63	24.45	25.19	21.68	1.37	0.12

Means were log transformed, analyzed, and back-transformed for reporting
To account for concentrations below detectable limit, all values were increased by 1 for log transformation
ψBaseline is defined as serum collected prior to application of stressors
[1]Stressors included 60-min trailer haul, 15-min forced exercise, and 2-h elevated head tie

Table 2.11 Effect of sampling hour on serum TNF-α of yearling horses before and after three types of stressors[1]

	Sampling hour				*P*-value
Cytokine	**Baseline[ψ]**	**1**	**6**	**SEM**	**Hour**
TNF-α, pg/mL	11.51	6.16	14.81	1.89	0.12

Means were log transformed, analyzed, and back-transformed for reporting
ψBaseline is defined as serum collected prior to application of stressors
[1]Stressors included 60-min trailer haul, 15-min forced exercise, and 2-h elevated head tie

Table 2.12 Effect of sampling hour on serum cortisol of yearling horses before and after three types of stressors[1]

	Sampling hour							*P*-value
Item	**Baseline[ψ]**	**1**	**6**	**12**	**24**	**48**	**SEM**	**Hour**
Cortisol, ng/mL	23.77	9.84[a]	4.05[b]	35.13[c]	19.00*[d]	13.94[e]	1.20	< 0.01

Means were log transformed, analyzed, and back-transformed for reporting
[abcd]Means with different superscripts within hour within serum parameter are different ($P > 0.05$)
*Denotes that means are similar to baseline within a serum parameter ($P > 0.05$)
ψBaseline is defined as serum collected prior to application of stressors
[1]Stressors included 60-min trailer haul, 15-min forced exercise, and 2-h elevated head tie

Table 2.13 Effect of sampling hour on serum prostaglandin E_2 of yearling horses before and after three types of stressors[1]

	Sampling hour				*P*-value
Item	**Baseline[ψ]**	**1**	**6**	**SEM**	**Hour**
PGE_2, pg/mL	26.40	27.04	21.97	1.2	0.24

Means were log transformed, analyzed, and back-transformed for reporting
ψBaseline is defined as serum collected prior to application of stressors
[1]Stressors included 60-min trailer haul, 15-min forced exercise, and 2-h elevated head tie

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Chapter 3 - A Review of the Literature-Silage

3.1 Introduction

Preservation of forage by ensiling is essential to many cattle feeding programs around the world. Because of its availability year round, ensiled crops allow producers to provide their livestock with nutritional feed regardless of harvest conditions, while also maximizing nutrient recovery of whole crops instead of specific portions, such as the grain (Aragon, 2012). The practice of ensiling is based on fermentation, in which bacteria degrade available sugars to organic acids, gases, and alcohols, thus preserving and improving nutritional value of forage. Optimization of this process can be accomplished by application of homofermentative and heterofermentative lactic acid bacteria (LAB) to the forage at harvest. These additives aid in rapid reduction of pH of the silage mass, inhibiting deleterious microbial activity and increasing nutritive value and stabilization of silage quality once exposed to air. Bacterial additives have been utilized for decades, but their effectiveness depends on proper management of variables involved in the ensiling process.

3.2 The Ensiling Process

Silage is the resulting product after fermentation of a high-moisture crop (McDonald et al., 1991). This practice is based on microbial fermentation of forage in an anaerobic environment, ultimately preserving the nutritional value of the crop. The four phases are: aerobic or packing phase, acidification or fermentative phase, storage phase, and feed-out phase.

Ensiling begins as the silo, bunker, or silage bag is packed with a high-moisture finely chopped forage. Oxygen is still present and many processes and microorganisms are active. Plant respiration degrades plant nutrients by degrading CHO through glycolysis and the Krebs cycle, yielding ATP, carbon dioxide, water, and heat (McDonald et al., 1991). Obligate and facultative

aerobic bacteria utilize available sugars for growth, and other microorganisms, like fungi, metabolize nutrients to produce alcohol, heat, and gas. Plant enzymes, such as proteases and carbohydrases, are also active, degrading plant material (Pahlow et al., 2003). These activities continue until the environment becomes anaerobic and the pH has dropped to a level inhibitory to enzymatic activity, generally < 6 (Oude Elferink et al., 1999).

Optimizing the compaction density of forage during the ensiling process is vital to nutrient retention, as dense packing eliminates residual oxygen. An anaerobic and acidic environment is usually reached within several hours of the silo being sealed, but it can be delayed by inadequate sealing and packing (Driehuis et al., 2001).

Once the environment becomes anaerobic, the activity of facultative and obligate anaerobic microorganisms increases. Water soluble carbohydrates (WSC) are used for primary fermentation by bacteria that ferment these sugars to organic acids. Secondary fermentation by other microorganisms, like fungi and enterobacteria, also uses WSC. Accumulation of organic acids during primary fermentation leads to decreased pH. This acidic environment inhibits secondary fermentation and ultimately preserves nutritive value of the forage. Fermentation of WSC continues and lactic acid accumulates until the pH decreases to 3.5 and 5.0 (Driehuis and Elferink, 2000). If pH decline is sluggish or added bacteria do not outcompete other microorganisms, deleterious microorganisms can proliferate. These undesirable microorganisms can result in spoiled forage and a reduction in feed quality. The fermentation phase can last anywhere from days to months, depending on harvest conditions, efficiency of ensiling, LAB concentration, and DM content of the forage (Driehuis and Elferink, 2000).

After microbial activity has been inhibited by a sufficiently low pH, the storage phase commences (Driehuis and Elferink, 2000; Arriola et al., 2011). Because of the acidity, the

growth and activity of harmful microorganisms, such as clostridia, bacilli, and yeast are inhibited (Ávila et al., 2009). Lactic acid bacteria (LAB) and acid tolerant enzymes are still active but at a greatly reduced rate. Lactic acid bacterial populations normally undergo at least a 3-log reduction due to acidity of their own end-products (Driehuis et al., 1999; Pahlow et al., 2003). This low-grade enzymatic activity is responsible for the continued decline in WSC concentrations as storage continues.

Once the silo is opened and oxygen is reintroduced, there is an increase in the activity of previously dormant microorganisms. Of these, acid-tolerant yeasts are the main initiators of aerobic spoilage (Moon, 1983; Driehuis et al., 1997) as they ferment preservation acids (i.e. lactic acid) to ethanol and carbon dioxide. This consumption of organic acids leads to increased pH, allowing increased activity of acid-sensitive microorganisms, which subsequently furthers spoilage. Though this is a natural process and occurs to some degree in all silage, reducing the activity of aerobic microorganisms as much as possible is essential (Driehuis and Elferink, 2000).

The time-frame required to generate high-quality silage depends on a multitude of factors, including initial crop DM, packing and sealing efficiency, and WSC concentration. While each variable is individually important, proper management of all factors potentiates production of high quality silage.

3.3 Factors Impacting Silage Fermentation

Moisture and Packing Density

Moisture content in forages harvested for silage is critical. While LAB require moisture to exert their preservative effects (McDonald et al., 1991) excess moisture predisposes silage to spoilage by detrimental microorganisms such as clostridia. Effluent losses may also increase,

causing managerial and nutritional consequences (Muck, 2010). Moisture content of silage is correlated with forage pH; crops with greater DM and elevated osmotic pressure tend to have greater pH (Woolford and Sawczyc, 1984). Drier silages (> 40% DM) have fewer active LAB, which delays pH decline and forage preservation (Weinberg and Muck, 1996). Drier silage is also subject to excessive heating (McDonald and Whittenbury, 1973). Silages with decreased DM are less likely to experience severe temperature fluctuations (Muck, 2004).

Packing density is heavily influenced by the crops DM at harvest and chop length. Drier silage is difficult to pack, leaving more residual oxygen and potentially prolonging the aerobic phase. Whole-plant corn harvested at optimum DM (35%) and chop length (19 mM) helps optimize bacterial fermentation (Aragon, 2012).

Water Soluble Carbohydrates

Readily hydrolyzable sugars in silage are classified as WSC and include monosaccharides, disaccharides, oligosaccharides, and fructans. During acidification, continuous competition occurs for use of these sugars between desirable and undesirable microorganims. Their utilization and subsequent fermentation into organic acids continues until the accumulation of end products inhibits microbial activity. Use of WSC by other microorganisms, such as yeasts, can result in spoilage, as their fermentative end products, like ethanol, reduce nutritive value of the forage, especially if produced in excess. A dramatic decline in WSC concentration is indicative of excessive plant respiration and secondary fermentation. Conversely, small decreases in WSC correlate with increased organic acids (i.e. lactic acid and acetic acid), suggesting that respiration and detrimental microbial activity was controlled, thus maintaining or improving nutritive value of the silage (Weinberg et al., 1993).

3.4 Secondary Fermentation

Interruption of 1 of the 4 ensiling phases results in reduced relative feed value. McDonald et al. (1991) noted that a prolonged aerobic phase, due to delay of sealing after chopping and packing results in reduced WSC concentrations in silage. This occurs due to secondary fermentation in which detrimental microorganisms utilize WSC or fermentative end products, which ultimately reduces silage quality by using dry matter and decreasing acid content, which allows for harmful microbial activity to increase. Microorganisms involved in secondary fermentation include clostridia, yeasts, molds, acetic acid bacteria, enterococci, and bacilli, and are all indicative of spoilage (Muck and Pitt, 1994).

Clostridia

While all bacteria require moisture to grow, detrimental microorganisms thrive in moist environments, such as crops low in DM. Clostridia are obligate anaerobes that utilize CHO to produce butyric acid and ferment amino acids to ammonia, thus increasing silage pH, degrading protein, and increasing DM losses (Danner et al., 2003; Muck, 2010). Clostridial fermentative activity also produces water and carbon dioxide, exacerbating damage to silage quality (Driehuis and Elferink, 2000).

Clostridia proliferate at pH above 4.0 and are stimulated by elevated storage temperatures, low WSC concentration, and crops that have elevated buffering capacities (McDonald et al., 1991). Crops with greater buffering capacities requires greater lactic acid accumulation to decrease pH to levels inhibitory to clostridia (McDonald et al., 1991). These characteristics make clostridia inhibition difficult in wet forages as crops with elevated moisture require greater acid concentrations to reduce pH (Weinberg and Muck, 1996). Crops like alfalfa

are more prone to clostridial damage when ensiled compared to corn because of its high buffering capacity and low concentration of WSC (McDonald et al., 1991).

Yeasts and Molds

Yeast species responsible for aerobic deterioration of silage are diverse and tolerant to various conditions (Muck, 2010). Concentrations of this eukaryotic microorganism within a silo can be as great as 1 x 10^7 cfu/g DM, with *Candida*, *Pichia*, and *Cryptococcus* being predominant (Jonsson and Pahlow, 1984). Yeasts are most active at a pH between 3.5 to 6.5, but some species can be active at a pH of 2 (McDonald et al., 1991). These microorganisms compete with bacteria for substrates and ferment sugar and lactic acid to carbon dioxide and water. This causes silage pH to increase, allowing activation of other microorganisms that are inhibited at a low pH (Driehuis et al., 2001). Evidence of yeast activity includes elevated pH and decreased lactic and acetic acid concentrations (Arriola et al., 2011).

Facultative anaerobic yeasts are active at a low pH when there are large concentrations of WSC available. These yeasts consume sugars like their aerobic counterparts but ferment them into ethanol, decreasing the DM yield of silage (Driehuis and Elferink, 2000). Due to their acid tolerant nature, these yeasts can remain active and continue to ferment WSC to ethanol even after the inhibition of LAB (Hafner et al., 2013). This yeast activity is generally limited, but it explains why small amounts of ethanol can be found in silage that has undergone adequate fermentation.

Molds are slow-growing organisms typically found in the late stages of aerobic spoilage and concentrations are minimal unless silos are ineffectively sealed. Molds are generally of minimal concern during silage production but mycotoxins produced by molds can be harmful to livestock consuming the feed (Muck, 2010). Potentially resulting in diminished rumen function

or disease, reducing mycotoxins in silage is important to maintaining the health of cattle consuming this forage (Storm et al., 2008).

Bacilli and Enterobacteria

Bacilli can be aerobic or anaerobic and are found during fermentation and feed-out phases of silage production. Bacilli ferment sugars and organic acids to lactate, acetate, butyrate, 2,3-butanediol, and ethanol. They are credited with producing silage that is "slimy" when active together with yeast and acetic acid bacteria at pH's > 4.5. Bacilli are responsible for a secondary heat wave in the feed-out phase, as CHO metabolism produces heat as an end product (McDonald et al., 1991).

Enterobacteria are facultative anaerobes found in silage due to contamination with soil or feces (Aragon, 2012). Enterobacteria are not usually of concern when silage is properly packed and pH $\leq$ 4.5 (Heron et al., 1993). But, in an aerobic environment, these bacteria compete for WSC and produce acetic acid, succinic acid, and 2,3-butanediol (Donald et al., 1995). Enterobacteria cause 2 problems: they utilize sugars and they produce gas in the form of nitrous oxide (Muck, 2010).

3.5 Bacterial Inoculants

Proper management of the 4 phases of ensiling is key to preventing secondary fermentation, thus bacterial additives are used to promote efficient. While these additives increase cost to the producer, the payoff can be worthwhile. Use of bacterial inoculants to enhance preservation, inhibit secondary fermentation, and improve relative feeding value of silage has been practiced for decades (Aksu et al., 2004).

Lactic Acid Bacteria

Several species of bacteria are used as silage inoculants, but LAB are predominant and consist of *Lactobacillus*, *Leukonostoc*, *Pediococcus*, *Lactocccus* and *Streptococcus* (Todar, 2008). Many LAB are homofermenters and produce lactic acid as a result of sugar fermentation. Conversely, some LAB are heterofermentative and ferment sugar to acetic acid, carbon dioxide, and ethanol, in addition to lactic acid (Todar, 2008). With a pKA of 3.86, lactic acid will donate a proton at a very acidic pH, allowing the silage mass to undergo a rapid decline in pH. Compared to other organic acids, like acetic acid that has a pKa of 4.75, lactic acid has potent acidifying properties and is generally considered to be the most important organic acid involved in silage production.

Epiphytic LAB are pre-existing bacteria present on the plant prior to harvest, while additive LAB are applied topically to the plant at harvest. Regardless of origin, the ability of LAB to consume available WSC and produce acidic end products such as lactic acid make them important to silage production. Epiphytic LAB are found in greater concentrations on damaged plants and Visser et al. (1986) speculated that these bacteria are part of the naturally occurring protective mechanisms. Epiphytic LAB comprise 1% of the normal epiphytic microflora and are not homogenously distributed throughout the crop mass (Aragon, 2012). Their concentration varies within species of plant, stage of maturity, and growing and harvesting environment (Pahlow et al., 2003). As a result, spontaneous but incomplete fermentation can occur and results in increased DM losses due to heating and spoilage by harmful epiphytic microflora. Therefore, reliance on natural silage preservation by epiphytic bacteria is not recommended.

Generally consisting of natural, isolated strains, LAB inoculants are added to forages before ensiling to outcompete resident epiphytic microflora for substrates and ultimately

dominate fermentation (Driehuis et al., 1999; Muck, 2010). McDonald et al. (1991) and Henderson (1993) reported that epiphytic microfloral populations can be overwhelmed if inoculants contain at least 1 x 10^5 to 10^6 cfu/g DM. At this concentration, pH declines more quickly and to a greater magnitude, there is a greater lactic acid to acetic acid ratio (L: A), and DM recovery improves.

All LAB behave in the same general manner but can be classified as being homolactic or heterolactic. These LAB are further categorized by fermentation patterns and end products.

Homolactic LAB

Bacteria that ferment a substrate to a single type of acid are classified as homolactic. These LAB ferment hexoses to lactic acid as the primary end product. These bacteria are most effective when applied to forage at a rate of 10^5 to 10^6 cfu/g DM (Weinberg and Muck, 1996) and have been used for decades to increase the nutritive value of forage, enhance pH decline, and prevent secondary fermentation (Muck, 2010).

Homolactic LAB can be further classified as obligately homofermentative or facultatively heterofermentative as some LAB exhibit different fermentative behavior under certain conditions. Obligate homofermentative LAB, such as *Pediococcus pentosaceus* and *Enterococcus faecalis* ferment hexoses only to lactic acid because they lack phosphoketolase, preventing them from fermenting pentoses (Muck, 2010). Utilizing the Embden-Meyerhof pathway (i.e. glycolysis), these organisms ferment 1 mole of glucose to 2 moles of lactic acid without the production of carbon dioxide (Kung et al., 2003).

Lactobacillus plantarum, a rod shaped, gram positive bacterium, is often used as an inoculant due to its vitality in diverse environments, especially its efficiency at decreasing forage pH even in aerobic conditions (Kung et al., 2003; Condon, 1987). While in environments with

plentiful glucose, this bacterium exhibits obligate homofermentative behavior but in low glucose environments, *L. plantarum* can display facultative heterofermentative properties, fermenting pentoses into lactic acid, carbon dioxide and acetic acid or ethanol via the pentose-phosphate pathway. This fermentation pathway is not as energetically efficient as carbon dioxide and ethanol are additionally produced (Holzer et al., 2003).

Unique in its aerotolerance, *L. plantarum* can utilize Mn to rid its cells of oxygen, thus reducing reactive oxygen species and allowing itself to thrive in aerobic environments, though its growth is greater in anaerobic environments (Archibald and Fridovich, 1981). Silages from crops treated with *L. plantarum* at harvest have less DM loss, decreased pH during fermentation and storage, and greater lactic acid concentrations (Ely et al., 1981).

Shortcomings of Homofermentative Lactic Acid Bacteria

Upon feeding, silage will be exposed to air. Thus, aerobic stability is of great concern to producers. Even in circumstances where silage is removed for feeding and bags are "re-sealed," the instance of air entering the silage mass and inducing a microbiological reaction is guaranteed. Aerobic instability is indicated by increased temperature and spoilage (Ashbell et al., 2002) while stability is quantified by length of time between exposure to the aerobic environment until silage heats to 2° C above ambient temperature (Ranjit and Kung, 2000). This heating is due to spoilage that occurs due to secondary fermentation when organic acids, mainly lactic acid, are fermented. This decreases the acidity within the silage bunk and increases pH (Courtin and Spoelstra, 1990). Spoilage is detrimental to silage quality and nutritional value (Basso et al., 2014).

Homofermentative LAB are common inoculants as they are affordable and promote efficient silage preservation through the storage phase, but silages inoculated with this bacterium

undergo rapid deterioration in the feed-out phase. This aerobic instability is predominately due to activity of aerobic yeasts that were inhibited within the acidic, anaerobic conditions of the silo and the lack of anti-mycotic organic acids in the silage mass to prevent the proliferation of these microorganisms (Driehuis et al., 2001). Lactic acid, while essential for rapid preservation of forage, is produced at the expense of other organic acids, especially those such as acetic acid, that have anti-mycotic properties. Therefore, elevated lactic acid concentrations compared to anti-fungal organic acids and large amounts of residual WSC predispose silage to aerobic instability, as both lactic acid and WSC are substrates of detrimental fungi (Weinberg et al., 1993). While *L. plantarum* effectively promotes silage fermentation, once exposed to air, silage inoculated with *L. plantarum* undergoes rapid deterioration. Therefore, sole inoculation with *L. plantarum* can have a detrimental impact on the aerobic stability of silage.

The efficacy of homofermentative LAB is variable and these bacterium perform better when applied to some crops compared to others. Oliveira et al. (2016) conducted a meta-analysis of the efficacy of homofermentative and facultative heterofermentative inoculants at improving fermentation, aerobic stability and silage and resulting dairy cattle performance. While inoculation with aforementioned bacteria improved the fermentation of grasses and legumes, fermentation of corn, sorghum, and sugarcane silage unaffected. Additionally, inoculation did not improve aerobic stability for any crop. Oliveira et al. (2016) postulated that lack of detectable differences in inoculated corn, sorghum, and sugarcane silages was likely due to the low innate buffering capacity, large WSC concentrations, and epiphytic bacterial populations present on these plant species. Indeed, grass and legume silages have greater buffering capacities, less epiphytic microflora, and decreased WSC concentrations.

Heterolactic LAB

Heterolactic LAB use is increasing due to its ability to promote aerobic stability, as it more effectively prevents aerobic yeast activity. Heterolactic LAB and homolactic LAB both convert glucose found in WSC to lactic acid; however, heterolactic LAB are classified as obligately heterofermentative because they ferment 1 mole of glucose into 1 mole of lactic acid, 1 mole of carbon dioxide, and 1 mole of ethanol or 1 mole of acetic acid via the pentose-phosphate pathway. While this fermentative pathway results in greater DM loss when compared to that of homolactic LAB, it is still insignificant to total silage production (Weinberg and Muck, 1996). Filya and Sucu (2007) inoculated wheat, sorghum, and corn silage with additives including *L. buchneri.* They observed that inoculation with *L. buchneri* slightly increased DM losses, but noted that the elevated lactic acid concentrations resulting from fermentation of these cereal grain silages generally have that leave them especially vulnerable to aerobic instability. Therefore, while slight DM losses do occur, *L. buchneri* is considered to provide a "protective" effect on silage mass, preventing aerobic instability (Filya and Sucu, 2007).

Acetic acid is lipophilic, or able to dissolve in lipids, which allows it to enter cellular membranes of fungi in its undissociated form. Once inside the cell, the acid disassociates, releasing protons, acidifying the cytoplasm, and killing the microorganism (Guldfeldt and Arneborg, 1998; Danner et al., 2003). The ability of acetic acid to inhibit microorganisms involved in secondary fermentation is dependent on the concentration of undissociated acids. Large concentrations of this organic acid, such as those found in an acidic silage environment, better inhibit harmful microorganisms (Muck, 2010). When acetic acid makes up 1.5 to 3% of the DM of silage, yeast activity is greatly decreased (Pahlow et al., 2003).

The most common heterofermentative LAB used today is *Lactobacillus buchneri* which is unique in that it can further metabolize 1 mole of lactic acid to 0.48 moles of acetic acid, 0.48 moles of 1, 2-propanediol, and 0.04 moles of ethanol in low glucose situations; though utilization rate is slower when glucose is fermented. This process generates carbon dioxide as a waste product (Driehuis et al., 1999) and is influenced by pH and temperature. Lactic acid degradation by *L. buchneri* is optimized at pH < 4.3 and at temperatures of 15 to 25° C (Oude Elferink et al., 2001).

Inoculation of silage with *L. buchneri* results in decreased yeast and mold counts when compared to un-inoculated silage (Ranjit and Kung, 2000). Improvement in aerobic stability was reported when corn was inoculated with *L. buchneri* and ensiled for 3 mo as demonstrated by decreased lactic acid concentration but increased acetic and propionic acid and 1-propanol concentration compared to initial organic acid and alcohol levels (Driehuis et al., 1999). Inoculation of corn silage with *L. plantarum* and *L. buchneri* or *L. buchneri* alone resulted in reduced yeast growth and improved pH stability compared to a control during an aerobic stability test (Weinberg et al., 2002). Weinberg et al. (2002) also reported increased acetic acid in wheat silages inoculated with *L. buchneri* compared to a control. Interestingly, mold was only present on wheat silages inoculated with *L. plantarum*, which had elevated lactic acid concentrations compared to other silages. Similarly, Filya and Sucu (2007) inoculated wheat, sorghum, and corn silages with a combination of *L. plantarum* and *L. buchneri* or *L. buchneri* alone and found that aerobic stability increased in all treatments as acetic acid concentrations increased and yeast counts decreased. The greatest lactic acid concentrations were found in silages inoculated with *L. plantarum*. Mari et al. (2009) applied *L. buchneri* to corn and reported increased 1,2-propanediol

concentrations compared to control silages and less yeast. As a result, aerobic stability improved when compared to control silages

The stabilizing effects of *L. buchneri* compared to *L. plantarum* inoculated silages or a control (no inoculum) were evaluated by Ranjit and Kung (2000). Compared to untreated silages, all inoculated silages displayed increased stability but silages inoculated with heterofermentative LAB at a rate of 1×10^6 cfu/g DM were more stable than control or homofermentative inoculated silages.

Silage quality can also be impacted by inoculation rate with *L. buchneri* as demonstrated by Ranjit and Kung (2000). They observed that increasing *L. buchneri* dosage from 1×10^5 to 1×10^6 cfu/g further improved aerobic stability. Driehuis et al. (1999) reported that an inoculation rate of 4×10^4 cfu/g DM to 2×10^5 cfu/g DM increased aerobic stability of whole crop corn silage. Taylor and Kung (2002) showed similar benefits when *L. buchneri* was used to inoculate high moisture corn, but they utilized a greater inoculation rate of 5×10^5 cfu/g.

Driehuis et al. (1999) reported increased propionic acid concentrations after corn silage was inoculated with *L. buchneri* and assumed that the 1,2-propanediol produced by fermentation of lactic acid served as an intermediary to the production of propionic acid. It was not until Krooneman et al. (2002) described the activity of *Lactobacillus diolivorans*, a heterolactic LAB, and its ability to ferment 1, 2-propanediol into 1-propanol and propionic acid that this mechanism was elucidated. *Lactobacillus diolivorans* appears to be closely associated with *L. buchneri* and its utilization of 1,2-propanediol allows this bacterium to proliferate and produce anti-mycotic acids, further decreasing proliferation of harmful microorganisms (Moon, 1983; Oude-Elferink et al., 1999). In situations where *L. diolivorans* is not a part of the inoculant but

propionic acid and 1-propanol are still present, it is assumed that *L. diolivorans* exists as a component of the epiphytic bacterial population.

Inoculation with mixtures of Homolactic and Heterolactic LAB

Lactobacillus buchneri exerts its effects on silage preservation at a slower rate than the homofermentative LAB *L. plantarum*. Because of this, sole inoculation of silage with *L. buchneri* does not induce the same rapid decrease in pH, thus increasing the time required to move silage from the aerobic and fermentation phases into the storage phase. Therefore, recent research has focused on developing silage inoculants that utilize both LAB species to promote an efficient fermentation process.

Demonstrated in grass (Driehuis et al., 2001) and corn silage (Huisden et al., 2009), inoculation with a combination of *L. plantarum* and *L. buchneri* increases speed of fermentation and aerobic stability by decreasing yeast and mold proliferation thus reducing subsequent DM losses through spoilage. When inoculated silage was subsequently fed to calves, DM intake, DM digestibility, average daily gain and rumen pH was elevated. It was thus apparent that this combination of LAB improved nutritive value of the forage, thus increasing animal productivity (Khuntia and Chaudhary, 2002).

The combination of *L. plantarum* and *L. buchneri* can be an effective inoculation strategy. There is an apparent symbiotic relationship between *L. plantarum* and *L. buchneri*. *Lactobacillus plantarum*, by rapid pH decline, decreases the competition between *L. buchneri* and other harmful microorganisms for WSC as substrates. This allows *L. buchneri* to efficiently utilize lactic acid for acetic acid production (Weinberg et al., 2002; Filya et al., 2003; Hu et al., 2009). Additive *L. diolivorans* also has the potential to further benefit the ensiling process if used

alongside *L. plantarum* and *L. buchneri*. Its ability to produce additional anti-mycotic end products would lend additional preservation benefits and increase aerobic stability.

Protein degradation is also reduced with application of combined LAB strains. When applied alone, *L. buchneri* inoculated silages have greater ammonia-N concentrations, indicative of protein degradation, compared to silages inoculated with a combination of *L. plantarum* and *L. buchneri* (Filya, 2003). While combinations of *L. buchneri* and other homofermentative LAB, such as *P. pentosaceus*, have been investigated, some results have been negative. Studies conducted by Adosogan (2004) and Kleinschmit and Kung (2006a) reported *P. pentosaceus* was ineffective or inconsistent at improving aerobic stability.

Many strains of microorganisms secrete antibacterial peptides, or bacteriocins, that inhibit other microorganisms by altering cellular membranes and interfering with membrane potential (Gollop et al., 2005). This property was displayed when *L. plantarum*-inoculated grass silage improved animal performance, regardless of the quality of the silage produced (Keady et al., 1994); however, feeding silage immediately after inoculation with LAB did not alter digestibility. Keady et al. (1994) postulated that crop fermentation may bestow benefits to the animal aside from improving nutritive value of silage. Yildirim et al. (2002) discussed the same bacteriocin producing ability in *L. buchneri*. Therefore, it appears that both facultative heterofermenters and obligate heterofermenters can potentially be utilized to alter feeding value of silage by conferring a probiotic effect (Gollop et al., 2005).

3.6 Ideal Silage

In the U.S., it is generally recommended that corn to be ensiled should be harvested at a DM content of approximately 35%. A 65% moisture level provides bacteria with a suitable environment for growth and potentiates the ability of bacterial inoculants to quickly dominate

fermentation processes. Ideally, epiphytic LAB of harvested crops would be less concentrated than bacteria in the silage inoculant. This would allow for a decline in pH due to efficient production of lactic acid by homofermentative LAB and acetic acid by heterofermentative LAB.

When both homofermentative and heterofermentative LAB are utilized together in silage inoculants, the ratio of lactic acid compared to acetic acid should be approximately 1 (Kung and Stokes, 2011). The addition of bacterial inoculants can promote a productive ensiling process, but failure to properly manage other variables has the potential to deregulate the entire process. Producers should ensure that silage is rapidly and efficiently packed, use bacterial inoculants that promote fermentation, maintain an anaerobic environment, and use of heterolactic LAB to maintain stability upon feed-out.

Summary

Silage production is a delicate practice. This multi-step process involves numerous variables; some that can be controlled by management and others that cannot. Therefore, optimum silage production schematics can vary between producers. Because of this variation, it is impossible to assign a one-size-fits-all silage production strategy, but careful management of these steps and application of bacterial inoculants can optimize the forage conservation process. Future research should investigate strategies to better overwhelm epiphytic bacteria or determine ways that epiphytic bacteria can be utilized for the betterment of the preservation process. While inoculation with appropriate bacteria increases the likelihood of success in many ensiling situations, it is not a fail-safe strategy.

Chapter 4 - Fermentation Characteristics and Aerobic Stability of Inoculated Corn Silages

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Abstract

An experiment was conducted to evaluate the efficacy of 3 different bacterial inoculants in altering fermentation characteristics and aerobic stability of whole-plant corn silage. Treatments consisted of *Lactobacillus plantarum* and *Lactobacillus buchneri* (LPB), a proprietary blend of *L. plantarum*, *L. buchneri*, and *Lactobacillus diolivorans* (LPBD), and *L. diolivorans* (LD). Inoculants were applied as a mist post-harvest and forage was individually packed into 56.8-L experimental silos. Silos were weighed on d 0, 7, 14, 21, 28, 39, 90, and 120 to measure respirative losses and sampled on d 0,14, 39, 90, and 120 to characterize fermentation parameters (DM, aNDF, ADF, ash, starch, and WSC) and for analysis of silage extract (VFA, lactate, alcohols). Interactions between treatment and sampling day were detected on d 39 and d 90 for both propionate and 1,2-propanediol concentrations ($P < 0.04$). Total VFA and acetate were elevated ($P = 0.01$) in LPBD silages compared to LD. Silages inoculated with LD had the greatest ($P < 0.01$) concentration of 1-propanol. When subjected to an aerobic stability test, lactate: acetate was elevated ($P < 0.02$) in CON silages compared to LPB and LPBD. While silages did undergo compositional changes, these alterations did not appear to increase aerobic stability. Utilizing ruminal fluid, an *in vitro* fermentation study was conducted with silage removed from sampling day barrels. There were differences between sampling days ($P < 0.05$) in IVDMD and gas production. IVDMD was elevated in cultures containing forages ensiled for 90 d ($P < 0.03$). The acetate: propionate ratio was elevated ($P = 0.01$) in cultures containing silage from d 14 and d 90 compared to silages sampled on d 39 ($P < 0.05$). Sampling day had a tendency ($P < 0.09$) to impact butyrate concentrations and butyrate was less in silage from d 90 compared to d 14 ($P < 0.03$). Differences between silage treatments were only detected for gas production ($P = 0.01$) and control silages produced the least amount of gas compared to other

treatments (P = 0.01). There were only small differences when silages were subjected to an aerobic stability test suggesting they underwent similar fermentation and spoilage patterns. This was likely due to a large population of epiphytic bacteria on the initial forage.

Introduction

Preserving whole-plant cereal grains allows for year-round feeding and improved relative feed value (RFV) of the crop. Use of inoculants in silage production, a common practice in dairy and beef production, can be a key strategy to further enhance nutritional value while reducing waste.

Silage inoculants generally consist of bacterium isolated from the crop species. Corn plant inoculants normally consist of lactic acid bacteria (LAB) isolated from the plant surface which is then purified and concentrated. Bacterial inoculants are generally effective at dominating the fermentation process while rapidly decreasing forage mass pH and maintaining or improving nutritional value (Muck, 2010). While bacterial inoculants promote an effective fermentation process, issues arise during feed-out in the form of spoilage when homofermentative LAB are the only species utilized (Wilkinson and Davies, 2013). Therefore, inclusion of other bacterial species in inoculants has expanded into the use of *Lactobacillus buchneri*, a heterofermentative LAB that produces organic acids that provide silage with added stability when exposed to air (Oude Elferink et al., 2001). Use of *L. diolivorans* is of particular interest as an inoculant due to its ability to utilize 1,2 propanediol produced from anaerobic fermentation of lactic acid by *L. buchneri* and produces propionic acid as well as 1-propanol (Krooneman et al., 2002). By providing an additional specie that produces anti-mycotic organic acid, application of *L. diolivorans* has the potential to increase the productivity of silage inoculants and lend additional stability to silage at feed-out. Therefore, the objective of this

experiment sought to evaluate the effects of *L. diolivorans* alone or as an addition to a *L. plantarum* and *L. buchneri* in an inoculant when added to whole-plant corn silage, ensiled for 14, 39, 90, or 120 d, and then subjected to an aerobic stability test.

Experiment 1

Materials and Methods

Experimental Design

Experiment 1 consisted of a split plot design with a 4 x 5 factorial treatment arrangement and silo as the experimental unit. Factors consisted of inoculant (negative control or 3 different test inoculants) and sampling day (day 0, 14, 39, 90, and 120). Each of the factorial combinations were prepared in quadruplicate.

Treatments and Silage Preparation

Whole-plant corn silage was harvested at approximately 35% DM and transported by truck to a concrete slab for preparation. Upon delivery, 500 lb aliquots of silage were topically inoculated by a misting spray gun. Treatments consisted of a control (water), *Lactobacillus plantarum* and *Lactobacillus buchneri* (LPB; Provita Supplements, Omagh, Ireland), *L. plantarum, L. buchneri, and Lactobacillus diolivorans* (LPBD; Provita Supplements, Omagh, Ireland), and *L. diolivorans* (LD; Provita Supplements, Omagh, Ireland). All inoculants were supplied in a limestone carrier. To prepare each inoculant, 2 g of inoculant powder was combined with 100 mL of reverse osmosis (RO) water and 22.7 mL of this inoculant mixture was combined with 300 mL of RO water to bring the total volume of inoculant and carrier liquid to 322.7 mL. Inoculant was added to each 500 lb batch of forage in a uniform manner while silage was constantly mixed. Silage was treated and mixed on tarps specific to their treatment to prevent cross contamination. Application dosage of inoculants LPB and LPBD was

approximately 5 x 10^5 CFU of bacteria/g of fresh forage. Inoculant LD provided approximately 3 x 10^5 CFU of bacteria/g of fresh forage.

Each batch of treated silage was transferred into sixteen, 56.8-L silos (Grief Inc., Delaware, OH) resulting in 45.4 kg of wet forage per silo. Silos were packed using a hydraulic press, covered with a plastic bag, and sealed with a lid containing a spring-loaded ring. Each lid was outfitted with a Bunsen type valve. Silos to be opened on d 120 were outfitted with a stainless-steel thermocouple probe (Pace Scientific, Inc., Model PT-960 Mooresville, NC) placed in the geometric center of the silo. Ambient temperature was recorded by placing a thermocouple probe in an empty experimental silo. Silos were stored in an open-air building covered by a roof.

Silage Composition

Silos were weighed on d 0, 7, 14, 21, 28, 39, 90, and 120 to quantify weight change due to respiration within the silos. On initial silage-packing day (d 0), samples of fresh corn forage were taken prior to each batch being divided into their respective barrels.

On d 0, 14, 39, 90, and 120, sixteen silos (4 silos from each treatment) were opened, contents were homogenized, and approximately 0.90 kg of homogenized silage was collected from each silo and placed into bags that sealed with a zipper and subsequently frozen (-20°C). Once in the lab, samples were transferred into 8.57 x 6.35 x 33.02 cm vacuum seal foil bags (Uline, S-18228SIL, Pleasant Prairie, WI), vacuum sealed and frozen (-20°C) for long-term storage. An additional 0.25 kg of silage was collected into a metal cup and pressed to obtain a liquid extract which was immediately analyzed for pH using a handheld pH meter (Thermo Scientific Orion 3-star portable pH meter, Waltham, MA). The liquid extract was transferred into two, 5-mL aliquots, one of which contained 1 mL of 25% (w/v) m-phosphoric acid. Samples were then frozen (-20°C) for later analysis.

Aerobic Stability

On d 14, 39, 90, and 120, an additional 5 kg of silage was removed from opened silos and placed, without packing, into an 18-L polyethylene bucket (The Home Depot, #05GLHD2, Cobb County, GA) outfitted with a foam insulator (Leaktite, #211306, Leominster, MA). A hole was cut into the top and bottom of the container to allow for oxygen exposure and air movement. A stainless-steel thermocouple probe (Pace Scientific, Inc., Model PT-960 Mooresville, NC) was placed through the top of each container and into the geometric center of the silage mass. Temperature readings were collected once per h for 14 d. Length of time until aerobic stability containers reached unstable temperatures were evaluated by finding a baseline temperature where all containers had similar temperatures and quantifying the length of time that it took for each barrel to have a temperature increase of more than 2°C above this baseline. A temperature increase above this point was considered outside the threshold of aerobically stable. Ambient temperature was collected by placing a thermocouple probe in a bucket as described above. Weights were recorded at the beginning of the aerobic stability test (d 0), after 7 d of the aerobic stability test, and after d 14 of the aerobic stability test. After 14 d of aerobic exposure, 1 kg of silage was collected from each container and placed into plastic zipper bags and frozen (-20°C). Once in the lab, samples were vacuum sealed as previously described and frozen (-20°C). Additionally, liquid extract was obtained as previously described when aerobic stability containers were opened.

Yeast and Mold Counts

On silo opening d 14, 39, 90, and 120, 50 g of silage was collected from each of the 16 silos opened and used for quantification of yeast and mold concentrations. Twenty-five grams of silage were combined with 230 mL of sterile, quarter strength lactated Ringer's solution (Oxoid

BR52: Unipath, Basingstoke, UK) into sterile 7.5 x 12" Whirlpak bags (Nasco, B01318, Fort Atkinson, WI) and homogenized using a Pulsifier II (Microbiology International, Frederick, MD) for 1 min. Resulting liquid was used to perform serial dilutions. Spread plating was conducted with a malt extract agar (Oxoid CM59, Thermo Fischer, Waltham, MA) acidified with 85% (w/w) lactic acid. Dilutions were plated between -1 and -8 log reductions in duplicate and plates were incubated at 32°C for 48 h. Plates containing between 15 and 300 colonies of yeast and mold were enumerated and counts were averaged between both plates.

Characterization of Fresh and Ensiled Forage

Analytical Procedures

Approximately 200 g of forage were weighed into plastic bags, left unsealed and placed into the drying chamber of a lyophilizer (SP Scientific, Warminster, PA; Genesis Model 35EL) programmed with a 200 mTorr vacuum, -20°C shelf temperature, and -80°C condenser chamber temperature. After 240 h, forages were removed, air equilibrated, weighed to determine DM, and ground through a 1-mm screen using a Wiley Mill (Thomas Scientific, Swedesboro, NJ; Model 4).

Ground, lyophilized forages were used to determine neutral detergent fiber using alpha amylase (aNDF; Goering and van Soest, 1970) and acid detergent fiber (ADF;Goering and van Soest, 1970) using the Ankom filter bag technique (Method 13; Ankom Technologies, Macedon, NY). Neutral detergent fiber and ADF analyses were sequentially conducted. Ash content was measured by placing approximately 1.0 g of ground, lyophilized forage into an aluminum pan and heating in a 450°C muffle oven for 8 h.

Ground, lyophilized forages were used to determine starch content of the forages as described by Saldana et al. (1989) and Richards et al. (1995) and water-soluble carbohydrate

content (WSC). Water-soluble carbohydrate analysis was performed by Rock River Labs (Watertown, WI) utilizing the procedures described in Doubois et al. (1956) and Hall (2013).

Fermentative End Products

Volatile Fatty Acids (VFA)

Acidified liquid extract collected after inoculation and after silo opening was transferred to microcentrifuge tubes and centrifuged for 15 min at 14,000 x g. Supernatant was removed and transferred to 2-mL glass chromatography vials. Volatile fatty acid concentrations were determined using an Agilent 7890A gas chromatograph (Agilent Technologies, Santa Clara, CA) equipped with a flame ionization detector and capillary column (Nukol 15m x 0.5mm x 0.5 µm film thickness; Supelco Analytical, Bellefonte, PA) using H as the carrier gas at a flow rate of 3.4 mL/min and a 10:1 split flow with a 1 µL split injection. Oven temperature began at 80°C and increased by 10°C/min to reach a final temperature of 200°C. Inlet and detector temperature were both maintained at 300°C. Volatile fatty acid concentrations were quantified by comparison to known standards (Volatile Fatty Acid Standard Mix; Sigma-Aldrich, St. Louis, MO).

Non-Volatile Organic Acids (nonVFA)

Lactate concentration was analyzed using acidified liquid extract following conversion to volatile methyl derivatives. Derivatization was carried out by combining 1 mL acidified extract with 0.4 mL of 50 % sulfuric acid and 2 mL methanol in 16 x 125 mm screw-topped culture tubes. Tubes were capped with Teflon-lined screw caps and incubated in a 55°C water bath for 30 min. Tubes were removed from the bath and 1 mL chloroform and 2 mL distilled water were added. Tubes were vortexed to homogenize contents, and centrifuged at 500 x g for 5 min. The bottom chloroform layer was removed and transferred to 2-mL glass chromatography vials. Quantification was carried out using an Agilent 7890A gas chromatograph (Agilent

Technologies, Santa Clara, CA) equipped with a flame ionization detector and capillary column (Nukol 15 m x 0.5 mm x 0.5 µm film thickness; Supelco Analytical, Bellefonte, PA) using hydrogen as the carrier gas at a flow rate of 4.7 mL/min and an 80:1 split flow with a 1 µL split injection. Oven temperature began at 50°C and increased by 15°C/min to reach a final temperature of 200ºC. Inlet and detector temperature were both maintained at 300° C. Lactate concentrations were quantified by comparison to known standards (Non-Volatile Fatty Acid Standard Mix; Sigma-Aldrich, St. Louis, MO).

Alcohols

Concentrations of ethanol, 1-propanol, and 1, 2-propanediol, and 2,3-butanediol were quantified. Non-acidified liquid extracts were transferred to 2 mL microcentrifuge tubes, spun at 15,000 x g for 15 min, and clear supernatant was removed and transferred to a second microcentrifuge tube and spun at 15,000 x g for 10 min. Clear supernatant was removed and placed into 2-mL glass chromatography vials. Quantification was carried out using a Hewlett Packard 5890 gas chromatograph equipped with a flame ionization detector and capillary column (Agilent DB-624 UI 30 m x 0.53 mm x 3.0 µm, Agilent Technologies, Santa Clara, CA). Helium was the carrier gas at a constant flow rate of 30 cm/s and a 1:10 split flow with a 1 µL split injection was used. Oven temperature began at 40° C for 5 min and increased to 260° C at a rate of 10 C/min before being held at 260° C for 3 min. Inlet temperature was maintained at 250° C and detector temperature was 300° C. Alcohol concentrations were quantified by comparison to individual standards purchased from Sigma-Aldrich (St. Louis, MO).

Statistical Analyses

Data were analyzed using proc GLIMMIX of SAS (Version 9.4) for comparisons of the variables of treatment, sampling day, aerobic stability day. The model consisted of fixed effects

of treatment, sampling day, and interaction between treatment and sampling day. The random effect was replicate. The experimental unit was silo. A $P < 0.05$ was considered to be significant and a tendency was noted if $0.05 < P < 0.10$. The Kenward-Rogers 2 correction was utilized for estimation of degrees of freedom. Differences between the least squares means were determined in SAS by the use of the PDiff option.

Results

Data presented compare sampling d 14, 39, and 90. Sampling d 120 occurred during December in Northeast Kansas, with an ambient high temperature of 4.5°C (40.2°F) and low of -7.39°C (18.7°F; Fig. 4.1). Because experimental silos were stored in an uninsulated building, forage stored in silos for 120 d were frozen to a depth of 13 cm and therefore excluded from analysis.

Characterization of Fresh Forage

All forage was harvested from the same field and sampled for analysis after treatments were applied (Table 4.1). These values served as a baseline to characterize initial crop variability. There were no detectable differences ($P > 0.05$) between fresh forage samples.

Characterization of Ensiled Forage-

Fiber Analyses

Dry matter was similar ($P = 0.42$; Table 4.2) between treatments but different between sampling days ($P < 0.01$). Silos opened on d 90 had elevated DM ($P < 0.01$; 37.83%) compared to d 14 (36.54%) and 39 (36.12%). Neutral detergent fiber was similar across treatments but different ($P < 0.01$) between sampling days with d 14 (36.98%) and 39 (36.49%) having greater ($P < 0.01$) aNDF compared to d 90 (32.41 %). Similarly, ADF ($P < 0.01$) was similar across treatments on d 14 (19.62%) and d 39 (19.53%) compared to d 90 (17.18%). Ash content varied

between treatments ($P = 0.05$) and sampling days ($P = 0.049$). Control silages had reduced ($P = 0.01$; 4.11%) ash compared to LPBD (4.34%) and LPB (4.31%) silages and LPBD silages had elevated ($P = 0.04$) ash compared to LD (4.19%).

Starch content was different between sampling days ($P = 0.04$). Starch content on d 90 was greater ($P = 0.04$; 38.95%) than d 14 (36.20%) and d 39 (35.83%). There were no differences detected ($P = 0.33$) in WSC content between treatments or sampling days.

Composition of Liquid Extracted from Silage

Silage pH was greatest across all treatments on d 14 (3.59; $P < 0.01$; Table 4.2) and lowest on d 39 (3.52; $P < 0.01$). Total VFA was elevated ($P < 0.04$) in LPB silages (116.87 mM) compared to LD (107.67 mM). Silages inoculated with LPBD (123.19 mM) had greater ($P < 0.02$) total VFA compared to CON (111.21 mM) and LD (107.67 mM). Silages sampled on d 14 (105.53 mM) had less ($P < 0.01$) total VFA compared to d 39 (117.57 mM) and d 90 (121.11 mM). Acetate differed between treatments ($P < 0.01$) and sampling days ($P < 0.01$). Silage inoculated with LPB and LPBD had greater ($P < 0.01$) acetate (114.20 and 119.92 mM, respectively) than that treated with LD (104.79 mM). Silage treated with LPBD had greater ($P < 0.01$) acetate when compared to CON (107.68 mM). All silages on d 14 had less ($P < 0.01$; 101.60 mM) acetate than on d 39 (114.09 mM) and d 90 (119.25 mM). Propionate was elevated ($P < 0.02$) in LPBD (0.73 mM) compared to CON (< 0.01 mM), LPB (< 0.01 mM), and LD (0.43 mM) on d 39 (Table 4.2). On d 90, LPBD had greater ($P < 0.04$; 1.25 mM) propionate than those treated with CON (0.12 mM), LPB (0.23 mM), and LD (0.59 mM). Averaged across sampling days, propionate was elevated ($P < 0.03$) in silages inoculated with LPBD (0.67 mM) compared to LPB silages (0.08 mM) and CON silages (0.21 mM). On d 90, propionate was elevated ($P < 0.02$; 0.55 mM) compared to d 14 (0.13 mM). Butyrate concentration was similar

between treatments and sampling days ($P > 0.05$). Lactate and lactate: acetate were greater ($P < 0.04$; $P < 0.01$) on d 14 (122.33 mM; 1.22) compared to d 39 (108.43 mM; 0.96) and d 90 (107.63 mM:0.92) respectively.

Silages sampled on d 14 (139.08 mM) had elevated ($P < 0.01$) ethanol compared to d 39 (80.36 mM) and d 90 (76.57 mM). Silages inoculated with LPBD had elevated (0.98 mM: $P < 0.02$) 1-propanol concentrations compared to LPB (0.11 mM) silages. Silages inoculated with LD (1.89 mM) had elevated ($P < 0.02$) 1-propanol compared to LPB (0.11 mM), CON (0.28 mM), and LPBD (0.98 mM). There was a treatment × sampling day interaction for 1,2-propanediol. On sampling d 39, LPB and LPBD (23.41 mM and 19.67 mM respectively) silages had elevated ($P < 0.01$) 1,2-propanediol compared to CON (5.63 mM) and LD (3.35 mM). Forages inoculated with LPB (38.11 mM) and LPBD (37.73 mM) had greater ($P < 0.01$) 1,2-propandiol compared to CON (14.71 mM) or LD (7.45 mM) on d 90. Control (7.61 mM) and LD (3.87 mM) contained less ($P < 0.01$) 1,2-propanediol than LPBD (20.23 mM) and LPB (21.72 mM) across treatments. Concentration of 1,2-propanediol was elevated ($P < 0.01$) on d 90 (24.50 mM;($P < 0.01$) compared to d 39 (13.02 mM) and d 14 (2.55 mM). Silages sampled on d 39 had greater ($P < 0.01$) 1,2-propanediol compared to d 14. Concentrations of 2,3-butanediol varied between sampling days only when averaged across treatments ($P < 0.01$) and was most elevated on sampling d 90.

Microbiological Analyses

Growth of yeast and molds was inconsistent within and between sampling days. While some silos had plentiful growth, there was little to no growth detected in other replications ($P > 0.05$).

Respiration Losses

Weight retention in individual silos tended to be impacted by treatment ($P = 0.053$) and sampling day ($P < 0.01$; Table 4.3). Control silos (99.52%) tended ($P = 0.09$) to retain more weight across sampling days than LPB (99.42%) treated silos and retained more weight ($P = 0.02$) than LPBD inoculated silages (99.37%). Silos inoculated with LD (99.50%) had greater ($P < 0.03$) weight retention than LPBD silos (99.37%).

Silos lost weight after the initial measurement on d 7 as silos were lighter ($P < 0.01$) on d 21, 28, 39, 90, and 120 with d 14 values intermediate ($P = 0.07$) to those on d 7. Day 14 had greater ($P < 0.01$) weight retention than d 28, 39, 90, and 120 but were intermediate ($P = 0.12$) to weights recorded on d 21. Silos weighed on day 21 had greater ($P < 0.01$) weight retention than silos weighed on d 28, 39, 90, and 120. Silos weighed on d 28 had elevated ($P = 0.03$) weight retention compared to silos weighed on d 90 but were similar ($P = 0.57$) to silos weighed on d 39. Silos weighed on d 39 had a tendency ($P = 0.09$) to be heavier than those weighed after 90 d of ensiling.

Aerobic Stability

All results described below will be after exposure to air for 14 d. Silage pH was elevated ($P < 0.01$; Table 4.4) in silos sealed for 90 d (5.46) across treatments compared to silage ensiled for 14 d (4.90). Total VFA were greater ($P < 0.01$; 80.29 mM) in forages ensiled for 14 d compared to forages ensiled for 39 (22.83 mM) and 90 d (17.77 mM). Acetate and propionate were elevated ($P < 0.01$) in silages ensiled for 14 d (53.30 mM; 20.03 mM) compared to forage ensiled for 39 (10.58 mM; 3.09 mM) and 90 d (9.36 mM; 2.10 mM) respectively. Butyrate was elevated ($P < 0.04$) in forage ensiled for 39 d (7.08 mM) compared to forages ensiled for 14 d (3.92 mM) and 90 d (4.32 mM). Butyrate tended ($P = 0.053$) to be different between treatments.

Control silages had elevated ($P < 0.05$; 5.46 mM) butyrate compared to LPB silages (3.21 mM). LPBD silages had elevated ($P < 0.03$; 5.66 mM) butyrate compared to LPB silages. LD silages had elevated ($P < 0.05$; 6.10 mM) butyrate compared to LPB silages.

Lactate was similar between treatments and sampling days ($P = 0.19$) but lactate: acetate ratios differed between treatments ($P = 0.02$) and sampling days ($P < 0.01$). Control silages had elevated ($P < 0.04$) lactate: acetate (1.04) compared to LPB (0.56) and LPBD (0.72) silages and LD silages tended ($P < 0.06$) to have greater lactate: acetate (0.83) compared to LPB (0.56). Compared to d 39 (1.15) and d 90 (0.99), d 14 (0.21) had the lowest ($P < 0.01$) lactate: acetate ratio.

Ethanol was similar between treatments and sampling days ($P = 0.33$), but both 1-propanol and 1,2-propanediol varied between sampling days ($P < 0.01$). Concentrations of 1-propanol and 1,2-propanediol were both elevated ($P < 0.02$) in silages that had been ensiled for 14 d (0.83 mM; 0.43 mM) compared those ensiled for 39 d (0.28 mM; 0.10) and 90 d (0.06 mM; 0.14 mM).

An interaction between treatment, sampling day, and aerobic stability day was detected ($P < 0.01$) for 1,2-propanediol. Silages inoculated with LPBD and LPB had elevated ($P < 0.01$) 1,2-propanediol compared to CON and LD silages after forages were ensiled for 39 d and before exposure to the aerobic stability test but there were no differences when the aerobic stability days were considered. The same effect was displayed in silages ensiled for 90 d ($P < 0.01$). Concentrations of 2,3-butanediol were only different between sampling days ($P < 0.01$) and were elevated in forages ensiled for 14 d and then subjected to the aerobic stability test. The interaction between treatment, sampling day, and aerobic stability day was also analyzed. Concentrations of 1,2-propanediol were the only differences detected when this three-way

interaction was evaluated but because the differences were explained by the statistical differences displayed when the treatment and sampling day interaction was evaluated, these data are not reported.

Weight retention in aerobic stability containers were similar ($P > 0.05$; Table 4.5) when the interactions between treatment and sampling day, treatment and aerobic stability day, and treatment, sampling day, and aerobic stability day were evaluated. Temperature during aerobic stability tests was only impacted by hour ($P < 0.01$; Fig. 4.2, 4.3, 4.4). When a baseline temperature was considered for aerobic stability containers (d 14: 24.78°C, d 39: 21.08°C, and d 90:15.28°C) and the length of time until containers exceeded 2°C above this baseline temperature, there were no differences detected ($P > 0.05$; Table 4.6, 4.7, 4.8) in length of time to reach an unstable temperature between treatments.

Experiment 2

Materials and Methods

Silage removed from silos on sampling days 0, 14, 39, and 120 and frozen in vacuum-sealed foil bags were utilized to evaluate *in vitro* fermentation characteristics by mixed ruminal microorganisms. Twenty-five g of thawed silage (9 g DM) were placed in 500-mL Pyrex screw-topped bottles containing 325 mL of artificial saliva (McDougall's buffer) and 25 mL of strained ruminal fluid from donor steers consuming silage. Bottles were assessed for pH, de-gassed with N, and capped with an Ankom pressure monitoring module and placed in a shaking incubator (New Brunswick Scientific; Model G25; New Brunswick, NJ) at 39ºC for 48 h. Pressure readings were taken every 15 min to quantify production of fermentation gases by ruminal microbes. At completion of the fermentation period, terminal pH was recorded, and 4-mL of liquid were removed and combined in a 4:1 ratio with meta-phosphoric acid. Samples were

frozen and later analyzed for VFA in the laboratory as previously described. Contents were emptied into individual metal pans and dried at 105 ºC for 48 h and used to calculate *in vitro* dry matter disappearance (IVDMD).

Results

Less gas was produced when ruminal microorganisms were fed control silages ($P < 0.01$; Fig. 4.5) compared to all other treatments. Silages inoculated with LPBD (5,073 mL) and LD (5,197 mL) produced more ($P < 0.01$) gas than LPB (4,977 mL), and LD silages produced more ($P < 0.01$) gas than LPBD. Cultures had similar gas production from h 1 through h 10 ($P > 0.05$) but were different ($P < 0.02$) from h 11 through the end of the 48-h incubation period. There was an interaction between sampling day and treatment ($P < 0.01$) and sampling day and time ($P < 0.01$), but no interaction was found between sampling day, treatment, and time ($P = 1.0$).

There were no differences ($P > 0.05$) detected between treatments for pH, total VFA, acetate, and propionate (Table 4.9). *In vitro* dry matter disappearance (IVDMD) was impacted by sampling day ($P < 0.01$) and was greatest ($P < 0.02$) in cultures containing silage sampled on d 90. Butyrate was elevated in silages from sampling day 14 compared to d 90 ($P < 0.03$). Concentration of butyrate tended to be impacted by treatment ($P = 0.09$) with CON silages producing greater butyrate compared to LD ($P < 0.04$). Acetate: propionate ratio was elevated in cultures that contained silage from opening d 14 compared to d 39 ($P < 0.05$) and in cultures containing silage from d 90 compared to d 39 ($P < 0.01$).

Discussion

Because inadequate fermentation and instability results in spoilage, nutrient loss, and reduced profitability, bacterial inoculants are commonly utilized to combat these issues. Easy to apply and non-hazardous, inoculants commonly consist of a single homofermentative bacteria

such as *L. plantarum* or a combination containing heterofermentative bacteria, like *L. buchneri*. This combination results in a rapid pH decline due to lactic acid production from fermentation of WSC. Additionally, spoilage at feed-out is reduced due to increased acetic and propionic acid from fermentation of WSC by heterofermentative *L. buchneri*. Recently, the ability of *Lactobacillus diolivorans*, a heterofermentative LAB, to utilize 1,2-propanediol, an end product of *L. buchneri* fermentation of LA has been elucidated. This discovery has roused the possibility that this LAB could further increase aerobic stability as it ferments this end product to anti-mycotic 1-propanol and propionate (Krooneman et al., 2002).

In the current study, similarities between treatment and sampling day may be due to epiphytic bacteria on plant surfaces prior to harvest which dominated the fermentation process. Rooke and Hatfield (2003) discussed that uninoculated silages should undergo homolactic fermentation, declining pH and increased lactic acid concentrations compared to acetic acid. Treatment differences were detected between silages when ensiled forages were evaluated for several parameters, such as acetate, propionate, and total VFA, indicating that silage composition did change based bacterial inoculants utilized but these differences were not profound. Similar compositional changes suggest that silages underwent similar fermentation patterns regardless of inoculation, especially indicated by the lack of differences in lactate concentration and the lactate: acetate ratio. Arriola et al. (2011) observed similar results and confirmed the presence of epiphytic bacteria by PCR analysis whereby all silages, even silages that were not inoculated with obligate heterofermentative bacteria contained *L. buchneri*. While all silages contained similar quantities of lactic acid, acetic acid and total VFA were elevated in silages inoculated with the combination of *L. plantarum, L. buchneri, and L. diolivorans* compared to CON and LD silages. Elevated acetic and total VFA concentrations are indicative of heterolactic fermentation

but were similar to the composition of LPB silages. Reich and Kung (2010) found that a combination of *L. buchneri* and *L. plantarum* increased acetic acid in corn silage and silages inoculated with these same bacteria behaved similarly in the current study. Filya and Sucu (2007) also observed similar results; wheat, sorghum, and maize silages contained greater acetic acid when inoculated with a combination of *L. plantarum* and *L. buchneri* compared to a control.

In the present study, propionic acid was greatest in silages inoculated with the 3-bacterium mixture on d 39 compared to CON and LPB but were similar to LD inoculated silages. This suggests that *L. diolivorans* alone was as effective as LPBD at producing propionate after 39 d but after 90 d, the 3-bacterium mixture was most effective at generating propionate.

Lactobacillus buchneri exerts its beneficial effects at a slower rate than *L. plantarum* (Oude-Elferink et al., 2001; Kleinschmidt and Kung, 2006a), which limits its use to aerobic stabilization rather initiation of fermentation. Schmidt et al. (2009) demonstrated this when proliferation of *L. buchneri* in inoculated alfalfa silage was not evident until d 45, when an increase in acetic acid and 1,2-propanediol was detected. Corresponding results were found in the current study where *L. diolivorans* behaved similarly to *L. buchneri*, as propionate concentration was elevated on d 39 in LD silages.

Maintenance of aerobic stability is arguably the one of the most important functions of silage inoculants. If silage is well-preserved but spoils rapidly when exposed to oxygen, feeding value decreases, along with potential for profitability due to lactate-assimilating yeast activity (Woolford et al., 1990). The first indication of aerobic instability is an increase in temperature due to water and carbon dioxide production from the metabolism of organic acids and WSC (Phalow et al., 2003). This temperature fluctuation is quantified by comparison to ambient temperature. In the present study, ambient temperatures were collected utilizing an empty

container and therefore were not reflective of actual ambient temperatures of the silage masses, preventing meaningful comparison of temperature changes.

Other indications of instability upon exposure to oxygen are increased pH, reduced weight retention, and changes in organic acid concentrations. In the current study, LPBD silages produced elevated concentrations of acetate compared to CON and LD silages and LPBD and LPB produced greater propionic acid suggesting that aerobic stability should have been enhanced. However, there were no differences in pH, temperature, and weight change during the aerobic stability test. Also, yeast and mold counts were analogous between treatments throughout the trial. Similarly, Weinberg et al. (2002) observed that control corn silages contained greater lactic acid:acetic acid compared to silages inoculated with *L. plantarum* and *L. buchneri,* indicative of homolactic fermentation. However, they did not observe differences in yeast and mold concentrations, suggesting that aerobic exposure similarly impacted silage stability retention. Silva et al. (2017) found that *L. buchneri* had no effect on acetic acid or yeast counts when applied to corn silage at a rate of 1×10^5 cfu/g DM. Authors postulated that their inoculation rate may have been too low to overwhelm native bacteria which may help explanation of results in the present study. When subjected to an aerobic stability test, LPB and LPBD silages were not different from each other or CON or LD. Additionally, the lack of interaction between treatments, sampling days, and aerobic stability days shows that certain parameters were variable when treatments, sampling days, and aerobic stability days were considered but silages were similar when all main effects were evaluated as part of the treatment by sampling day by aerobic stability day three-way interaction.

There is also the possibility that other bacteria may have been a contributing factor. Weiss et al. (2016) suggested that decreased aerobic stability along with reduced yeast counts

may be due to the activity of acetic acid bacteria, which convert ethanol to acetic acid (Spoelstra et al., 1988). This bacterium was not quantified in the present study, but the lack of differences between ethanol and acetate concentrations when the interaction between treatment and sampling days were considered could indicate that this bacterium was present and active in all silages.

Inoculation did not affect aNDF content of corn silages compared to CON which agrees with findings from Reich and Kung (2010) who used *L. plantarum* and *L. buchneri* as corn plant inoculants and observed similar aNDF in inoculated and uninoculated silages. In the present study, ADF tended to be less in CON than all other silages. This contradicts Reich and Kung (2010) who reported no differences in ADF between silages inoculated with a combination of *L. buchneri* and *L. plantarum* or *L. buchneri* in combination with 2 different homofermentative inoculants compared to control. Additionally, differences in WSC were not detected in the present study but were reduced when Reich and Kung (2010) inoculated silages with *L. buchneri + L. plantarum* and compared to a control.

When *L. plantarum* is utilized, DM losses are expected to improve (Da Silva et al., 2014), but when combined with *L. buchneri*, losses increase slightly due to the carbon dioxide production as a result of *L. buchneri* activity. Fortunately, these losses are normally inconsequential compared to losses associated with uninoculated silage (Weinberg and Muck, 1996). In the current study, DM was unaffected by treatments and it is therefore likely that epiphytic bacterial activity altered the fermentation process or *L. buchneri* inoculation rate was not enough to alter DM retention.

Bacteria utilized in inoculants in the present study were likely overwhelmed by epiphytic bacterial on the forage or not viable, as the majority of parameters measured were only different between sampling days. This could be attributed to the inability of the inoculant bacteria to

dominate the fermentation process due to insufficient inoculation rates or un-viable bacteria. This may have also been due to delays between chopping, packing, and ensiling, thus allowing native bacteria to dictate fermentation. Woolford and Pahlow (1997) discussed the "chopper inoculation phenomenon," in which epiphytic LAB population increase on surface of crops by up to 100-fold after chopping. Greater epiphytic concentrations are also found on areas of crops that had been damaged (Visser et al. 1986). Therefore, limitations of this study included the lack of bacteria, yeast, and mold enumeration of the fresh forage following field harvest. It is possible that PCR analysis of fresh forage could have confirmed the assumption that epiphytic microflora was plentiful at initiation of the study and could explain the lack of profound, consistent effects.

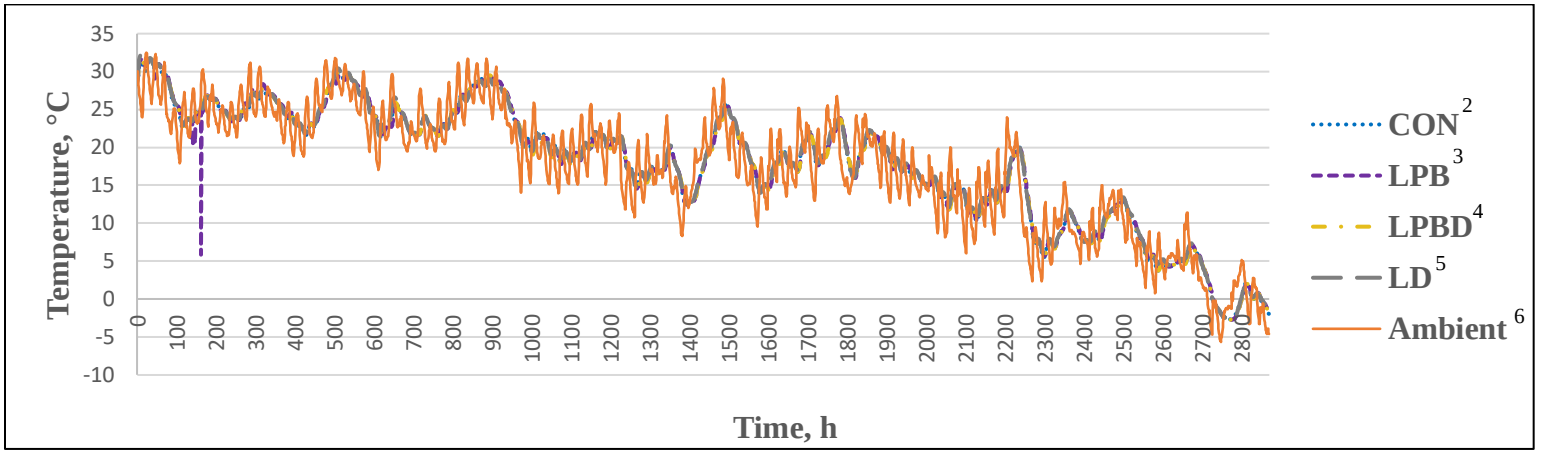
In experiment 2 when silages were incubated with rumen fluid, all treatments yielded similar pH, VFA and IVDMD, but gas production was different between treatments. Control silages produced the least gas, indicating that they underwent less extensive fermentation by mixed ruminal microbes. These findings further support the conclusion that *L. diolivorans*, *L. plantarum*, and *L. buchneri*, either in isolation or mixture, did not improve the relative feed value of corn silage at the inoculation rates utilized in this experiment.

Summary

Bacterial inoculation is a common method utilized to improve the relative feed value of silage. While silage production is a delicate process, use of these additives potentiates the success of the practice. While bacterial additives were utilized in the current study, there were not large differences detected between characteristics measured as indicated by the lack of interaction between treatments and sampling days. This suggests that other factors likely dominated the trial, such as a large epiphytic microflora population on fresh forage. This conclusion is further supported by the lack of differences when silages were subjected to an

aerobic stability test and confirms that all silages likely underwent similar fermentation and spoilage patterns. Based on these data and previous experiments reporting similar results, it appears that native microflora populations should be examined. While epiphytic bacteria can vary due to environmental conditions, better understanding of this native bacterial population will enhance our ability to use bacterial inoculants to improve silage production practices.

Figure 4.1 Effect of inoculant treatment on silage mass temperature[1]



[1]Temperatures were collected every hour for 120 d via thermocouple probe inserted in center of empty experimental silo.
[2]No inoculant added to forages at ensiling.
[3]Inoculant contained *Lactobacillus plantarum* and *L. buchneri* applied at a rate of 5 x 10^5 CFU of bacteria/g of fresh forage.
[4]Inoculant contained *L. plantarum*, *L. buchneri*, and *L. diolivorans* applied at a rate of 5 x 10^5 CFU of bacteria/g of fresh forage.
[5]Inoculant contained *L.diolivorans* applied at a rate of 3 x 10^5 CFU of bacteria/g of fresh forage.
[6]Temperatures collected from empty experimental silo.

Table 4.1 Characteristics of chopped whole-plant corn after chopping and application of treatment

	Treatment					*P*-value
Item	**CON[1]**	**LPB[2]**	**LPBD[3]**	**LD[4]**	**SEM**	**Trt[5]**
DM, %	38.40	38.03	37.79	38.52	0.360	0.42
aNDF[6], % DM	34.90	36.86	36.59	36.12	0.796	0.46
ADF, % DM	18.73	19.19	19.49	19.10	0.718	0.90
Ash, % DM	4.16	4.18	4.33	4.0	0.144	0.56
Starch, % DM	38.40	38.13	36.61	39.32	2.275	0.82
WSC[7], % DM	9.56	9.13	9.02	10.39	0.876	0.16
pH	5.14	5.10	5.14	5.12	0.060	0.93
Total VFA, mM	13.63	12.60	12.62	12.15	1.208	0.76
Acetate, mM	3.42	3.04	3.24	2.84	0.561	0.80
Propionate, mM	< 0.01	< 0.01	< 0.01	< 0.01	-	-
Lactate, mM	1.08	< 0.01	1.28	< 0.01	0.842	0.44
Lactate: acetate	0.20	< 0.01	0.19	<0.01	0.137	0.44
Ethanol, mM	5.34	4.25	4.95	3.86	1.460	0.25
1-propanol, mM	0.019	0.020	0.024	0.018	0.005	0.34
1,2-propanediol, mM	0.37	0.37	0.57	0.35	0.102	0.30
2,3-butanediol, mM	0.44	0.44	0.65	0.40	0.177	0.36

[1]No inoculant added to forages at ensiling.
[2]Inoculant contained *Lactobacillus plantarum* and *L. buchneri* applied at a rate of 5 x 10^5 CFU of bacteria/g of fresh forage.
[3]Inoculant contained *L. plantarum*, *L. buchneri*, and *L. diolivorans* applied at a rate of 5 x 10^5 CFU of bacteria/g of fresh forage.
[4]Inoculant contained *L.diolivorans* applied at a rate of 3 x 10^5 CFU of bacteria/g of fresh forage.
[5]Treatment.
[6]Neutral detergent fiber determined using alpha-amylase.
[7]Water soluble carbohydrates.

Table 4.2 Effect of inoculant treatment and sampling day on fermentation characteristics of whole plant corn silage

	Sampling day															
	14				39				90							
	Treatment				Treatment				Treatment					*P*-Value		
Item	CON[1]	LPB[2]	LPBD[3]	LD[4]	CON	LPB	LPBD	LD	CON	LPB	LPBD	LD	SEM	Trt[5]	SD[6]	Trt × SD[7]
DM, %	36.88	36.65	35.96	36.65	36.34	35.80	35.99	36.34	38.11	37.80	38.06	37.33	0.500	0.42	< 0.01	0.69
aNDF[8], % DM	36.03	36.90	37.53	37.46	35.43	37.39	36.94	36.20	31.47	31.57	33.57	33.03	1.184	0.23	< 0.01	0.96
ADF, % DM	18.39	19.69	20.14	20.25	18.77	19.93	19.71	19.70	16.59	16.92	17.95	17.27	0.713	0.10	0.01	0.96
Ash, % DM	4.02	4.20	4.24	4.11	4.17	4.33	4.31	4.27	4.12	4.38	4.67	4.18	0.089	0.01	0.05	0.85
Starch, % DM	36.77	35.87	35.74	36.41	37.01	32.55	36.01	37.75	39.39	36.60	38.39	41.43	1.894	0.17	0.04	0.85
WSC[9], % DM	1.78	2.89	1.87	2.22	2.15	2.70	2.67	2.95	2.49	3.05	3.02	1.45	0.656	0.52	0.33	0.15
pH	3.60	3.59	3.60	3.60	3.51	3.53	3.52	3.52	3.56	3.55	3.56	3.56	0.010	0.96	< 0.01	0.88
Total VFA, mM	107.55	101.54	113.44	99.58	115.43	120.92	124.38	109.54	110.66	128.16	131.74	13.90	5.534	0.01	< 0.01	0.63
Acetate, mM	102.43	98.21	109.59	96.20	111.48	117.95	120.71	106.21	109.13	126.45	129.46	111.97	5.346	0.01	< 0.01	0.69
Propionate, mM	0.51[a]	< 0.01[a]	< 0.01[a]	< 0.01[a]	< 0.01[a]	< 0.01[a]	0.73[b]	0.43[a]	0.12[a]	0.23[a]	1.25[b]	0.59[a]	0.230	0.03	0.04	0.03
Butyrate, mM	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	0.12	< 0.01	< 0.01	0.07	0.13	0.00	0.00	0.049	0.44	0.25	0.55
Lactate, mM	119.82	116.11	129.20	124.21	110.77	103.50	113.24	106.20	108.24	94.88	98.42	128.99	9.621	0.57	0.04	0.35
Lactate: acetate	1.19	1.20	1.20	1.29	1.00	0.89	0.94	1.00	1.00	0.75	0.77	1.17	0.112	0.23	0.01	0.63
Ethanol, mM	128.98	122.55	134.15	170.63	72.97	86.02	76.62	85.83	88.90	58.08	75.13	84.19	0.739	0.37	< 0.01	0.61
1-propanol, mM	0.41	0.06	0.82	1.62	0.09	0.12	1.04	1.85	0.35	0.16	1.10	2.21	0.342	< 0.01	0.45	0.86
1,2-propanediol, mM	2.48	3.64	3.29	0.80	5.63[a]	23.41[b]	19.67[b]	3.35[a]	14.71[a]	38.11[b]	37.73[b]	7.45[a]	3.291	< 0.01	< 0.01	< 0.01
2, 3-butanediol, mM	2.40	2.10	2.25	1.95	2.83	2.73	2.84	2.29	3.44	3.30	3.28	3.40	0.281	0.37	< 0.01	0.82

[ab]Means within a row, within a sampling day, with different superscripts differ ($P < 0.05$).
[1]No inoculant added to forages at ensiling.
[2]Inoculant contained *Lactobacillus plantarum* and *L. buchneri* applied at a rate of 5 x 10^5 CFU of bacteria/g of fresh forage.
[3]Inoculant contained *L. plantarum*, *L. buchneri*, and *L. diolivorans* applied at a rate of 5 x 10^5 CFU of bacteria/g of fresh forage.
[4]Inoculant contained *L. diolivorans* applied at a rate of 3 x 10^5 CFU of bacteria/g of fresh forage.
[5]Treatment.
[6]Sampling day.
[7]Interaction between treatment and sampling day.
[8]Neutral detergent fiber determined using alpha-amylase.
[9]Water soluble carbohydrates.

Table 4.3 Effect of inoculant treatment and weigh day on weight retention of silos containing whole plant corn silage

		Weigh day							*P*-value		
Treatment	**Trt Mean**	**7**	**14**	**21**	**28**	**39**	**90**	**SEM**	**Trt[5]**	**Day[6]**	**Trt × Day[7]**
CON[1]	99.52*[a]	99.65	99.62	99.49	99.48	99.46	99.40	0.078	0.053	< 0.01	0.99
LPB[2]	99.42[a,b]	99.56	99.50	99.47	99.36	99.33	99.29				
LPBD[3]	99.37[b]	99.53	99.48	99.43	99.32	99.27	99.16				
LD[4]	99.50[a]	99.66	99.57	99.56	99.42	99.44	99.36				
Weigh Day	-	99.60[A]	99.54[A,B]	99.49[B]	99.39[C]	99.37[C,D]	99.30[D]	0.049	-	< 0.01	-

* Shown as percentage of the initial weight, which is considered 100%.
[ab]Treatment means with different superscripts differ ($P < 0.05$).
[ABCD]Weigh day means with different superscripts differ ($P < 0.05$).
[1]No inoculant added to forages at ensiling.
[2]Inoculant contained *Lactobacillus plantarum* and *L. buchneri* applied at a rate of 5 x 10^5 CFU of bacteria/g of fresh forage.
[3]Inoculant contained *L. plantarum*, *L. buchneri*, and *L. diolivorans* applied at a rate of 5 x 10^5 CFU of bacteria/g of fresh forage.
[4]Inoculant contained *L. diolivorans* applied at a rate of 3 x 10^5 CFU of bacteria/g of fresh forage.
[5]Treatment.
[6]Weigh day.
[7]Interaction between treatment and weigh day.

Table 4.4 Effect of inoculant treatment and sampling day on aerobic stability of whole plant corn silages after exposed to air for 14 d

	Sampling day															
	14				39				90							
	Treatment				Treatment				Treatment					*P*-value		
Item	CON[1]	LPB[2]	LPBD[3]	LD[4]	CON	LPB	LPBD	LD	CON	LPB	LPBD	LD	SEM	Trt[5]	SD[6]	Trt × SD[7]
pH	4.85	4.61	5.14	5.00	5.05	5.50	4.87	5.18	5.49	5.82	5.07	5.46	0.298	0.84	0.02	0.29
Total VFA, mM	92.62	78.85	64.82	84.89	24.01	19.57	30.01	17.74	19.07	8.88	21.62	21.52	11.662	0.83	< 0.01	0.74
Acetate, mM	64.43	59.76	37.68	51.33	12.26	8.14	14.27	7.65	8.68	3.94	13.96	10.84	8.794	0.83	< 0.01	0.49
Propionate, mM	20.55	15.05	19.65	24.88	2.99	3.50	4.19	1.67	2.48	1.39	3.08	1.43	4.203	0.87	< 0.01	0.87
Butyrate, mM	4.57	1.78	4.54	4.76	6.97	5.75	9.18	6.43	4.84	2.10	3.26	7.09	1.873	0.05	0.04	0.71
Lactate, mM	10.45	9.04	8.78	8.52	11.86	6.33	14.45	9.25	12.01	2.89	11.80	6.84	2.684	0.19	0.49	0.54
Lactate: acetate	0.18	0.16	0.27	0.24	1.32	0.85	0.98	1.43	1.61	0.65	0.89	0.82	0.24	0.02	< 0.01	0.46
Ethanol, mM	1.05	0.52	0.53	0.72	0.86	0.51	0.91	0.55	0.86	0.82	0.51	0.21	0.231	0.29	0.73	0.38
1-propanol, mM	1.06	0.43	0.93	0.89	0.27	0.31	0.47	0.06	0.09	0.04	0.04	0.06	0.310	0.80	< 0.01	0.88
1,2-propanediol, mM	5.52	5.00	6.34	5.72	1.21	1.12	1.86	1.06	2.11	0.97	1.78	2.48	1.640	0.92	< 0.01	0.99
2,3-butanediol, mM	7.10	9.72	2.86	3.88	2.28	1.14	3.34	1.40	0.63	0.20	0.10	0.35	2.001	0.70	< 0.01	0.35

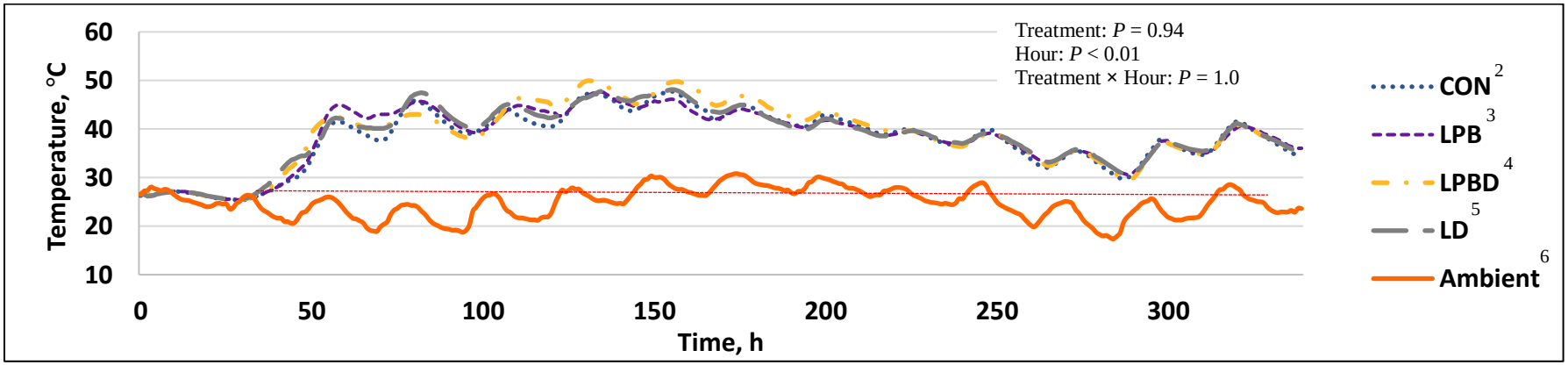
[1]No inoculant added to forages at ensiling.
[2]Inoculant contained *Lactobacillus plantarum* and *L. buchneri* applied at a rate of 5 x 10^5 CFU of bacteria/g of fresh forage.
[3]Inoculant contained *L. plantarum*, *L. buchneri*, and *L. diolivorans* applied at a rate of 5 x 10^5 CFU of bacteria/g of fresh forage.
[4]Inoculant contained *L diolivorans* applied at a rate of 3 x 10^5 CFU of bacteria/g of fresh forage.
[5]Treatment.
[6]Sampling day.
[7]Interaction between treatment and sampling day.

Table 4.5 Effect of inoculant treatment, sampling day, and aerobic stability day on weight reduction of silos containing whole-plant corn silage

	Sampling day									
	14		39		90					
	Aerobic stability day							*P*-value		
Treatment	7	14	7	14	7	14	SEM	Trt × SD[5]	Trt × AS day[6]	Trt × SD × AS day[7]
CON[1]	96.69*	93.97	98.47	96.30	98.73	96.87	0.820	0.46	0.67	0.76
LPB[2]	96.25	93.13	98.32	96.28	98.78	96.32				
LPBD[3]	95.30	92.12	98.71	96.79	98.85	96.88				
LD[4]	98.13	95.32	98.46	96.36	98.95	96.73				

*Shown as percentage of the initial weight, which is considered 100%.
[1]No inoculant added to forages at ensiling.
[2]Inoculant contained *Lactobacillus plantarum* and *L. buchneri* applied at a rate of 5 x 10^5 CFU of bacteria/g of fresh forage.
[3]Inoculant contained *L. plantarum*, *L. buchneri*, and *L. diolivorans* applied at a rate of 5 x 10^5 CFU of bacteria/g of fresh forage.
[4]Inoculant contained *L.diolivorans* applied at a rate of 3 x 10^5 CFU of bacteria/g of fresh forage.
[5]Treatment × sampling day interaction.
[6]Treatment × aerobic stability day interaction.
[7] Interaction between treatment, sampling day, and aerobic stability day.

Figure 4.2 Effect of inoculant treatment and time on temperatures[1] of whole plant corn silage ensiled for 14-d and subjected to a 14-d aerobic stability test



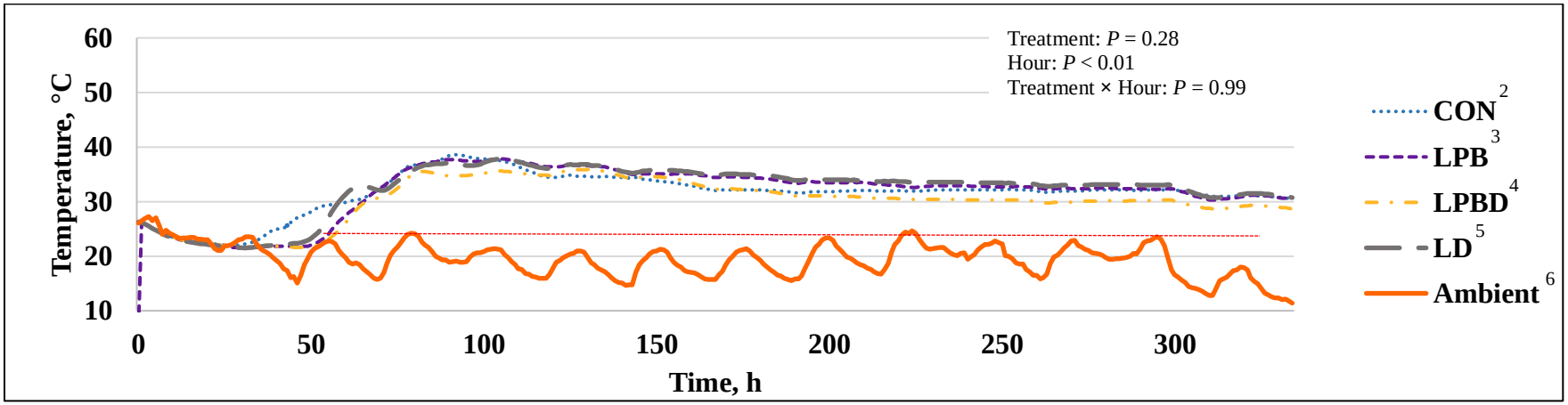
Horizontal dotted line indicates 2^{0}C temperature elevation above baseline of 24.78^{0}C.
[1]Temperature was recorded once per h throughout 14-d aerobic stability test via thermocouple probe inserted in center of aerobic stability container.
[2]No inoculant added to forages at ensiling.
[3]Inoculant contained *Lactobacillus plantarum* and *L. buchneri* applied at a rate of 5 x 10^5 CFU of bacteria/g of fresh forage.
[4]Inoculant contained *L. plantarum*, *L. buchneri*, and *L. diolivorans* applied at a rate of 5 x 10^5 CFU of bacteria/g of fresh forage.
[5]Inoculant contained *L.diolivorans* applied at a rate of 3 x 10^5 CFU of bacteria/g of fresh forage.
[6]Temperatures collected from empty experimental silo.

Table 4.6 Effect of inoculant treatment on aerobic stability after a 14-d ensiling period[1]

	Treatment					*P*-value
Item	**CON[2]**	**LPB[3]**	**LPBD[4]**	**LD[5]**	**SEM**	**Trt[6]**
Hours until 2^{0}C above baseline[7]	13.25	10.75	10.75	9.75	3.733	0.92

[1]Length of time until heating to above 2^{0}C above baseline when whole-plant corn forage was ensiled for 14-d and then subjected to a 14-d aerobic stability test.
[2]No inoculant added to forages at ensiling.
[3]Inoculant contained *Lactobacil1lus plantarum* and *L. buchneri* applied at a rate of 5 x 10^5 CFU of bacteria/g of fresh forage.
[4]Inoculant contained *L. plantarum*, *L. buchneri*, and *L. diolivorans* applied at a rate of 5 x 10^5 CFU of bacteria/g of fresh forage.
[5]Inoculant added contained *L.diolivorans* applied at a rate of 3 x 10^5 CFU of bacteria/g of fresh forage.
[6]Treatment.
[7]Temperature was recorded once per h throughout 14-d aerobic stability test via thermocouple probe inserted in center of aerobic stability container.

Figure 4.3 Effect of inoculant treatment and time on temperatures[1] of whole plant corn silage ensiled for 39-d and subjected to a 14-d aerobic stability test



Horizontal dotted line indicates 2^{0}C temperature elevation above baseline of 21.08^{0}C.

[1]Temperature was recorded once per h throughout 14-d aerobic stability test via thermocouple probe inserted in center of aerobic stability container.

[2]No inoculant added to forages at ensiling.

[3]Inoculant contained *Lactobacillus plantarum* and *L. buchneri* applied at a rate of 5 x 10^5 CFU of bacteria/g of fresh forage.

[4]Inoculant contained *L. plantarum*, *L. buchneri*, and *L. diolivorans* applied at a rate of 5 x 10^5 CFU of bacteria/g of fresh forage.

[5]Inoculant contained *L.diolivorans* applied at a rate of 3 x 10^5 CFU of bacteria/g of fresh forage.

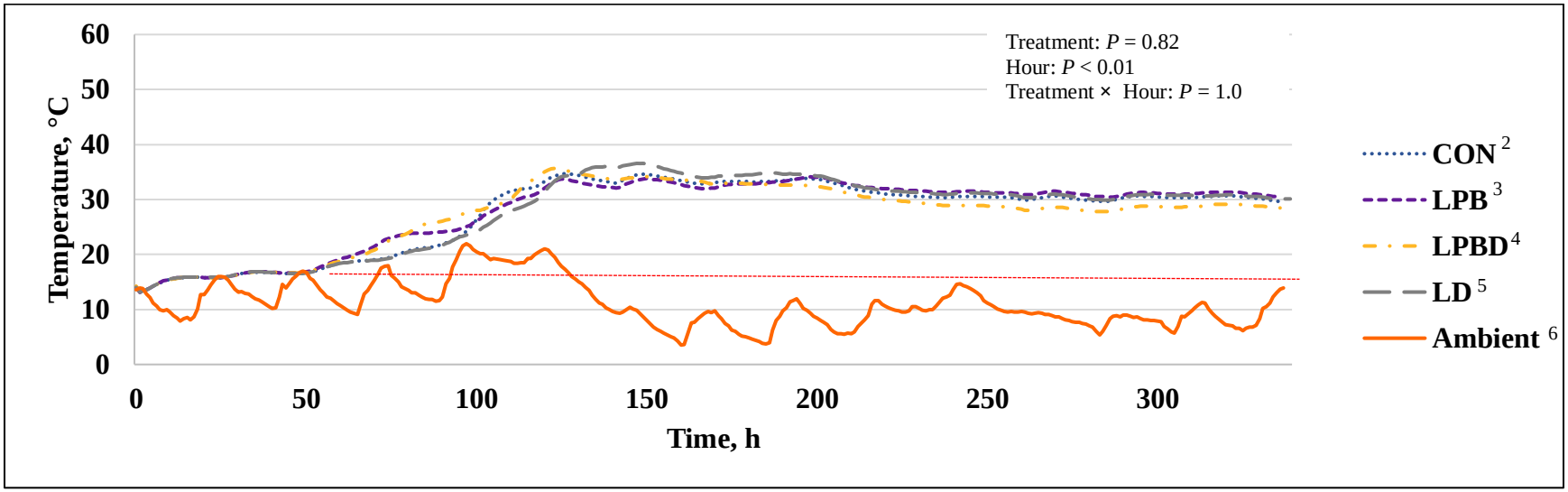
[6]Temperatures collected from empty experimental silo.

Table 4.7 Effect of inoculant treatment on aerobic stability after a 39-d ensiling period[1]

	Treatment					*P*-value
Item	**CON[2]**	**LPB[3]**	**LPBD[4]**	**LD[5]**	**SEM**	**Trt[6]**
Hours until 2^0C above baseline[7]	25.00	32.25	33.75	31.50	5.21	0.66

[1]Length of time until heating to above 2^0C above baseline when whole-plant corn forage was ensiled for 39-d and then subjected to a 14-d aerobic stability test
[2]No inoculant added to forages at ensiling.
[3]Inoculant contained *Lactobacil1lus plantarum* and *L. buchneri* applied at a rate of 5 x 10^5 CFU of bacteria/g of fresh forage.
[4]Inoculant contained *L. plantarum*, *L. buchneri*, and *L. diolivorans* applied at a rate of 5 x 10^5 CFU of bacteria/g of fresh forage.
[5]Inoculant added contained *L.diolivorans* applied at a rate of 3 x 10^5 CFU of bacteria/g of fresh forage.
[6]Treatment.
[7]Temperature was recorded once per h throughout 14-d aerobic stability test via thermocouple probe inserted in center of aerobic stability container.

Figure 4.4 Effect of inoculant treatment and time on temperatures[1] of whole plant corn silage ensiled for 90-d and subjected to a 14-d aerobic stability test



Horizontal dotted line indicates 2^{0}C temperature elevation above baseline of 15.28^{0}C.

[1]Temperature was recorded once per h throughout 14-d aerobic stability test via thermocouple probe inserted in center of aerobic stability container.

[2]No inoculant added to forages at ensiling.

[3]Inoculant contained *Lactobacillus plantarum* and *L. buchneri* applied at a rate of 5 x 10^5 CFU of bacteria/g of fresh forage.

[4]Inoculant contained *L. plantarum*, *L. buchneri*, and *L. diolivorans* applied at a rate of 5 x 10^5 CFU of bacteria/g of fresh forage.

[5]Inoculant contained *L.diolivorans* applied at a rate of 3 x 10^5 CFU of bacteria/g of fresh forage.

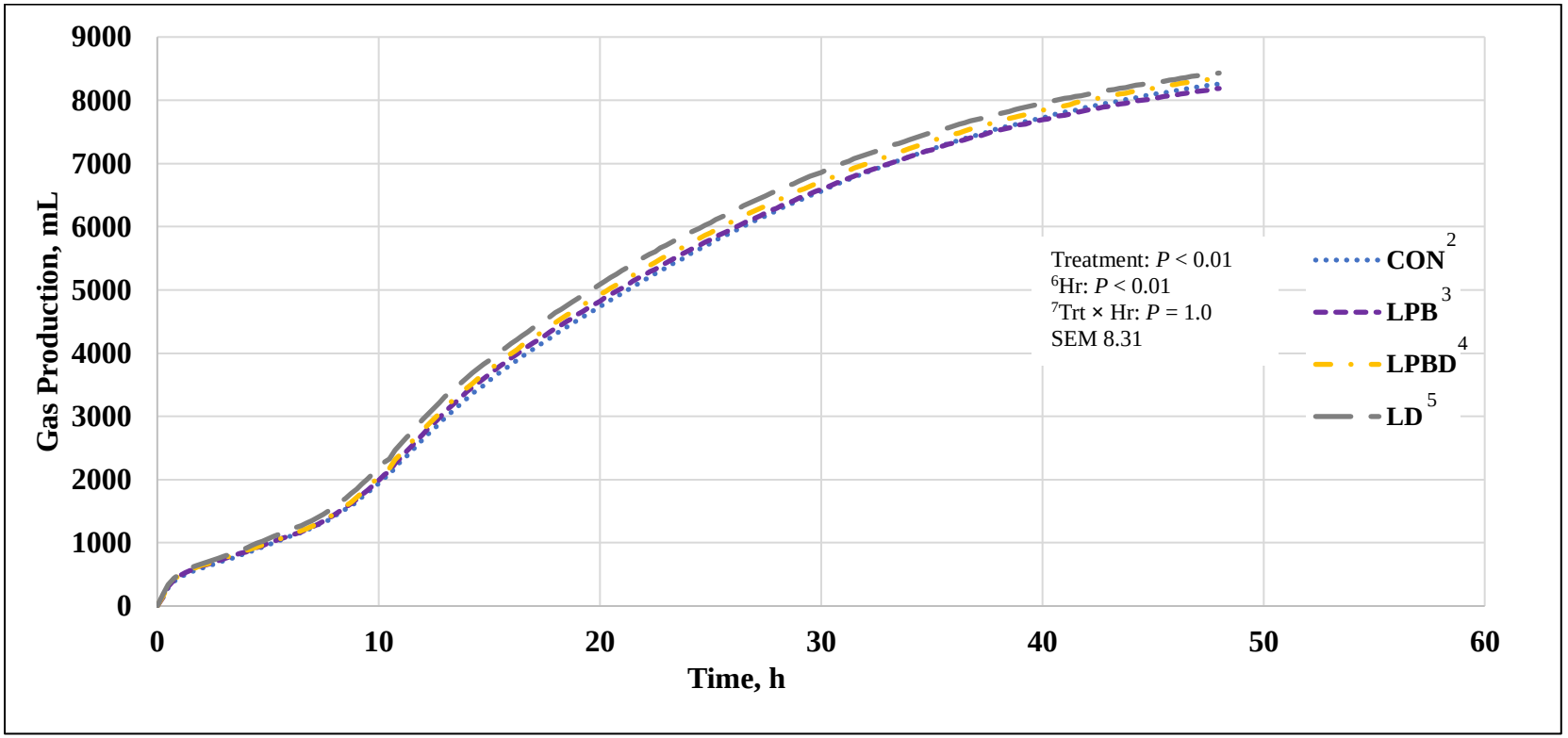
[6]Temperatures collected from empty experimental silo.

Table 4.8 Effect of inoculant treatment on aerobic stability after a 90-d ensiling period[1]

	Treatment					*P*-value
Item	**CON[2]**	**LPB[3]**	**LPBD[4]**	**LD[5]**	**SEM**	**Trt[6]**
Hours until 2^0C above baseline[7]	27.95	26.20	26.70	28.95	3.90	0.79

[1]Length of time until heating to above 2^0C above baseline when whole-plant corn forage was ensiled for 90-d and then subjected to a 14-d aerobic stability test
[2]No inoculant added to forages at ensiling.
[3]Inoculant contained *Lactobacil1lus plantarum* and *L. buchneri* applied at a rate of 5 x 10^5 CFU of bacteria/g of fresh forage.
[4]Inoculant contained *L. plantarum, L. buchneri,* and *L. diolivorans* applied at a rate of 5 x 10^5 CFU of bacteria/g of fresh forage.
[5]Inoculant added contained *L.diolivorans* applied at a rate of 3 x 10^5 CFU of bacteria/g of fresh forage.
[6]Treatment.
[7]Temperature was recorded once per h throughout 14-d aerobic stability test via thermocouple probe inserted in center of aerobic stability container.

Figure 4.5 Effect of inoculant treatment and time on *in vitro* fermentation gas production[1]



Data presented are gas production of cultures averaged within treatment and presented across 48 h of *in vitro* fermentation.
[1]Incubated with mixed ruminal microorganisms for 48 h.
[2]No inoculant added to forages at ensiling.
[3]Inoculant contained *Lactobacillus plantarum* and *L. buchneri* applied at a rate of 5 x 10^5 CFU of bacteria/g of fresh forage.
[4]Inoculant contained *L. plantarum*, *L. buchneri*, and *L. diolivorans* applied at a rate of 5 x 10^5 CFU of bacteria/g of fresh forage.
[5]Inoculant contained *L.diolivorans* applied at a rate of 3 x 10^5 CFU of bacteria/g of fresh forage.
[6]Hour of fermentation.
[7]Interaction between treatment and hour of fermentation

Table 4.9 Effect of inoculant treatment, sampling day, and interaction between inoculant and sampling day on *in vitro* fermentation characteristics[1]

	Sampling day															
	14				39				90							
	Treatment				Treatment				Treatment					*P*-Value		
Item	CON[2]	LPB[3]	LPBD[4]	LD[5]	CON	LPB	LPBD	LD	CON	LPB	LPBD	LD	SEM	Trt[6]	SD[7]	Trt × SD[8]
Terminal pH	5.86	5.95	5.99	5.86	5.79	5.99	5.93	5.79	5.91	5.90	6.01	5.84	0.305	0.89	0.94	1.00
IVDMD[9], %	45.19	40.86	39.56	42.00	39.75	39.19	36.75	38.36	45.67	46.72	45.89	43.25	0.021	0.37	< 0.01	0.74
Total VFA, mM	7.17	5.75	6.83	6.00	6.15	5.95	6.38	6.37	6.40	6.34	5.96	5.75	0.801	0.23	0.49	0.68
Acetate, mM	3.48	2.90	3.35	3.10	2.92	2.95	3.09	3.00	3.29	3.20	3.07	2.92	0.406	0.46	0.30	0.64
Propionate, mM	2.43	1.94	2.27	1.94	2.18	2.04	2.20	2.01	2.10	2.13	1.93	1.89	0.317	0.20	0.57	0.76
Butyrate, mM	1.00	0.72	0.95	0.75	0.80	0.74	0.84	0.74	0.78	0.75	0.73	0.72	0.141	0.07	0.09	0.48
A:P[10]	0.06	0.06	0.06	0.06	0.06	0.05	0.06	0.06	0.06	0.06	0.06	0.06	0.004	0.29	< 0.01	0.42

[1]Data collected following 48 h incubation of silage with mixed ruminal microorganisms.
[2]No inoculant added to forages at ensiling.
[3]Inoculant contained *Lactobacillus plantarum* and *L. buchneri* applied at a rate of 5 x 10^5 CFU of bacteria/g of fresh forage.
[4]Inoculant contained *L. plantarum*, *L. buchneri*, and *L. diolivorans* applied at a rate of 5 x 10^5 CFU of bacteria/g of fresh forage.
[5]Inoculant contained *L.diolivorans* applied at a rate of 3 x 10^5 CFU of bacteria/g of fresh forage.
[6]Treatment.
[7]Sampling day.
[8]Interaction between treatment and sampling day.
[9]In vitro dry matter disappearance.
[10]Acetate:propionate.

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