Nutritional factors affecting animal growth and activity of gut microorganisms

by

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Abstract

Three studies were conducted as part of this graduate program. The first study was to evaluate effects of *Nannochloropsis* biomass on growth performance, fatty acid profile of leg quarters, and histopathology of skeletal muscle when fed to broiler chicks. Day-old male Ross-708 broiler chicks (n = 576) were separated into 72 battery pens (8 birds/pen). Pens (experimental unit) were blocked by tier location within battery and randomly assigned to 1 of 4 dietary treatments consisting of either 0% (control), 2%, 4%, or 10% algae. All diets, formulated to be isocaloric and isonitrogenous, were fed *ab libitum* for 18 d. On d 18, pen weights were recorded and 2 birds per pen were sacrificed for fatty acid, histopathology, and toxicology analyses. Data were analyzed using PROC GLIMMIX with fixed effect of treatment, random effects of battery and tier, and pen as the experimental unit. When compared to control, 2% and 4% algae did not impact average daily gain ($P = 0.1084$) or average daily feed intake ($P = 0.9998$). However, 10% algae decreased ($P = 0.0003$) feed efficiency when compared to all other treatments. Fatty acid profile of leg quarters improved with algae supplementation at all levels as demonstrated by linear increases ($P < 0.0001$) in polyunsaturated and total n-3 fatty acids. There was no impact of treatment on necrosis of muscle fibers ($P = 0.3189$). Arsenic, cadmium, lead, and thallium were below detectable limits in the liver for all treatments. Supplementation of *Nannochloropsis* algae to broiler chicks improved fatty acid profile of muscle tissue without negatively affecting growth performance when fed at 2 and 4% of total diet.

A second study was conducted to assess impact of various carbohydrates on fermentation by bovine ruminal microorganisms. To do this, ruminal fluid was collected and used to inoculate fermentation bottles containing buffer, forage, and a supplemental carbohydrate source. Bottles contained 5 g of forage (prairie hay, smooth bromegrass hay, or alfalfa) and 0.1 g of

supplemental carbohydrate (glucose, fructose, galactose, xylose, mannose, arabinose, rhamnose, lactose, sucrose, melibiose, maltose, isomaltose, trehalose, stachyose, raffinose, raw corn starch, hydrolyzed corn starch, cellulose, sugar cane molasses, or beet molasses). Bottles were equipped with continuous gas pressure monitors and incubated in duplicate for 48 h using the ANKOM RF System (ANKOM Technology, Macedon, NY). Bottles were then opened and terminal pH, *in vitro* dry matter disappearance (IVDMD), *in vitro* neutral detergent fiber disappearance (IVNDFD), and volatile fatty acid (VFA) concentrations were measured. Data were analyzed using the PROC GLIMMIX procedure for terminal pH, IVDMD, IVNDFD, and VFA's with fixed effects of carbohydrate source, forage type, and interaction. Total gas production data were analyzed via NLIN and the MIXED procedure. Total gas production was not affected by carbohydrate source in cultures containing prairie hay or bromegrass ($P \geq 0.06$). However, total gas production increased with cellulose, galactose, and hydrolyzed corn starch ($P \leq 0.05$) in cultures containing alfalfa. Terminal pH and IVDMD were not affected by carbohydrate source in cultures containing prairie hay, bromegrass, or alfalfa. There was no impact of carbohydrate source on IVNDFD in cultures with prairie hay and alfalfa. However, cultures containing bromegrass had a greater IVNDFD with isomaltose and galactose when compared to control ($P = 0.038$). Several changes were observed in cultures supplied with prairie hay. Cellulose reduced acetate compared to control ($P = 0.003$), while the control had a greater A:P ratio when compared to trehalose, sucrose, raffinose, melibiose, mannose, maltose, lactose, glucose, galactose, fructose, and cellulose ($P \leq 0.05$). Meanwhile, butyrate concentration was greater ($P = 0.010$) in cultures containing arabinose, and cellulose led to a greater concentration of valerate ($P = 0.007$) and caproate ($P = 0.003$) when compared to control. Control cultures had greater methane production compared to those with cellulose, fructose, and isomaltose ($P = 0.001$).

Finally, total VFA concentration was greater with maltose when compared to the control with prairie hay alone ($P = 0.015$). In cultures containing bromegrass, a greater A:P ratio was produced in cultures containing arabinose, glucose, isomaltose, lactose, melibiose, molasses, raffinose, rhamnose, sucrose, and trehalose when compared to control ($P < 0.0001$). In cultures containing alfalfa, there was a difference in A:P ratio between the control and all treatments ($P = 0.006$). Because most alterations to fermentative parameters were observed with prairie hay, it seems that carbohydrates have most potential to improve digestion of poor-quality forages.

In vitro fermentation systems allow researchers to mimic the equine hindgut while providing quick, affordable, and publishable data. In these systems, the substrate:buffer ratio has a profound impact on fermentation parameters. The objective of the third experiment was to evaluate varying substrate levels, thus altering the substrate:buffer ratio for its effect on culture pH, gas production, dry matter disappearance (DMD), neutral detergent fiber disappearance (NDFD), and volatile fatty acid (VFA) concentrations. Cecal fluid was collected from 4 cecally cannulated Quarter horses and used to inoculate fermentation bottles containing buffer and substrate. Substrate consisted of bromegrass or corn included at 0.5, 1, 1.5, 2, 3, or 4 g/flask (as-fed basis) while buffer and inoculant remained constant at 125 and 25 mL/flask, respectively. Flasks were equipped with continuous gas pressure monitors utilizing the ANKOM RF Gas Production System (ANKOM Technology; Macedon, NY) and placed into a shaking incubator for 48 hours at 39°C. Data were analyzed using mixed models with fixed effects of treatment, time, and the treatment x time interaction, repeated measure of time, and random effects of run x horse within run. Differences in total gas production, VFA production, and terminal pH were evident for substrate level and substrate type ($P < 0.0001$). At 1.5 g or 2.0 g substrate, there appeared to be a shift in slope as the incremental increase in many response variables began to

decrease as more substrate was provided. Thus, cultures with > 2 g substrate may have been inhibited by buffer exhaustion or end-product accumulation. The composition of fermentative end products is impacted by substrate concentration, and for cultures containing 125 mL of buffer and 25 mL of cecal inoculum, we would recommend using no more than 2 g of substrate.

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Dedication

I dedicate this Master's Thesis to my parents, Dr. Astrid Gutsmann and Dr. Craig Conrad. Thank you for raising me to follow my heart and endless encouragement throughout my graduate career.

Chapter 1 - Literature Review

Introduction

In this thesis, 3 separate experiments with 3 different species are described. This literature review serves as an introduction to components that were touched throughout the studies described herein. This review will include protein, lipid, and carbohydrate classifications; poultry digestion with a focus on proteins and lipids; ruminant and equine digestion of carbohydrates; microalgae as a feed additive; and usage of *in vitro* techniques in nutritional experiments.

A broiler diet typically contains wheat, barley, or corn, all of which contain sufficient starch to lead to increased fat content in the meat (NRC, 1994). A newer approach to feeding broilers is to utilize alternative feedstuffs to improve fatty acid (FA) profiles in meat, thus altering carcass characteristics. One such feed ingredient is microalgae, and recently published reports verify its efficacy as a feedstuff (Skrede et al., 2011; Stamey et al., 2012; Lemahieu et al., 2013). Some species of microalgae have been used to replace soybean meal as a protein source. Protein is important for synthesis of tissues necessary for growth, reproduction, and development (Beski et al., 2015). In addition, improving FA profiles in dietary meat sources has been shown to be beneficial to human health (Covington, 2004).

Gut bacteria employ a multitude of metabolic pathways to convert feedstuffs into a variety of end products. In both equine and ruminant diets, carbohydrates are provided through concentrates and forages. Carbohydrates (structural and non-structural) are the primary substrate utilized by microbes and contribute approximately 60 to 75% of digestible energy in the bovine rumen (Sutton, 1979) and 30 to 80% of digestible energy in the equine cecum (Glinksy et al., 1976; Vermorel et al., 1997a). There are many similarities in fermentative characteristics and

microbial populations of the equine cecum and bovine rumen. Most differences in digestive function between the two species are due to relative differences in location of the fermentative organ.

While microbial fermentation in the rumen has been studied extensively, reports pertaining to cecal fermentative parameters are still sparse. Many reports regarding fermentation characteristics in cattle and horses describe data obtained through *in vitro* techniques. While many of these researchers have utilized rumen inoculum, most *in vitro* experiments aimed at characterizing cecal fermentation have employed fecal inoculum. However, fecal inoculum does not accurately duplicate fermentative characteristics when compared to those obtained with cecal inoculum (Douthit et al., 2019; Sorenson et al., 2021).

Protein Structure

Monogastrics have specific dietary requirements for both crude protein (CP) and essential amino acids (EAA). Crude protein is the measurement of total protein content and can be calculated by multiplying elemental N by 6.25. This way of calculating CP assumes protein to be 16% N (Mariotti et al., 2008), as most amino acids contain approximately 16% N (Moore et al., 2010). Both protein quality and concentration affect growth performance and emission of N. By matching amino acid composition to the animal's requirements, N excretion can be minimized (Kim, 2014).

Proteins are comprised of amino acid chains called polypeptides (≥ 4 amino acids), tripeptides (3 amino acids), and dipeptides (2 amino acids). In addition to number of amino acids that make up a protein, structural shape plays an important role in how proteins are utilized. Amino acids are linked in a primary chain through peptide bonds. Peptide chains are folded into

secondary (2D) or tertiary (3D) shapes anchored by H bonds (Boyer, 2005). The gut can only absorb proteins when they are broken down into amino acids or short peptides. Thus, the bonds must be broken for the body to absorb the amino acids. While H bonds can be broken by low pH (strong acidity), peptide bonds require specific enzymes (Raymond, 2006).

Amino acids are building blocks for all tissues and are the backbone of every metabolic function in the body. There are approximately 20 amino acids that serve as building blocks of protein and peptides (Raymond, 2006). In any animal, amino acids can be divided into 2 categories: non-essential and essential. Non-essential amino acids can be synthesized by an organism. In contrast, essential amino acids cannot be synthesized and must be provided in the animal's diet. Amino acid requirements vary with age and productivity (NRC, 1994).

Protein Digestion

Digestion and Absorption in Poultry

Broilers are unique as they cannot chew food. Instead, feed lands in the crop after passing through the mouth and esophagus. Feed is stored in the crop and is available for digestion. Next, feed arrives at a broiler's proventriculus, which is sometimes referred to as the "true stomach". The proventriculus is where digestion is initiated by hydrochloric acid (HCl) and gastrointestinal enzymes. Protein is denatured in the proventriculus by HCl, wherein strong acidity denatures proteins resulting in a loss of 3D structure. Further degradation of the 2D peptide structure requires specific peptidases to break down the protein into smaller polypeptide units (Boyer, 2005). As chyme continues to pass through the digestive tract, feed enters a specialized, muscular sack known as the gizzard. The gizzard mixes and grinds feed into smaller particle sizes to allow for easier digestion (Jacob et al., 2011). Grinding in the gizzard and churning

movements of the proventriculus mix H ions with pepsin to aid in the breakdown of proteins. Eventually, digesta is released into the small intestine in miniscule quantities (Svihus, 2014). An unique aspect to broiler digestive physiology is the ability to reverse peristalsis (Sacranie et al., 2012). Ravindran et al. (2016) summarize reverse peristalsis as the ability of the digestive tract to become shorter and increase retention time, thus providing additional time for digestion. Refluxes occur with each duodenal contraction, and the second reflux moves the digesta from the small intestine back into the gizzard (Lentle et al., 2013).

Partially digested proteins and peptides continue into the duodenum. Fingerlike projections called villi increase surface area of the small intestine to increase absorptive capabilities (Caspary, 1992). Pancreatic ducts help transfer bicarbonate from pancreatic secretions to the small intestine to neutralize acidity of arriving chyme. This ultimately prevents acidic chyme from damaging the internal intestinal lining. While the increase in pH denatures pepsin (Piper and Fenton, 1965), pancreatic juices include other proteases which denature protein further. These proteases include trypsinogen, chymotrypsin, proelastase, and procarboxypeptidase. They allow for further digestion of protein in the duodenum and upper jejunum, where the greatest proportion of protein digestion occurs. These enzymes can be classified as exopeptidases and endopeptidases. Exopeptidases are enzymes that cleave the end of an amino acid chain and are primarily located in the small intestine. Endopeptidases add a water molecule to internal peptide bonds to break the chain. The three most common endopeptidases are pepsin, trypsin, and chymotrypsin (Ritchie, 2013). Trypsin then binds chymotrypsin to convert it into the active state of chymotrypsin; proelastase into elastase; and procarboxypeptidase into carboxypeptidasen. Trypsin, chymotrypsin, and elastase continue to

break down remaining protein and polypeptides into smaller chains with two to six amino acids in a process called proteolysis (Boyer, 2005).

While most protein is digested and absorbed in the small intestine, a small amount reaches the large intestine. Those proteins include elastin and collagen from sloughed cells, dead bacteria, and various pancreatic enzymes. In the large intestine, "left over" protein is degraded by colonic bacteria, which colonic epithelial cells then absorb and utilize. Remaining residues pass through the cloaca and finally exit at the vent. (Silk et al., 1989; Apajalahti and Vienola, 2016).

Apparent protein digestibility reflects the difference between N contained in feed versus what is excreted in feces. Digestible protein refers to the "disappearance" of protein as it travels through the gastrointestinal (GI) tract Fuller and Reeds, 1998). Metabolic fecal N arises from normal metabolism of tissue protein and includes N from sloughed cells located in the intestinal lining and from residues of digestive enzymes and other substances secreted into the lumen of the GI tract (Leibach and Ganapathy, 1996).

Amino acids are absorbed from the lumen of the GI tract through different mechanisms: (1) active transport, (2) passive diffusion, (3) facilitated diffusion, (4) co-transport, and (5) endocytosis (Choudhuri and Chanderbhan, 2016). Active transport involves the movement of molecules across the cellular membrane against the concentration gradient (i.e. low to high concertation; Schultz and Curran, 1970). Active transporters involve carrier proteins and require energy to move molecules. Passive diffusion is the movement of molecules from a greater concentration to a lower concentration. With facilitated diffusion, the movement of molecules from a higher concentration to a lower concentration is accomplished by utilizing a carrier protein within the cellular membrane. Co-transport uses the movement of 1 molecule from

greater to lower concentration to power the movement of another molecule from lower to higher concentration. Finally, endocytosis, the intake of matter by a cell through invagination of its membrane to form a vacuole, requires energy (Doherty and McMahon, 2009). The brush border membrane in the small intestine contains at least 2 active transport systems. The first is for neutral amino acids and the other is for basic amino acids (Pond et al., 2005). Following absorption, amino acids circulating in the blood are transported to the liver.

Protein Metabolism in Poultry

The liver is the most important organ for the degradation of amino acids (Conde and Scornik, 1976; Pösö and Mortimore, 1984). Amino acids are degraded according to structure and purpose. Degradation requires many enzymes including transaminase, decarboxylase, arginase, and synthase, which remove amino groups from the carbon skeleton for glucose production (glucogenesis), lipogenesis, carbon dioxide production, and fabrication of energy (Qaid and Al-Garadi, 2021). Proteases cleave proteins and regulate metabolic pathways (Ward, 2011).

Proteins are synthesized from free amino acids circulating in the blood or accumulating within tissues (Dublecz, 2011). Dietary amino acids are used to build muscle, as membrane glycoproteins, and as precursors for deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) (Wu, 2013). Protein synthesis occurs inside cells and includes transcription, translation, and post-translation events such as protein folding and proteolysis. Transcription includes encoding the sequence of the protein in a trinucleotide code. The trinucleotide code is translated by RNA polymerase to follow the genetic code. Ribosomes then translate the codons to their respective amino acids (Clancy and Brown, 2008). Following translation, protein folding and proteolysis occurs. Protein folding is when the peptide chains fold into secondary and tertiary structures. The

utilization of amino acids is described as the equilibrium between anabolism and catabolism. Anabolism refers to assimilation of amino acids into tissues. Whereas catabolism refers to the breakdown of proteins to produce urea (Qaid and Al-Garadi, 2021).

Amino acids regulate cellular metabolism and homeostasis. For example, arginine plays a role in growth (Visek, 1984), secretion of hormones such as insulin, glucagon production, and production of other hormones that regulate protein, lipid, and glucose metabolism (Wu and Self, 2011). Arginine impacts protein metabolism in mammals by regulating signaling pathways. Leucine increases protein synthesis and prevents proteolysis in skeletal muscles. Alanine inhibits pyruvate kinases, thereby regulating gluconeogenesis and glycolysis to ensure net glucose production is stable. Glutamate and aspartate aid in regulating glycolysis. Glutamine and arginine help keep the urea cycle in an active state (Morris, 2002).

During metabolization N, O, C, and H are utilized for energy through gluconeogenesis (Obeid et al., 2005; Baum et al., 2006). Because the body is unable to store the basic form of N, excess N is excreted in the form of ammonia. Animals have evolved to eliminate toxic ammonia from the system by diluting it and excreting it, specifically as urea or uric acid. In poultry, N is excreted as uric acid. Feeding excessive protein in diets can cause increased N excretion, a multitude of health issues, and has environmental impacts (Chung and Baker, 1992). Excessive production of uric acid can cause an accumulation of ammonia in blood, causing disease, and ultimately increasing mortality. By constraining amino acid concentration in diets, uric acid secretion can be reduced to improve the overall health of the bird (Chung and Baker, 1992).

Lipid Classification

Lipids play an integral role in the health and physiology of an animal (Brindley, 1984). Lipids include a variety of chemical compositions that are insoluble in water. These structures include glycolipids, sterols, and triacylglycerols. Triacylglycerols make up > 95% of all dietary lipids. Triacylglycerols have a glycerol backbone plus 3 fatty acid attachments. Lipids supply energy for daily maintenance and bodily functions. Feeding lipids slows the rate of passage which enhances digestion for the bird (Mateos and Dell, 1981). Lipids are a source of essential fatty acids, serve as transporters for fat-soluble vitamins, and serve as integral components of cellular membranes (Mersmann, 2011).

Enser (1984) defined the term “fat” to describe lipids in a solidified form and “oil” to describe lipids in a liquid state. Fatty acids may be saturated or unsaturated. Saturated fatty acids are solids at room temperature due to a high melting point. Carbon molecules in saturated fatty acids are linked by single bonds. Unsaturated fatty acids are characterized by double bonds. Monosaturated fatty acids have a single double bond and polysaturated fatty acids contain 2 or more double bonds. Unsaturated fats are liquid at room temperature (Enser, 1984).

Lipid Digestion

Digestion and Absorption in Poultry

Breakdown of lipids, lipolysis, occurs in the small intestine. The entry of lipids into the small intestine stimulates cholecystokinin, which regulates the secretion of pancreatic lipase and bile (Krogdahl, 1985). Pancreatic lipase is secreted from the pancreas and bile salts are expelled from the gall bladder. In poultry, pancreatic lipase can be inhibited by large concentrations of

bile salts (Bosc-Bierne et al., 1984). However, colipase can help regulate lipase activity and aid in triglyceride emulsification (Borgstrom, 1980).

While fats are insoluble in water, bile is amphiphilic, as it contains a nonpolar, hydrophobic region and a polar, hydrophilic region, which, in combination with phospholipids, enables emulsification of triglycerides (Krogdahl, 1985). Triglycerides are broken down by pancreatic lipase into glycerol and free fatty acids (FFA). The hydrolysis of triglycerides into glycerol and fatty acids creates a useable source of energy for the organism (Schweiger et al., 2014). Products of lipolysis are incorporated into bile salt micelles. Micelles are composed of bile salts, monoglycerides, fatty acids, unsaturated long-chain fatty acids (polyunsaturated), and phospholipids. The hydrophobic core of the micelle can solubilize long-chain saturated fatty acids, diglycerides, fat-soluble vitamins, and cholesteryl esters. Micelles facilitate fat absorption by providing dense concentrations of lipids in layers adjacent to mucosal cells (Krogdahl, 1985). Decreased pH causes lipids to separate from bile salts before absorption occurs and causes disaggregation of micelles. Disaggregation of micelles allows for increased surface area, enhancing the effectiveness of pancreatic and intestinal lipases, resulting in hydrolysis of triglycerides and FFA (Pond et al., 2005).

The jejunum of the small intestine is the primary location of lipid absorption, while the ileum is important for absorption of linoleic, steric, and palmitic acid (Hurwitz et al., 1973; Whitehead and Fisher, 1975). Passage of lipids and absorption rate depends on chain length and saturation (Bierbach et al., 1979). Short-chain FA and monoglycerides are absorbed passively from the intestinal lumen into the blood by intestinal cells (Pond et al., 2005). Long-chain saturated FA and diacylglycerols require solubilization in the hydrophobic region of the micelles, which are then transported inside intestinal cells (Davenport, 1980).

Inside the intestinal cell, triglycerides are reconstructed from long-chain FA and monoglycerides, combined with esterified cholesterols, lipoproteins, and phospholipids. The combination forms chylomicrons that are secreted into portal circulation and then called portomicrons (Hermier, 1997). Portomicrons are transported to liver and other tissues in the body, where lipids are then metabolized as an energy source or stored as adipose tissue (Akiba et al., 1982).

Importance of Fatty Acids to Human and Animal Health

Since the 1929 discovery that fatty acids are important to rat health (Burr and Burr, 1929) and the discovery of linoleic acid in 1930 (Burr and Burr, 1930), there have been many researchers evaluate effects of FA on the body. In 1950, the ability to convert linolenic acid to another omega-3 fatty acid was demonstrated in animals (Widmer and Holman, 1950). Three years later, Hammond and Lundberg (1953) identified that omega-3 fatty acid as docosahexaenoic acid (DHA). Even though the importance of dietary FA was recognized in the 1930's and the identification of omega-3 FA occurred in the 1950s and 1960s, most of the key studies involving the importance of FA on health were not conducted until the 1970s. Benolken et al. (1973) examined the interaction between DHA and retinal visual response. This report stimulated further investigative studies on the impact of DHA on other physiological concerns. In the late 1980s, Kerby and Steward (1988) confirmed that eicosapentaenoic acid (EPA), DHA, and linoleic acids can be used as treatments for heart disease, obesity, depression, and contain anti-inflammatory properties.

It is now known that omega-3 (n-3) and omega-6 (n-6) fatty acids are essential for health and optimal physiological functions in animals and humans. Fish and plant oils are primary

dietary sources of the n-3 fatty acids EPA and docosahexaenoic acid (DHA). Consumption of diets with increased concentrations of long chain n-3 fatty acids improves immune and cardiovascular function in humans (Kris-Etherton et al., 2002) and carcass and reproductive performance in cattle (Mattos et al., 2000; Petit et al., 2002).

Poultry products have become a source of long-chain polyunsaturated fatty acids (LC-PUFAs) in human diets in the U.S. and Europe. Givens and Gibbs (2008) summarized that individual daily intake of EPA + DHA from animal products in human diets is roughly 230 mg, with poultry meat providing the largest contribution with 74 mg. Over the last 100 years, increased consumption of meat that is high in n-6 fatty acids has shifted the ratio of n-6 to n-3 from 1:1 to approximately 10:1 in humans on typical Western diet (Kris-Etherton et al., 2000). Within the last 30 years, poultry products have become a major source of fatty acid consumption in human diets (Alagawany et al., 2019).

Feeding products with increased n-3 content not only benefits bird health, in terms of improving hydrolysis and absorption of lipoproteins, but also improves environmental health by reducing dust from feed particles in bird houses (Nobakht et al., 2011). Dietary fatty acid composition is reflected by FA composition of broiler breast, thigh, and skin tissues (Marion and Woodroof, 1963). Increasing dietary EPA and DHA also increases EPA and DHA in depot fat and muscle lipids (Neudoerffer and Lea, 1967). Dietary inclusion of microalgae in birds improves fatty acid profiles of meat by increasing n-3 FA and needs to be further investigated.

Carbohydrate Classification

Carbohydrates (CHO) serve as the primary energy source to herbivores. Carbohydrates are classified according to chemical structure and how they affect the animal (Pond et al. 2005). Van Soest (1963) developed a method to distinguish between structural and non-structural

carbohydrate (NSC). Structural carbohydrates include neutral detergent fiber (NDF), which contains cellulose, hemicellulose, and lignin, and acid detergent fiber (ADF), which include only cellulose and lignin. Nonstructural carbohydrate components include starches, sugars, and fructans (Van Soest et al., 1991). Non-fiber carbohydrates have been described as components not in the NDF portion, such as soluble fibers, pectic substances, 1-3 and 1-4 β-glucans, and organic acids (Hall, 2003). Hall (2003) further explains that while monosaccharides, maltose, and starch can be digested by enzymes, other NSC such as fructans, β-glucans, and pectic substances cannot be hydrolyzed by enzymes and thus are considered dietary fiber.

Classification of carbohydrates based on digestibility characteristics has become more common in recent years. While NFC may be an appropriate term to utilize in analyzing digestion in ruminants as all components are fermented by ruminal microbes, NFC's are digested in multiple locations in the horse. Because of this, Hoffman et al. (2001) proposed a way to classify carbohydrates that is more descriptive of equine digestion. Instead of the conventional NDF, NSC, and NFC terms, Hoffman et al. (2001) implemented the terms hydrolysable carbohydrates (CHO-H), rapidly fermentable (CHO-F_r) and slowly fermentable carbohydrates (CHO-F_s). Shirazi-Beechy (2008) found that in the small intestine, CHO-H are hydrolyzed to produce glucose. Meanwhile, cecal and colonic microbiota ferment CHO-F_r and CHO-F_s to produce organic acids (Glinksy et al., 1976; Vermorel et al., 1997).

Carbohydrate Digestion

Digestion and Absorption in the Ruminant

Mechanical digestion of carbohydrates begins with mastication in the mouth. From there, broken-down feed particles enter the rumen, where peristalsis and antiperistalsis take place. Peristalsis moves feed particles from anterior to posterior regions of the rumen. Antiperistaltic contraction is responsible for regurgitation and eructation. Regurgitation is the act of moving feedstuffs back through the esophagus into the mouth for further mechanical breakdown (Clark, 1956). Ruminants do not have salivary amylase to aid in breakdown of carbohydrates (McDougall, 1948). Instead, microbes in the rumen are responsible for breakdown of carbohydrates. Microbes utilize special enzymes that breakdown feedstuffs to yield end products, such as volatile fatty acids (VFA), that are utilized by the animal (NRC, 2016).

Ruminants can digest a wider range of carbohydrates than monogastrics. This is attributed to an extensive microbial population (bacteria, fungi, protozoa, yeast, and archaea) within the rumen. The first step of carbohydrate digestion in the rumen is anaerobic fermentation. While protozoa and fungi contribute to carbohydrate digestion, most fermentation is bacterial. This process hydrolyzes polysaccharides into hexoses and pentoses. From there, hexoses and pentoses are converted to acetic acid, propionic acid, butyric acid, and methane (Sutton, 1971).

Diets high in starch lead to decreased degradation of NDF due to a decrease in fibrolytic bacteria (Nozière et al., 1996). Compared to NDF, starch is more rapidly degraded in the rumen (Offner et al., 2003) and amylolytic bacteria increase activity with greater starch availability (Nozière and Michalet-Doreau, 1997). Amylolytic bacteria play a key role in degradation of starch molecules, as they produce amylase enzymes required to break down starch. Individual

populations of microbes do not produce all enzymes required for fermentation (Huntington, 1997). This means that a consortium of bacterial species is needed to complete fermentation (Horton, 2017; Cotta, 1992). When there are large amounts of starch and sugars ingested, pH decreases drastically, lactate accumulates, and acidosis can occur.

Rate and degree of carbohydrate digestion are affected by many different factors, including type of substrate, amount of intake, and particle size (Lecher-Doll et al., 1991). These factors affect the ruminal population and products formed during fermentation (Huntington, 1997). Structural carbohydrates (found primarily in roughages) influence rumination, motility, and salivation (Nocek and Russell, 1988). Long forage particles also stimulate reticulorumen contractions and help increase rate of passage for digesta. Since NDF is digested more slowly than starch and sugar, inclusion of forage in feedlot diets decreases rate of carbohydrate digestion and prevents large decreases in ruminal pH (NRC, 2016). While adding roughage may help stimulate production of saliva, which helps to buffer the acidity in the rumen, including feed additives like prebiotics may help further decrease lactic acid accumulation during fermentation (Jung et al., 1993).

Impact of Prebiotics on Microbial Fermentation in the Rumen

Prebiotics are organic compounds that serve as food to targeted microbial populations. They have been used to change activity of intestinal microbiota to favor beneficial microbial communities, thus yielding health benefits to the animal (Schrezenmeir and de Vrse, 2001). In cattle, prebiotics include carbohydrate constituents such as dietary fiber with low digestibility or oligosaccharides (Uyeno et al., 2015). Gibson et al. (2010) stated that for a carbohydrate to be classified as a prebiotic, the compound must be resistant to low pH, fermentable by microbiota,

and increase growth and activity of desired bacteria. Prebiotic properties are influenced by length of the sugar chain and whether the chain consists of glucose, galactose, fructose, mannose, or xylose (Nagaraja and Lechtenberg, 2007).

When consumed in sufficient amounts, prebiotics can stimulate growth of microbes (Gaggla et al., 2010). Stimulating growth of beneficial microbiota can increase resistance to bacterial infections and stimulate immune responses. For example, prebiotics may increase lactic acid bacteria (LAB) like lactobacilli and bifidobacteria. These bacterial communities produce antimicrobial compounds and have been noted to decrease growth of *Escherichia coli* (Shu and Gill, 2002) and *Salmonella* app. (Gill et al., 2001).

While prebiotics have potential to improve microbial balance in the rumen and thus enhance productivity (Uyeno et al., 2015), there is a need for studies to better define the microbiome's response to specific prebiotics. Obtaining deeper insights into the functionality of prebiotics in cattle and may improve overall growth efficiency, productivity, and animal health.

Equines

Foregut Digestion and Absorption

Mechanical digestion of feedstuffs begins with mastication in the mouth, where the horse utilizes maxillary and mandibular cheek teeth (Dacre, 2006). While chewing, saliva is produced by the parotid, mandibular, and sublingual glands (Alexander, 1966). Horses produce small amounts of α-amylase in their saliva to aid in the initial breakdown of carbohydrates (Eckersall et al., 1985). Once particle size is decreased from mastication, moistened feed travels through the esophagus into the stomach.

In the stomach, there is limited carbohydrate digestion and absorption due to rapid rate of passage (Argenzio et al., 1974). The stomach is the smallest organ in the digestive tract and its function includes mixing. Secretion of HCl decreases pH and stimulates hydrolysis of some carbohydrates (Strauch et al., 2017). Once feed passes from the stomach, it enters the small intestine.

The pancreas produces amylase to break down starch into the most basic form of glucose to allow the animal to utilize it (Shirazi-Beechey, 2008). Pancreatic amylase is the primary enzyme responsible for breaking down starch in the small intestine by cleaving α-1,4 linkages (Gray, 1992). Liberated glucose is then transported across the intestinal lining by a sodium-glucose co-transporter (Moran et al., 2010). Horses have limited secretion of α-amylase (Kiensle, 1994). Potter et al. (1994) noted that the upper limit of starch digestion in the small intestine is between 2 to 4 g starch • kg BW^{-1} • $meal^{-1}$, verifying that starch in large quantities can exceed the digestive capacity of the small intestine.

Hindgut Digestion and Absorption

Overflow of starch from the small intestine is susceptible to microbial fermentation in the hindgut. The hindgut is comprised of the cecum and large colon, which collectively accounts for about 60% volume of the total gastrointestinal tract (Hintz and Cymbaluk, 1994). Undigested carbohydrates that enter the cecum affect cecal microbial populations (Warzecha et al., 2017) and production of individual VFAs (Hintz et al., 1971; Medina et al., 2002; Sadet-Bourgeteau et al., 2017). Amylolytic bacteria rapidly ferment high starch concentrates and produce large quantities of organic acid. When a horse is not adjusted to a high-starch diet, buildup of lactic acid can decrease cecal pH, and development of cecal acidosis can occur (Bailey et al., 2003; Al

Jassim and Andrews, 2009). In horses that have been adapted to high-starch diets, lactate is metabolized to propionate and butyrate (Millinovich et al., 2007) by bacteria like *Lactobacillus* and *Streptococcus* (Warzecha et al., 2017).

Reduced pH promotes protonation and increases the rate of VFA absorption (Bergman, 1990). Protonated VFAs are absorbed across epithelial tissues via passive diffusion (Argenzio et al., 1974; Hintz et al., 1978). Acetate is used directly by tissues as an energy source via oxidation (Janson and Lindberg, 2012), propionate is gluconeogenic (Larsen and Kristensen, 2013), and butyrate is metabolized further in epithelial tissues and is an energy source for enterocytes (Daly and Shirazi-Beechy, 2006).

Microalgae as a Feed Source

The term algae signifies simple marine plants that contain chlorophyll and utilize sunlight. There are multiple species of algae, but there are 2 primary classifications based on dimension: macroalgae, which refers to macroscopic algae, and microalgae, which refers to minuscule algae. Microalgae have been a promising food source, as they have been reported to contain proteins, lipids, vitamins, and antioxidants (Sathasivam et al., 2017). Nutrient variability between microalgae species can be vast. For example, protein, lipid, and carbohydrate content can range between 6 to 71%, l3 to 40%, and 4 to 64%, respectively (Koyande et al., 2019). Microalgae have been used in human diets for thousands of years (Caporgno et al., 2019). Because some species have substantial protein content, they have potential as an alternative protein source in animal feed. Utilizing algae as a source of protein is still a relatively new idea and has several advantages in terms of minimal land requirements and freshwater necessities (Caporgno et al., 2019).

The species *Chlorell*a and *Arthrospir*e have amino acid profiles similar to those found in eggs and soybeans (Becker, 2007), making them high quality protein sources. In addition, microalgae are promising sources of polyunsaturated fatty acids such as alpha-linolenic acid (ALA), EPA, and DHA (Raposo et al., 2016). Many microalgae such as *Spirulina, Chlorella*, *Tetraselmis*, *Nannochlorpsis*, *Nitzchia*, and *Navicula* have been evaluated for their impact on ruminants, pigs, poultry, and other animals. Some examples include the impact of dietary *Lithothamnium* on milk protein, fat, and lactose in dairy cows (Wu et al., 2015). Algaes have improved egg quality, fatty acid profiles in egg yolk, and laying performance in chickens (Englmaierova et al., 2013; Halle et al., 2009; Swiatkiewiez et al., 2020; Pulz and Gross, 2004). Furthermore, carcass characteristics and meat quality improvements were noted in rabbits fed *Spirulina platensis* (Peiretti and Meineri, 2011). *Laminaria digitata* increased average daily gain (ADG) in pigs, as well as DHA levels in pig loins (He et al., 2001; Sardi et al., 2006). Even though there are improvements noted when adding algae to other livestock rations, the focus of algae supplementation has been in poultry (Becker, 2007) as a feed source of protein (Spolaore et al., 2006).

Even with growing interest and research on growth performance and characteristics of animals fed algae, there are possible risks of toxic metals, allergenicity, cyanotoxins, pathogens, radioisotopes, and toxic synthetic compounds (Wells et al., 2017). Because of these toxicity concerns with some algae species, there are international and national regulations (Sporlaore et al., 2006; Gouveia et al., 2008). In the United States, the Food and Drug Administration (FDA) is responsible for the regulatory status of algae products and additives, which have Generally Recognized as Safe (GRAS) status. Each heavy metal is considered safe for consumption if concentrations are below a threshold that is specified in part per million (ppm), which varies

depending on the property of the metal (fda.gov). In the European Union (EU), guidelines regarding the use of algae for feed is regulated by the European Food Safety Authority (EFSA; van der Spiegel et al., 2013). This requires that algae that has not been used for food by EU countries before 1997 needs to undergo safety testing (Regulation (EC) N0 258/97; Rychen et al., 2016). Species such as *Spirulla, Chlorella, Dunaliella, Crypthecodinium chonii,* and *Arthospira* have been recognized as safe (GRAS) by the FDA and the EFSA. Interestingly, the WHO has suggested *Spirulla* algae be added to National Aeronautics and Space Administration's (NASA) food for astronauts because it is reported that *Spirulla* has the recommended nutrients and is small and compact for space travel (Karkos et al., 2010).

There are various species of microalgae that have been studied, but of particular relevance to research conducted as part of this thesis is *Nannochloropsis*. *Nannochloropsis* is a marine unicellular algae commonly cultivated for growing the microscopic animal rotifer. Algae has recently become a focus for lipid and biofuel research because *Nannochloropsis* algae grows rapidly in freshwater ponds and seawater (Ma et al., 2014). It is enriched with PUFAs, such as EPA, and has high yields of lipids, roughly 60% of dry weight (Rodolfi et al., 2009). Traditionally, *Nannochloropsis* has been used as feed for aquatic animals and terrestrials (Gouveia et al., 2008). Dietary *Nannochloropsis* has shown promise in enhancing FA composition in meat of poultry, cattle, and swine. Researchers utilizing *Nannochloropsis* have tested FA enrichment in poultry products like eggs and meat (Nitsan et al., 1999; Lemahieu et al., 2013; Gatrell et al., 2015; Gatrell et al., 2017) and toxicology of the algae (Markovits et al., 1992; Kagan and Matulka, 2015; Kafie et al, 2012; Nuño et al., 2012; Kagan et al., 2014). There is no evidence that *Nannochloropsis* poses a safety concern when fed to various animals, and

supplementation of *Nannochloropsis* algae leads to improvement of FA profile by increasing the amount of n-3 in meat tissue.

In Vitro Culture Techniques for Nutritional Research

In the early 1900's, fistulation and cannulation of animals became popular techniques to facilitate study of gastrointestinal compartments of animals. This is done by surgically opening a small passage between the organ and the outside of the animal. Cannulation is an essential procedure to research fermentation of feedstuffs and effects of new feed sources (Castillo and Hernández, 2021). Over the last 40 years, ethical concerns regarding animal welfare have developed, and society has demanded improved treatment of animals used for scientific purposes. Therefore, *in vitro* methods for research are preferable to using live animals when appropriate. Utilizing *in vitro* experiments in nutritional research has several advantages over *in vivo* experiments including (1) cheaper costs, (2) greater experimental power, (3) rapid results, and (4) reduced live animals on trial (Graudejus et al., 2018).

In vitro systems are designed to duplicate the environment of the gastrointestinal tract while allowing researchers to control various parameters (Mould et al., 2005) In order to mimic gastrointestinal juices, buffer must have chemical properties required for microflora to flourish. McDougall's buffer is commonly utilized and incorporates sodium bicarbonate as the buffering compound (McDougall, 1948) and provides similar compounds to saliva to aid microbial fermentation (Camacho et al., 2019).

In vitro systems have been valuable in understanding complex processes throughout digestion and in monitoring microbial fermentation characteristics of ruminal and cecal contents. The first documented *in vitro* system of Tilley and Terry (1963) contained ruminal fluid in sealed

tubes with rubber corks fitted with Bunsen release values. Czerkawski & Breckenridge (1977) developed an *in vitro* system that allowed microbes in ruminal fluid to grow constantly. Later, Pell and Schofield (1993) developed mechanized gas sensors to record continuous gas production. *In vitro* models have evolved to more closely mimic gut environments to allow microbial communities to flourish. Data generated in these systems must be interpreted in context of an *in vitro* environment, however. The rumen is anerobic and facilitates a variety of complex interactions between microbes. This can be difficult to replicate with exact precision as more than 70% of microbes cannot be cultivated successfully (Matthews et al., 2019). In addition, providing feedstuffs to cecal inoculum directly can lead to inaccuracies. The cecum's location and fermentation of feedstuffs in the horse occurs after digestion and absorption in the stomach and small intestine. Thus, application of feedstuffs directly to cecal fluid and its microbes is functionally different than what happens in the horse. Therefore, utilizing *in vitro* systems is more useful in helping researchers identify fermentation trends, rather than nutritional impact on the animal.

Summary

In summary, there are numerous nutritional factors affecting animal growth and activity of gut microorganisms. Protein, lipids, and carbohydrates play major roles in nutrition and impact various mechanisms in the animal. Protein is a primary focus in broiler diets due to an exponential growth rate. Lipids supply energy for maintenance and function. In addition, n-3 lipids improve FA profile in meat, which can be beneficial to consumer health.

Algae has proven to be a viable replacement for soybean meal in poultry diets. Algae can be used to improve FA profile in meat without severe detrimental effects to growth performance.

Since algae is a newer feed source, understanding the effects on the bird and human consumer are important. Furthermore, some algae species have toxic properties and are regulated for animal and human consumption. Many species of algae have been intensely researched; however, effects of *Nannochloropsis* algae are unclear because of varying results observed by different researchers. *Nannochloropsis* may have the ability to increase fatty acid content in broiler meat without jeopardizing growth performance when used as a protein replacement for soybean meal. Due to potential toxicity, the amount of *Nannochloropsis* supplemented must be limited.

Carbohydrates provide energy to the animal and influence fermentation characteristics of the gastrointestinal microbiome. While both ruminal and cecal microbes utilize carbohydrates well, there are issues that arise when these animals are fed high-starch diets, such as declining pH, acidosis, and potential fatalities to the animal.

In vitro experiments have become more prevalent in the last 20 years and can reduce the number of live animal trials while generating data regarding fermentative characteristics. While utilizing an *in vitro* experiment does not exactly replicate *in vivo* environment, it is a valuable tool to predict trends. Using *in vitro* techniques to measure effects of supplements such as prebiotics in ruminal fluid can help characterize how treatments affect fermentation parameters, without jeopardizing health of the animal.

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Chapter 2 - Effect of Dietary *Nannochloropsis* Biomass on Growth Performance and Tissue Characteristics of Broiler Chicks

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Abstract

This study was conducted to evaluate the effects of *Nannochloropsis* biomass on growth performance, fatty acid profile of leg quarters, and histopathology of skeletal muscle when fed to broiler chicks. Day-old male Ross-708 broiler chicks (n = 576) were separated into 72 battery pens (8 birds/pen). Pens (experimental unit) were blocked by tier location within battery and randomly assigned to 1 of 4 dietary treatments consisting of either 0% (control), 2%, 4%, or 10% algae. All diets, formulated to be isocaloric and isonitrogenous, were fed *ab libitum* for 18 d. On d 18, pen weights were recorded and 2 birds per pen were sacrificed for fatty acid, histopathology, and toxicology analyses. Data were analyzed using PROC GLIMMIX with fixed effect of treatment, random effects of battery and tier, and pen as the experimental unit. When compared to control, 2% and 4% algae did not impact average daily gain ($P = 0.1084$) or average daily feed intake ($P = 0.9998$). However, 10% algae decreased ($P = 0.0003$) feed efficiency when compared to all other treatments. Fatty acid profile of leg quarters improved with algae supplementation at all levels as demonstrated by linear increases ($P < 0.0001$) in polyunsaturated and total n-3 fatty acids. There was no impact of treatment on necrosis of muscle fibers ($P = 0.3189$). Arsenic, cadmium, lead and thallium were below detectable limits in the liver for all treatments. Supplementation of *Nannochloropsis* algae to broiler chicks improved fatty acid

profile of muscle tissue without negatively affecting growth performance when fed at 2 and 4% of total diet.

Introduction

Due to an expanding human population and diminishing agricultural land, alternative feedstuffs have received considerable attention over the past several decades[1]. Microalgae has been identified as an innovative and unconventional source of protein and energy in livestock diets[1-4]. *Nannochloropsis,* a genus of algae grown in fresh water, differs from other microalgae due to an absence of *chlorophyll* a and *chlorophyll* b[5]. This single cell organism can provide higher-quality protein when compared to more traditional plant-based ingredients due to its greater concentration of essential amino acids. Similarly, its greater concentration of n-3 fatty acids provides a value-added source of energy and modulates the fatty acid profile of animal products such as eggs, milk, and meat when fed to livestock[2-4, 6-9].

Broiler chicks are the most efficient and fastest growing commercial species in the world[10]. An ingredient that improves the fatty acid profile of chicken meat while contributing to bird performance could have profound impacts on the poultry industry. This would provide health benefits to those consuming chicken, thus increasing consumer demand. Becker[1] and Gatrell et al.[11] reported that broiler chicks supplemented with dietary *Nannochloropsis* algae had increased polyunsaturated and total n-3 fatty acids in breast and thigh meat. Similarly, Nitsan et al.[12] and Tao et al.[13] reported that dietary inclusion of *Nannochloropsis* increased the concentration of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in breast and thigh meat. Omega-3 fatty acids have anti-inflammatory properties and may decrease the risk of cardiovascular disease, insulin resistance, and obesity in people[14-17]. While fatty acid

supplements for people consist of plant oils and fish oil, poultry products have become a primary source of long chain polyunsaturated fatty acids (LC-PUFA) for the consumer[9].

Researchers have reported *Nannochloropsis oceanica* supplementation had no detrimental effects on bird health when fed at levels as high as 16% of the diet[12,18]. Supplementation of *Schizochytrium* algae[19, 20] and *Kappaphycus alvarezii1*[21] algae also had no effect on growth performance in broiler chickens. However, supplementation with some species of algae is detrimental to growth performance, with reduced feed efficiency reported with 10% *Staurosira* algae[17] and 20%[22] and 21%[23] *Spirulina* algae.

The purpose of this study was to evaluate the effect of *Nannochloropsis* supplementation on growth performance (average daily gain, dry matter intake, feed efficiency, morbidity, and mortality), fatty acid profile of leg quarters, and toxicology and histopathology of tissue in broiler chicks.

Materials & Methods

All procedures related to housing, care, and handling of birds were approved by the Kansas State University Institutional Animal Care and Use Committee. This experiment was performed as a randomized complete block design with 18 replicates of 4 treatments. Treatments consisted of dietary inclusion level of *Nannochloropsis* algae at 0, 2, 4, and 10% of total diet. Cage was the experimental unit (n = 72), with each cage initially containing 8 birds (576 total birds).

Birds and Facilities

Day old male Ross-708 broiler chicks were obtained from Aviagen® (Sallisaw, OK) and transported to the Kansas State University Poultry Research Center (Manhattan, KS). Chicks were housed in battery cages (119.38 cm x 236.22 cm) equipped with mesh wire flooring and trough-style feeders and waterers. Batteries consisted of 6 tiers with 4 cages per tier and were housed in a temperature and ventilation-controlled building. Birds were inspected and counted by a technician twice daily.

Diets

Diets (Table 2.1) were formulated to be isocaloric and isonitrogenous and included 4 rates of *Nannochloropsis* algae (Naturally Better Omega-3 Technology (NBO3), Manhattan, KS): 0 (control), 2, 4, and 10% algae. Diets were manufactured at the O. H. Kruse Feed Technology and Innovation Center (Kansas State University, Manhattan, KS). Corn was ground to 700 μm using a roller mill. Final mash was steam conditioned with a 25.5 x 140-cm twin staff pre-conditioner (Model 150; Wenger, Sabetha, KS) and pelleted (1012-2 HD Master Model; California Pellet Mill, Crawfordsville, IN) through a 0.4 x 3.175 cm die. Diets were analyzed via wet chemistry by Dairy One, Inc. (Ithaca, NY; Table 2.2) and for fatty acid content by NBO3 Technologies (Manhattan, KS; Table 2.3).

Birds were provided *ab libitum* access to feed and water throughout the duration of the study. On d 18, residual feed was removed from each feeder and weighed. The following calculations were performed.

Equation 2.1. Total feed consumed per pen = ***Feed issued – feed recovered***

Equation 2.2. Daily intake per bird = ***Total feed consumed ÷ [final head count in pen x total days on feed]***

Cage Weights

On d 1, eight birds were placed into a tared box (50 cm x 34 cm x 40 cm) and, if needed, reassigned to obtain a collective weight of 371 ± 6 g per pen. On d 18, birds were counted, placed into a tared box, and weighed to determine ending cage weight, which was used to calculate average daily gain (ADG) using the following equation.

Equation 2.3. Average daily gain (ADG) = ***[initial pen weight – ending pen weight] / [final head count in pen x total days on feed]***

Fatty Acid Analyses

On d 18, 1 chick from each cage was euthanized by cervical dislocation. Legs and thighs were removed and transported to NBO3 (Manhattan, KS) for measurement of fatty acid methyl esters (FAME) via a HP 7890B gas chromatographer with flame ionization detector equipped with a SP-2560 capillary column (100 m x 0.25 mm x 0.2 µm film; Supelco, Inc., Bellefonte, PA). Injection port and detector temperatures were 250° C with a flow rate of 1 mL N/min, split ratio of 100:1, and 1 µL injection volume. Oven temperature began at 100° C with a 5-min hold, increased 3° C/min to 240° C, and was held at 240° C for 20 min. Concentration of individual FAMES were calculated using a 37 fatty acid methyl ester (FAME) standard (Supelco, Inc., Bellefonte, PA). Additional standards (Matreya, LLC., #1254, 1255, 1256, 1257, 1263, 1267)

were used to identify peaks for conjugated linoleic acids (CLAs), C18:1*cis*-11 and C18:1*trans*-11.

Histopathology

On d 18, 1 chick from each cage was sacrificed by cervical dislocation and immediately transported to the Kansas State University Veterinary Diagnostic Laboratory (Manhattan, KS). A technician blinded to treatment collected representative samples of cardiac muscle (sections containing left ventricular free wall, interventricular septum, and right ventricular free wall), brain (cerebrum, cerebellum), lung, liver, spleen, kidney, skeletal muscle (pectoral), duodenum, pancreas, colon, and cecum. Tissues were collected directly into 10% neutral buffered formalin and fixed for at least 48 h prior to trimming into cassettes for routine processing. Samples were processed with a tissue processor and embedded in paraffin wax. Three sections of each tissue were cut on a microtome and mounted on glass slides prior to staining with hematoxylin and eosin (HE).

Histologic analyses were carried out at 200x and 400x magnification using a standard bright field Olympus microscope. Necrosis of skeletal myofibers within pectoral muscles was observed in birds of all groups; these lesions ranged in severity from mild to moderate. A scoring system was developed (Table 2.4) to describe necrosis of muscle to aid in semi-quantitative assessment of these lesions for statistical analysis. Briefly, the total number of individual necrotic and degenerate skeletal myofibers were assessed in representative tangential sections of skeletal muscle. A total of five 200x fields of view were quantified. A score was assigned to each bird on a scale of 0 to 10 (0 to 33.5% of necrotic skeletal myofibers) per field of view (Figure 2.1; 300 skeletal myofibers per field).

Toxicology

On d 18, chicks sacrificed for histopathology and toxicology were immediately transported to the Kansas State University Veterinary Diagnostic Laboratory (Manhattan, KS). Liver tissue was digested using trace metal grade nitric acid in preparation for heavy metal determination. Samples were analyzed for arsenic (As), cadmium (Cd), lead (Pb), and titanium (Ti) using an ICP-MS (PerkinElmer, NexION 350X, Waltham, Massachusetts). Bismuth, germanium, and rhodium served as internal standards. All runs included a control of bovine liver trace elements and methylmercury in muscle tissue standards (freeze-dried; National Institute of Standards and Technology Gaithersburg, MS) as appropriate for verification of instrument accuracy.

Statistical Analyses

Data were analyzed using PROC GLIMMIX procedure of SAS (v. 9.4, SAS Institute, Inc., Cary, NC). Fixed effect was treatment, random effect was block, and pen was the unit. Treatments were blocked by battery and tier. Linear and quadratic contrasts were used. Significance was declared if $P \leq 0.05$ and a tendency was denoted by $0.05 < P \leq 0.10$. The PDiff option in SAS was used to determine the differences among least squares means.

Results

Growth Performance

Average daily gain ($P = 0.1084$) and average daily feed intake (ADFI; $P = 0.998$) were similar across all treatments (Table 2.5). Feed efficiency (FE; gain:feed) decreased with 10%

algae when compared to all other treatments ($P = 0.0003$). Mortality was unaffected by treatment ($P = 0.3134$).

Fatty Acid Analyses

All fatty acid response variables were affected by treatment (Table 2.6; $P \leq 0.0038$). Percentage of total fatty acid, saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and total n-6 fatty acids were similar among birds consuming 0, 2, and 4% algae ($P > 0.05$) but increased ($P < 0.0001$) in those consuming 10% algae. Percentage of polyunsaturated fatty acids (PUFA), eicosapentaenoic acids (EPA), docosapentaenoic acids (DPA), docosahexaenoic acid (DHA), and total n-3 fatty acids increased linearly ($P < 0.0001$) and quadratically ($P < 0.0001$) with increasing algae supplementation. Total n-6:n-3 decreased linearly ($P < 0.0001$) as inclusion level of algae increased.

Histopathology & Toxicology

Significant histologic lesions were not observed in the following tissues regardless of treatment: brain, lung, spleen, kidney, duodenum, pancreas, colon, and cecum. Birds in all treatment groups including control (0% algae) had mild multifocal aggregates of granulocytes present within various regions of the myocardial interstitium, as well as mild peri-biliary lymphocytes and rarely heterophils; however, all were within normal limits.

All birds, regardless of treatment, had skeletal muscle necrosis in pectoral tissue, characterized by myofiber swelling, hypereosinophilia, sarcoplasmic fragmentation, nuclear pyknosis, and rarely mineralization. Skeletal muscle lesions scores ranged in severity from 1 to 7

on a scale of 0 to 10. There was no effect of treatment on necrosis and degeneration of muscle fibers (P = 0.3189; Figure 2.2).

Hepatic concentrations of As, Cd, Pb, and Ti were below detectable limits with all treatments. Detection limits were as follows: As < 2 ppm, Cd < 0.5 ppm, Pb < 0.5 ppm, and Ti < 2.5 ppm.

Discussion

Dietary inclusion of *Nannochloropsis* in a chick starter ration at 2, 4, or 10% of total diet had neither beneficial nor detrimental effects on ADG, ADFI, or mortality when compared to a control. Broiler growth responses to dietary algae are often affected by feeding rates, with negative effects observed when algae intake is high[10,18]. Others have demonstrated no effect on growth performance in broilers in response to *Nannochloropsis* supplementation up to 8% inclusion; however, ADG and G:F decreased in chicks consuming 16% algae[10, 18]. Tao et al.[12] reported growth performance of broiler chickens fed 10% *Nannochloropsis* algae for 6 wk was similar to control. Ross and Dominy[22] found that isonitrogenous diets containing 5, 10, and 15% *Spirulina plantensis* algae had no effect on ADG of broilers compared to a control. When included at 20% of the diet, ADG was reduced as early as the first week. Chicks fed 10 and 20% algae also grew slower during the third week in comparison to chicks in the control group. Evans et al.[23] fed isocaloric diets with or without *Spirulina plantensis* algae to broiler chickens and reported decreased ADG and ADFI with 21% algae. Likewise, Austic et al.[17] found reduced ADG, ADFI, and FE when including more than 7.5% *Staurosia* algae in broiler chick diets. In the current study, there was reduced FE in birds consuming 10% *Nannochloropsis*. Gatrell et al.[18] also found reduced FE in birds consuming a diet containing 16% *Nannochloropsis*.

In the current study, total n-3 fatty acids, DPA, and EPA concentrations in leg quarters increased linearly with increasing inclusion rates of algae, which is consistent with previous reports. Gatrell et al.[10] and Tao et al.[12] reported greater concentrations of EPA, DHA, and total n-3 fatty acids in thigh and breast meat of broiler chickens consuming *Nannochloropsis* algae when compared to those on control diets. Other species of algae have elicited similar effects. Dietary inclusion of *Schizochytrium* algae increased DHA, PUFA, and total n-3 fatty acid concentrations of breast meat[19, 24]. Likewise, *Crypthecodinium cohnii* algae fed to Muscovy ducks increased DHA, PUFA, and total n-3 fatty acid concentrations of breast meat[13]. The diets, including those with *Nannochloropsis*, utilized in this experiment did not contain DPA or DHA. Consequently, increased n-3 fatty acids in tissue of birds fed *Nannochloropsis* was synthesized by the bird from PUFA and EPA provided in diets. Desaturation of PUFA produces EPA, which is then elongated to DPA, and then to tetracosapentaenoic acid. Tetracosapentaenoic acid then undergoes desaturation which eventually leads to synthesis of DHA[25]. Clearly, dietary inclusion of algae improves fatty acid profile of poultry meat. By supplementing this sustainable ingredient, producers can raise and potentially market a value-added product to consumers. Humans consuming increased levels of EPA and DHA have reduced risks of certain metabolic diseases and meta-inflammation[26].

Dietary inclusion of *Nannochloropsis* algae at all levels had no effect on histopathology parameters, including necrotic myofibers and lesion scores. Additionally, consumption of *Nannochloropsis* algae had no effect on tissue toxicology as assessed by liver concentrations of As, Cd, Pb, and Ti. Similar findings have been reported in rats. *Nannochloropsis* algae had no effect on hematological indices in Sprague-Dawley rats when fed at up to 10% of total diet[27]. Furthermore, Kafaie et al.[28] found no pathological changes or heavy metal toxicity in Sprague-

Dawley rats fed 0, 3, 6, 12 g *Nannochloropsis*/kg body weight (BW). Kagan et al.[29] showed that rats consuming diets at up to 10 mL/kg BW/rat of *Nannochloropsis* algae had no abnormal pathological lesions or heavy metal toxicity. Similar findings have been reported with a variety of other algae species. Neumann at al.[30] evaluated the effects of *Chlorella vulgaris*, *Phaeodactylum tricornutum*, *and Nannochloropsis* at 0, 5, 15, and 25% of total diet in C57BL/6 mice and found no histological or toxicological abnormalities in liver, gastrointestinal tract, heart, or kidneys due to treatment. Venkataraman et al.[31] supplemented *Spirulina plantensis* algae (40 g/kg BW and 170 g/kg BW) to broiler chicks for 12 wk and reported normal histopathology in liver, heart, and kidneys. In fact, Venkataraman et al.[31] found that hepatic metal concentrations were greater in birds consuming the control diet, which consisted of groundnut cake, vitamins/minerals, and fish meal, compared to birds consuming algae-based diets. Based on these results, there is no apparent immediate health risk to birds consuming *Nannochloropsis* algae. Similarly, meat harvested from algae-fed birds does not pose a heavy metal toxicity threat to the human food supply chain.

Conclusion

Dietary inclusion of *Nannochloropsis* algae at up to 4% of total diet in broiler chicks improves the fatty acid profile of leg quarters without negatively affecting growth performance or histopathology and toxicology of tissue. While diets with 10% *Nannochloropsis* algae further improved the fatty acid profile of leg quarters, G:F was reduced. Thus, *Nannochloropsis* algae can be used as a supplement to increase n-3, EPA, and DHA content in poultry products. High inclusion rates reduce feed efficiency. As a result, producers should consider whether decreased feed efficiency is offset by increased market value. Poultry meat with enhanced n-3 content

could be perceived by consumer to have greater health benefits than conventional poultry. If margins are tight and feed efficiency cannot be sacrificed, then producers may want to limit inclusion to 4%.

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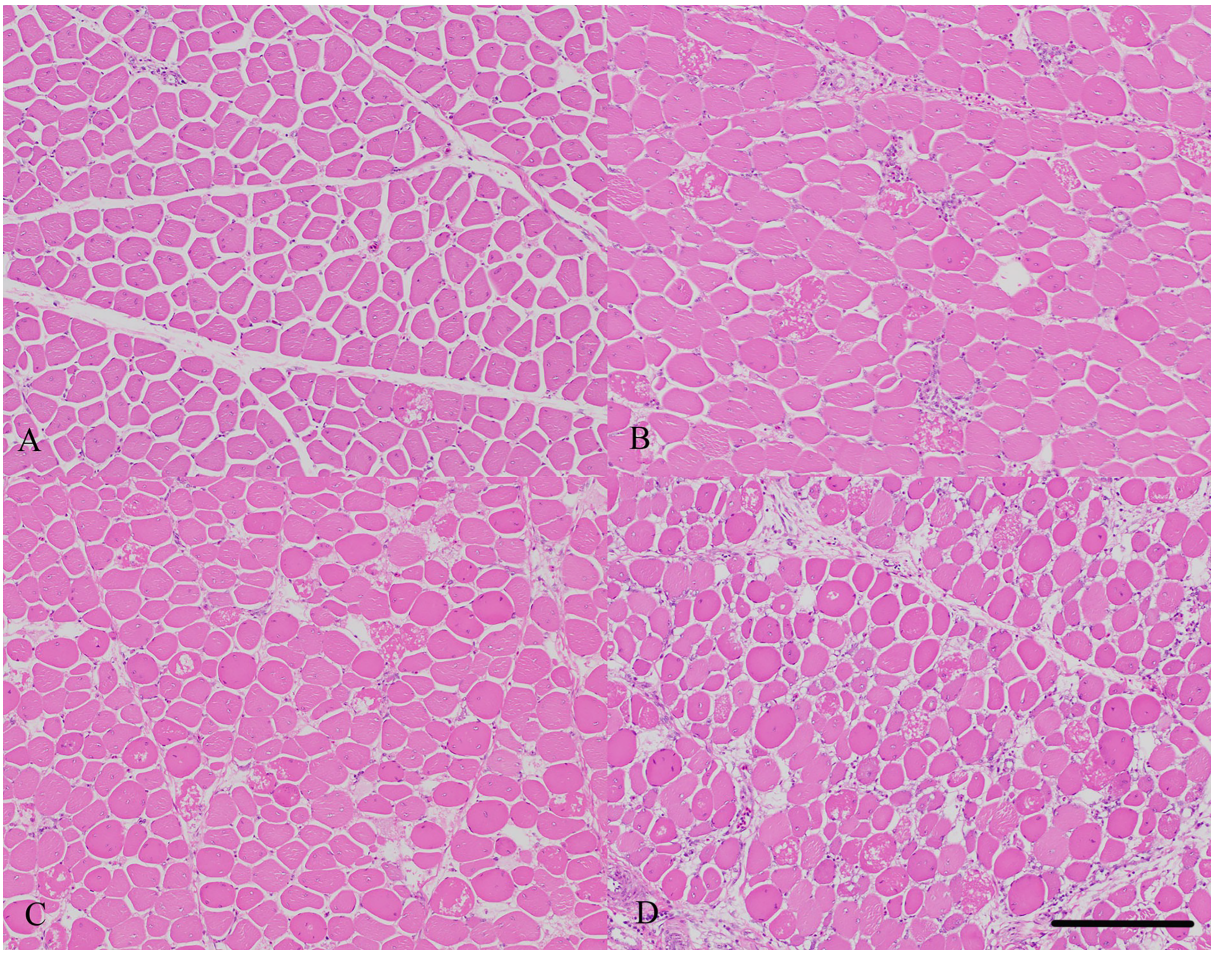


Figure 2.1. Skeletal muscle lesions: A scoring system was developed to assess necrosis in muscle tissues where a 0 indicated no necrosis and a 10 indicated 30% necrosis. Scores of 1 (panel A), 3 (panel B), 5 (panel C), and 7 (panel D) are shown. All images taken at 200 x magnification, bar = 200 μm.

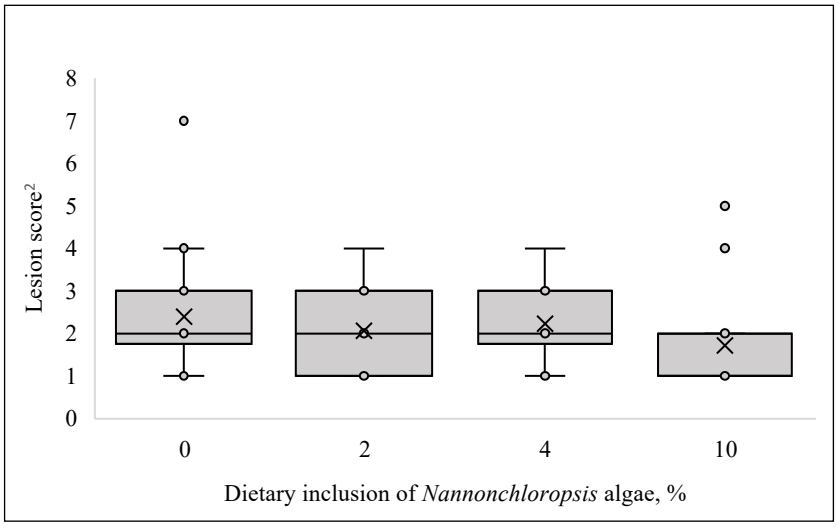


Figure 2.2. Effect of *Nannochloropsis* algae[1] supplementation on degeneration and necrosis of pectoral skeletal muscle in broiler chick

[1]*Nannochloropsis* algae, NBO3 Technologies, Manhattan, KS

[2]See table 2.3 for lesion scoring scheme

Table 2.1. Composition of experimental diets[1]

	Nannochloropsis algae[2], %			
Ingredient, %	0	2	4	10
Corn	56.67	56.68	56.69	56.49
Soybean meal, 48%	35.91	34.10	32.28	26.88
*Nannochloropsis a*lgae[2]	0.00	2.00	4.00	10.00
Soybean oil	2.92	2.92	2.92	3.01
Limestone	1.58	1.59	1.60	1.63
Salt	0.72	0.56	0.40	0.00
Monocalcium phosphate, 21%	1.49	1.46	1.43	1.34
L–Lysine hydrochloride	0.28	0.28	0.28	0.26
DL-Methionine	0.14	0.14	0.13	0.12
L-Threonine	0.04	0.03	0.03	0.01
Vitamin and mineral premix[3]	0.25	0.25	0.25	0.25

[1]Diets were pelleted through a 3-mm die, cooled, crumbled, and provided *ad libitum.*
[2]*Nannochloropsis* algae, NBO3 Technologies, Manhattan, KS
[3]Nutrablend Poultry VTM premix, Neosho, MO

Table 2.2. Proximate analysis (dry matter basis) of experimental diets[1]

	Nannochloropsis algae[2], %			
Item	0	2	4	10
Crude protein, %	24.30	24.10	23.30	24.30
Acid detergent fiber, %	6.30	3.50	3.30	3.10
Neutral detergent fiber, %	11.30	8.60	7.80	8.00
ME[3], Mcal/kg	3.46	3.50	3.51	3.52
Calcium, %	1.14	1.17	1.41	1.41
Phosphorus, %	0.86	0.84	0.86	0.88
Magnesium, %	0.20	0.19	0.19	0.19
Potassium, %	1.11	1.09	1.08	1.15
Sodium, %	0.35	0.33	0.37	0.37
Iron, mg/kg	358.00	392.00	490.00	585.00
Zinc, mg/kg	133.00	210.00	268.00	261.00
Copper, mg/kg	21.00	20.00	23.00	21.00
Manganese, mg/kg	125.00	155.00	199.00	180.00
Molybdenum, mg/kg	0.90	1.10	1.10	1.30

[1]Analysis conducted by Dairy One, Inc., Ithaca, NY

[2]*Nannochloropsis* algae, NBO3 Technologies, Manhattan, KS

[3]ME = Metabolizable energy

Table 2.3. Proximate fatty acid analysis of experimental diets[1]

	Nannochloropsis algae[2], %			
Item	0	2	4	10
Total fatty acid, %	5.73	5.70	5.79	6.59
Total calculated fat, %	6.37	6.33	6.44	7.32
SFA[3], %	0.81	0.84	0.88	0.95
MUFA[4], %	2.60	2.56	2.68	2.94
PUFA[5], %	2.32	2.29	2.23	2.69
Total n-3 fatty acids, %	0.34	0.37	0.39	0.58
Eicosapentaenoic acid (C20:5 n-3), %	0.00	0.04	0.07	0.21
Docosapentaenoic acid (C22:5 n-3), %	0.00	0.00	0.00	0.00
Docosahexaenoic acid (C22:6 n-3), %	0.00	0.00	0.00	0.00
Total n-6 fatty acids, %	1.97	1.89	1.80	2.09
Total n-6:n-3	5.71	5.06	4.57	3.58

[1]Analysis conducted by NBO3 Technologies, Manhattan, KS
[2]*Nannochloropsis* algae, NBO3 Technologies, Manhattan, KS
[3]Total Saturated Fatty Acid = [C8:0] + [C10:0] + [C12:0] + [C14:0] + [C16:0] + [C17:0] + [C18:0] + [C20:0] + [C22:0] + [C24:0]
[4]Total Monounsaturated Fatty Acid = [C14:1] + [C16:1] + [C16:1n-10] + [C18:1] + [C18:1n-7] + [18:1n-9] + [C18:1n-12] + [C20:1] + [C20:1n-9] + [C20:1n-11] + [C22:1] + [C24:1]
[5]Total Polyunsaturated Fatty Acid = [C16:3] + [C18:3] + [C18:4] + [C20:3] + [C20:4] + [C20:5] + [C21:5] + [C22:5] + [C22:6] + [C24:5] + [C24:6]

Table 2.4. Scoring system utilized for histopathological analysis of pectoral skeletal muscle[1]

Necrotic myofibers, #[2]	Myofibers affected, %[3]	Score[4]
0	0	0
1 to 10	0.50 to 3.49	1
11 to 20	3.50 to 6.49	2
21 to 30	6.50 to 9.99	3
31 to 40	10.0 to 13.49	4
41 to 50	13.50 to 16.49	5
51 to 60	16.50 to 19.99	6
61 to 70	20.00 to 23.49	7
71 to 80	23.50 to 26.49	8
81 to 90	26.50 to 29.99	9
91 to 100	30.00 to 33.50	10

[1]Pectoral muscle of broiler chicks
[2]Number of myofibers affected per 200 x field in 5 random fields.
[3]Percent of myofibers affected per 200 x field (300 fibers per field) in 5 random fields.
[4]A single score was recorded for each bird representing the mean score assigned to each of five 200 x fields

Table 2.5. Effect of *Nannochloropsis* algae supplementation on broiler chick growth performance.

	Nannochloropsis algae[1], %					P-Value		
Item	0	2	4	10	SEM	Treatment	Linear	Quadratic
Average daily gain, g/d	39.01	38.97	39.31	37.66	0.53	0.1084	0.1049	0.1169
Average daily feed intake, g/d	48.62	48.61	48.56	48.55	0.60	0.9998	0.9276	0.9980
Gain: feed	0.80[a]	0.80[a]	0.81[a]	0.78[b]	0.0063	0.0003	0.0047	0.0045
Mortality, %	1.39	3.47	0.69	4.17	1.59	0.3134	0.4115	0.6456

[1]*Nannochloropsis* biomass, NBO3 Technologies, Manhattan, KS

[a, b] Means within a row with differing superscripts are different, $P \leq 0.05$

Table 2.6. Effect of *Nannochloropsis* algae on fatty acid profiles of thigh tissue of broiler chicks.

	Nannochloropsis algae[1], %					P - Value		
Item	0	2	4	10	SEM	Treatment	Linear	Quadratic
Total fatty acid, %	6.10[a]	6.03[a]	6.30[a]	7.31[b]	0.20	< 0.0001	< 0.0001	0.0100
SFA[2], %	1.56[a]	1.52[a]	1.55[a]	1.78[b]	0.05	0.0038	0.0049	0.0168
MUFA[3], %	2.99[a]	2.96[a]	3.07[a]	3.57[b]	0.11	0.0009	0.0006	0.0262
PUFA[4], %	1.55[a]	1.55[a]	1.67[b]	1.96[c]	0.04	< 0.0001	< 0.0001	0.0007
Total n-3 fatty acids, %	0.24[a]	0.26[b]	0.32[c]	0.41[d]	0.0072	< 0.0001	< 0.0001	< 0.0001
Eicosapentaenoic acid (C20:5 n-3), %	0.01[a]	0.021[b]	0.035[c]	0.084[d]	0.0013	< 0.0001	< 0.0001	< 0.0001
Docosapentaenoic acid (C22:5 n-3), %	0.02[a]	0.03[b]	0.04[c]	0.06[d]	0.001	< 0.0001	< 0.0001	< 0.0001
Docosahexaenoic acid (C22:6 n-3), %	0.02[a]	0.02[a]	0.03[b]	0.04[c]	0.0013	< 0.0001	< 0.0001	0.1373
Total n-6 fatty acids, %	1.29[a]	1.27[a]	1.33[a]	1.52[b]	0.03	< 0.0001	< 0.0001	0.002
Total n-6:n-3	5.40[a]	4.91[b]	4.22[c]	3.71[d]	0.02	< 0.0001	< 0.0001	0.7215
Eicosapentaenoic acid (C20:5n-3), mg / 4 oz. serving	11.54[a]	24.51[b]	42.44[c]	96.66[d]	1.46	< 0.0001	< 0.0001	< 0.0001
Docosapentaenoic acid (C22:5n-3), mg / 4 oz. serving	25.91[a]	34.33[b]	44.82[c]	67.65[d]	0.99	< 0.0001	< 0.0001	< 0.0001
Docosahexaenoic acid (C22:6n-3), mg / 4 oz. serving	21.65[a]	25.33[b]	30.46[c]	39.83[d]	1.29	< 0.0001	< 0.0001	0.0286
Total n-3 fatty acids, mg/ 4 oz. serving	271.29[a]	293.21[a]	358.10[b]	464.63[c]	7.91	< 0.0001	< 0.0001	< 0.0001

[1]*Nannochloropsis* algae, NBO3 Technologies, Manhattan, KS

[a,b,c,d] Means within a row without common superscripts are different P < .0001

[2]Total Saturated Fatty Acid = [C8:0] + [C10:0] + [C12:0] + [C14:0] + [C16:0] + [C17:0] + [C18:0] + [C20:0] + [C22:0] + [C24:0]

[3]Total Monounsaturated Fatty Acid = [C14:1] + [C16:1] + [C16:1n-10] + [C18:1] + [C18:1n-7] + [18:1n-9] + [C18:1n-12] + [C20:1] + [C20:1n-9] + [C20:1n-11] + [C22:1] + [C24:1]

[4] Total Polyunsaturated Fatty Acid = [C16:3] + [C18:3] + [C18:4] + [C20:3] + [C20:4] + [C20:5] + [C21:5] + [C22:5] + [C22:6] + [C24:5] + [C24:6]

Chapter 3 - Carbohydrate Sources as Prebiotics to Stimulate Fermentation by Mixed Ruminal Microbes: Impact on in Vitro Fermentation and End-Product Formation

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Abstract

To assess the impact of various carbohydrates on fermentation by bovine ruminal microorganisms. To do this, ruminal fluid was collected and used to inoculate fermentation bottles containing buffer, forage, and a supplemental carbohydrate source. Bottles contained 5 g of forage (prairie hay, smooth bromegrass hay, or alfalfa) and 0.1 g of supplemental carbohydrate (glucose, fructose, galactose, xylose, mannose, arabinose, rhamnose, lactose, sucrose, melibiose, maltose, isomaltose, trehalose, stachyose, raffinose, raw corn starch, hydrolyzed corn starch, cellulose, sugar cane molasses, or beet molasses). Bottles were equipped with continuous gas pressure monitors and incubated in duplicate for 48 h using the ANKOM RF System (ANKOM Technology, Macedon, NY). Bottles were then opened and terminal pH, *in vitro* dry matter disappearance (IVDMD), *in vitro* neutral detergent fiber disappearance (IVNDFD), and volatile fatty acid (VFA) concentrations were measured. Data were analyzed using the PROC GLIMMEX procedure for terminal pH, IVDMD, IVNDFD, and VFAs with fixed effect of carbohydrate source x forage. Total gas production data were analyzed via NLIN and the MIXED procedure. Total gas production was not affected by carbohydrate source in

cultures containing prairie hay or bromegrass ($P \geq 0.06$). However, total gas production increased with cellulose, galactose, and hydrolyzed corn starch ($P \leq 0.05$) in cultures containing alfalfa. Terminal pH and IVDMD were not affected by carbohydrate source in cultures containing prairie hay, bromegrass, or alfalfa. There was no impact of carbohydrate source on IVNDFD in cultures with prairie hay and alfalfa. However, cultures containing bromegrass had a greater IVNDFD with isomaltose and galactose when compared to control ($P = 0.038$). Several changes were observed in cultures supplied with prairie hay. Cellulose reduced acetate compared to control ($P = 0.003$), while the control had a greater A:P ratio when compared to trehalose, sucrose, raffinose, melibiose, mannose, maltose, lactose, glucose, galactose, fructose, and cellulose ($P \leq 0.05$). Meanwhile, butyrate concentration was greater ($P = 0.010$) in cultures containing arabinose, and cellulose led to a greater concentration of valerate ($P = 0.007$) and caproate ($P = 0.003$) when compared to control. Control cultures had greater methane production compared to those with cellulose, fructose, and isomaltose ($P = 0.001$). Finally, total VFA concentration was greater with maltose when compared to the control with prairie hay alone ($P = 0.015$). In cultures containing bromegrass, a greater A:P ratio was produced in cultures containing arabinose, glucose, isomaltose, lactose, melibiose, molasses, raffinose, rhamnose, sucrose, and trehalose when compared to control ($P < 0.0001$). In cultures containing alfalfa, there was a difference in A:P ratio between the control and all treatments ($P = 0.006$). Because most alterations to fermentative parameters were observed with prairie hay, it seems that carbohydrates have most potential to improve digestion of poor-quality forages.

Introduction

The complex ruminal environment is continuously changing in response to the composition of substrates present. Bacteria, archaea, protozoa, and fungi respond to dietary alterations, which are reflected in microbial metabolism. Because carbohydrates comprise 60 to 70% of typical diets for cattle, it is important to understand how carbohydrates and sugars alter ruminal function (Varga et al., 2019). Carbohydrate type drives rate of fermentation and concentration of varying end products (Russell, 1984). The energy disbursed by microbial growth is directly linked to digestion of starches, sugars, and pectin (Hoover and Strokes, 1991).

Ruminal bacteria employ a multitude of metabolic pathways and have the capability to convert substrates into a variety of end products, which impacts the proportion of energy harvested. For example, bacterial metabolism of a single carbohydrate can lead to increased production of methane and other end products not used by the animal, while other end products may be metabolized and are a valuable source of energy. By considering what is known about fermentation and digestion of various substrates, it is possible to make predictions of animal performance based on dietary composition (Russell, 1984).

In the rumen, nonstructural carbohydrates are fermented rapidly. When cattle are fed diets that contain glucose, sucrose, and fructose, there is rapid production of volatile fatty acids (VFAs) and a decline in pH (Eldsen, 1945). However, fermentation varies between monosaccharides and disaccharides (Oba, 2010). Weisbjerg et al. (1998) reported lactose had a slower fermentation rate when compared to sucrose and glucose. Sutton (1968) intubated glucose, fructose, galactose, xylose, and arabinose in the rumen of sheep and found glucose and fructose were almost completely fermented and resulted in greater VFA production compared to other carbohydrates. Likewise, Cullen et al. (1986) reported that pentoses (arabinose, ribose, and

xylose) did not reduce ruminal pH as drastically as hexose sugars (fructose, glucose, and galactose) in sheep. Furthermore, fermentation of hexoses and disaccharides (maltose, sucrose and cellobiose) yielded greater concentrations of lactate (Dunlop, 1972). Acidosis results from the accretion of organic acids, including lactate, that causes a decline in pH sufficient to impact microbial activity, ruminal function and the overall health of the animal (Nagaraja and Lechtenberg, 2007; Xu and Ding, 2011).

Major modifiers of ruminal fermentation are pH and turnover rate, which are affected by nutritionally associated features like feed intake, feeding strategies, and forage quality (Hoover & Strokes, 1991). Forage quality and digestibility are critical to ruminants, given that forage is typically a major component of the diet. Forage quality can be assessed by determining the amount of supplementation required to meet the animal's requirement for energy and protein (Buxton et al., 1996). There is an inverse relationship between neutral detergent fiber (NDF) and forage digestibility. Neutral detergent fiber and lignin content increase as forages become more mature (Merchen and Bouriquin, 1994). Most legumes (alfalfa) have increasing NDF and lignin content in stems as the forage matures, but a similar increase is not observed in leaves. Thus digestibility of stems decreases with maturity, whereas leaves stay relatively digestible (NRC, 2016). Van Soest (1994) reported that grass hays contain more NDF than legumes; however, legumes have greater lignin content. Leng (1990) further defined a low-quality forage as less than 55% digestible and deficient in crude protein (CP). Brito et al. (2009) found that dairy cows had greater dry matter intake (DMI), total tract digestibility of organic matter, and ruminal pH when consuming forage with more water-soluble carbohydrates (WSC). In order to minimize the need for supplementation, it is critical to maximize forage quality. When forage and concentrate are fed together, there are associative effects based on digestibility and metabolic interactions

between the two (Merchen and Bouriquin, 1994; Dixon and Stockdale, 1999). Forage intake is positively associated with rumination time, while intake of starch negatively impacts rumination (Byskov et al., 2015). Inclusion of forage into a high starch diet leads to slower digestion of carbohydrates and decreased rate of VFA production (Yang and Beuchemin, 2006). Because forage intake is positively associated with rumination, there is generally a corresponding increase in methane production (Nagaraja and Lechtenberg, 2007).

Methane comprises about 8% of greenhouse gas emissions on Earth and cattle produce about a quarter of that percentage (Varga et al., 2019). On average, about 8 to 12% of digestible energy in cattle diets is converted to methane gas (Tamminga, 1992) and the amount of methane produced by cattle can approach 400 L per day. In recent years, there has been focused research on methane production by altering dietary variables (Ellis et al., 2007). Swift et al. (1948) identified a correlation between carbohydrate intake and methane production. Czerkawski and Breckenridge (1969) verified methane production was not due to type of carbohydrate, but rather amount. Later, Moe and Tyrrell (1979) reported that methane production is influenced by amount of intake and total digestibility of the carbohydrates. Blaxter and Wainman (1964) reported that forage to grain ratio also has an impact on methane production. As forage increases, methane production increases. Forages decrease rate of fermentation, leading to increased methane production. Diets rich in starch, soluble sugars, and legume-type forages can reduce methane production.

In vitro methods have been developed to assess ruminal digestibility and fermentation characteristics (Judd and Kohn, 2018). *In vitro* studies have been recognized as affordable alternatives to conducting live animal studies to characterize fermentation parameters. In addition, using an *in vitro* system allows for a greater number of samples to be evaluated in a

shorter time frame than a live trial, making this an efficient use of time and financial resources. It also reduces the number of animals required for research purposes.

The environment of the rumen impacts health of the animal. Prebiotics can be used to decrease pathogenic microbes and prevent toxins from crossing the epithelium (Uyeno et al., 2015). The most common prebiotics are oligosaccharides or carbohydrates that include dietary fiber and can aid in digestibility (Uyeno et al., 2015). Prebiotics serve as a food source to desired microbial populations that yield health benefits to the host (Schrezenmeir and de Vrse, 2001). The purpose of this study was to assess the impact of supplementing different carbohydrate sources on digestibility and fermentation parameters within *in vitro* cultures of ruminal microorganisms fed low, moderate, and high-quality forages.

Methods & Materials

Animals

All animal procedures were approved by the Kansas State University Institutional Animal Care and Use Committee prior to initiation. Two ruminally cannulated Holstein-Jersey cross steers served as donors of ruminal inoculum. Animals were housed in a dry lot pen (50 m^2/steer) and maintained on 17 kg (as-fed) total mixed ration (TMR; Table 3.1).

Experimental Design

This experiment consisted of a split-split-plot design whereby the whole plot consisted of forage (warm season grasses native to Kansas [prairie hay], Smooth Bromegrass hay [bromegrass], and alfalfa), subplot of inoculant donor (steer 1 and steer 2), and sub-subplot of carbohydrate source. Experimental unit at the whole plot, subplot, and sub-subplot level

consisted of run (n = 6), run x steer (n = 12), and fermentation vessel (n = 264), respectively. Each run consisted of a control containing only forage, 20 carbohydrate sources mixed with forage, and blanks without forage or added carbohydrate.

Culture Preparation

Prairie hay, bromegrass, and alfalfa were ground using a Wiley mill (1-mm screen; Thomas Scientific, Philadelphia, PA). Following protocols previously established at Kansas State University (Fehlberg et al., 2018), 5 g dry matter (DM) forage were added to flasks. Then, 0.1 g carbohydrate (DM) was added to 250-mL fermentation flasks (#7056; ANKOM Technology, Macedon, NY). Flasks were placed in a 39°C incubator approximately 12 h before ruminal inoculum was added. Buffer (McDougall's Artificial Saliva) was mixed according to McDougall (1948) but CaCl was excluded. Buffer was refrigerated 12 h before being added to samples. Four hours before inoculation, calcium chloride was added. Buffer pH was corrected to 7.0 with hydrochloric acid (HCl) or sodium hydroxide (NaOH). Buffer was then added to each flask at 125 mL per vessel and warmed to 39°C.

In Vitro Fermentation

Ruminal fluid was collected from 2 fistulated steers housed at the Kansas State University Beef Cattle Research Center via gravity flow through the fistula. Fluid was strained through 8 layers of cheese cloth into 4-L pre-warmed, insulated thermoses. Rumen fluid was transported to the pre-harvest laboratory and re-strained through 8 layers of cheesecloth into separatory funnels. Funnels were degassed with N_2 and placed in an 39°C incubator (Isotemp 550D Incubator Oven, Fischer Scientific, Hampton, NH) for 1 h to allow fluids to separate.

During this time, the computer was started, and modules were checked to ensure they were working properly. The RF Gas production system software (ANKOM Technology, Macedon, NY) was opened, gas release was set to 34.5 kilopascal (kPa), and all modules were confirmed to have pressure of ±0.75 kPa. Then the shaking incubator (G25; New Brunswick Scientific Inc., New Brunswick, NJ) was turned on and heated to 39°C.

Following fluid separation in separatory funnels, the microbe-rich liquid layer was collected into flasks corresponding with the different steers, and pH (Thermo Orion Star A121; Thermo Fisher Scientific Inc., Chelmsford, MA) was measured. Twenty-five milliliters of microbe-rich liquid was placed into prepared bottles with hay, CHO, and buffer. pH of individual bottles was measured and recorded as initial pH. Bottles were gassed with N_2, capped with a pressure monitoring module (RF Gas Production System; ANKOM Technology, Macedon, NY) and placed in the shaking incubator. Start time was recorded for each individual bottle. Samples were monitored every 3 to 6 h to check battery levels and to monitor pressure. Batteries were changed as needed and samples were shaken every 12 h to allow microbes to access substrate that may have been stuck to the bottle.

Bottles were removed from the shaking incubator 48 h later and pressure was manually released by removing the modules. Four milliliters of fermented liquid were removed from each bottle and combined with 1 mL 25% (w/v) meta-phosphoric acid in falcon tubes. Culture pH was measured in fermentation bottles, and remaining contents from each bottle were poured into pre-weighed aluminum pans. Bottles were rinsed with deionized (DI) water to loosen any remaining contents which were transferred into the corresponding aluminum pan. Aluminum pans were placed in a 55°C oven and dried for 2 to 5 d.

Dry Matter Disappearance Measurement

After 2 to 5 d in a 55°C oven, aluminum pans were weighed, and dry matter was placed in a small plastic bag. Samples were stored at room temperature storage. Dry matter disappearance (IVDMD) was determined using the equation:

Equation 3.1. *In vitro* dry matter disappearance (IVDMD).

$$\frac{(Ws - Wp) - [(Wds - Wp) - (Wc - Wp)]}{(Ws - Wp)} \; x \; 100$$

Ws: wet sample and pan weight;

Wds: dried sample and pan weight;

Wc: dry blank sample and pan weight.

Neutral Detergent Fiber Analysis

From each sample, 0.5 g (± 0.05) DM was weighed and placed into an ANKOM Fiber Analyses bag and into a NDF ANKOM Fiber Analyses appliance. Neutral detergent fiber was determined using the Goering and Van Soest Method (1970). Each run contained an empty fiber bag to determine baseline changes in weight unassociated with fermentation. Percent IVNDFD was determined with the equation:

Equation 3.2. *In vitro* neutral detergent fiber disappearance (IVNDFD).

$$\frac{Wf - (Wds - Wb)}{Wf} \; x \; 100$$

Wf: grams DM weighed into fiber bag;

Wds: grams NDF of dry sample plus bag weight;

Wb: bag weight in grams.

Preparation for VFA Analyses

One milliliter of meta-phosphoric acid (25% wt/v; 25 g in 100 mL DI water) was combined with 4 mL fermented ruminal fluid from each bottle after 48 h of fermentation and placed into a -24°C freezer. Frozen samples were later thawed to room temperature and homogenized (Vortex-Genie 2; Scientific Industries, Bohemia, NY). One milliliter of each sample was collected into centrifuge tubes and centrifuged at 16,000 x g for 15 min (Eppendorf 5415 C; Brinkmann Instruments, Inc., Westbury, NY). About 1 mL supernatant was placed into gas chromatography vials with snap caps and placed onto an Agilent 7890A gas chromatograph (GC; Agilent Technologies, Santa Clara, CA) fitted with a Nukol capillary column (#25326 Supelco columns; Sigma-Aldrich, St. Louis, MO) and flame ionization detector (FID). Initial oven temperature was set to 70°C and increased by 30°C/minute until final temperature was 190°C. Inlet and FID temperature were set at 300°C. Hydrogen was the carrier gas, with internal pressure maintained at 1.0746 pounds per square inch (psi) and flow rate of 5.2814 mL/minute. Injection volume per sample was 0.2 µL with an injection split ratio of 10:1. Standards (Supelco Volatile Fatty Acid Standard Mix; Sigma-Aldrich, St. Louis, MO) containing acetate, propionate, isobutyrate, butyrate, isovalerate, and valerate were used to quantify the volatile fatty acids measured.

Methane Calculations

Methane production was calculated using the following equation (Moss et al., 2000):

Equation 3.3. Methane production.

$$0.50C2 - 0.25C3 + 0.50C4$$

C2: is mmol acetate;

C3: is mmol propionate;

C4: is mmol butyrate.

Statistical Analyses

Terminal pH, IVDMD, IVNDF, and VFA data were analyzed using Proc GLIMMIX in SAS (Version 9.4). The models for terminal pH, IVDMD, IVNDFD, and VFA production included fixed effects of carbohydrate type, forage, and their interaction.

Gas Production

For each vessel, gas pressure (psi) from 0.25 h to 48 h in 0.25 h intervals was fitted by 1) logistic growth curve and 2) log-logistic growth curve. The non-linear model for the logistic growth curve is given by:

Equation 3.4. Cumulative gas production for logistic growth curve.

Total gas production per substrate weight (t) = $\frac{K}{1+\exp\left(-\beta\left(t-t_{half}\right)\right)} + \varepsilon$

K: maximum capacity at the stationary stage;

t_{half}: time to reach half of K;

β: logistic growth rate; β > 0;

ε: error term.

Equation 3.5. The non-linear model for the log-logistic growth curve is given by:

Total gas production per substrate weight (t) = $\frac{K}{1+(\frac{t}{t_{half}})^{-r}} + \varepsilon$

K: maximum capacity at the stationary stage;

t_{half}: time to reach half of K;

r: log-logistic growth rate; shape parameter for log-logistic curve; $r > 0$;

ε: error term.

Parameters of each fitted curve were estimated by minimizing the non-linear least squares.

Blank cultures had no gas production and served as a negative control. The corresponding 12 samples were excluded from curve fitting. When pressures of a vessel decreased after gas production reached the stationary stage, specifically, after 47 h, these values were removed from curve fitting. Pressure reduction at earlier timepoints indicated leakage and data from the samples were excluded from curve fitting. Data from certain combinations of forage, animal and carbohydrate were missing. Altogether, 210 log-logistic growth curves were fitted. Comparison of mean square errors (MSE) of the 2 models revealed that the logistic model performed better for prairie hay with MSE ranging from 0.07 to 0.87 (psi^2) and log-logistic model performs better

for alfalfa and bromegrass with MSE ranging from 0.13 to 1.60 (PSI^2). Therefore, cumulative gas production was fitted with the logistic growth curve for vessels containing prairie hay and log-logistic growth curve for vessels containing alfalfa and bromegrass. Parameters including K and t_{half} have the same interpretation across models. Interpretation of β and r is model-specific.

Experimental unit was bottle. Curve parameters were all positive. Their estimates first underwent natural-log transformation to better achieve normality and were then analyzed, separately for each parameter, under the linear mixed model. For K, t_{half} and r, fixed effects of the model include forage, animal, carbohydrate, and forage x carbohydrate interaction. Random effects include run (whole plot error), run x animal (subplot error), run x animal x carbohydrate (sub-subplot error). For β, prairie hay is the only level of forage; fixed effects of the model therefore reduced to animal and carbohydrate.

The effect of forage for each carbohydrate was assessed via back-transformed least squares means (corresponding to medians of a given curve parameter) and back-transformed mean differences (corresponding to ratios of medians of the curve parameter). Comparisons between 2 levels of forage for a given carbohydrate were carried out using the 2-sided t-test at the 0.05 level. Multiplicity adjustment was not applied. Statistical analyses were performed using Statistical Analysis Software (SAS version 9.4; Cary, NC) NLIN procedure and MIXED procedure.

Results

Total Gas Production

Values for total gas production (K) for each carbohydrate within forage were compared to the appropriate control sample (forage with no added carbohydrate). With prairie hay (Table

3.2), there were no differences ($P > 0.05$) in total gas production when comparing any supplemental carbohydrates to control, but there was a trend for increased gas production with molasses ($P = 0.069$). When analyzing the effects of carbohydrates with bromegrass hay (Table 3.3), none differed from control ($P = 1.000$). There were differences in total gas production observed with cultures containing alfalfa (Table 3.4). Gas production increased when cellulose ($P = 0.022$), galactose ($P = 0.039$) and hydrolyzed corn starch ($P = 0.050$) were added to cultures. No other carbohydrates altered gas production in cultures provided alfalfa ($P \geq 0.060$). However, there were trends for gas production to increase with beet molasses ($P = 0.081$), glucose ($P = 0.087$), raw corn starch ($P = 0.092$), stachyose ($P = 0.078$), and sucrose ($P = 0.060$).

Terminal pH, IVDMD, IVNDF

There was no treatment effect on pH in cultures provided prairie hay (Table 3.5; $P = 0.46$), bromegrass (Table 3.6; $P = 0.14$), or alfalfa (Table 3.7; $P = 0.32$). There was no treatment effect on IVDMD with prairie hay ($P = 0.42$), bromegrass ($P = 0.17$), or alfalfa ($P = 0.42$). There was no effect of carbohydrate on IVNDFD with prairie hay ($P = 0.39$) or alfalfa ($P = 0.90$). However, in cultures provided bromegrass, there was a treatment effect ($P = 0.038$). Galactose and isomaltose enhanced IVNDFD ($P < 0.05$).

Volatile Fatty Acid (VFAs)

Cultures containing prairie hay (Table 3.8) responded to varying carbohydrate sources with altered VFA production. There was a treatment effect on acetate ($P = 0.003$). The A:P ratio ($P < 0.05$) decreased with cellulose, fructose, galactose, glucose, lactose, maltose, mannose, melibiose, raffinose, sucrose and trehalose compared to control. Butyrate concentration was

greater with arabinose ($P < 0.05$) compared to control. There was no effect of treatment on isobutyrate ($P = 0.408$) or isovalerate ($P = 0.896$). In cultures provided with prairie hay, supplementation with galactose, isomaltose and trehalose generated more valerate when compared to control ($P < 0.05$). Caproate concentration was greater ($P < 0.05$) in cultures containing galactose, isomaltose, lactose, rhamnose, and trehalose when compared to the control. Methane production ($P < 0.05$) decreased with the addition of cellulose, fructose, and lactose when compared to control. Maltose supplementation was associated with a greater concentration of total VFAs when compared to control ($P < 0.05$).

There was no treatment effect on acetate ($P = 0.889$), propionate ($P = 0.935$), isobutyrate ($P = 0.9414$), butyrate ($P = 0.360$), isovalerate ($P = 0.578$), valerate ($P = 0.118$), caproate ($P = 0.3127$), methane ($P = 0.64$), or total VFA ($P = 0.950$) in cultures containing bromegrass (Table 3.9). In cultures provided bromegrass, there was a treatment effect on A:P ratio ($P < 0.0001$). The addition of arabinose led to a greater A:P ratio than control, and glucose, isomaltose, lactose, melibiose, raffinose, rhamnose, sucrose, and trehalose all led to decreased A:P ratio compared to control ($P < 0.05$).

Treatment had no effect on acetate ($P = 0.50$), propionate ($P = 0.41$), isobutyrate ($P = 0.45$), butyrate ($P = 0.323$), isovalerate ($P = 0.708$), valerate ($P = 0.645$), caproate ($P = 0.2462$), methane ($P = 0.400$) or total VFA ($P = 0.540$) in cultures containing alfalfa (Table 3.10). However, there was an effect on A:P ratio with all treatments decreased from control ($P < 0.05$) except raw corn starch.

Discussion

This experiment was designed to compare the effect of supplementing 22 different carbohydrates on fermentation of 3 types of hay by ruminal microbes in an *in vitro* setting. Supplemental carbohydrate sources have been used as prebiotics to increase the supply of substrate for lactic acid and other beneficial bacteria. Lactic acid bacteria (LAB) can produce antioxidants, organic acids, antimicrobial compounds, and improve microbial balance in the gut (Aswathy et al., 2008). Lactic acid bacteria can tolerate sudden decreases in pH and may even limit the possibility of certain diseases in feedlot cattle (Nocek and Kautz, 2006). In addition, increasing desirable bacteria can improve fiber digestion as LAB can degrade high-fiber feedstuffs efficiently (Chung et al., 2011) and have positive effects on other digestive properties (Uyeno et al., 2015).

Forage quality can be defined by palatability, voluntary intake, digestibility, and nutritional content. Palatability is influenced by moisture, smell, and taste. Intake refers to how much the animal consumes willingly if provided unlimited access. Digestibility describes how easily the forage is digested as it passes through the gastrointestinal tract. Nutritional content refers to chemical composition of cellular and structural components. Animals fed low-quality forage often need to be supplemented with concentrate to meet their energy requirements, whereas a high-quality forage can often provide enough energy for the animal alone. Legumes are generally considered higher quality than grasses due to less fiber, reduced NDF, and greater CP concentration (Ball et al., 2001). Rate of passage, ruminal digestion and degradation are affected by NDF (NRC, 2016). Another factor affecting forage quality of grass hays is whether it is comprised of cool-season or warm-season cultivators. Cool-season species, such as bromegrass, are generally higher in quality as they are more easily digested by the animal and

have greater CP content (Ball et al., 2001). In locally grown hay obtained by our laboratory, NDF has ranged from 39.7 to 47.2% in alfalfa, 58.6 to 63.3% in bromegrass, and 66.4 to 68.2% in prairie hay. Crude protein has ranged from 19.3 to 22.4% in alfalfa, 7.10 to 9.6% in bromegrass, and 4.35 to 4.42% in prairie hay (Jordan et al., 2017; Sorenson et al., 2020; Fehlberg et al., 2018; Reeg et al., 2015). In this study, a legume (alfalfa) was used as a high-quality forage, a cool-season grass (smooth bromegrass hay) was used as a medium quality hay, and a warm-season grass (prairie hay) was used as a low-quality forage.

In cultures containing alfalfa, the addition of cellulose, galactose, and hydrolyzed corn starch led to greater gas production compared to control. Conrad et al. (1958) found that adding various carbohydrates to washed alfalfa fiber increased gas production. In the current study, there were no changes in digestibility parameters, pH, or VFA accumulation. The A:P ratio was greater in the control that was not provided additional CHO. Others have found that carbohydrate and starch fermentation in the rumen lead to greater propionate production and less methane production because of a shifting A:P ratio (Hengarty, 1999; Janssen, 2010). However, in the current study, likely because carbohydrate treatments were added in small amounts, there were no differences in propionate detected despite the change in A:P ratio.

In cultures provided with bromegrass hay, treatment did not affect gas production, dry matter disappearance, or pH. However, there was greater NDF digestibility with isomaltose and galactose compared to the control. It is not immediately apparent why isomaltose, α-1, 6 linkage glucopyranosyl-glucose disaccharide, and galactose, a monosaccharide, both had this effect (Yang et al., 2016). There were no treatment effects on VFA production in cultures provided with bromegrass hay, but the addition of CHO influenced A:P ratio.

In cultures provided with prairie hay, there were no changes with treatment in gas production or digestibility parameters. However, carbohydrate supplementation influenced acetate, butyrate, valerate, caproate, methane, and total VFAs. Nilsson et al. (2010) found that a common fermentation product produced when prebiotics are administered is butyrate, which can increase the production of anti-inflammatory cytokines. In the current study, supplementation of arabinose increased butyrate concentration relative to the control. Heldt et al. (1998) conducted an *in vitro* study utilizing low-quality forages and supplemented glucose, fructose, galactose, arabinose, xylose, lactose, maltose, and sucrose on a 4:1 forage carbohydrate ratio (0.00063 g carbohydrate per 1 mL of fluid). Similar to the current study where 0.00066 g carbohydrate per 1 mL of fluid was provided, these sugars led to increased VFA production, however Heldt et al. (1998) also observed a corresponding decrease in pH (< 6.2) and subsequently depressed forage digestion, which contrasts with the current findings.

Prebiotics can influence microbial activity and are considered beneficial to health of the animal (Uyeno et al., 2015). Some prebiotics are classified as dietary fiber and are highly fermentable. They are resistant to gastric acidity, resistant to hydrolysis by mammalian enzymes, and are selected to stimulate growth or activity of a desired bacteria (Gibson et al., 1995). Śliżewska and Chlebicz-Wójcik (2020) utilized glucose, maltodextrin, inulin, corn starch, apple pectin, and β-glucan as prebiotics and found that all 5 prebiotic sources led to similar growth of *Lactobacillus* spp. Gang et al. (2020) and Cao et al. (2011) reported that using prebiotics could increase LAB, which could improve fermentative quality and reduce methane emission. Furthermore, increasing LAB populations leads to improved NDF digestibility in diary calves and has resolved diarrhea (Renaud et al., 2019). Weinberg et al. (2007) reported in the first 24 h of an *in vitro* study, that an increase in LAB improved DMD, but not NDFD, in cultures with

ruminal inoculum. However, after 48 h there were no major effects on DMD or NDFD. While carbohydrate treatment in the current study did not influence digestibility at 48 h in cultures provided low-quality hay, they did change fermentation characteristics as reflected in VFA profiles. These changes were probably not directly due to fermentation of the carbohydrates themselves, as similar effects were not seen with other hay types. Instead, microbial activity was likely altered by the added carbohydrate sources. Including prebiotics in the diet would increase the bacteria like *Lactobacillus* spp. and alter the microbial activity (Holscher, 2017), which may increase fiber degradation, although this may have only been apparent prior to the 48 h termination of this experiment. Dietary fibers like maltose, trehalose, melibiose, raffinose, stachyose, arabinose, xylose, mannose, rhamnose, lactose, sucrose, fructose, galactose, and glucose are readily fermented by the microbiota (Holscher et al., 2015) and can improve fermentation of poor-quality forages. Whereas, insoluble fibers, such as cellulose, are poorly fermented by microbes, increase rate of passage, and thus reduce the availability for bacterial fermentation (Titgemeyer et al., 1991). In the current study, treatment with cellulose reduced VFA concentrations when compared to some of the other carbohydrate treatments.

Ruminants can utilize fibrous carbohydrates well due to the rumen's microbial communities (Taube et al., 2013); however, forage quality impacts ruminal fermentation. High quality forage can provide all nutrients needed for microbial growth and activity, therefore prebiotics are not likely to impact fermentation characteristics profoundly. However, low-quality forages are deficient in nutrients and energy. Feeding a low-quality forage alone will generally inhibit animal growth and negatively affect microbial growth or activity (Leng, 1990). Thus, adding supplementation to a low-quality forage, like a prebiotic, can cause significant

fermentation responses by providing support to targeted microbial communities that is simply not available in the hay supply (Lee et al., 1987).

Terminal pH and total VFAs are closely related in fermentation (Beuvink & Spoelstra, 1992). Achenbach et al. (2011) reported fermentation of carbohydrates leads to rapid production of VFA, which decrease ruminal pH. Carbohydrate additives often cause a decrease in pH in ruminal cultures provided with forage (Fondevila et al., 1994; Strizler et al., 1998) and pH is typically the main parameter that determines stability of the rumen (Beuvink & Spoelstra, 1992). The primary substrates utilized by ruminal microbes are carbohydrates and approximately 75 to 85% of feed energy is converted to VFA, with the remainder lost as heat and methane (Sutton, 1979). Cullen et al. (1986) demonstrated that 1.5 to 1.98 g of arabinose, ribose, xylose, glucose, galactose, fructose, maltose, and raffinose decreased pH in ruminal cultures with 30 mL of buffer/rumen fluid mixture without added forage, which equates to about 0.05 g per 1 mL of fluid. Dennis et al. (1981) observed 3 g glucose in 30 mL buffer/rumen fluid mixture (about 0.1 g per 1 mL of fluid), 20 g glucose in 200 mL buffer/rumen fluid mixture (about 0.1 g per 1 mL of fluid), and 4 g of arabinose, galactose, fructose, lactose, mannose, sucrose, sorghum grain, corn, and wheat in 40 mL of buffer/rumen fluid (about 0.1 g per 1 mL of fluid) mixture decreased pH as well. If provided in sufficient amounts, metabolism of the NSC itself generally leads to increased VFA production and subsequently decreased pH. In this study, the carbohydrate amount is small enough (0.00066 g per 1 mL of fluid) that metabolism of the carbohydrate itself would have minimal effect on fermentation. Instead, the goal was to enhance fermentation by providing substrate to target microbial populations and thus alter fermentation by changing microbial activity.

Conclusion

It is apparent that supplemental sugars can affect forage utilization (Heldt et al., 1998). Use of prebiotics generally increase LAB populations and researchers have found that increased LAB, such as *Lactobacillus* and *Bifidobacterium*, improves fiber digestibility of both high-starch and high-fiber diets in multiple species (Coverdale, 2016; Uyeno et al., 2015; Heldt et al., 1998). With the doses used in this experiment (0.00066 g carbohydrate per 1 mL of fluid), there were various effects noted with all hay types. However, changes in fermentative activity were most notable with prairie hay. Further refinement of dosage provided or timing of samples would further enhance our understanding of their effects in the rumen. Based on these findings, it appears that prebiotics have most potential to alter fermentative activity when ruminal microbes are provided low-quality hay.

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Table 3.1. Composition of total mixed ration (TMR) fed to cannulated steers[1,2].

Ingredient	Amount, %
Corn silage	46.67
Sweet bran[3]	19.67
Dry-rolled corn	16.67
Prairie hay	14.67
Supplement[4]	2.00

[1]Steers serving as ruminal fluid donors were fed 17 kg (as-fed) TMR head/d.
[2]Mixed at Dairy Teaching and Research Center, Kansas State University, Manhattan, KS.
[3]Cargill Incorporated, Minneapolis, MN.
[4] Supplement contained urea, limestone, potassium chloride, vitamin and mineral mix, vitamins A and E, and Rumensin (Elanco Animal Health, Greenfield, IN). Manufactured at O. H. Kruse Feed Technology Innovation Center, Kansas State University Manhattan, KS.

Table 3.2. Total gas production (*K*; mL) of ruminal microbes supplied with prairie hay and an additional carbohydrate source.

	K[1]	
Carbohydrate	Mean (± SE)	*P*-Value[Δ]
Arabinose	321.4 ± 20.7	0.326
Beet Molasses	317.4 ± 24.7	0.643
Cellulose	328.7 ± 19.3	0.107
Fructose	328.7 ± 19.3	0.103
Galactose	315.4 ± 20.7	0.502
Glucose	325.4 ± 24.7	0.410
HCS[2]	326.7 ± 25.3	0.370
Isomaltose	315.4 ± 20.7	0.500
Lactose	322.7 ± 20.7	0.290
Maltose	329.4 ± 25.3	0.318
Mannose	323.4 ± 18.7	0.180
Melibiose	254.1 ± 19.3	0.999
Molasses	338.1 ± 22.0	0.069
Raffinose	328.1 ± 19.3	0.114
Raw CS[3]	324.7 ± 24.7	0.429
Rhamnose	327.4 ± 18.7	0.119
Stachyose	325.4 ± 21.3	0.236
Sucrose	299.4 ± 22.7	0.988
Trehalose	324.7 ± 24.7	0.419
Xylose	326.7 ± 21.3	0.210
Control	272.7 ± 16.0	--

[Δ]*P*-value reflects difference between sample and control.
[1]Total gas production.
[2]Hydrolyzed corn starch.
[3]Raw corn starch.

Table 3.3. Total gas production (*K*; mL) of ruminal microbes supplied with bromegrass and an additional carbohydrate source.

	K[1]	
Carbohydrate	Mean (± SE)	*P*-Value[Δ]
Arabinose	399.4 ± 23.3	1.000
Beet Molasses	424.8 ± 27.3	1.000
Cellulose	436.1 ± 28.0	1.000
Fructose	413.4 ± 24.0	1.000
Galactose	422.1 ± 27.3	1.000
Glucose	414.8 ± 24.0	1.000
HCS[2]	435.4 ± 33.3	1.000
Isomaltose	413.4 ± 31.3	1.000
Lactose	421.4 ± 24.7	1.000
Maltose	408.7 ± 26.7	1.000
Mannose	425.4 ± 24.7	1.000
Melibiose	420.8 ± 24.7	1.000
Molasses	416.1 ± 26.7	1.000
Raffinose	425.4 ± 24.7	1.000
Raw CS[3]	416.1 ± 24.0	1.000
Rhamnose	420.8 ± 24.7	1.000
Stachyose	412.1 ± 24.0	1.000
Sucrose	410.1 ± 26.7	1.000
Trehalose	422.1 ± 24.7	1.000
Xylose	410.7 ± 26.7	1.000
Control	415.4 ± 24.0	--

[Δ]*P*-value reflects difference between sample and control.
[1]Total gas production.
[2]Hydrolyzed corn starch.
[3]Raw corn starch.

Table 3.4. Total gas production (*K*; mL) of ruminal microbes supplied with alfalfa and an additional carbohydrate source.

	K[1]	
Carbohydrate	Mean (± SE)	*P*-Value[Δ]
Arabinose	498.8 ± 28.7	0.132
Beet Molasses	514.1 ± 33.3	0.081
Cellulose	522.1 ± 30.0	0.022
Fructose	502.1 ± 29.3	0.109
Galactose	524.8 ± 34.0	0.039
Glucose	504.8 ± 29.3	0.087
HCS[2]	512.1 ± 30.0	0.050
Isomaltose	477.4 ± 28.0	0.463
Lactose	513.4 ± 39.3	0.185
Maltose	509.4 ± 32.7	0.109
Mannose	485.4 ± 37.3	0.602
Melibiose	505.4 ± 29.3	0.084
Molasses	429.4 ± 24.7	1.000
Raffinose	490.8 ± 28.7	0.224
Raw CS[3]	504.1 ± 29.3	0.092
Rhamnose	466.8 ± 27.3	0.716
Stachyose	506.8 ± 29.3	0.078
Sucrose	510.1 ± 29.3	0.060
Trehalose	498.1 ± 32.0	0.216
Xylose	467.4 ± 29.3	0.697
Control	418.1 ± 24.0	--

[Δ]*P*-value reflects difference of carbohydrate source against the blank control.
[1]Total gas production.
[2]Hydrolyzed corn starch.
[3]Raw corn starch.

Table 3.5. Terminal pH, *in vitro* dry matter disappearance, and *in vitro* neutral detergent fiber disappearance in ruminal cultures provided prairie hay and an additional carbohydrate source.

	Item		
Carbohydrate	Terminal pH	IVDMD (%)[1]	IVNDFD (%)[2]
Arabinose	5.77	40.00	47.62
Beet Molasses	5.78	41.53	48.13
Cellulose	5.78	47.03	49.03
Fructose	5.78	49.79	50.12
Galactose	5.87	42.29	48.54
Glucose	5.77	40.70	47.87
HCS[3]	5.78	40.07	47.50
Isomaltose	5.74	40.37	48.25
Lactose	5.78	42.94	48.19
Maltose	5.78	46.60	48.67
Mannose	5.79	47.39	49.05
Melibiose	5.78	46.21	47.64
Molasses	5.77	40.86	46.03
Raffinose	5.80	44.61	48.79
Raw CS[4]	5.76	46.63	49.67
Rhamnose	5.77	41.01	49.46
Stachyose	5.76	49.21	47.93
Sucrose	5.79	48.48	48.58
Trehalose	5.82	46.47	49.05
Xylose	5.71	41.06	48.28
Control	5.80	52.89	48.91
SEM	0.09	5.19	1.67
P-Value	0.46	0.42	0.39

[a,b,c,d,e,f,g]Means in the same column with different superscripts differ ($P < 0.05$)
[1]In vitro dry matter disappearance.
[2]In vitro neutral detergent fiber disappearance.
[3]Hydrolyzed corn starch.
[4] Raw corn starch.

Table 3.6. Terminal pH, *in vitro* dry matter disappearance, and *in vitro* neutral detergent fiber disappearance in ruminal cultures provided bromegrass and an additional carbohydrate source.

	Item		
Carbohydrate	Terminal pH	IVDMD (%)[1]	IVNDF (%)[2]
Arabinose	5.29	47.52	45.05^{abcde}
Beet Molasses	5.31	43.18	43.22^{ef}
Cellulose	5.29	47.13	45.58^{abcd}
Fructose	5.29	46.86	45.44^{abcde}
Galactose	5.29	47.30	46.18^{ab}
Glucose	5.29	47.34	44.52^{bcdef}
HCS[3]	5.30	47.21	45.28^{abcde}
Isomaltose	5.29	47.47	46.79^{a}
Lactose	5.28	47.56	45.02^{abcde}
Maltose	5.30	47.26	45.51^{abcd}
Mannose	5.29	51.01	43.69^{cdef}
Melibiose	5.29	46.47	44.41^{bcdef}
Molasses	5.30	48.27	44.46^{bcdef}
Raffinose	5.29	47.44	45.46^{abcde}
Raw CS[4]	5.28	47.31	42.41^{f}
Rhamnose	5.29	46.95	45.75^{abc}
Stachyose	5.29	46.99	45.62^{abc}
Sucrose	5.30	47.12	45.52^{abcd}
Trehalose	5.29	47.08	43.36^{def}
Xylose	5.31	47.08	44.51^{bcdef}
Control	5.31	51.30	43.76^{cdef}
SEM	0.01	1.97	0.84
P-Value	0.14	0.17	0.04

[a,b,c,d,e,f,g]Means in the same column with different superscripts differ ($P < 0.05$)
[1]In vitro dry matter disappearance.
[2]In vitro neutral detergent fiber disappearance.
[3]Hydrolyzed corn starch.
[4] Raw corn starch.

Table 3.7. Terminal pH, *in vitro* dry matter disappearance, and *in vitro* neutral detergent fiber disappearance in ruminal cultures provided alfalfa and an additional carbohydrate source.

	Item		
Carbohydrate	Terminal pH	IVDMD (%)[1]	IVNDFD (%)[2]
Arabinose	5.97	47.19	38.3
Beet Molasses	5.99	46.49	37.48
Cellulose	5.96	51.08	37.98
Fructose	5.95	52.24	38.94
Galactose	5.98	46.34	36.76
Glucose	5.96	47.96	37.39
HCS[3]	5.97	46.53	36.77
Isomaltose	5.94	48.54	37.87
Lactose	5.98	48.98	37.74
Maltose	5.95	45.94	38.19
Mannose	5.95	48.85	38.41
Melibiose	5.95	51.48	37.66
Molasses	5.98	47.29	37.47
Raffinose	5.93	47.47	37.30
Raw CS[4]	5.97	46.30	37.07
Rhamnose	6.00	48.80	36.82
Stachyose	5.95	46.43	37.08
Sucrose	5.7	45.90	36.75
Trehalose	5.71	46.62	36.36
Xylose	5.98	47.73	37.43
Control	6.09	44.63	39.33
SEM	0.08	1.99	3.10
P-Value	0.32	0.42	0.90

[a,b,c,d,e,f,g]Means in the same column with different superscripts differ ($P < 0.05$)
[1]In vitro dry matter disappearance.
[2]In vitro neutral detergent fiber disappearance.
[3]Hydrolyzed corn starch.
[4] Raw corn starch.

Table 3.8. Concentration of volatile fatty acids (mmol) in ruminal cultures supplied with prairie hay and a supplemental carbohydrate source.

	Item (mmol)									
Carbohydrate[Ω]	Acetate	Propionate	AP Ratio[1]	Isobutyrate	Butyrate	Isovalerate	Valerate	Caproate	Methane	Total VFAs
Arabinose	1.92^{ab}	0.87	2.20^{abc}	0.006	0.185^{a}	0.0013	0.021^{abcde}	0.004^{abcde}	28.369^{ab}	3.009^{ab}
Beet Molasses	1.84^{abc}	0.83	2.23^{abc}	0.006	0.166^{abc}	0.0014	0.021^{abcde}	0.003^{e}	26.949^{abcde}	2.869^{abcd}
Cellulose	1.60^{d}	0.81	1.98^{def}	0.002	0.143^{ef}	0.0008	0.016^{f}	0.003^{e}	22.782^{g}	2.580^{e}
Fructose	1.65^{de}	0.87	1.90^{f}	0.005	0.153^{cdef}	0.0008	0.019^{cdef}	0.003^{e}	23.306^{g}	2.698^{cde}
Galactose	1.75^{bcde}	0.89	1.97^{def}	0.005	0.158^{bcdef}	0.0009	0.024^{a}	0.006^{a}	24.913^{defg}	2.836^{abcde}
Glucose	1.77^{abcde}	0.86	2.06^{cdef}	0.006	0.161^{bcde}	0.0010	0.019^{cdef}	0.003^{e}	25.605^{bcdefg}	2.824^{abcde}
HCS[2]	1.74^{cde}	0.83	2.09^{abcde}	0.004	0.166^{abc}	0.0010	0.019^{def}	0.004^{cde}	25.318^{cdefg}	2.766^{bcde}
Isomaltose	1.95^{a}	0.86	2.27^{ab}	0.004	0.176^{ab}	0.0013	0.024^{ab}	0.006^{ab}	28.795^{a}	3.021^{ab}
Lactose	1.63^{de}	0.79	2.07^{cdef}	0.005	0.139^{f}	0.0010	0.019^{def}	0.006^{abc}	23.347^{g}	2.583^{e}
Maltose	1.94^{a}	0.94	2.08^{bcdef}	0.005	0.168^{abc}	0.0013	0.023^{abcd}	0.004^{cde}	27.885^{abc}	3.080^{a}
Mannose	1.68^{cde}	0.81	2.07^{cdef}	0.003	0.153^{cdef}	0.0012	0.020^{bcdef}	0.003^{e}	24.309^{efg}	2.674^{cde}
Melibiose	1.65^{de}	0.79	2.06^{cdef}	0.002	0.143^{def}	0.0010	0.018^{ef}	0.004^{de}	23.674^{gf}	2.607^{de}
Molasses	1.85^{abc}	0.82	2.28^{a}	0.005	0.164^{bcd}	0.0012	0.022^{abcde}	0.004^{de}	27.297^{abcd}	2.870^{abcd}
Raffinose	1.70^{cde}	0.87	1.95^{ef}	0.006	0.158^{bcdef}	0.0008	0.021^{abcde}	0.004^{bcde}	24.264^{efg}	2.765^{bcde}
RCS[3]	1.68^{cde}	0.78	2.16^{abcd}	0.003	0.159^{bcdef}	0.0008	0.019^{def}	0.005^{abcde}	24.595^{defg}	2.642^{cde}
Rhamnose	1.77^{abcde}	0.84	2.11^{abcde}	0.004	0.154^{cdef}	0.0007	0.021^{abcde}	0.006^{abc}	25.630^{bcdefg}	2.798^{bcde}
Stachyose	1.73^{cde}	0.84	2.09^{abcde}	0.006	0.156^{cdef}	0.0007	0.021^{abcde}	0.003^{e}	24.947^{defg}	2.755^{bcde}
Sucrose	1.73^{cde}	0.87	1.98^{def}	0.003	0.156^{cdef}	0.0007	0.020^{abcde}	0.004^{bcde}	24.639^{defg}	2.789^{bcde}
Trehalose	1.74^{bcde}	0.89	1.97^{def}	0.003	0.163^{bcd}	0.0081	0.024^{abc}	0.005^{abcd}	24.890^{defg}	2.825^{abcde}
Xylose	1.83^{abc}	0.88	2.10^{abcde}	0.005	0.161^{bcde}	0.0011	0.020^{abcde}	0.003^{e}	26.513^{abcdef}	2.903^{abc}
Control[Δ]	1.79^{abcde}	0.78	2.28^{a}	0.004	0.159^{bcdef}	0.0014	0.019^{def}	0.003^{e}	26.417^{abcdef}	2.757^{bcde}
SEM	0.166	0.095	0.119	0.002	0.018	0.0007	0.007	0.003	2.598	0.264
P-Value	0.003	0.077	0.001	0.408	0.010	0.8960	0.023	0.001	0.001	0.015

[a,b,c,d,e,f,g]Means with in the same column with different superscripts differ ($P < 0.05$).
[Ω]Carbohydrate source was provided at 0.1 g DM.
[1]Acetate: propionate ratio.
[2] Hydrolyzed corn starch.
[3] Raw corn starch.
[Δ]Control contained 5 g DM forage with no added CHO source.

Table 3.9. Concentration of volatile fatty acids (mmol) in ruminal cultures supplied with bromegrass and a supplemental carbohydrate source.

	Item (mmol)									
Carbohydrate[Ω]	Acetate	Propionate	AP Ratio[1]	Isobutyrate	Butyrate	Isovalerate	Valerate	Caproate	Methane	Total VFAs
Arabinose	2.055	1.180	1.735^{a}	0.004	0.281	0.0021	0.043	0.003	29.68	3.57
Beet Molasses	2.012	1.231	1.638^{bcde}	0.005	0.256	0.0028	0.037	0.002	28.10	3.55
Cellulose	2.005	1.220	1.643^{bcd}	0.005	0.241	0.0028	0.034	0.002	27.80	3.51
Fructose	2.046	1.252	1.633^{bcde}	0.005	0.269	0.0024	0.042	0.002	28.72	3.62
Galactose	1.946	1.182	1.645^{bcd}	0.005	0.246	0.0021	0.036	0.002	27.23	3.42
Glucose	1.937	1.204	1.608^{de}	0.005	0.255	0.0024	0.037	0.002	27.03	3.44
HCS[2]	1.936	1.149	1.683^{ab}	0.004	0.252	0.0023	0.036	0.002	27.43	3.38
Isomaltose	2.003	1.259	1.588^{ef}	0.005	0.266	0.0026	0.038	0.002	27.86	3.58
Lactose	1.985	1.236	1.603^{de}	0.005	0.239	0.0024	0.035	0.001	27.30	3.50
Maltose	1.951	1.194	1.638^{bcde}	0.004	0.248	0.0021	0.038	0.002	27.24	3.44
Mannose	2.028	1.257	1.618^{cde}	0.005	0.258	0.0025	0.037	0.002	28.17	3.59
Melibiose	1.912	1.196	1.603^{de}	0.004	0.235	0.0021	0.035	0.002	26.33	3.39
Molasses	2.036	1.214	1.678^{b}	0.004	0.259	0.0024	0.038	0.002	28.71	3.56
Raffinose	2.036	1.272	1.600^{de}	0.005	0.253	0.0027	0.036	0.002	28.10	3.61
Raw CS[3]	2.089	1.252	1.665^{bc}	0.005	0.280	0.0029	0.039	0.002	29.63	3.67
Rhamnose	1.849	1.203	1.538^{f}	0.004	0.239	0.0021	0.037	0.001	25.27	3.34
Stachyose	2.053	1.258	1.630^{bcde}	0.005	0.269	0.0028	0.039	0.002	28.78	3.63
Sucrose	1.961	1.216	1.610^{de}	0.005	0.257	0.0024	0.039	0.002	27.37	3.48
Trehalose	1.904	1.186	1.610^{de}	0.004	0.249	0.0025	0.037	0.002	26.52	3.38
Xylose	1.988	1.215	1.638^{bcde}	0.004	0.260	0.0024	0.039	0.002	27.88	3.51
Control[Δ]	1.950	1.175	1.665^{bc}	0.005	0.236	0.0029	0.033	0.002	27.16	3.40
SEM	0.129	0.049	0.061	0.0007	0.024	0.0006	0.004	0.0008	2.24	0.20
P-Value	0.889	0.935	< 0.0001	0.9414	0.360	0.5780	0.118	0.3127	0.64	0.95

[a,b,c,d,e,f,g]Means with in the same column with different superscripts differ ($P < 0.05$).
[Ω]Carbohydrate source was provided at 0.1 g DM.
[1]Acetate: propionate ratio.
[2] Hydrolyzed corn starch.
[3] Raw corn starch.
[Δ]Control contained 5 g DM forage with no added CHO source.

Table 3.10. Concentration of volatile fatty acids (mmol) in ruminal cultures supplied with alfalfa and a supplemental carbohydrate source.

	Item (mmol)									
Carbohydrate[Ω]	Acetate	Propionate	AP Ratio[1]	Isobutyrate	Butyrate	Isovalerate	Valerate	Caproate	Methane	Total VFAs
Arabinose	2.26	1.03	2.198^{bcd}	0.019	0.21	0.015	0.071	0.0038	33.21	3.60
Beet Molasses	2.24	1.05	2.143^{cd}	0.019	0.20	0.016	0.071	0.0035	32.68	3.60
Cellulose	2.22	1.03	2.165^{bcd}	0.019	0.20	0.016	0.068	0.0035	32.39	3.55
Fructose	2.20	1.01	2.180^{bcd}	0.017	0.19	0.014	0.069	0.0035	32.03	3.50
Galactose	2.34	1.10	2.138^{cd}	0.020	0.22	0.016	0.075	0.0038	34.22	3.77
Glucose	2.16	1.01	2.148^{bcd}	0.172	0.19	0.015	0.069	0.0035	31.42	3.46
HCS[2]	2.31	1.05	2.203^{bc}	0.021	0.23	0.017	0.074	0.0043	34.12	3.70
Isomaltose	2.11	1.01	2.098^{cd}	0.017	0.19	0.014	0.066	0.0035	30.56	3.41
Lactose	2.25	1.03	2.178^{bcd}	0.018	0.19	0.015	0.068	0.0035	32.79	3.58
Maltose	2.29	1.08	2.115^{cd}	0.019	0.21	0.016	0.073	0.0038	33.23	3.69
Mannose	2.13	1.02	2.103^{cd}	0.016	0.18	0.014	0.066	0.0033	30.69	3.43
Melibiose	2.31	1.06	2.178^{bcd}	0.019	0.20	0.016	0.070	0.0035	33.67	3.68
Molasses	2.40	1.12	2.156^{bcd}	0.022	0.22	0.017	0.075	0.0040	35.03	3.86
Raffinose	2.17	1.06	2.063^{d}	0.018	0.18	0.014	0.068	0.0043	30.92	3.51
Raw CS[3]	2.30	1.01	2.283^{ab}	0.020	0.22	0.017	0.069	0.0048	34.27	3.64
Rhamnose	2.23	1.09	2.063^{d}	0.020	0.20	0.017	0.069	0.0040	31.92	3.62
Stachyose	2.23	1.05	2.123^{cd}	0.018	0.20	0.014	0.069	0.0035	32.23	3.58
Sucrose	2.23	1.03	2.155^{bcd}	0.018	0.20	0.016	0.072	0.0035	32.56	3.58
Trehalose	2.27	1.05	2.170^{bcd}	0.020	0.21	0.016	0.071	0.0035	33.28	3.64
Xylose	2.13	0.98	2.180^{bcd}	0.016	0.18	0.013	0.065	0.0033	30.88	3.38
Control[Δ]	2.02	0.89	2.408^{a}	0.017	0.16	0.014	0.054	0.0018	29.65	3.16
SEM	0.11	0.05	0.119	0.003	0.04	0.002	0.006	0.0007	2.19	0.18
P-Value	0.50	0.41	0.006	0.450	0.323	0.708	0.645	0.2462	0.40	0.54

[a,b,c,d,e,f,g]Means with in the same column with different superscripts differ ($P < 0.05$).
[Ω]Carbohydrate source was provided at 0.1 g DM.
[1]Acetate: propionate ratio.
[2] Hydrolyzed corn starch.
[3] Raw corn starch.
[Δ]Control contained 5 g DM forage with no added CHO source.

Chapter 4 - Evaluation of Substrate to Buffer Ratio for In Vitro Cultures Inoculated with Equine Cecal Contents

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Abstract

In vitro fermentation systems allow researchers to mimic the equine hindgut while providing quick, affordable, and publishable data. In these systems, the substrate: buffer ratio has a profound impact on fermentation parameters. The objective of the third experiment was to evaluate varying substrate levels, thus altering the substrate: buffer ratio, for its effect on culture pH, gas production, dry matter disappearance (DMD), neutral detergent fiber disappearance (NDFD), and volatile fatty acid (VFA) concentrations. Cecal fluid was collected from 4 cecally cannulated Quarter horses and used to inoculate fermentation bottles containing buffer and substrate. Substrate consisted of bromegrass or corn included at 0.5, 1, 1.5, 2, 3, or 4 g/flask (as-fed basis) while buffer and inoculant remained constant at 125 and 25 mL/flask, respectively. Flasks were equipped with continuous gas pressure monitors utilizing the ANKOM RF Gas Production System (ANKOM Technology; Macedon, NY) and placed into a shaking incubator for 48 hours at 39°C. Data were analyzed using mixed models with fixed effects of treatment, time, and treatment x time interaction, repeated effect of time, and random effects of run x horse within run. Differences in total gas production, VFA production, and terminal pH were evident for substrate level and substrate type ($P < 0.0001$). At 1.5 g or 2.0 g substrate, there appeared to be a shift where the incremental increase in many response variables began to decrease as more

substrate was provided. Thus, cultures with > 2 g substrate may have been inhibited by buffer exhaustion or end-product accumulation. The composition of fermentative end products is impacted by substrate concentration, and for cultures containing 125 mL of buffer and 25 mL of cecal inoculum, we would recommend using no more than 2 g of substrate.

Introduction

In vitro fermentation systems are used to assess dietary manipulations in livestock by mimicking in vivo processes and thus reduce the need for live animals for research. In vitro systems have been available for nearly 45 years and have undergone improvements in design to better mimic natural digestive properties (Duarte et al., 2017). In vitro experiments are faster and less expensive than live feeding trials (Getachew et al., 2014) while allowing for characterization of fermentative parameters (Storm et al., 2012). For example, digestibility of feedstuffs, gas production, and volatile fatty acid (VFA) production (Goering and Van Soest, 1970) can be assessed in these experiments.

One of the first in vitro fermentation systems that accommodated multiple samples simultaneously was designed by Tilley and Terry in 1963. It incorporated oven dried forage, McDougall's buffer solution, rumen fluid, tubes sealed with rubber corks fitted with Bunsen release values, and acid-pepsin to check bacterial activity. In 1970, Goering and Van Soest modified Tilley and Terry's procedure by replacing acid-pepsin with a neutral detergent fiber (NDF) digestion step, which allowed a more accurate method of determining true digestibility. Seven years later, Czerkawski & Breckenridge (1977) developed a continuous culture system, called the RUSITEC (Rumen Simulation TEChnique), that allowed microbes to grow continuously. This system allowed researchers to study ruminal microbes and fermentative

parameters at various time points throughout the fermentative process, whereas previous systems only allowed for assessment of parameters after the experiment was terminated. Menke et al. (1979) created a method of estimating feed value from gas production. Theodorou et al. (1991) developed a method of measuring accumulation of gas in a small space. Later, Pell and Schofield (1993) computerized gas sensors to record accumulation of gas. Using computerized gas sensors, Schofield and Pell (1993) discovered a linear relationship between gas production and NDF disappearance. Cone et al. (1996) verified the accuracy of automated equipment in measuring gas production. The ANKOM RF system contains modules that measure gas pressure in individual bottles, providing a simple and repeatable procedure to estimate the extent of feed degradation (Mould and Nordheim, 1998).

With increased usage of *in vitro* systems over the last century, it has been documented that substrate to buffer ratios (Henderson et al., 2015) affect fermentative parameters. Pell and Scholfield (1993) suggested that a fixed rate of 100 mg of forage and 50 mL of rumen inoculant was sufficient to maximize fiber digestion. In studies conducted using the ANKOM RF System in our laboratory at Kansas State University, 140 mL of buffer and 10 mL of inoculant is typically used, but substrate level has varied. Horton et al. (2018) used 3 g of corn with ruminal fluid and Fehlberg et al. (2018) used 5 g of dried forage with equine cecal inocula. Utilizing too little substrate might cause the microbial population to be substrate limited, meaning there is not sufficient food to support microbial activity throughout the culture period. Conversely, too much substrate might indirectly inhibit microbial activity due to buffer exhaustion, meaning there is not sufficient buffer to maintain suitable pH due to over-accumulation of fermentative end products. Thus, substrate: buffer ratio utilized in experiments must be optimized so differences

noted can be attributed to treatment effects and are not due to substrate depletion or buffer exhaustion.

The goal of this experiment was to utilize the ANKOM RF Gas Production System (ANKOM Technology; Macedon, NY) to determine the optimal substrate: buffer ratio for equine experiments. This was accomplished by evaluating fermentative parameters of equine cecal inocula with different substrate: buffer ratios by characterizing terminal pH, gas pressure, dry matter disappearance (DMD), neutral detergent fiber disappearance (NDFD), and volatile fatty acid (VFA) concentrations.

Materials and Methods

Animals

All animal procedures were approved by Kansas State University Institutional Animal Care and Use Committee. Four cecally cannulated American Quarter Horses were used as donors of cecal fluid. Horses were housed at the Equine Research and Teaching Center in a drylot. Horses were offered 1.3 kg (as-fed) pelleted feed (Table 4.1) head/day, *ad libitum* Bromegrass grass hay, *ad libitum* water, and a salt block.

Experimental Design

This study implemented a split-plot design with a 2 x 7 factorial treatment structure. Ground corn and ground smooth bromegrass (brome) hay were utilized at 7 levels: 0 g, 0.5 g, 1 g, 1.5 g, 2 g, 3 g, and 4 g per sample flask. Three horses per run were utilized as cecal fluid donors. There was a total of 42 samples per run, with a total of 4 runs.

Sample Preparation

Twenty-four hours before inoculation, whole corn and bromegrass hay were ground to 1 mm in a Wiley Mill, weighed, and added to 250-mL fermentation flasks (#7056; ANKOM Technology, Macedon, NY). Flasks were placed in a 39°C incubator and left over night. McDougall's Artificial Saliva (McDougall, 1948) was prepared with sodium bicarbonate ($NaHCO_3$), sodium phosphate (Na_2HPO_4), potassium chloride (KCl), sodium chloride (NaCl), and magnesium sulfate ($MgSO_4$-$7H_2O$). Urea was excluded from the buffer because horses are hindgut fermenters who do not benefit from nonprotein nitrogen (NPN; NRC, 2007).

Calcium chloride ($CaCl_2$) was added to the artificial saliva on the morning of inoculation and the saliva solution was warmed to 39°C. pH of the artificial saliva was measured and adjusted to 7.0 using hydrochloric acid (HCl) and sodium hydroxide (NaOH) as needed. Three 1-L thermoses were filled with hot water and transported to the Kansas State University Equine Teaching and Research Center. Hot water was removed from thermoses and cecal fluid was collected via gravitational flow from 3 horses (1 L per horse). Fluid was strained through cheesecloth into the pre-labeled pre-warmed thermoses.

In vitro Fermentation

Cecal fluid was transported back to the laboratory and re-strained through 8 layers of cheesecloth into separatory funnels. Funnels were degassed with N_2 and placed in a 39°C incubator (Isotemp 550D Incubator Over, Fischer Scientific, Hampton, NH) for 1 h to allow fluids to separate. Meanwhile, the software for the RF Gas Production System (ANKOM Technology, Macedon, NY) was opened, gas release was set to 34.5 kilopascal (kPa), and all

modules were confirmed to have pressure of 0 ± 0.75 kPa. A shaking incubator (G25; New Brunswick Scientific Inc., New Brunswick, NJ) was turned on and heated to 39°C.

After separation of cecal fluid, the microbe-rich liquid layer was collected into separate flasks and mixed with the artificial saliva in a 5:1 (buffer:cecal fluid) ratio. The mixture was dispersed at 140 mL per sample into bottles. The bottles were made anaerobic using N_2 gas, capped with a gas pressure monitoring module (RF Gas Production System; ANKOM Technology, Macedon, NY), and placed in the shaking 39°C incubator. Start time was recorded for each individual sample, modules were checked every 3 to 6 h, and batteries were changed as needed. Total gas production was measured every 0.25 h up to 48 h.

Fermentation was terminated about 48 h after each start time. Samples were removed from the shaking incubator and pressure was manually released by removing the modules. Four milliliters of fermented liquid were removed from each bottle and placed with 1 mL meta-phosphoric acid in a falcon tube and stored at -24°C until VFA analysis. pH (Thermo Orion Star A121; Thermo Fisher Scientific Inc., Chelmsford, MA) was measured in each fermentation bottle and remaining contents were emptied into pre-weighed aluminum pans. Fermentation bottles were rinsed with deionized (DI) water to facilitate transfer of all contents to aluminum pans.

Dry Matter Disappearance

Aluminum pans containing samples were dried in a 55°C oven for 3 d. Pans were weighed and dry matter was scraped into a small plastic bag and kept at room temperature until all samples were collected. Dry matter disappearance (DMD) was determined using equation 4.1.

Equation 4.1. Dry matter disappearance.

$$Ws - (Wds - Wp)$$

Ws: initial weight of samples weighed into flasks (i.e., 1 g, 2 g, 3 g, etc.);

Wds: weight of dried sample and pan;

Wp: denotes empty pan weight.

Neutral Detergent Fiber Analysis

After drying, 0.5 g (± 0.05) DM of each sample was placed into an ANKOM Fiber Analyses bag. Samples were placed into an NDF ANKOM Fiber Analyses appliance. Neutral detergent fiber (NDF) was determined using the Goering and Van Soest Method (1970). Each run contained an empty fiber bag to determine baseline changes in weight unassociated with fermentation and a control bag of unfermented corn and bromegrass hay (initial NDF). In vitro neutral detergent fiber disappearance (NDFD) was determined with equation 4.2.

Equation 4.2. Neutral detergent fiber disappearance.

$$\text{Initial NDF} - ((Ws \times (Wf / (Wds - Wb) \times 100))$$

Wf: g weighed into fiber bag;

Wds: g NDF of dry sample plus bag weight;

Wb: bag weight in grams;

Ws: calculated dry matter in initial samples.

VFA Analyses

Samples with meta-phosphoric acid were thawed at room temperature and homogenized (Vortex-Genie 2; Scientific Industries, Bohemia, NY). One milliliter was collected into centrifuge tubes and centrifuged at 16,000 x g for 15 min (Eppendorf 5415 C; Brinkmann Instruments, Inc., Westbury, NY). About 1 mL of supernatant was placed into gas chromatography vials with snap caps and placed onto an Agilent 7890A gas chromatograph (Agilent Technologies, Santa Clara, CA) fitted with a Nukol capillary column (#25326 Supelco columns; Sigma-Aldrich, St. Louis, MO) and flame ionization detector (FID). Initial oven temperature was set at 70°C and increased by 30°C per minute until final temperature of 190°C. The inlet and FID temperature were set to 300°C. Hydrogen was the main carrier gas, internal pressure was maintained at 1.0746 psi and the flow rate was 5.2814 mL/minute. Injection volume per sample was 0.2 μL with injection split ratio of 10:1.

Statistical Analyses

The whole plot, animal (1, 2, 3, 4), was the treatment; run (run 1, run 2, run 3, run 4) was the blocking factor for assigning treatment, and run x animal was the experimental unit (a total of 12 levels). Each animal was included in 3 of the 4 runs in a balanced-incomplete manner. For subplot, treatment was the combination of substrate (bromegrass, corn) and level (0 g, 0.5 g, 1 g, 1.5 g, 2 g, 3 g, 4 g) and experimental unit was bottle, i.e., run x animal x substrate x level (a total of l68 levels).

Processing of Gas Production Data

For each bottle, total gas production per substrate weight (mL/g) collected from 0.25 h to 48 h in a 0.25 *r* interval was fitted by the logistic-growth curve and log-logistic growth curve. The non-linear model for the logistic growth curve is given by:

Equation 4.3. Logistic growth curve for total gas production per substrate weight.

$$\text{Total gas production per substrate weight } (t) = \frac{K}{1+\exp\left(-\beta\left(t-t_{half}\right)\right)} + \varepsilon$$

K: maximum capacity at the stationary stage;

t: total time;

t_{half}: time to reach half of *K*;

β: logistic growth rate; β > 0;

ε: error term.

Equation 4.4. Log-logistic growth curve for non-linear model for the is given by:

$$\text{Total gas production per substrate weight } (t) = \frac{K}{1+\left(\frac{t}{t_{half}}\right)^{-r}} + \varepsilon$$

K: maximum capacity at the stationary stage;

t: total time;

t_{half}: time to reach half of *K*;

r: log-logistic growth rate; shape parameter for log-logistic curve; *r* > 0;

ε: error term.

Parameters of each fitted curve were estimated by minimizing the non-linear least squares. To assure convergence, the values for *K* were bounded above by 240 (mL/g) and values

for t_{half} were bounded below by 3 h and above by 40 h. Note that the curve for total gas production (mL) has the same parameter values except its maximum capacity being K x substrate weight.

Vessels at level 0 g of substrate had no gas production and served as a negative control. The corresponding 12 vessels were excluded from curve fitting. When pressures of a vessel decreased after gas production reached the stationary stage, specifically, after 47 h, these values were removed from curve fitting. Data from certain combinations of substrate, level, animal, and run were missing. Altogether, total gas productions of 129 vessels were subjected to curve fitting. Comparison of mean square errors (MSE) of the 2 models indicates that, in general, the logistic model performed better for corn with MSE ranging from 1.2 to 174.5 $((mL/g)^2)$ and log-logistic model performed better for bromegrass with MSE ranging from 0.8 to 82.6 $((mL/g)^2)$. Therefore, total gas production was fitted with the logistic growth curve for vessels containing corn and log-logistic growth curve for vessels containing bromegrass. Parameters including K and t_{half} have the same interpretation across models. Interpretation of β (logistic growth rate) and r (log-logistic growth rate) is model-specific.

Statistical Method

Curve parameters were all positive. Their estimates first underwent natural-log transformation to better achieve normality and were then analyzed, separately for each parameter, under the linear mixed model. For K, K x substrate weight, t_{half}, and r fixed effects of the model included run, animal, substrate, level, and substrate x level interaction. Random effects included run x animal (whole plot error) and run x animal x substrate x level (subplot error). For

β and *r*, there was only 1 substrate and fixed effects of the model reduced to run, animal and level.

The effect of level for each substrate was assessed via back-transformed least squares means (corresponding to medians of a given curve parameter) and back-transformed mean differences (corresponding to ratios of medians of the curve parameter). Comparisons between 2 levels of a given substrate were carried out using the 2-sided t-test at the 0.05 level. Multiplicity adjustment was not applied. Statistical analyses were performed using Statistical Analysis Software (SAS version 9.4; Cary, NC) NLIN procedure and MIXED procedure. Results are presented both on a total accumulation and per gram of substrate basis.

Results

Gas Production

Total gas production (*K*; Table 4.2) was affected by substrate type ($P < 0.001$) and substrate level ($P < 0.001$). Gas production increased as more substrate was provided, and more gas was produced with corn compared to bromegrass. There was no interaction between substrate type and level ($P = 0.290$). T_{half} gas production was affected by substrate type ($P = 0.014$) and substrate level ($P < 0.001$) and there was a tendency for an interaction between substrate type and level ($P = 0.073$) with corn samples reaching t_{half} more quickly when less substrate was provided, but this difference disappeared at greater substrate values. Beta was affected by substrate level ($P < 0.001$) with an increase from 0.5 g to 4 g corn. *R* was affected by substrate level ($P < 0.001$) with an increase from 0.5 g to 4 g bromegrass.

Dry Matter and Neutral Detergent Fiber Disappearance

There were no differences due to substrate level on DMD when considered on a per gram of substrate basis (Table 4.3 and Fig. 4.1; $P = 0.561$). However, there was a difference due to substrate type ($P < 0.001$) as there was greater DMD in corn compared to bromegrass. When evaluated on the basis of total substrate basis, DMD increased with both substrates as substrate level increased (Table 4.4 and Fig. 4.2; $P < 0.001$), but there was increased DMD with corn compared to bromegrass ($P < 0.001$). There also was a substrate type x substrate level interaction ($P < 0.001$), DMD increased as substrate level increased, but corn increased at a greater magnitude compared to bromegrass.

When evaluated on a per gram of substrate basis, there was an effect of substrate type ($P < 0.001$) with greater NDFD in corn samples. Additionally, there was a tendency for NDFD to increase as substrate level increased ($P = 0.096$). When evaluated on the basis of total substrate provided, NDFD increased with both substrate types as substrate level increased ($P < 0.001$), but there was increased NDFD with corn compared to bromegrass ($P < 0.001$). There also was a substrate type x substrate level interaction ($P < 0.001$), whereby, there was a larger increase in NDFD as substrate level increased in corn than there was with bromegrass.

Volatile Fatty Acids

While total acetate increased with increasing substrate levels ($P < 0.001$), there was a decrease in acetate concentration per gram of substrate as substrate levels increased ($P < 0.001$). There were greater concentrations of acetate with corn compared to bromegrass ($P = 0.002$). There was greater propionate production as substrate levels increased ($P < 0.001$), while there was a decrease in propionate concentration per gram of substrate as substrate levels increased (P

= 0.014). There were greater concentrations of propionate with corn compared to bromegrass ($P < 0.001$). When comparing A:P ratio on a per gram basis, A:P ratio decreased with increasing substrate levels ($P < 0.001$) and on a total basis, there was an increase in A:P ratio as substrate levels increased ($P < 0.001$). There were greater A:P ratios with bromegrass compared to corn ($P < 0.001$). When compared on a total accumulation basis, there was an interaction between substrate type and level ($P < 0.001$), an effect of substrate type ($P < 0.001$), and an effect of substrate level ($P < 0.001$) on A:P ratio. Butyrate concentration increased as substrate level increased in both substrate types ($P < 0.001$)., with corn having greater butyrate production than bromegrass. There were no differences in butyrate concentration per gram of substrate as substrate level increased ($P = 0.361$). There were greater concentrations of butyrate with corn compared to bromegrass ($P < 0.001$).

Total VFA increased with increasing substrate level ($P < 0.001$). On a per gram of substrate basis, there was a decrease in total VFA concentration as substrate level increased ($P < 0.001$). Total VFA concentration was greater with corn compared to bromegrass ($P < 0.001$).

Terminal pH

Terminal pH decreased as substrate level increased ($P < 0.001$) and was reduced in corn compared to bromegrass ($P < 0.001$).

Discussion

To our knowledge, this is the first report evaluate the optimal substrate to buffer ratio for culturing equine cecal microbes while using the ANKOM RF system. When plotting the response variables for total accumulation on a line graph, there appears to be a change of slope

for most response variables with both substrates when the substrate level was 1.5 or 2 g. This is particularly evident for acetate, propionate, butyrate, total VFA, and pH. When plotting the response variables in a bar chart on a per gram basis, there also appears to be changes that occur when substrate levels are 1.5 or 2 g. While we did not have enough statistical power to confirm this, we suspect that when using the ANKOM RF System with 25 mL cecal fluid and 125 mL buffer, approximately 2 g of substrate is the maximum amount that can be provided before creating a buffer-limited environment. A follow up experiment with more substrate levels (i.e., 1.4, 1.5, 1.6 g substrate etc.) would be appropriate to enable a breakpoint analysis to test when the slope changes between 2 points to better define optimal substrate level.

As expected, total gas production increased as substrate level increased, with greater gas production by microbes provided corn. Cornou et al. (2013) also found that gas production was consistently reduced in sheep ruminal microbes cultured with hay compared to those provided corn. Cone et al. (1996) reported similar findings in sheep ruminal microbes cultured with grass hay (rye grass) when compared to microbes provided corn silage and corn cob mix. Judd and Kohn (2018) also reported increased gas production with increasing substrate levels. Utilizing Hungate's method of culturing anerobic bacteria and 2 levels of substrate (0.05 g vs. 0.1 g), they reported gas production was greater in samples with more hay.

Corn is considered more completely fermentable than bromegrass (Hyslop et al., 1999). Our results support this, as gas production, DMD, NDFD, and VFA parameters were all greater with corn compared to bromegrass. Hungate (1966) discovered high-corn diets produced greater amounts of propionate relative to forage-based diets in ruminants. Hamilton et al. (2017) reported diets with corn residue resulted in greater DMD when compared to diets with bromegrass when cultured in rumen fluid. Medina et al. (2002) and Sadet-Bourgeteu et al. (2017)

fed cecally cannulated horses large amounts of corn and generated increased propionate concentrations, thereby reducing the A:P ratio in horses fed corn vs. hay. As expected, in the current experiment all VFA, including propionate, were increased and the A:P ratio was decreased when cecal microbes were provided corn versus bromegrass.

Corn and other high starch concentrates lead to decreased cecal pH as starch is rapidly fermented by lactate-producing bacteria. In addition, larger quantities of starch are more rapidly fermented by the cecal microbes and cause an increase in VFA production. Together, the increase in VFA production and the increase of lactate generated by lactate-producing bacteria result in a rapid decrease in cecal pH (Medina et al., 2002; Biddle et al., 2013; Ochonski et al., 2021). Fonevila et al. (2008) compared fermentative parameters with varying amounts of substrate (alfalfa) versus inocula (rumen) and found more substrate with more ruminal inocula resulted in the lowest pH and VFA concentration (mmol/g of substrate). Judd and Kohn (2018) determined that pH decreased with increased hay concentration (0.05 g vs 0.10 g Timothy hay) because more VFAs were produced from greater amount of substrate. We also observed declining pH as more substrate was provided. This could be due to insufficient buffer to support continued microbial fermentation of the increased substrate, or it could simply be due to the accumulation of VFA.

Conclusion

It appears that in cultures containing 125 mL of buffer and 25 mL of cecal inoculum, no more than 2 g of substrate should be provided in each flask when utilizing the ANKOM RF system. This will allow microbes to utilize substrate without overwhelming the system due to insufficient buffer to support microbial growth and activity. If the amount of substrate provided is too low, fermentative activity will be decreased and substrate degradation will fall within a

narrow range of whatever the inoculum proportion may be (Getachew et al., 1998), making it difficult to detect treatment differences. Conversely, using too much substrate can cause a decrease in inoculum proportion. In these instances, rapid fermentation and accumulation of end products may overwhelm the buffering capacity of the system leading to decreased pH and ultimately reducing microbial activity and fermentation. A follow up experiment with a breakpoint analysis would more narrowly define the optimal substrate level.

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Table 4.1. Proximate analysis of pelleted concentrate fed to cannulated horses during cecal fluid collection period.

Item	As Fed
Dry matter, %	88.7
Crude protein, %	15.0
ADF[1], %	14.4
NDF[2], %	30.2
Starch, %	16.8
DE[3], mcal/lb	1.2

[1]Acid detergent fiber
[2]Neutral detergent fiber
[3]Digestible energy

Table 4.2. Gas production using a type 3 test of fixed effects.

		Item (± SE)				
	Substrate Level, g	***K*[1], mL**	***K*/Sub wt[2], mL/g**	**T_{half}[3], h**	**Beta[4]**	***R*[5]**
Bromegrass	0.5	85 ± 9[a]	171 ± 19[a]	4.8 ± 1.1[a]	-	0.385 ± 0.033[a]
	1	144 ± 16[b]	144 ± 16[a]	16.0 ± 3.6[b]	-	0.566 ± 0.048[b]
	1.5	151 ± 16[b]	100 ± 10[b]	13.4 ± 2.8[b]	-	0.565 ± 0.046[b]
	2	233 ± 22[c]	116 ± 11[b]	28.5 ± 5.5[c]	-	0.648 ± 0.048[c]
	3	279 ± 26[c]	93 ± 9[b]	24.2 ± 4.6[c]	-	0.825 ± 0.061[d]
	4	382 ± 38[d]	96 ± 9[b]	23.0 ± 4.6[c]	-	1.032 ± 0.080[e]
Corn	0.5	98 ± 10[a]	196 ± 19[a]	11.5 ± 2.3[a]	0.0557 ± 0.0063[a]	-
	1	177 ± 18[b]	177 ± 18[a]	25.1 ± 5.0[b]	0.0637 ± 0.0072[a]	-
	1.5	263 ± 26[c]	175 ± 17[a]	24.7 ± 5.0[b]	0.0784 ± 0.0088[b]	-
	2	332 ± 33[c]	166 ± 16[a]	23.5 ± 4.7[b]	0.0954 ± 0.0107[b]	-
	3	465 ± 48[d]	155 ± 16[a]	25.1 ± 5.3[b]	0.0958 ± 0.0111[b]	-
	4	546 ± 52[d]	136 ± 13[ab]	22.5 ± 4.3[b]	0.1121 ± 0.0122[b]	-
P-Value	Substrate Type	< 0.001	< 0.001	0.014	-	-
	Substrate Level	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
	T x L[6]	0.29	0.29	0.073	-	-

[1]Total gas production.
[2]Total gas production / substrate weight.
[3]T_{half}, amount of time to reach half of *K*.
[4]Beta, logistic growth rate, model specific for corn substrate.
[5]R, log-logistic growth rate, model specific for bromegrass.
[6]Substrate type (T) and substrate level (L) interaction.
[a, b, c, d, e] Means within a column and substrate type without a common superscript are different, $P \leq 0.05$.

Table 4.3. Fermentation characteristics presented on a per gram of substrate basis in equine cecal cultures provided with 5:1 buffer: cecal fluid ratio.

		Item (± SE)							
	Substrate Levels, g	**DMD[1], g/g**	**NDFD[2], g/g**	**Acetate, mmol/g**	**Propionate, mmol/g**	**A:P Ratio[3], mmol/g**	**Butyrate, mmol/g**	**Total VFA[4], mmol/g**	**Terminal pH**
Bromegrass	0.5	0.438 ± 0.143	0.351 ± 0.0242	12.8 ± 1.4^{a}	7.4 ± 0.7	2.340 ± 0.078^{a}	0.89 ± 0.57	21.5 ± 2.2^{a}	7.073 ± 0.020^{a}
	1	0.314 ± 0.035	0.204 ± 0.0203	11.3 ± 0.8^{a}	7.3 ± 0.8	2.152 ± 0.146^{a}	0.98 ± 0.37	20.0 ± 1.7^{a}	6.840 ± 0.019^{b}
	1.5	0.315 ± 0.037	0.247 ± 0.0081	10.6 ± 0.8^{a}	7.5 ± 0.7	1.705 ± 0.043^{b}	0.93 ± 0.39	19.6 ± 1.7^{a}	6.693 ± 0.026^{c}
	2	0.319 ± 0.026	0.241 ± 0.0059	9.8 ± 0.9^{a}	7.5 ± 0.7	1.584 ± 0.037^{c}	1.16 ± 0.33	19.1 ± 1.8^{a}	6.526 ± 0.059^{d}
	3	0.294 ± 0.017	0.259 ± 0.0038	8.1 ± 0.8^{ab}	6.5 ± 0.9	1.490 ± 0.028^{d}	1.58 ± 0.20	17.1 ± 1.6^{a}	6.372 ± 0.052^{e}
	4	0.343 ± 0.034	0.304 ± 0.0033	6.9 ± 0.7^{bc}	5.8 ± 0.7	1.416 ± 0.034^{d}	1.73 ± 0.40	15.6 ± 1.0^{ab}	6.218 ± 0.065^{e}
Corn	0.5	0.457 ± 0.143	0.480 ± 0.0242^{a}	17.6 ± 1.4^{a}	11.8 ± 0.7^{a}	1.987 ± 0.078^{a}	2.69 ± 0.57^{a}	32.7 ± 2.2^{a}	6.887 ± 0.020^{a}
	1	0.463 ± 0.035	0.460 ± 0.0203^{a}	12.9 ± 0.8^{b}	12.3 ± 0.8^{a}	1.421 ± 0.146^{b}	2.73 ± 0.37^{a}	28.5 ± 1.7^{a}	6.661 ± 0.019^{b}
	1.5	0.514 ± 0.037	0.519 ± 0.0081^{a}	12.4 ± 0.8^{b}	13.3 ± 0.7^{a}	1.191 ± 0.043^{b}	3.60 ± 0.39^{a}	30.1 ± 1.7^{a}	6.397 ± 0.026^{c}
	2	0.508 ± 0.026	0.535 ± 0.0059^{ab}	11.9 ± 0.9^{b}	14.1 ± 0.7^{ab}	1.047 ± 0.037^{c}	3.88 ± 0.33^{ab}	31.0 ± 1.8^{a}	6.116 ± 0.059^{d}
	3	0.473 ± 0.017	0.516 ± 0.0038^{ab}	9.5 ± 0.8^{c}	12.8 ± 0.9^{ab}	0.918 ± 0.028^{d}	3.16 ± 0.20^{ab}	26.8 ± 1.6^{ab}	5.846 ± 0.052^{e}
	4	0.474 ± 0.034	0.521 ± 0.0033^{ab}	7.8 ± 0.7^{cd}	10.8 ± 0.7^{bc}	0.895 ± 0.034^{d}	3.22 ± 0.40^{ab}	23.7 ± 1.0^{b}	5.606 ± 0.065^{f}
P-Value	Substrate Type	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
	Substrate Level	0.561	0.096	< 0.001	0.014	< 0.001	0.361	< 0.001	< 0.001
	T x L[5]	0.775	0.521	0.244	0.477	0.489	0.265	0.666	< 0.001

[1]Dry matter disappearance.
[2]Neutral detergent fiber disappearance.
[3]Acetate to propionate ratio.
[4] Total volatile fatty acid concentration.
[5] Substrate type (T) and substrate level (L) interaction.
[a, b, c, d, e, f, g, h] Means within a column of a substrate type without a common superscript are different, $P \leq 0.05$.

Table 4.4. Fermentation characteristics presented as total accumulation in equine cecal cultures provided with 5:1 buffer: cecal fluid ratio (adjusted by blank only).

		Item (± SE)							
	Substrate Level, g	**DMD[1], g**	**NDFD[2], g**	**Acetate, mmol**	**Propionate, mmol**	**A:P Ratio[3]**	**Butyrate, mmol**	**Total VFA[4], mmol**	**Terminal pH**
Bromegrass	0.5	0.219 ± 0.143[a]	0.176 ± 0.024[a]	6.4 ± 1.400[a]	1.621 ± 0.700[a]	1.170 ± 0.078[a]	0.445 ± 0.570[a]	10.75 ± 2.200[a]	7.073 ± 0.020[a]
	1	0.314 ± 0.035[a]	0.204 ± 0.020[a]	11.3 ± 0.800[b]	7.300 ± 0.800[b]	2.152 ± 0.146[b]	0.980 ± 0.370[ab]	20.00 ± 1.700[b]	6.840 ± 0.019[b]
	1.5	0.473 ± 0.037[b]	0.371 ± 0.008[b]	15.9 ± 0.800[c]	11.250 ± 0.700[c]	2.560 ± 0.043[c]	1.400 ± 0.390[b]	29.40 ± 1.700[c]	6.693 ± 0.026[c]
	2	0.638 ± 0.026[c]	0.482 ± 0.006[c]	19.6 ± 0.900[d]	15.00 ± 0.700[d]	3.168 ± 0.004[d]	2.320 ± 0.330[c]	38.20 ± 1.800[d]	6.526 ± 0.059[d]
	3	0.882 ± 0.017[d]	0.777 ± 0.004[d]	24.3 ± 0.800[e]	19.500 ± 0.900[e]	4.470 ± 0.028[e]	4.740 ± 0.200[d]	51.30 ± 1.600[e]	6.372 ± 0.052[e]
	4	1.372 ± 0.034[e]	1.216 ± 0.003[e]	27.6 ± 0.700[f]	23.200 ± 0.700[f]	5.664 ± 0.034[f]	6.920 ± 0.400[e]	62.40 ± 1.000[f]	6.218 ± 0.065[e]
Corn	0.5	0.229 ± 0.143[a]	0.240 ± 0.024[a]	8.8 ± 1.400[a]	5.900 ± 0.700[a]	0.994 ± 0.078[a]	1.345 ± 0.570[a]	16.35 ± 2.200[a]	6.887 ± 0.020[a]
	1	0.463 ± 0.035[b]	0.460 ± 0.020[b]	12.9 ± 0.800[b]	12.300 ± 0.800[b]	1.421 ± 0.146[b]	2.730 ± 0.370[b]	28.50 ± 1.700[b]	6.661 ± 0.019[b]
	1.5	0.771 ± 0.037[c]	0.779 ± 0.008[c]	18.6 ± 0.800[c]	19.950 ± 0.700[c]	1.787 ± 0.043[c]	5.400 ± 0.390[c]	45.15 ± 1.700[c]	6.397 ± 0.026[c]
	2	1.016 ± 0.026[d]	1.070 ± 0.006[d]	23.8 ± 0.900[d]	28.200 ± 0.700[d]	2.094 ± 0.037[d]	7.760 ± 0.330[d]	62.00 ± 1.800[d]	6.116 ± 0.059[d]
	3	1.419 ± 0.017[e]	1.548 ± 0.004[e]	28.5 ± 0.800[e]	38.400 ± 0.900[e]	2.754 ± 0.028[e]	9.480 ± 0.200[e]	80.40 ± 1.600[e]	5.846 ± 0.052[e]
	4	1.896 ± 0.034[f]	2.084 ± 0.003[f]	31.2 ± 0.700[f]	43.200 ± 0.700[f]	3.580 ± 0.034[f]	12.880 ± 0.400[f]	94.80 ± 1.000[f]	5.606 ± 0.065[f]
P-Value	Substrate Type	< 0.001	< 0.001	0.002	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
	Substrate Level	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
	T x L[5]	< 0.001	< 0.001	0.852	< 0.001	0.489	< 0.001	< 0.001	< 0.001

[1]Dry matter disappearance.
[2]Neutral detergent fiber disappearance.
[3]Acetate to propionate ratio.
[4] Total volatile fatty acid concentration.
[5] Substrate type (T) and substrate level (L) interaction.
[a, b, c, d, e, f, g, h] Means within a column of a substrate type without a common superscript are different, $P \leq 0.05$.

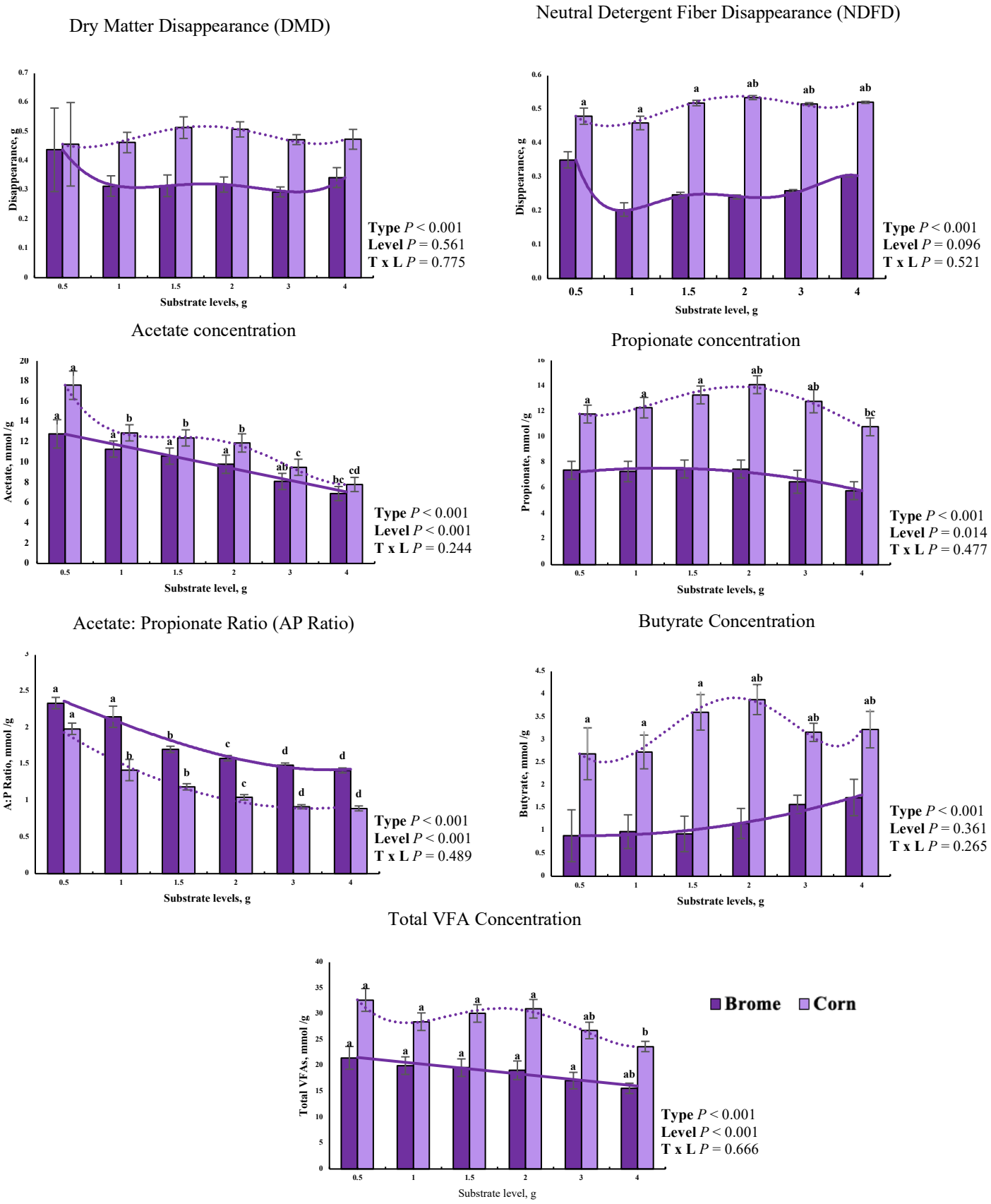


Figure 4.1. Fermentative characteristics on a per gram of substrate basis in equine cecal cultures provided with 5:1 buffer: cecal fluid ratio.

[a, b, c, d, e, f, g, h] Means within substrate type without a common superscript are different, $P \leq 0.05$

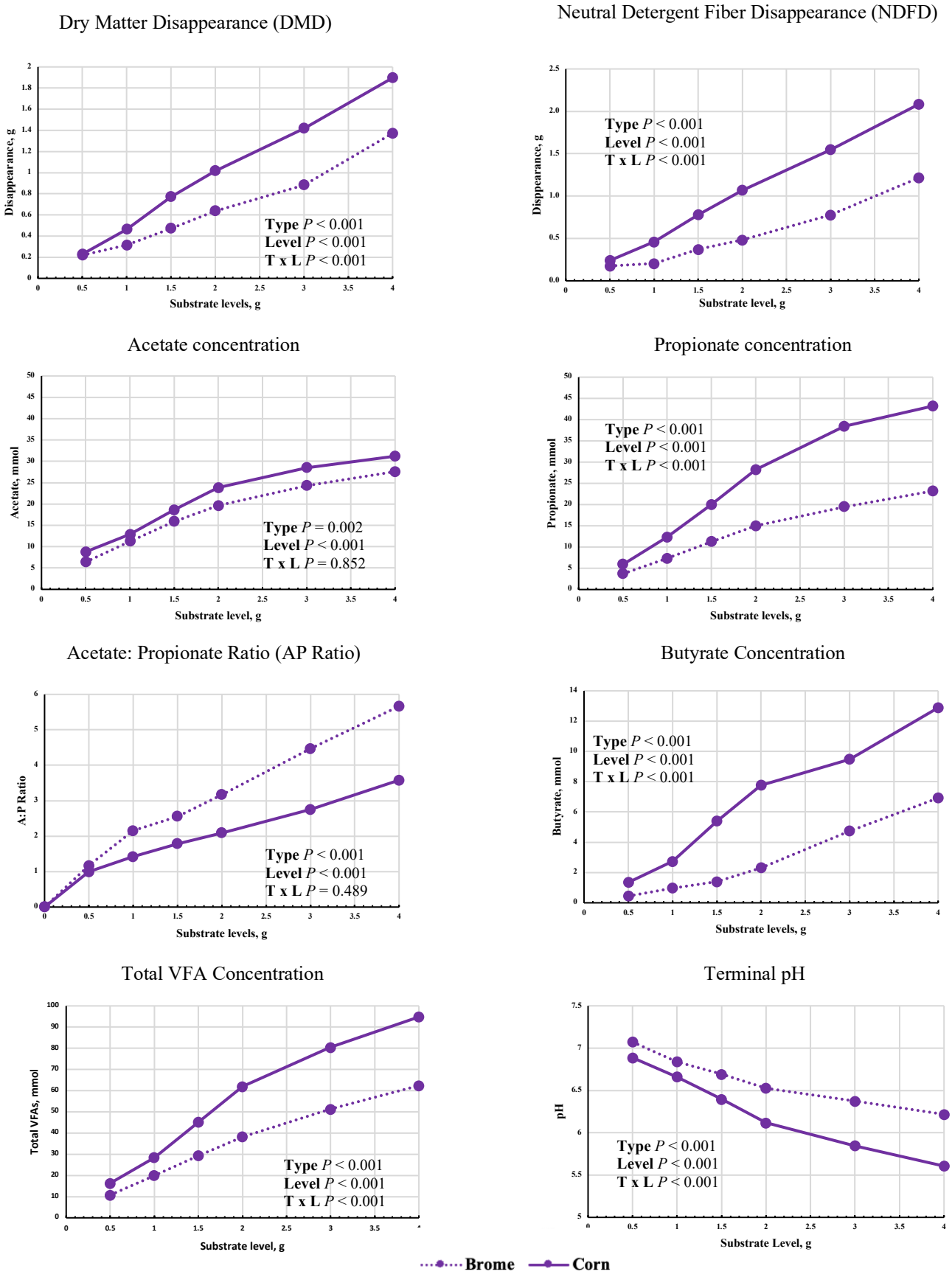


Figure 4.2. Total accumulation of fermentation end products in equine cecal cultures provided with 5:1 buffer: cecal fluid ratio.

[a, b, c, d, e, f, g, h] Means within substrate type without a common superscript are different, $P \leq 0.05$