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Influences of Red Clover Isoflavones on Mitigating Tall Fescue Toxicosis in Beef Cattle

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I am submitting herewith a thesis written by Emily Anne Melchior entitled "Influences of Red Clover Isoflavones on Mitigating Tall Fescue Toxicosis in Beef Cattle." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

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Influences of Red Clover Isoflavones on Mitigating Tall Fescue Toxicosis in Beef Cattle

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DEDICATION

This thesis is dedicated to all teachers, but especially my agriculture teachers, Debra Barry, and Brett Wheeler for your encouragement, and support to pursue my dreams; to Dr. Laura Flatow, for countless words of encouragement, laughter and running in sparkle skirts together; and to Joanne Duke, for your kindness and support of everything agriculture related.

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ABSTRACT

The United States is one of the leading producers of beef in the world, producing between 24-27 million pounds each year. To meet the demands of a growing global population, cattle producers are under increased pressure to efficiently produce meat. The climate in the Southeastern United States provides abundant precipitation which cattle producers may utilize for multi-seasonal grazing. While cattle graze on many different forage varieties, tall fescue *(Lolium arundinaceum (Darbyshire))* has been cultivated throughout the region as one of the most predominate forages. Tall fescue provides an abundant forage supply for livestock, and can be grazed in both the spring and fall cool season months.

Despite the advantages of an ample forage supply, when tall fescue is infected with an endophyte it can be harmful to livestock. This endophyte (*Epichloë coenophiala*) provides the tall fescue plant with disease resistance and drought tolerance. The endophyte also produces ergot alkaloid compounds which can be harmful to livestock when consumed. Overall reduced reproductive efficiency in males and females as well as reduced growth performance and heat tolerance are several symptoms of the ergot alkaloids. These symptoms are collectively known as tall fescue toxicosis, which can cost the beef cattle industry up to $2 billion in losses each year. In an effort to reduce these effects, the inclusion of legumes such as red clover has proven beneficial. Legumes contain unique compounds known as isoflavones, found in abundance in red clover. The objectives of the present study were to determine the beneficial physiological, behavioral and microbiological influence of red clover isoflavones on beef cattle experiencing tall

fescue toxicosis. Isoflavones improved fiber and protein disappearance post ruminal-fermentation compared to control treatments. Isoflavones reduced serum glucose, altered twenty-six ruminal bacterial taxa, but did not affect average daily gain, dry matter intake or serum prolactin levels. Additionally isoflavones did not significantly alter ruminal volatile fatty acid concentrations, pH, or behavioral patterns. The dosage used in the present studies were not sufficient to elucidate the same benefits cattle experience from consuming red clover in a tall fescue pasture, and thus subsequent dosing studies should be conducted.

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INTRODUCTION

The United States is one of the leading producers of beef in the world, producing between 24-27 million pounds each year (Comerford et al., 2016). To meet the demands of an exponentially growing global population, cattle producers are facing increased pressure to efficiently produce meat for consumers. The climate in the Southeastern United States provides abundant precipitation which cattle producers may utilize for almost year-round grazing. While cattle graze on many different forage varieties, tall fescue (*Lolium arundinaceum (Darbyshire))* has been cultivated throughout the region as one of the most predominate forages. Tall fescue provides an abundant forage supply for livestock, and can be grazed in both the spring and fall cool season months.

Despite the advantages of a longer grazing season and ample forage supply, when tall fescue is infected with an endophyte it can be harmful to livestock. This endophyte (*Epichloë coenophiala)* provides the tall fescue plant with disease resistance and drought tolerance. Despite the benefits associated with the endophyte, it produces ergot alkaloid compounds which can be harmful to livestock when consumed. Overall reduced reproductive efficiency in males and females as well as reduced growth performance and heat tolerance are several symptoms of the tall fescue endophyte. The culmination of these symptoms is known as tall fescue toxicosis, which can cost the beef cattle industry up to $2 billion in losses each year.

CHAPTER I
LITERATURE REVIEW

A version of this chapter has been submitted for publication by Emily A. Melchior and Phillip R. Myer to Journal of Applied Animal Research.

Introduction

Tall fescue (*Lolium arundinaceum (Darbyshire)*) is a cool season perennial grass and is the most common forage utilized by beef cattle operations in the mid-South region of the United States. The plant is known for its disease and insect resistance as well as prominent stand persistence throughout seasons of drought. Despite the nutritive benefits tall fescue provides, negative impacts on livestock growth and reproduction can occur when infected with the endophytic fungus *Epichloë coenophiala* (previously *Neotyphodium spp.).* In ruminants, consumption of the endophyte often results in the condition called fescue toxicosis. This condition is characterized by several symptoms including decreased weight gain, reduced conception rates, depressed feed intake, and increased blood pressure and body temperatures. Periods of extreme heat or cold will cause animals to exhibit more pronounced symptoms leading to an unthrifty appearance. These symptoms manifest in decreased productivity which costs beef producers across the United States over $2 billion annually. To meet the nutritional demands of a growing global population, cattle producers are under increased pressure to efficiently produce beef that is economical for its consumers. As many stocker and backgrounding operations utilize this forage, it is critical to identify economical and novel methods and management strategies that will reduce losses incurred by symptoms of fescue toxicosis.

While the physiological effects of the tall fescue endophyte are well known from decades of research, the effects of the endophyte upon the ruminant system as a whole

has yet to be determined. Behavioral changes from fescue toxicosis symptoms have been shown to negatively affect the health and nutrient intake of cattle (Howard et al., 1992). In an effort to cope with heat stress that is amplified by consumption of the endophyte, animals may spend increased amounts of time in shady areas of a pasture, or wading in pools of water or mud to alleviate this stress. As extreme environmental conditions exacerbate the effects of fescue toxicosis, the resultant behaviors should be elucidated more clearly to increase awareness of potential with tall fescue consumption in a pasture. Due to observance of these behaviors alongside physiological stressors, historically producers have used various mitigation methods, particularly the use of cool-season legumes such as red and white clover within these pastures to reduce these effects. Legumes are unique in their ability to fix-nitrogen in the soil, as well as containing compounds known as isoflavones.

The ruminal microbiome is critical to the health and nutritional status of the ruminant, as fermentation provides vital energy precursors. Without a healthy microbiome, the animal's ability to ferment forages like tall fescue, and other feedstuffs would be impaired. Yet, as concerning as the effects of endophyte-infected tall fescue are for reproduction and other physiological production parameters, little research exists as to the effect of the tall fescue endophyte on the rumen microbiome.

To that end, this review discusses fescue toxicosis caused by the *Epichloë* spp. derived ergovaline, its effects on the rumen microbial populations, as well as mitigation through grazing management, focusing primarily on the animal aspects and activities associated with these ergot alkaloids and clover isoflavones. Understanding the

mechanisms of how isoflavones alter biological processes, and result in reducing the observed symptoms associated with fescue toxicosis and mitigation of those symptoms will provide increased insight to this condition.

Tall Fescue Toxicosis

Physiological effects of consumption

Tall fescue (Lolium arundinceum Schreb.) is one of the most predominant forages utilized in beef cattle production, and is particularly popular in the south-eastern United States. This non-native, cool-season perennial grass occupies approximately 15 million hectares throughout the country (Keyser et al., 2011). Tall fescue is well known for its combination of drought resistance in humid environments, its insect and disease tolerance. (Hoveland, 1993), as well as its high forage quality, where digestible nutrients and protein are competitive with other native forages (Bush et al., 1973). The most common variety “Kentucky-31” was released as a tall fescue cultivar by the University of Kentucky in 1942. The popularity of this cultivar spread throughout the Southeast and Midwest during the 1940s and 1950s because it was easy to establish and manage for abundant forage production.

The ability of tall fescue to remain tolerant to stressors lies within the presence of the fungal endophyte *Epichloë coenophiala* (Leuchtmann et al., 2014; Young et al., 2014). The endophyte and tall fescue plant form a mutualistic relationship, improving the plant’s competitive advantage over surrounding forages even in harsh environmental conditions such as drought, and temperature extremes. The fungus persists throughout the plant

and produces ergot alkaloid compounds which are often concentrated in the seed head of tall fescue. Despite the advantages the endophyte provides the plant, cattle and horses grazing endophyte-infected tall fescue will often develop syndromes that reduce production, lactation and reproductive capacity due to these ergot alkaloids. (Ball et al., 1991; Waller, 2009). These concerns for reduced animal performance first gained attention in the 1950s, where Merriman reported a loss of appetite, reduced body weight and increased respiration rates (1955a). Reduced average daily gain, increased respiration rates and reduced reproductive efficiency became noted in cattle consuming endophyte-infected tall fescue compared to other forages and legumes (Blaser et al., 1956; Forney et al., 1969; Jacobson et al., 1970; Williams et al., 1972). Improved research efforts unearthed that the endophyte itself is not harmful, but is merely responsible for the production of ergot alkaloids throughout the plant. When consumed by livestock, these compounds bind to receptors found throughout the body. Most namely of these alkaloids is ergovaline, which is well known for its dopamine agonist (Yates et al., 1985; Lyons et al., 1986; Campbell et al., 2014), prolactin regulation (Strickland et al., 1994) and vasoconstrictive effects (Klotz et al., 2007; Foote et al., 2012).

In cattle, reduced overall gains, decreased reproductive performance, reduced neuroendocrine function and increased heat stress are exhibited when ergot alkaloids are consumed. It has been reported that adverse effects with gain may be related to reduced ruminal and gastrointestinal function and motility. Dry matter intake has been reduced in cattle and sheep consuming endophyte-infected tall fescue (Hemken et al., 1979; Hemken et al., 1981). Appetite suppression may be the result of poor thermoregulation

due to high ambient temperature stress (Beede, 1986), as well as the possible interaction of the ergot alkaloid with serotonin receptors (Dyer, 1993). Other researchers have suggested that the reduced weight gains exhibited by cattle consuming endophyte-infected tall fescue could not be completely accounted for by only reduced intake (Schmidt et al., 1993). Although differences in weight gain may not be entirely explained by heat stress or intake; altered rumen kinetics (Hannah et al., 1990) or reduced blood flow to the digestive tract (Rhodes et al., 1991; Klotz et al., 2007; Foote et al., 2012) will decrease gain, nutrient uptake and subsequent utilization.

The effects of vasoconstriction occur with both arteries and veins, becoming most apparent with extreme heat or cold stress. In periods of heat stress, ergot alkaloids interfere with the thermoregulatory mechanism by impeding heat dissipation that is dependent on circulating blood to the extremities and skin surface to be cooled (Walls et al., 1970). The combination of vascular constriction throughout the animal's body and the reduced ability to shed a winter hair coat provide the opportunity for cattle to undergo heat stress during the warmer summer months in the mid-South transition region. Hoveland et al. (1983) reported elevated rectal temperatures and increased respiration rates when cattle consuming endophyte were subjected to high ambient temperature. In a study conducted by Klotz et al. (2016), steer vasculature remained more constricted even after removal from toxic endophyte-infected tall fescue for 28 days, suggesting that reduced susceptibility to heat stress and increasing vascular activity requires at least 35 days on non-toxic tall fescue or another forage before returning to baseline levels. These studies provide evidence that the constriction of the animal's vasculature may be most

responsible for the characteristic symptoms of heat stress with fescue toxicosis. In addition to experiencing more pronounced heat stress, when cattle consume endophyte-infected tall fescue in periods of extreme cold temperatures, the vasoconstrictive effects of ergovaline may cause cattle to experience lameness, commonly known as fescue foot, as well as sloughing off the tips of tails and ears (Spiers et al., 1995) due to impaired vascular function in the extremities.

The reproductive effects of ergot alkaloid consumption in ruminants has focused a great deal on female reproductive physiology, of which ergot alkaloid consumption has been demonstrated to reduce conception, and lactation (Gay et al., 1988; Peters et al., 1992). Research into the reproductive effects of ergot alkaloids in male livestock has not been as well documented beyond influences on spermatozoa production. When exposed to ergot alkaloids, bulls have reduced fertility potential (Schuenemann et al., 2005b). A decrease in sperm concentration and increase in abnormal sperm collected from bulls grazing endophyte-infected tall fescue has also been reported (Pratt et al., 2015b), and the main implication from these is reduced bull fertility. In addition to reduced motility, sperm collected from bulls consuming endophyte-infected tall fescue pastures also had a slower velocity (Looper et al., 2009). The presence of the prolactin receptor in the testes and the presence of prolactin in seminal fluid has been demonstrated in cattle (Pratt et al., 2015a). Given the effects of ergot alkaloids on prolactin in the pituitary, this may be an area where ergot alkaloids could have an effect on male reproduction, as reduced serum prolactin is characteristic of fescue toxicity in the blood. Another aspect worth considering is the localized effect of vasoconstriction on the temperature of the testes, as

bulls consuming ergot alkaloids have a reduced scrotal temperature (Schuenemann et al., 2005a), possibly reducing motility of spermatozoa.

Consumption of endophyte-infected tall fescue alters several neuroendocrine pathways. The endophyte has been shown to reduce serum prolactin concentrations across species, in cattle (Hemken et al., 1979), sheep (Bond et al., 1981), and horses (McCann et al., 1992). Dopamine is the major prolactin-inhibiting hormone and the ability of the endophyte to reduce serum prolactin levels is due in part to the actions of ergot alkaloids on pituitary dopamine receptors (Goldstein et al., 1980; Civelli et al., 1993; Campbell et al., 2014). Administration of a dopamine antagonist to animals affected by fescue consumption increases the circulating levels of prolactin and demonstrates the dopaminergic effect of the alkaloids produced by the endophyte (Lipham et al., 1989). Prolactin, while pivotal for milk production and growth of mammary tissue, is also associated with the shedding or retention of a winter hair coat. Cattle grazing endophyte-infected tall fescue frequently maintain their winter coat, which is known to be a classic symptom of animal's experiencing summertime fescue toxicosis.

Ergot alkaloids and rumen microbiome

Ergot alkaloids are susceptible to microbial degradation in the rumen (Moyer et al., 1993) and in the earthworm intestine (Rattray et al., 2010). However, it is unknown at the present time which organisms within the rumen microbiome are responsible for the degradation. Tryptophan is essential for the biosynthesis of ergovaline (Garner et al., 1993; Roylance et al., 1994). The structure of tryptophan allows for the formation of the ergovaline ring structure (Figure 1) which has a similar backbone to several

catecholamines, including dopamine and serotonin. The presence of the detrimental ergot alkaloids is not unique to the tall fescue plant; perennial ryegrass produces an endophyte containing ergopeptides that provide similar symbiotic traits to the ergot alkaloids from tall fescue as well as in *Claviceps purpurea* and related species (Clay, 1988; Bush et al., 1997; Wang et al., 2004). Where fescue toxicosis impacts production throughout the United States, ryegrass toxicity is found commonly throughout Australia and New Zealand (Van Heeswijck et al., 1992; Easton, 1999), with detrimental effects persisting through consumption of a similar endophyte that infects tall fescue, *Neotyphodium lolii*. Ryegrass staggers has been well documented throughout literature, caused by consumption of endophyte-infected perennial ryegrass that produces the neurotoxin lolitrem B (Figure 2). Lolitrem B acts as a calcium activated potassium channel inhibitor, causing muscle tremors, stiff movements, and lateral recumbency (Tor-Agbidye et al., 2001; Imlach et al., 2008). In both ryegrass and tall fescue, the endophyte is not uniformly distributed throughout the plant, and grazing management strategies to reduce seed heads has proven effective at reducing instances of these toxicities.

In a recent in vitro study conducted by Harlow et al. (2017b), it was determined that ruminal hyper-ammonia producing bacteria (HAB), which are responsible for the deamination of amino acids and breakdown of peptides, could be responsible for the breakdown of ergovaline and ergopeptide compounds. HAB are unique that they utilize tryptophan to breakdown amino acids and produce ammonia at very high rates. Specifically, it was concluded that five bacteria found in the rumen were able to degrade ergovaline, all of which had characteristics of hyper-ammonia producing bacteria, lending

support that bacteria which degrade tryptophan, also have the ability to degrade ergovaline. Specifically, these bacteria were gram-positive and rod shaped, phylogenetically similar to *Clostridium botulinum*, a gram-positive organism, but non-toxic when ruminant animals consume this orally (Allison et al., 1976). While the majority of ruminal bacteria are designated gram-negative, an increase in the proportion of gram-positive bacteria is seen during the transition from forage based diets to increased concentrate diets. This shift is typically seen when calves from cow-calf and stocker operations make the transition to a common feedlot diet (Fernando et al., 2010). In higher grain diets and more readily digestible forages, bacterial numbers can increase 10-100 fold compared to when animals are fed a less digestible forage diet (Nagaraja, 2016). Harlow et al. also concluded that some *Prevotella* species contribute to the metabolism of ergovaline (2017b). As *Prevotella* species are relatively abundant in the rumen (Jami et al., 2012) by assisting in protein metabolism (Attwood et al., 1995), inducing a ruminal environment favorable to *Prevotella* and other HAB production could be beneficial at reducing instances of fescue toxicosis.

It has previously been determined that ergovaline can be degraded into lysergic acid (Figure 3), where it is then excreted in urine and feces, but where this degradation specifically occurs has not been ascertained (Hill et al., 2001; Schultz et al., 2006). The mechanism behind the conversion between ergovaline and lysergic acid has yet to be understood. De Lorme et al. suggest that ergovaline is first liberated from feed material and thus microbial action degrades it further to lysergic acid, acting on tissues throughout the body where it will eventually be excreted in urine and fecal material (2007). Klotz et

al. concluded that ergovaline is exponentially more powerful in stimulating vasoconstriction within in vitro bovine vasculature compared to lysergic acid, and more potent throughout host physiology as a whole (2006; 2007). Ruminal microorganisms interact with feedstuffs in three established ways: those within the ruminal fluid, those loosely attached to feed particles and those firmly attached to feedstuffs (Cheng et al., 1997), thus breakdown of feedstuffs is essential for several types of microorganisms to access nutrients. Consequently, potentially detrimental compounds such as ergot alkaloids including ergovaline and subsequently lysergic acid are also accessible by microorganisms.

When tall fescue seed was incubated with rumen fluid in an in vitro fermentation study conducted by Westendorf et al. (1992), the diet that had been previously incubated with fluid was less toxic to rats consuming the whole seed than those that were fed a non-ruminal fluid incubated fescue seed. In the same study, Westendorf et al. determined that rats consuming the rumen fluid inoculated endophyte-infected seed had improved gain: feed conversion compared to those with non-incubated seed (1992). This suggests that the rumen microbiota, are responsible for reducing some of the toxicity of the endophyte, in agreement with results by De Lorme et al (2007). However, the results from Westendorf et al. (1992), De Lorme et al. (2007) and the bacteria isolated by Harlow et al. (2017b), were accessed from rumen fluid conducted as an in vitro experiment, rather than in vivo where additional physiological parameters can be measured for efficacy.

As endophyte-infected tall fescue will continue to persist throughout the United States as an important forage base, it is paramount to understand its benefits as well as limitations.

The impacts of ergot alkaloids on livestock production have been problematic to producers for decades but also have prompted the development of several successful mitigation methods.

Mitigation Strategies: Novel Endophyte, Clovers and Isoflavones

Research on the effects associated with the consumption of endophyte-infected fescue has led to investigation of management strategies to improve production where replacement of tall fescue forage is not economically appropriate. As costs of replacing existing stands of endophyte-infected tall fescue can exceed $600/ha, additional management alternatives can be sought out (Kallenbach, 2015). Some of these strategies have included supplementation of vitamin E (Jackson et al., 1997), thiamine (Dougherty et al., 1991), and protein (Aiken et al., 2001) to rectify many of the concerns with fescue toxicosis.

The use of a novel endophyte-infected tall fescue plant provides similar nutritional value as the endophyte-infected plant, but without the deleterious effects of the endophyte. Cattle grazing novel endophyte-infected tall fescue experience increased growth rates and increased average daily gain without reduced prolactin, increased respiration rates and rectal temperatures (Nihsen et al., 2004; Beck et al., 2008; Hancock et al., 2009). While the novel endophyte proves useful in managing and reducing fescue toxicosis, it is not always the most economical solution. Gunter et al. (2004) estimated at least a three year span between introductions of the novel endophyte before yields were enough to become economically profitable. Zhuang et al. (2005) estimated that until a

pasture is 70% or more infected with the endophyte, it is not economically beneficial to utilize tall fescue with the novel endophyte.

Grazing management strategies of including rotation grazing and inclusion of legumes and other grasses within a tall fescue pasture have been used for decades to reduce instance of fescue toxicosis. Legumes have a distinctive benefit compared to other forage species, due to their ability to harness and fix nitrogen into the soil, improving soil fertility and nutrient value. Vincent (1980) and Young (1989) describe the relationship between legumes and nitrogen fixation as a result of the symbiotic soil microorganism *Rhizobium spp*. The bacteria form a symbiotic relationship with the legumes, creating small nodes on the root system of the legume. The plant will supply carbohydrates for the bacterium, and the *Rhizobium* spp. provide nitrogen fixation to the plant. In addition to benefitting both the bacterium and soil by this nitrogen fixation property, legumes are high in digestible nutrients and protein, increasing the quality of the pasture when included alongside grasses (Chestnut et al., 1991; Duranti et al., 1997). When conducting a study with sheep, Thornton and Minson calculated voluntary intake of legumes was 28% higher when compared to equally digestible grasses (1973). As they are more digestible and palatable, the use of lush pastures of legumes is cause for speculation on their use, as livestock can have a tendency to bloat with the consumption of such nutritive forages, particularly pastures of legumes with alfalfa and ladino clover (Bryant et al., 1960; Lees et al., 1981). Despite these concerns, the inclusion of legumes into forage stands presents great opportunity for improving forage nutritive value. Particularly in the mid-South region of the United States, forage recommendations may include planting red and

white clover or other cool-season legumes alongside tall fescue stands in a pasture, for the previously mentioned benefits.

In particular, red and white clover have proven to be most successful at increasing growth and overall gains, and mitigating fescue toxicosis (McLaren et al., 1983; Lusby et al., 1990; Chestnut et al., 1991; Beck et al., 2012). Lusby et al. (1990) found that the inclusion of red clover into stands of tall fescue not only reduced severity of fescue toxicosis (reduced rectal temperatures and respiration rates) but it improved average daily gain and carcass quality compared to cattle on tall fescue alone. Broderick (1995) noted that the protein content of legumes provided an excellent source of nitrogen for ruminants, citing that red clover compared to alfalfa was more efficient as a source of rumen un-degradable protein (RUP). Previous literature has indicated that the inclusion of legumes in tall fescue pasture will reduce the occurrence of fescue toxicosis by a dilution or competitive selection against tall fescue. However, recent research is indicating the legumes may reduce the severity of fescue toxicosis by other mechanisms.

Legumes, including clovers and soybeans, contain phenolic, phytoestrogen compounds known as isoflavones. These compounds are known for their use in human medicine for mitigation of menopausal symptoms in women (van de Weijer et al., 2002). While the isoflavones are considered nonsteroidal, they maintain a weak-moderate affinity for estrogen receptors, specifically estrogen receptor β (ER-β). Receptor ER-β is most widely expressed on non-reproductive tissues such as bone and blood vasculature where it mediates some of the growth-promoting effects of estrogen on non-reproductive tissues (Sunita et al., 2011). However Millington et al. (1964) noted an estrogenic effect

of wethers grazing red clover pastures. Adams (1995) suggested that fertility is only affected by cows and ewes consuming legumes, while males remain unaffected. When cows consumed solely red clover silage, a significant reduction in conception was noted until the silage was removed from the diet, and consequently the cows were able to conceive (Kallela et al., 1984), contrasting unaffected heifer fertility rates reported by Austin et al. (1982). In males, Lightfoot et al. (1967) noted failure of sperm transport from rams to ewes previously grazing legume pastures. Various reports indicate species differences of subterranean clover varieties contributing to these fertility differences rather than all clover or legume varieties (*Trifolium subterraneum L*.) (Cox et al., 1974; Hughes Jr, 1988). Nevertheless, the nutritive value red and white clover varieties add to pastures should be taken into account when addressing fertility concerns. Waghorn and McNabb (2003) suggested that reducing specific isoflavone consumption from clover varieties will have a beneficial effect on fertility. Contrary to a specific area of tall fescue where the endophyte is concentrated, isoflavones are found throughout the entire plant, rather than concentrated in the bloom or leaves (Table 1).

Four major forms of isoflavones found in red clover include biochanin A (Figure 4), formononetin, genistein, and daidzein. In ruminants, formononetin is metabolized to daidzein and further metabolized to equol. Biochanin A is metabolized to genistein and then further to p-ethylphenol (Braden, 1967; Dickinson et al., 1988). Equol has been suggested to have greater influence on bovine blood vasculature than its precursors. Red clover, the most commonly used commercial clover, contains biochanin A in the highest quantity. When dairy cows were fed on a white clover, red clover, lucerne, or chicory

pastures, equol from red clover was found in the highest concentrations throughout the body and discovered in the milk (Andersen et al., 2009). Sheep are more susceptible to formononetin compared to larger ruminants, having reduced fertility from lingering estrogenic effects.

It has been reported that when exposed to isoflavones, the vasculature will relax (Nevala et al., 1998; Simoncini et al., 2005), whereas when ergovaline from tall fescue is consumed vasculature will constrict due to binding on amide receptors (Rhodes et al., 1991; Aiken, 2009) It has been proposed that red clover isoflavones contribute to an antimicrobial effect within ruminal bacteria communities (Flythe and Kagan, 2010) and reduce production of ammonia from ruminal bacteria at a concentration of 30ppm (Flythe et al., 2013a). Interestingly, the hyper-ammonia producing bacteria are reduced in the presence of isoflavones, which are among the bacteria needed for ergovaline degradation as elucidated by Harlow et al. (2017b). It was further determined that when 30mg of biochanin A per liter of rumen was infused into the rumen of goats receiving endophyte-infected tall fescue seed, vasorelaxation and return to normal pulse were observed (Aiken et al., 2016). This has been associated with agonist activity at β-adrenergic receptors within the endothelium of the blood vessels which stimulates synthesis of nitric oxide that in turn will promote vasorelaxation. As vasoconstriction contributes to heat stress, promoting vasorelaxtion with inclusion of legumes may be a solution to understanding tall fescue toxicosis mitigation.

In an effort to greater understand the influence of these compounds on rumen microflora, Harlow et al. (2017a) used biochanin A in combination with dried distiller's

grains to determine amino acid degradation and performance of beef steers. They observed that biochanin A reduced HAB and had an additive effect on steer weight gain. Additionally, studies conducted by Harlow et al. targeted specific amylolytic or cellulolytic bacterial species and their sensitivities to biochanin A (Harlow, 2017; 2018). Three cellulolytic bacteria (*Fibrobacter succinogenes S85, Ruminococcus flavefaciens 8, and Ruminococcus albus 8)* and four amylolytic bacteria (*Strep. bovis JB1, Strep. bovis HC5, Lactobacillus reuteri, Selenemonas ruminatium* were reduced with exposure to biochanin A. However, the minimum inclusion amount of biochanin A in a diet has not been determined, and may have a much lower threshold than what has been previously used in diet formulation. Biochanin A may be the primary isoflavone to improve the performance of production animals. This adds further evidence that the use of isoflavones improve beef cattle efficiency, and future studies should be conducted to determine a minimum inclusion rate for positive results.

Conclusions and Future Directions

These preliminary studies indicate a change in vasculature and rumen microbial communities in response to red clover sourced isoflavones, but further studies need to be conducted to determine the effect on the entire rumen bacterial community and physiology in cattle with that specific concentration of isoflavones. It has yet to be elucidated if clover isoflavones can mitigate symptoms of fescue toxicosis in cattle, and if daily administration of isoflavones will cause any microbial dysbiosis within the rumen. Therefore, due to the prevalence of fescue toxicosis throughout the Southeast, there is a critical need to determine microbial and host-physiological responses to clover

isoflavones in cattle, possibly resulting in additional mitigation approaches to fescue toxicosis. Reducing any potential dysbiosis in rumen microbial populations that may occur due to consumption of endophyte-infected tall fescue may contribute to increased nutritional efficiency and subsequent health of the animal. Therefore, determining the effect of clover isoflavones on rumen microbial health and host physiology can provide further insights into combating fescue toxicosis.

As the inclusion of legumes into pastures provides many benefits for both soil fertility and forage quality, determining the minimum amount of isoflavones in a diet to sufficiently reduce symptoms of fescue toxicosis can be key to determining forage management practices. For example, not over- or under- seeding pastures with legumes can result in reduced productivity for the producer due to competition of other plants and upkeep expenses for these broadleaf plants. Determining the minimum amount of isoflavones in red clover that will reduce instances of fescue toxicosis, could improve forage recommendations to producers. The concerns with ergot alkaloid consumption on livestock will persist until the combined efforts of pasture and cellular/biochemical research approaches are met.

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Appendix A

Table 1**.** Averages of the total isoflavone concentration (mg/g) among plant parts and clover species (adapted from Butkute et al. (2014).

Plant Part	*Trifolium spp.*[1]				
	T. medium	*T. pretense*	*T. repens*	*T. pannonicum*	*T. rubens*
Stems	3.62	3.62	0.204	0.230	0.780
Leaves	7.54	2.74	0.191	0.274	0.493
Flowers	2.31	2.22	0.171	0.98	0.38

[1]Concentration of total isoflavones in mg/g

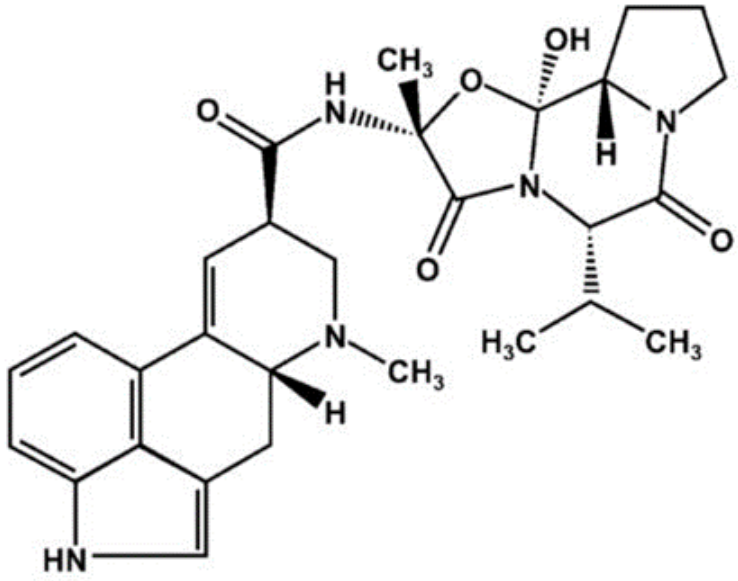

Figure 1 Structure of ergovaline, courtesy of Klotz et al. (2007).

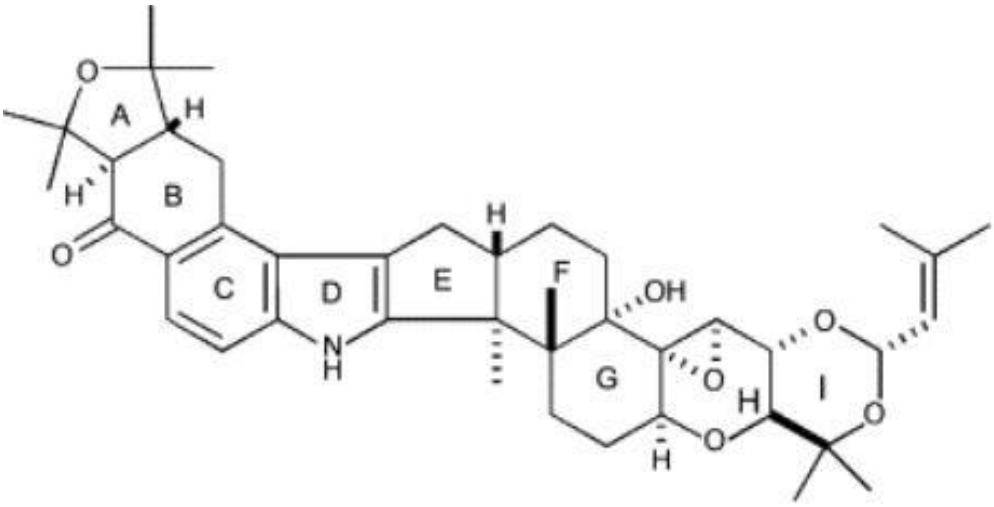

Figure 2 Structure of Lolitrem B, found commonly in perennial ryegrass. Image from Saikia et al. (2008**)**

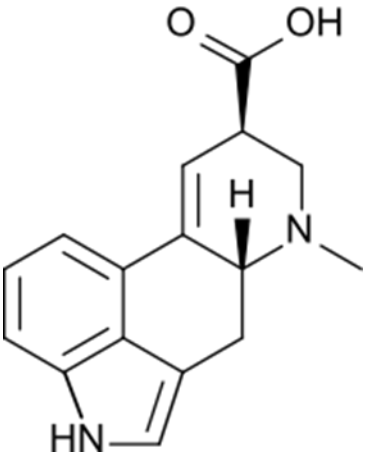

Figure 3 Structure of lysergic acid. Image adapted from (Pesqueira et al., 2014).

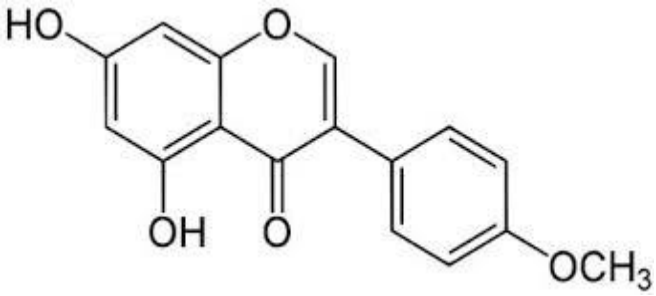

Figure 4. Structure of the isoflavone biochanin A. Image adapted from Saviranta et al. (2011)

CHAPTER II
EFFECTS OF ENDOPHYTE-INFECTED TALL FESCUE SEED AND RED CLOVER ISOFLAVONES ON RUMEN MICROBIAL POPULATIONS, FIBER DEGREDATION AND VOLATILE FATTY ACIDS *IN VITRO*

Abstract

Negative impacts of endophyte-infected tall fescue in livestock systems are responsible for over $2 billion in losses each year to producers. Influences of endophyte-infected tall fescue has been studied for decades but mitigation methods have not yet been clearly elucidated. Isoflavones found in red and white clover have been the subject of recent research regarding tall fescue toxicosis mitigation, but have yet to establish baseline concentrations that are effective. Therefore, the aim of this study was to evaluate the effect of ergovaline and red clover isoflavones on rumen microbial populations, fiber degradation and volatile fatty acids in an *in vitro* system. Using a dose of 1.10 mg × L^{-1}, endophyte-infected or endophyte-free tall fescue seed was added to ANKOM fiber bags with or without 2.19mg isoflavones in the form of a control, powder or pulverized tablet, resulting in a 2 × 3 factorial arrangements of treatments. Measurements of rumen pH, volatile fatty acids, bacterial taxa, as well as neutral detergent fiber, acid detergent fiber and crude protein disappearance were taken after 48h fermentation using the DAISY II system. NDF disappearance values were significantly altered by seed type and treatment ($P < 0.05$), and ADF disappearance values were significantly different among isoflavone treatments, as well as seed × treatment interaction ($P < 0.05$). A seed × treatment interaction was observed with respect to crude protein disappearance ($P < 0.05$). Bacterial taxa were significantly different among groups ($P = 0.03$). Rumen pH values were not significantly different between treatments groups ($P > 0.05$). As isoflavones benefitted fiber degradation with tall fescue seed, they may be a viable use for mitigating

tall fescue toxicosis, but further research should be conducted to determine physiological implications as well as microbiological changes in an *in vivo* experiment.

Introduction

Ergot alkaloid toxins found in the endophyte-infected tall fescue plant (*Lolium arundinaceum (Darbyshire))* contribute to the tolerance of drought, heat and disease in the plant, but are implicated in expensive production losses when grazed by livestock (Kallenbach, 2015). Concerns of the alkaloids manifest with reduced blood flow to the periphery, reduced reproductive efficiency and overall lower reported growth performance (Bush et al., 1970; Bush and Buckner, 1973; Ball et al., 1991). Of the alkaloids produced by endophyte-infected tall fescue, ergovaline is the most predominant of concern in the plant, which has the most detrimental effects on livestock (Strickland et al., 2011; Klotz and Nicol, 2016). Reducing the consumption of endophyte-infected tall fescue has proven to be a challenge for livestock producers, as it occupies roughly 15 million hectares in the United States (Kim Abney et al., 1993) with the majority in the east and southeast regions of the country. Replacing endophyte-infected varieties with novel and endophyte free varieties of tall fescue can abate toxicosis, but may not be a cost effective long term solution (Zhuang et al., 2005). However, the inclusion of cool-season legumes into tall fescue pastures is beneficial in reducing or mitigating tall fescue toxicosis symptoms (Lusby et al., 1990; McMurphy et al., 1990). Phytoestrogenic compounds found in red and white clover have been reported to improve overall performance when livestock are grazing tall fescue throughout the spring and summer months (Shappell et al., 2015; Aiken et al., 2016; Harlow et al., 2017a). Various isoflavones have been targeted for use

in human medicine for their beneficial effects on breast cancer, cardiovascular health as well as menopausal symptoms (van de Weijer and Barentsen, 2002; Beck et al., 2005; Cruz et al., 2006).

Ergot alkaloid pressure is reduced in the presence of ruminal fluid (Westendorf et al., 1992; De Lorme et al., 2007), indicating the ruminal microorganisms may be responsible for degradation of ergot alkaloids to less harmful compounds including lysergic acid. Harlow et al. (2017b) identified several hyper-ammonia producing bacteria capable of degrading ergovaline due to structural similarities. Isoflavones may benefit the ruminal microbiome in addition to the animal, improving fiber degradation, reducing several of the microorganisms responsible for lowered pH, and increased lactate production that manifests as ruminal acidosis (Harlow, 2017; Harlow, 2018).

This study examined the effect of endophyte-infected or endophyte free tall fescue ground seed with an isoflavone source on *in vitro* rumen fermentation, fiber degradation and rumen bacterial communities. The objectives of this study were to 1) determine if isoflavones added to tall fescue seed will significantly improve fiber degradation and rumen fermentation, and 2) determine if *in vitro* rumen bacterial populations are significantly altered with treatments. We hypothesized that the addition of isoflavones to endophyte-infected tall fescue seed will alter *in vitro* fermentation and rumen bacterial populations.

Materials and Methods

Study Design

A randomized incomplete block design with replication, blocking on run, was utilized. Within each run there were four jars each with different treatments. Jar was the experimental unity to which a treatment combination was applied. Treatments were arranged as a 3 x 2 factorial design with (1) Promensil® 80mg isoflavones tablet or (2) Promensil ® powder, and (3) control, receiving no isoflavones. Two types of seed were used with the treatments: (1) endophyte-infected tall fescue seed or (2) endophyte free tall fescue seed. The seed was coarsely ground using a Wiley Mill with a 5mm screen. Treatments were applied to ten fiber bags per jar. A total of five replications of the treatments was performed.

For the responses of neutral detergent fiber (NDF) and acid detergent fiber (ADF) disappearance, ten bags were included as sampling unites within each jar. For the response of crude protein disappearance, five bags per jar were used in each “boat” for analysis, for a total of two boats per jar as the sampling unit. For the responses of ruminal pH and volatile fatty acids (VFA), two samples per jar were obtained using a 15mL conical tube.

In vitro fiber and crude protein disappearance

The Daisy II in vitro fermentation system (ANKOM Corp., Fairport, NY) was utilized to determine the rate and extent of fiber disappearance of the endophyte-infected or endophyte free tall fescue seed with the addition of isoflavones from Promensil®. Each substrate (500 ± 40 mg) was weighed into artificial fiber bags (F57 fiber bags, ANKOM

Corp.), which were then heat sealed. Content included 0.4425g of either endophyte-infected (Kentucky 31) or endophyte-free tall fescue seed (Kentucky 32), and 0.0575g of Promensil® (tablet or powder, dry matter basis) to add to 500mg. The fiber bags were separated into four groups of 12 bags, with one treatment per jar, including two empty bags for correction, and placed into upright plastic containers. A total of 1600mL of rumen fluid was procured via aspiration from two fistulated Holstein heifers (Cherokee Farm, Knoxville, TN), and buffered using a 1:4 dilution of rumen fluid to buffer among the four jars. A total of 400mL were added to each ANKOM jar with 1600mL of buffer, and, if needed, adjusted to a pH of 6.8. The fiber bags, in addition to all liquid contents were added to the fermentation containers. Fermentation occurred for 48 h at 39°C (± 0.5°C).

Upon completion of the 48 h fermentation, rumen pH was measured and 15mL of rumen content was sampled and stored at -80°C until further processing. Analysis of neutral detergent fiber (aNDF) was obtained using α-amylase and sodium sulfite (Mertens, 2002), and acid detergent fiber (ADF) was determined using sulfuric acid-based detergent (Vogel et al., 1999). Both procedures were conducted using the ANKOM200 fiber analysis system (ANKOM Corp., Fairport, NY). Crude protein was determined by total nitrogen combustion analysis (LECO Instruments, Inc., St. Joseph, MI). Neutral detergent fiber, acid detergent fiber and crude protein were measured pre-and post-fermentation to evaluate disappearance.

Quantification of ergot alkaloids

Prior to the study, quantities of ergovaline and its epimer ergovalinine in fescue seed were determined using HPLC with fluorescence detection as described in Aiken et

al. (2009) with modifications described by Koontz et al. (2012). The endophyte-infected fescue seed had 2.94ppm of ergovaline plus ergovalinine (1.85 and 1.09ppm, respectively) and the endophyte free tall fescue seed had a total of 0ppm of alkaloids. Additionally, both seed varieties tested negative for the presence of the alkaloid ergotamine and its epimer ergotaminine. Seed was ground using a Wiley Mill to 5mm before 0.4425g of seed (1.3865×10^{-6}g ergot alkaloids) was included in each ANKOM fiber bag.

Quantification of isoflavones

Isoflavones were procured by grinding the product Promensil® (PharmaCare Inc.), an over-the-counter isoflavone supplement isolated from red clover. Quantification of isoflavones in Promensil®, including biochanin A, formononetin, genistein and daidzein, was performed similarly to those used by Aiken et al. (2016), using LC-MS rather than UV for detection. Briefly, isoflavone extracts were prepared by adding 7mL of 85% methanol in 0.5% acetic acid to ground samples in 50-mL conical polypropylene tubes. Samples were vortexed briefly and sonicated for 30 min at ambient temperature. Three milliliters of deionized water was added to each sample prior to being vortexed and centrifuged for 8 min at 2200 × g. The supernatant was filtered through a 0.45µm GHP membrane syringe filter. Extracts were diluted and flavone added as internal standard. One portion of each sample was analyzed as-extracted and a second portion was heated at 85°C for 5 h to hydrolyze isoflavone malonyl-glucosides to the corresponding isoflavone glucosides. Concentrations of biochanin a-malonyl-glucoside and formononetin-malonyl-glucoside were determined by difference between hydrolyzed and

un-hydrolyzed portions. Isoflavone extracts were analyzed by LC-MS on a Waters Acquity UPLC coupled to a Waters Synapt G2 (q-ToF) high resolution mass spectrometer. Chromatographic separation was obtained using a Waters BEH C18 UPLC column (1.7µm, 2.1mm x 150mm). The mobile phase employed a mixture of water containing 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B) in a linear gradient from 20% B to 80% B at a flow rate of 0.35mL/min. The high resolution mass spectrometer was operated in positive ion electrospray mode with a resolving power of ~14,000 and scanned from 100 to 1000Da in 0.3s. Leucine enkephalin was used to provide a lock mass (m/z 554.2615). Quantification of isoflavones was performed using QuanLynx software with a linear calibration curve and internal standard method. Extracted ion chromatograms with a mass window of 0.020Da around the accurate mass of each analyte were used to calculate peak areas. A total of 2.19mg of total isoflavones were provided per bag (0.92mg biochanin A, 0.89mg formononetin, 0.0038mg genistein and 0.0016mg daidzein), for a concentration of 1.10mg/L of rumen fluid and buffer. A total of 0.0575g of Promensil® pulverized tablet or powder was included in each ANKOM fiber bag.

VFA Analysis

A subsample of rumen content was aliquoted from each rumen content sample for VFA analysis using HPLC, similar to those in Harlow et al. (2017). Briefly, samples were analyzed for concentrations of acetate, propionate, butyrate, valerate, isovalerate/methylbutyrate (IVMB) using a Summit HPLC (Dionex; Sunnyvale, CA, USA) equipped with an anion exchange column (Aminex HP-87H; Bio-Rad, Hercules, CA, USA)

and UV detector. The eluting compounds were separated isocratically with an aqueous sulfuric acid solution (5 mM). The parameters included: injection volume 0.1 mL, flow rate 0.4 mL/min, and column temperature 50°C.

DNA extraction, PCR and sequencing

DNA was extracted from ruminal fluid post fermentation. The procedure of the DNA extraction method is similar to that described by Yu and Morrison (2004). After the chemical/mechanical cell lysis and isopropanol precipitation of nucleic acids, metagenomic DNA was purified with Rnase and proteinase K treatment, followed by the use of QIAamp columns from the Qiagen DNA Stool Mini Kit (Qiagen). Genomic DNA concentration was determined using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE), and verified using PicoGreen. Extractions were stored at -20˚C until sequencing library preparation. Bacterial 16S rRNA genes were PCR-amplified with dual-barcoded primers targeting the V4 region, as per the protocol of Kozich et al. (2013.) Amplicons were sequenced with an Illumina MiSeq using the 250-bp paired-end kit (v.2). Sequences were denoised, taxonomically classified using Greengenes (v. 13_8) as the reference database, and clustered into 97% similarity operational taxonomic units (OTUs) with the mothur software package (v. 1.39.5) (Schloss et al. 2009), following the recommended procedure (https://www.mothur.org/wiki/MiSeq_SOP; accessed November 2017).

Statistical Analyses

Study analyses included responses of NDF, ADF, CP, rumen pH and VFAs as well as ruminal bacterial communities. For the response of NDF and ADF, ten bags were included as sampling units within each jar. For the response of crude protein, 2 boats within each jar was the sampling unit. For the response of rumen pH and VFA, two ruminal fluid and buffer samples were collected per jar into 15mL conical tubes for analysis.

Samples were analyzed using the MIXED procedure of SAS 9.4 (SAS Inst. Inc., Cary, NC) to test treatment combinations. The model included the fixed effects of seed type, treatment, and treatment × seed interaction. The model also included random effects of run and run × seed × treatment. Least square means were compared using Fisher's LSSD. Effects were considered significant at a *P*-value of ≤ 0.05 with tendencies declared at *P*-values between 0.05 and 0.10.

For rumen bacterial communities, analysis was conducted in the R environment. Alpha diversity was estimated with the Shannon index on raw OTU abundance tables. The significance of diversity differences was tested with an ANOVA. To estimate beta diversity across samples, OTUs were excluded if occurring in fewer than 10% of the samples with a count of less than three and computed Bray-Curtis indices. Beta diversity, emphasizing differences across samples, was visualized using non-metric multidimensional (NMDS) ordination. Variation in community structure was assessed with permutational multivariate analyses of variance (PERMANOVA) with treatment group as the main fixed factor and using 4,999 permutations for significance testing.

Results and Discussion

NDF, ADF, and CP Disappearance

Endophyte-infected tall fescue seed had significantly different NDF disappearance compared to endophyte free tall fescue seed ($P = 0.003$, Table 2), Additionally, isoflavone treatment, regardless of in the tablet or powdered form, had significantly greater NDF disappearance than the control (Table 2). Previous research conducted by Harlow et al. (2018) and Flythe and Kagan (2010) noting improved fiber degradation and ammonia reduction utilized biochanin A as the primary isoflavone (30 mg L^{-1}), whereas the present study included 920 µg biochanin A per fiber bag (0.46 mg L^{-1}) for a total concentration of 1.10 mg total isoflavones per liter. As the results from this experiment were inconsistent with those achieved by Harlow et al (2018), the amount of total isoflavones (1.10 mg L^{-1}) in this experiment may have been insufficient to elucidate all the beneficial effects on fiber degradation.

ADF disappearance differed significantly with seed × treatment type ($P < 0.05$, Table 2). Overall, endophyte-free tall fescue seed and isoflavone treatments, regardless of form, had higher ADF disappearance post fermentation than endophyte-infected tall fescue seed (Table 2). A lower ADF disappearance was observed with endophyte-infected tall fescue seed with the powder isoflavone form, compared to the control or tablet treatments ($P < 0.05$).This increase in ADF disappearance is consistent with what Harlow (2018) observed, when the inclusion of the isoflavone biochanin A reduced overall ADF values post fermentation compared to the controls. As acid detergent fiber measures the amount of least digestible fiber component, the inclusion of isoflavones with tall fescue

seed in the present study increased indigestible fiber disappearance. ADF has an inverse relationship with energy digestibility, and thus a high ADF component indicates less digestible energy available to the animal. In the present study treatments with isoflavones resulted in a higher ADF disappearance, indicating possible improvement in available digestible and fermentable energy when included with tall fescue seed.

A significant difference was observed with a seed × treatment interaction (P = 0.0002) but was not observed with isoflavone treatment alone ($P > 0.05$) on overall crude protein disappearance. Endophyte-infected seed with the control treatment had significantly lower crude protein disappearance than endophyte-free tall fescue seed with the control (Table 2). This is inconsistent with most tall fescue and crude protein literature, where proteolytic bacteria including several species of hyper-ammonia producing bacteria are more readily able to degrade ergot alkaloid compounds (Harlow et al., 2017b). While these hyper-ammonia bacteria are able to degrade ergovaline, they are a source of inefficiency in the animal when dietary nitrogen is lost as ammonia. Antimicrobials, including ionophores and similar compounds, may be employed in the diet to combat this loss of nitrogen, selectively inhibiting some of the microflora and reducing wasteful byproducts of digestion (Chen and Russell, 1989; Tedeschi et al., 2003; Reynolds et al., 2014). Flythe et al. (2010; 2013b) examined red clover isoflavones as antimicrobials due to their resistance to amino-acid degradation and action on hyper-ammonia producing bacteria. A seed × treatment interaction improved crude protein disappearance with endophyte free tall fescue seed and isoflavones compared to endophyte-infected tall fescue seed and isoflavones. Although treatment of isoflavones

alone did not alter crude protein disappearance significantly (*P = 0.68)*, the present study may have provided insufficient levels of isoflavones to reduce overall amino-acid degradation from endophyte-free and endophyte-infected tall fescue seed.

Ruminal bacteria populations, VFA concentrations, and pH

After stringent sequence processing, a total of 408,813 high quality reads were obtained and averaged 17,034 ± 3180 per sample, which is a consistent depth for sequencing analyses from ruminal samples (Myer et al., 2015) . Number of observed OTUs totaled 48,867 and averaged 2,037 ± 169 per sample. PERMANOVA global pairwise comparisons indicated significant differences among treatment groups (R^2 = 0.32, *P* = 0.03). Shannon's Diversity Index by treatment group was not statistically significant (*P > 0.05*, Figure 5). Non-metric multidimensional scaling (NMDS) was utilized to analyze beta diversity (Figures 6, 7) where clusters of samples represent similarity of 16S rRNA bacterial genera by group based on rank. While treatment groups may have appeared to cluster similarly, there was no observable pattern between groups. Relative proportions of the genus level diversity between groups are represented in Figure 7. Overall, 35 genera were significantly different by seed, treatment or seed × treatment interaction (Table 4).

Concentrations of acetate, propionate, butyrate acetate:propionate, isovalerate/methylbutyrate (IVMB), and valerate were measured. Propionate concentrations were significantly reduced with endophyte-free tall fescue seed type (*P* = 0.0324, Table 3), but no effect of treatment or treatment × seed interaction was observed (*P* > 0.05). The acetate: propionate ratio had a tendency to be reduced (Table 3) with

endophyte-infected tall fescue seed type (P = 0.09). The lack of significant differences in VFA concentrations provides support that the treatments were not inhibiting *in vitro* rumen fermentation.

Rumen pH was not significant by seed type, treatment or seed × treatment interaction (P > 0.05, Figure 9), and average pH ranged from 6.97 -7.57. The donor animals (n = 2) used for rumen fluid procurement were not tall fescue-naïve animals, and consumed a forage-based diet. As these animals were not fescue-naïve it is possible that the rumen fluid obtained had been exposed to ergot alkaloid pressure previously, which may have affected results.

Conclusion

The combined results of this study indicate some moderate rumen fermentation changes may have occurred due to treatments, but these results may have been affected by several factors. Where fiber and crude protein degradation results were not consistent with previous *in vitro* studies, this may be the result of insufficient isoflavone administration to the *in vitro* fermentation system. As previous studies utilized a minimum of 30 mg L^{-1} of biochanin A to elucidate responses, the present study utilized 0.63 mg L^{-1} of biochanin A which may have not been enough to elicit a response. Ergot alkaloid pressure on rumen fermentation was not clearly elucidated in the present study. VFA concentrations, are indicative of rumen fermentation, which was not affected by each treatment. It is noteworthy that isoflavone administration did not alter VFA production, and that seed type was the driving factor in any of the changes in VFA concentrations. Foote et al. (2013) noted significant reductions in VFA flux and absorption in animals

administered endophyte-infected tall fescue seed, lending support that nutrient utilization and energy absorption may be further delayed when cattle experience tall fescue toxicosis. As propionate supplies up to 65% of the glucose carbon in cattle (Herbein et al., 1978) and livestock consuming endophyte-infected tall fescue produce lower levels of VFAs (Hannah et al., 1990), it is interesting that in the present study propionate was reduced in treatment with endophyte free tall fescue seed.

While rumen bacterial populations varied among groups, the rumen microbiome is a dynamic ecosystem that is affected by various environmental and dietary factors, and thus changes in the bacterial taxa among groups may not be solely indicative of treatment with fescue seed or isoflavone treatment. In the present study, bacterial taxa were only identified to the genus level. As other studies have noted specific bacterial species capable of ergovaline degradation and benefits from isoflavones, further research should be conducted to validate consistent shifts in the rumen microbiome from ergot alkaloid pressure. Further research should be conducted to determine overall livestock and specifically cattle performance with high ergot alkaloid pressure and mitigation of fescue toxicosis using isoflavones in a more controlled environment.

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Appendix B

Table 2. Effects of isoflavones and fescue seed type on neutral detergent fiber (NDF), acid detergent fiber (ADF) and crude protein (CP) disappearance after rumen fluid digestion (48 h)

Measurement (%)	Treatment[1]						SEM	P-value[3]		
	Control		Powder		Tablet					
	Fescue Seed[2]									
	E+	E-	E+	E-	E+	E-		Seed	Treatment	Seed × Treatment
NDF	9.11^{b}	12.52^{ab}	12.72^{ab}	15.84^{a}	12.74^{ab}	13.68^{a}	1.24	0.003	0.005	0.432
ADF	1.72^{c}	3.21^{bc}	7.01^{a}	2.19^{c}	1.27^{c}	5.91^{b}	1.03	0.36	0.009	< 0.001
CP	2.44^{d}	12.23^{a}	6.98^{bc}	7.33^{bc}	4.01^{cd}	9.08^{ab}	0.93	< 0.001	0.689	0.0002

[1]Treatment: Control (c), isoflavone powder (p), or isoflavone pulverized tablet (t)
[2]Seed type: KY 31 endophyte-infected (E+) tall fescue seed, KY 32 endophyte-free (E-) tall fescue seed
[3]Within a row, means among treatment groups are considered significant at $P < 0.05$, and trending at $P < 0.1$
[abcd] Significant differences represented by means separation letters

Table 3. Effects of isoflavones and fescue seed type on volatile fatty acid concentrations (mM) in the fluid phase of an in vitro rumen fluid digestion (48 h)

VFA[1]	Treatment[2]						SEM	P-value[4]		
	Control		Powder		Tablet					
	Fescue Seed[3]									
	E+	E-	E+	E-	E+	E-		Seed	Treatment	Seed × Treatment
Acetate	12.86	11.83	11.44	10.44	12.23	10.27	1.55	0.14	0.46	0.91
Propionate	7.96	6.09	6.00	5.52	7.57	5.52	0.95	0.03	0.31	0.64
Butyrate	2.00	1.65	2.00	1.44	1.73	1.64	0.43	0.18	0.90	0.81
IVMB	0.24	0.28	0.18	0.12	0.22	0.21	0.33	0.99	0.97	0.98
Valerate	0.36	0.15	0.21	0.21	0.27	0.26	0.11	0.21	0.69	0.29
A:P	1.60	1.96	1.91	1.99	1.73	1.98	0.21	0.09	0.62	0.73

[1] Concentration in mmol L^{-1}

[2] Treatment: Control, isoflavone powder, or isoflavone pulverized tablet

[3]Seed type: KY 31 endophyte-infected (E+) tall fescue seed, KY 32 endophyte-free (E-) tall fescue seed

[4]Within a row, means among treatment groups are considered significant at $P < 0.05$, trending at $P < 0.1$

[abcd] Significant differences represented by means separation letters

Table 4. Effects of isoflavones and fescue seed type on relative abundance of significant *in vitro* rumen bacterial taxa

Classification	Percentage of total sequences[1]						Effect	SEM	*P*-value[4]
	Treatment[2]								
	Control		Powder		Tablet				
	Fescue Seed[3]								
	E+	E-	E+	E-	E+	E-			
Actinomycetales_unclassified	0.0011^{b}	ND^{b}	0.013^{ab}	0.0027^{b}	0.012^{ab}	0.019^{a}	Treatment	0.0047	0.018
Alphaproteobacteria_unclassified	0.012^{bc}	0.017^{abc}	0.0255^{ab}	ND^{c}	0.0203^{abc}	0.037^{a}	Seed × Treatment	0.0071	0.020
Anaeroplasma	0.054^{ab}	0.067^{ab}	0.0233^{b}	0.075^{ab}	0.044^{b}	0.108^{a}	Seed × Treatment	0.019	0.037
Anaerovibrio	0.074^{bc}	0.014^{c}	0.244^{a}	0.104^{bc}	0.158^{ab}	0.096^{bc}	Seed	0.0447	0.027
Arcobacter	0.928^{bc}	0.277^{c}	1.44^{b}	2.34^{a}	1.39^{b}	0.574^{bc}	Seed × Treatment	0.2937	0.015
Bacteria_unclassified	4.06^{b}	5.56^{a}	5.54^{a}	4.16^{ab}	4.23^{ab}	5.49^{ab}	Seed × Treatment	0.4920	0.015
Bacteroidetes_unclassified	8.77^{d}	13.96^{a}	12.62^{ab}	10.11^{cd}	11.26^{bc}	11.58^{abc}	Seed × Treatment	0.8247	0.001
Bulleidia	0.079^{b}	0.21^{a}	0.11^{b}	0.12^{b}	0.086^{b}	0.096^{b}	Seed × Treatment	0.0219	0.019
Christensenellaceae_unclassified	0.81^{b}	1.61^{a}	0.742^{b}	0.801^{b}	0.721^{b}	0.941^{b}	Seed	0.1092	0.031
Clostridiales_unclassified	3.33^{b}	5.60^{a}	4.18^{ab}	3.92^{b}	3.22^{b}	3.98^{b}	Seed	0.2961	0.039
Corynebacterium	0.041^{c}	0.065^{bc}	0.131^{a}	0.109^{ab}	0.066^{bc}	0.076^{bc}	Seed	0.0014	0.009
Endomicrobia_unclassified	ND^{b}	0.0115^{a}	ND^{b}	0.0015^{b}	0.0015^{b}	0.0056^{ab}	Seed × Treatment	0..0024	0.007

Table 4. Continued.

Classification	Percentage of total sequences[1]						Effect	SEM	*P*-value[4]
	Treatment[2]								
	Control		Powder		Tablet				
	Fescue Seed[3]								
	E+	E-	E+	E-	E+	E-			
Gammaproteobacteria_unclassified	0.018^{cd}	0.06^{ab}	0.056^{abc}	0.013^{d}	0.043^{bcd}	0.083^{a}	Seed × Treatment	0.0152	0.006
Megasphaera	0.117^{a}	0.018^{b}	0.0411^{b}	0.0431^{b}	0.0344^{b}	0.0286^{b}	Seed × Treatment	0.0502	0.008
Methylobacillus	ND	0.0014	0.0043	0.0042	ND	ND	Treatment	0.0051	0.020
ML615J.28_unclassified	0.027^{b}	0.065^{b}	0.109^{b}	0.123^{b}	0.123^{b}	0.456^{a}	Treatment	0.3030	0.009
Moraxella	0.0092^{ab}	0.0012^{b}	0.0142^{ab}	0.0029^{b}	0.027^{a}	0.0088^{ab}	Seed	0.0038	0.030
Olsenella	0.0292^{b}	0.148^{a}	0.053^{b}	0.038^{b}	0.034^{b}	0.039^{b}	Seed × Treatment	0.0233	0.021
p.75.a5	0.028^{c}	0.103^{a}	0.047^{bc}	0.071^{ab}	0.063^{abc}	0.071^{ab}	Seed	0.0134	0.004
Peptoniphilus	0.0069^{ab}	ND^{b}	ND^{b}	0.0146^{a}	0.0063^{ab}	0.0044^{b}	Seed × Treatment	0.0033	0.011
Peptostreptococcaceae_unclassified	0.0578^{a}	0.0088^{c}	0.0024^{c}	0.0278^{bc}	0.0373^{ab}	0.0162^{bc}	Seed × Treatment	0.0094	0.003
Planococcaceae_unclassified	0.095^{a}	0.0014^{b}	0.0044^{b}	0.0118^{b}	0.007^{b}	0.0136^{b}	Seed × Treatment	0.0168	0.010
Proteiniclasticum	1.45^{ab}	0.223^{c}	2.25^{a}	1.29^{b}	1.27^{b}	1.35^{ab}	Seed	0.1770	0.011
Proteiniphilum	0.097^{ab}	0.027^{ab}	0.0203^{ab}	0.0069^{b}	0.124^{a}	0.0148^{b}	Seed	0.0203	0.037
Proteobacteria_unclassified	0.157^{b}	0.196^{b}	0.272^{ab}	0.192^{b}	0.263^{ab}	0.378^{a}	Treatment	0.0332	0.021
Pseudobutyrivibrio	0.496^{bc}	0.237^{c}	1.11^{ab}	0.831^{abc}	1.24^{a}	0.681^{abc}	Treatment	0.1568	0.020
Ruminococcaceae_unclassified	2.18^{cd}	3.45^{a}	2.75^{b}	2.04^{d}	2.20^{cd}	2.66^{bc}	Seed × Treatment	0.1852	0.002
Schwartzia	0.0334^{bc}	0.0087^{c}	0.0766^{ab}	0.079^{a}	0.065^{ab}	0.060^{ab}	Treatment	0.0108	0.004
Selenomonas	0.219^{bc}	0.159^{c}	0.459^{abc}	0.629^{a}	0.543^{ab}	0.514^{ab}	Treatment	0.0842	0.012

Table 4. Continued.

Classification	Percentage of total sequences[1]						Effect	SEM	*P*-value[4]
	Treatment[2]								
	Control		Powder		Tablet				
	Fescue Seed[3]								
	E+	E-	E+	E-	E+	E-			
SR1_unclassified	0.230^{bc}	0.0901^{c}	0.903^{ab}	0.950^{a}	0.954^{a}	0.607^{abc}	Treatment	0.236	0.0104
Succiniclasticum	0.672^{b}	1.67^{a}	0.662^{b}	0.774^{b}	0.627^{b}	0.632^{b}	Seed × Treatment	0.191	0.0349
Succinivibrio	0.002^{b}	0.014^{a}	ND^{b}	0.004^{ab}	0.0015^{b}	0.0088^{ab}	Seed	0.1670	0.027
Tenericutes_unclassified	1.21^{cd}	1.01^{d}	2.43^{a}	1.32^{bcd}	2.14^{ab}	2.07^{abc}	Seed	0.2967	0.008
Treponema	0.0078^{a}	ND^{b}	ND^{b}	0.0028^{b}	ND^{b}	0.0031^{b}	Seed × Treatment	0.0011	< 0.001
Wautersiella	0.445^{b}	0.797^{b}	0.865^{ab}	0.709^{b}	0.722^{b}	1.31^{a}	Seed × Treatment	0.153	< 0.001

[1]Data shown as LS means
[2]Treatment: Control, isoflavone powder, or isoflavone pulverized tablet
[3]Seed type: KY 31 endophyte-infected (E+) tall fescue seed, KY 32 endophyte-free (E-) tall fescue seed
[4]Within a row, means among treatment groups are considered significant at P < 0.05, trending at P < 0.1
^{abcd} Significant differences represented by means separation letter

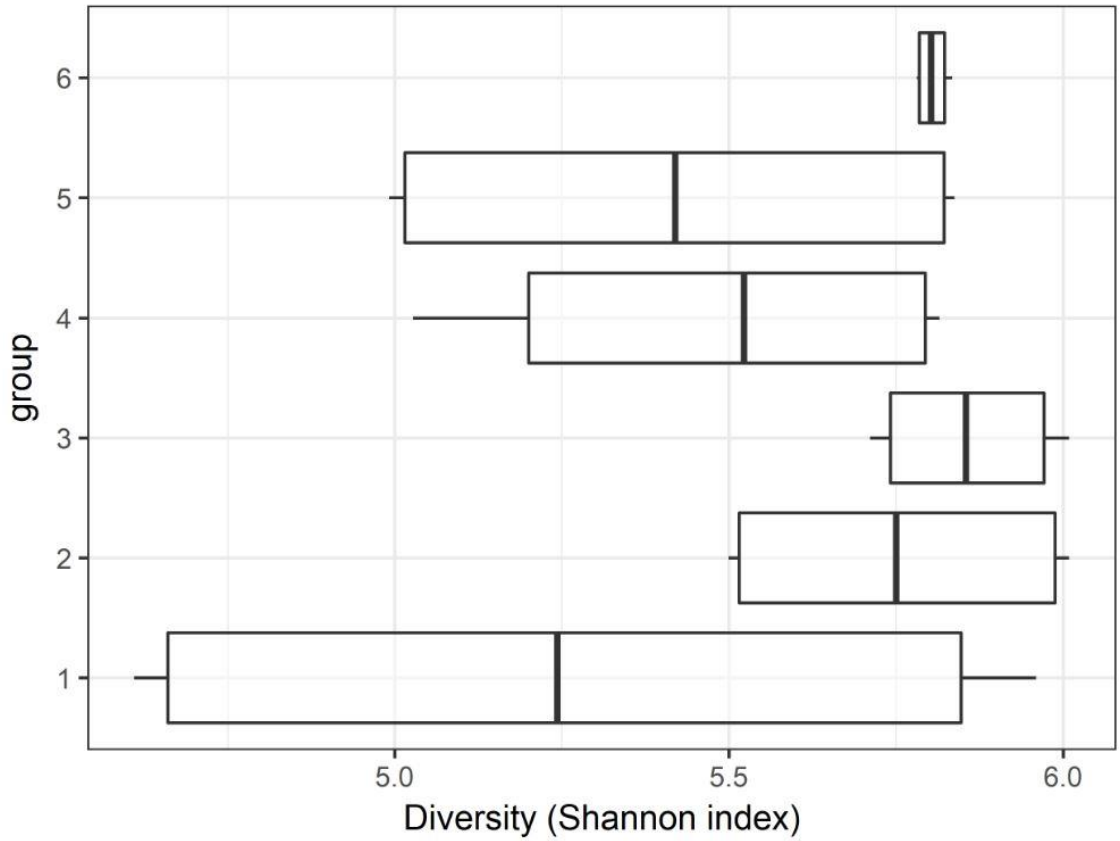

Figure 5. Shannon's Diversity Index box plot of bacterial species diversity across treatment groups. Group 1: Endophyte-infected tall fescue seed. Group 2: Endophyte-free tall fescue seed. Group 3: Endophyte-infected tall fescue seed with isoflavone powder. Group 4: Endophyte-infected tall fescue seed with isoflavone tablet. Group 5: Endophyte-free tall fescue seed with isoflavone powder. Group 6: Endophyte-free tall fescue seed with isoflavone tablet.

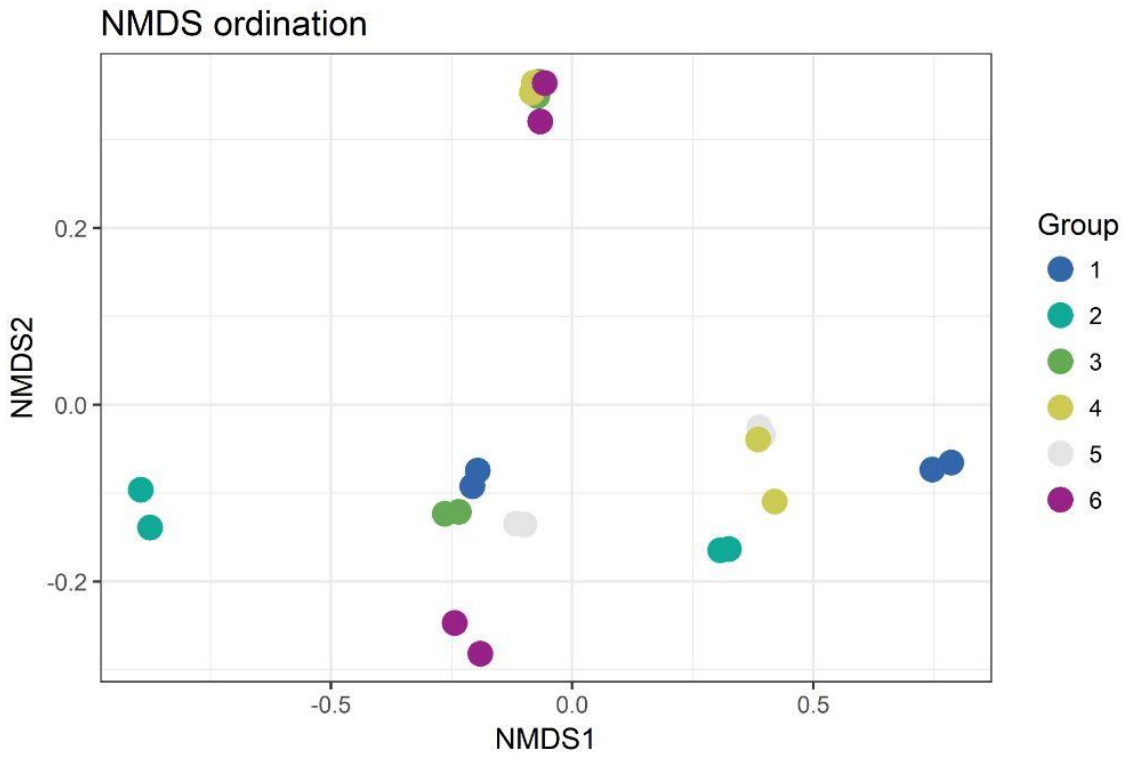

Figure 6. NMDS ordination plot grouped by treatment. Points represent taxa within treatment groups. Similar clustering indicates similar taxa within treatment groups. Group 1: Endophyte-infected tall fescue seed. Group 2: Endophyte-free tall fescue seed. Group 3: Endophyte-infected tall fescue seed with isoflavone powder. Group 4: Endophyte-infected tall fescue seed with isoflavone tablet. Group 5: Endophyte-free tall fescue seed with isoflavone powder. Group 6: Endophyte-free tall fescue seed with isoflavone tablet.

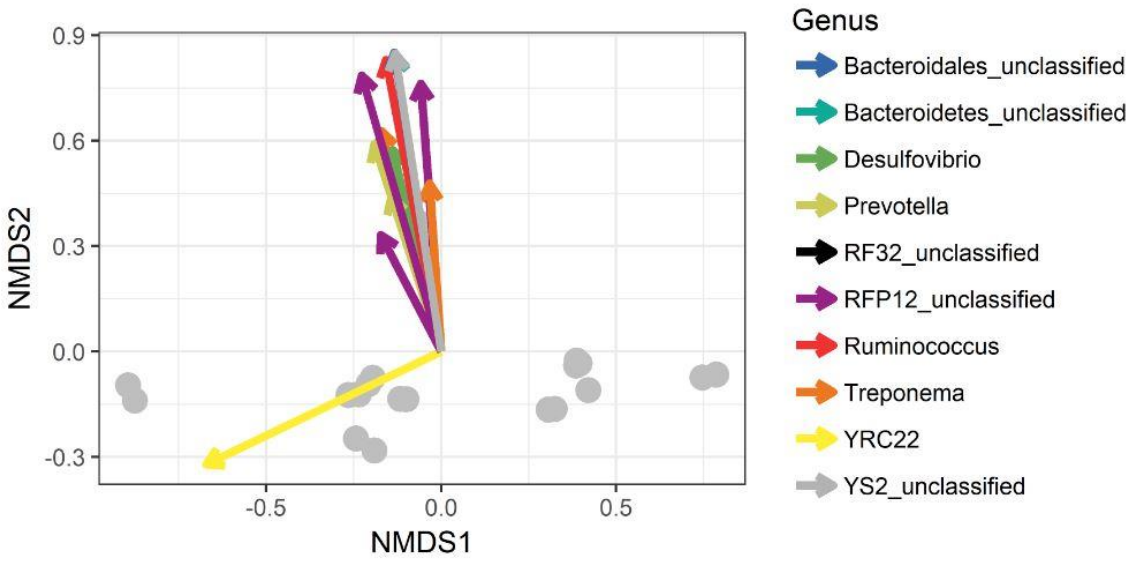

Figure 7**.** NMDS ordination plot indicating significant taxa at the genus level that influenced major bacterial shifts among treatments.

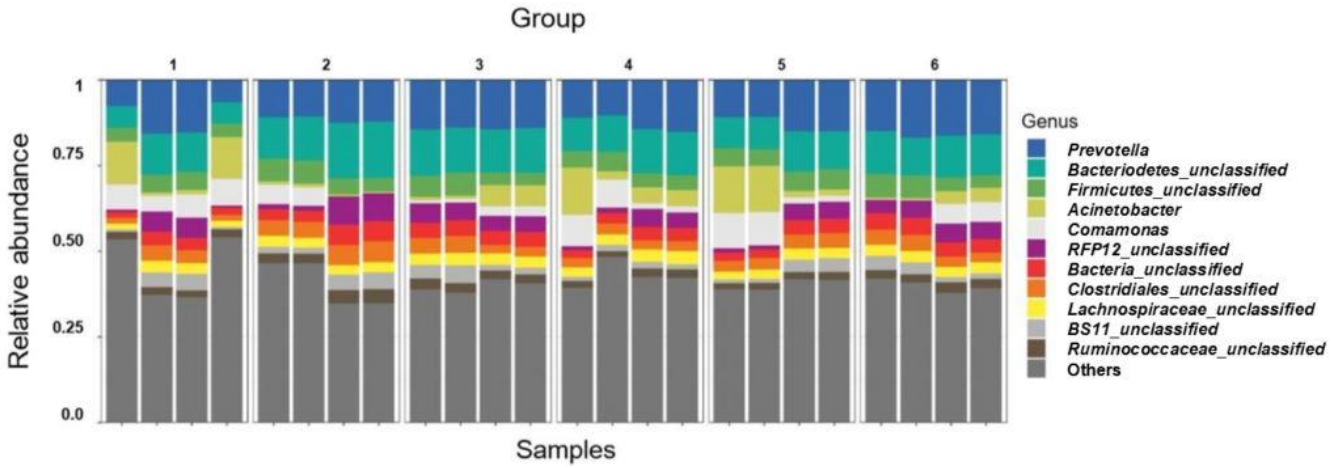

Figure 8. Taxonomic profiles of the relative proportions of bacterial communities by genus, grouped by treatment. Group 1: Endophyte-infected tall fescue seed. Group 2: Endophyte-free tall fescue seed. Group 3: Endophyte-infected tall fescue seed with isoflavone powder. Group 4: Endophyte-infected tall fescue seed with isoflavone tablet. Group 5: Endophyte-free tall fescue seed with isoflavone powder. Group 6: Endophyte-free tall fescue seed with isoflavone tablet.

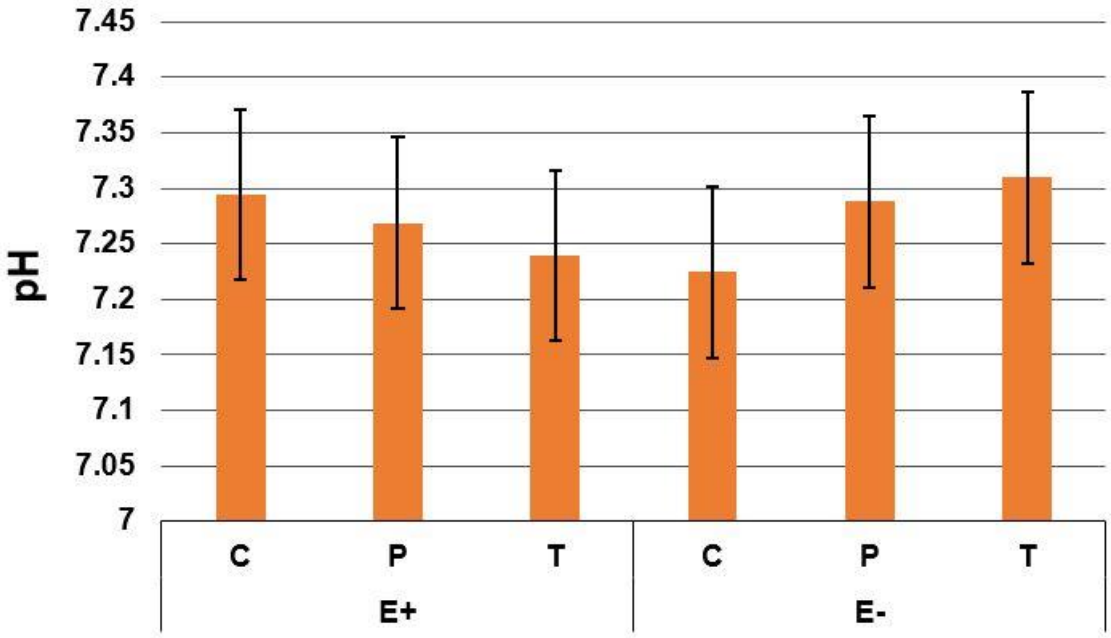

Figure 9. Mean ruminal pH by endophyte-infected (E+) and endophyte free (E-) seed type and treatment type. No significant differences in ruminal pH by seed, treatment or seed × treatment ($P > 0.05$). Error bars represent SEM.

CHAPTER III EFFECTS OF ENDOPHYTE-INFECTED TALL FESCUE AND RED CLOVER ISOFLAVONES ON RUMEN MICROBIAL POPULATIONS AND BEEF CATTLE

Abstract

Symptoms resulting from tall fescue toxicosis are responsible for over $2 billion in losses for beef cattle producers across the United States, including reduced reproductive performance and reduced overall gain and feed efficiency. The toxic alkaloids produced by the endophyte within tall fescue are responsible for vasoconstriction, reduced serum prolactin and reduced production in cattle. The inclusion of mixed forages, especially red clover, into pastures has shown to mitigate some of these effects. Clovers contain phytoestrogenic compounds such as isoflavones, which may play a role in reducing these symptoms. The present study utilized forty Angus steers to determine if daily supplementation with a red clover-isolated isoflavone feed additive would reduce the symptoms of tall fescue toxicosis at a physiological level and within the rumen microbial environment over a 21-day period. Angus steers were stratified among treatments based upon their single-nucleotide polymorphism genotype at the DRD2 receptor. Treatments were designed as a 2×2 factorial arrangement within a completely randomized design, consisting of tall fescue seed type (endophyte-infected tall fescue seed vs endophyte free tall fescue seed) supplemented with and without the isoflavone additive. Serum prolactin concentration was decreased with consumption of endophyte-infected tall fescue seed, but was increased with consumption of endophytes free tall fescue seed (*P = 0.023)*. Average daily gain was significantly reduced with endophyte-infected tall fescue seed (*P = 0.0029*). Isoflavones significantly reduced serum glucose levels compared with control animals (*P = 0.03).* Twenty-eight ruminal bacteria taxa shifted as a result of seed type or isoflavone treatment. Feeding isoflavones to ruminants may be a viable option to alleviate

symptoms of fescue toxicosis and promote livestock growth and performance in the future.

Introduction

Endophyte-infected tall fescue *(Lolium arundinaceum Schreb.)* is a cool season perennial grass and one of the most common forages utilized by beef cattle operations in the mid-South region; providing an abundant supply of forage for roughly 8.5 million cattle throughout the majority of the growing season (Hoveland, 1993). Despite the nutritive quality of endophyte-infected tall fescue, negative impacts on production may occur when livestock graze tall fescue infected with the endophytic fungus *Epichloë coenophiala.* Numerous studies have outlined the detrimental effects of the ergot alkaloid compounds produced by the native endophyte on milk quality and production (Peters et al., 1992; Brown et al., 1996), reproduction (Porter and Thompson, 1992; Schuenemann et al., 2005) and overall weight gain and forage intake (Bush and Buckner, 1973; Goetsch et al., 1987; Nihsen et al., 2004). The combined effects of these reduced production parameters contribute to an average of a $2 billion loss for beef producers in the United States (Hancock and Andrae, 2009; Kallenbach, 2015) alone, not including the detrimental effects of the ergot alkaloids and similar loline compounds on a global level.

The fungal endophyte produces several ergot-alkaloid compounds, but primarily ergovaline. Ergovaline acts as an α-2 adrenergic agonist on blood vessels causing vasoconstriction and can similarly bind dopamine receptor D2 (DRD2) on the anterior pituitary gland, reducing serum prolactin levels (Hurley et al., 1980; Dyer, 1993; Oliver, 2005). Campbell et al. (2014) determined that a single nucleotide polymorphism (SNP) at

the DRD2 gene loci can dictate increased or reduced susceptibility to fescue toxicosis in Angus cattle, potentially improving herd resistance to the endophyte through genetic selection. Cattle with the genotype SNP “AA" were found to have increased resistance to fescue toxicosis, whereas “AG” were found to have moderate resistance, and cattle with “GG” were most susceptible to fescue toxicosis (Campbell et al., 2014).

When exposed to a diet containing ergovaline at toxic levels, vasoconstriction in the extremities and digestive system can cause hyperthermia and hypersalivation in cattle (Oliver et al., 1993). These symptoms are often observed in concert with increased rectal temperatures, reduced feed intake and growth, as well as behavioral changes. Vasoconstriction from ergot alkaloid consumption includes constriction to the ruminal artery and vein (Foote et al., 2011), and this could result in reduced nutrient absorption and post-absorptive metabolism. Numerous efforts have been made to reduce the manifestations of fescue toxicosis on the animal’s physiology (Lusby et al., 1990; Koontz et al., 2012; Jia et al., 2015; Klotz, 2015; Green et al., 2017), but limited research has been conducted on the effects of ergovaline on ruminant microbial communities. Recently, Harlow et al. (2017b) conducted an *in vitro* study with purified rumen fluid where several communities of hyper-ammonia producing bacteria (HAB) were able to degrade ergovaline, which are the same communities of bacteria that are able to degrade tryptophan. While this study indicates potential host-microbe symbiotic mechanisms for mitigating fescue toxicosis, further studies need to be conducted to determine additional mitigation attempts through functionality and health of the rumen.

One of the most common strategies utilized to mitigate the effect of fescue toxicosis involves mixing tall fescue pastures with other forages, particularly legumes such as alfalfa and clovers, to dilute the effect of the infected endophyte. Legumes are relatively high quality forages that contain phytoestrogenic compounds known as isoflavones. These compounds are utilized in human medicine for mitigation of menopausal symptoms in women. While the isoflavones are nonsteroidal, they maintain a weak affinity for estrogen receptors; specifically estrogen receptor β (ER-β). Receptor ER-β is most widely expressed on non-reproductive tissues such as bone and blood vasculature, which mediates some of the growth-promoting effects of estrogen on non-reproductive tissues (Sunita and Pattanayak, 2011). Four major isoflavones found in red clover are biochanin A, formononetin, genistein, and daidzein. In ruminants, formononetin is metabolized to daidzein and further metabolized to equol. Biochanin A is metabolized to genistein and then further to p-ethylphenol (Braden, 1967; Dickinson et al., 1988). When dairy cows grazed white clover, red clover, lucerne (alfalfa), or chicory pastures, red clover produced the highest concentrations of equol, which subsequently was found throughout the body and discovered in the milk (Andersen et al., 2009). In addition, vasculature has been reported to relax after exposure to isoflavones (Nevala et al., 1998). Therefore, red clover isoflavones may selectively have an antimicrobial effect within the ruminal bacterial community. The isoflavone biochanin A inhibits gram-positive hyper-ammonia producing bacteria (Flythe and Kagan, 2010) and reduce production of ammonia from ruminal bacteria at a concentration of 30ppm (Flythe et al., 2013a). Biochanin A is also a selective inhibitor of certain amylolytic and cellulolytic bacteria

(Harlow, 2017; Harlow, 2018). It was further determined that when 30mg of biochanin A per liter of rumen was ruminally-dosed into goats consuming endophyte-infected tall fescue seed, vasodilation and return to normal blood flow rates was observed (Aiken et al., 2016). This has been associated with agonist activity at β-adrenergic receptors within the endothelium of the blood vessels, which stimulates synthesis of nitric oxide that will promote vasodilation (Wu et al., 2010). These studies indicate a change in vasculature and rumen microbial communities in response to red clover-sourced isoflavone. However further studies need to be conducted to determine their effects on the entire rumen bacterial community and whole physiology in cattle. We hypothesized that supplementation of isoflavones to beef cattle consuming endophyte-infected tall fescue seed will mitigate reductions in growth performance, as indicated by increased average daily gain and dry matter intake and which will influence rumen microbial populations. Determining if ergovaline contributes to rumen microbial dysbiosis and diminished functional capacity as well as the effect of isoflavones on these physiological parameters will provide increased insight to producers managing fescue toxicosis in their herds.

Materials and Methods

All animal handling and experimental procedures were conducted in accordance with guidelines set by the University of Tennessee Institutional Animal Care and Use Committee. This experiment was conducted at the Plateau Research and Education Center in Crossville, TN.

Experimental design and treatment stratification

The study was designed as a completely randomized block design. Treatments were designed in a 2×2 factorial arrangement, where steers received a treatment assignment and commencement of either: (1) endophyte-infected tall fescue seed alone (E+), (2) endophyte free tall fescue seed alone (E-), (3) E+ with isoflavones (E+ P), or (4) E- with isoflavones (E- P). Prior to initiation of the study, 36 purebred Angus steers (approximately 8 months of age; 250 (± 20 kg) were stratified by genotype at the DRD2 receptor using methods similarly to those established by Campbell et al. (2014). Briefly, genomic DNA was isolated from 5–10 tail hair follicles of steers using Quickextract (Epicentre, Cambridge, UK). Genomic amplification was then performed on the isolated DNA samples using the GenomiPhi V2 DNA amplification kit (GE Healthcare, Piscataway, NJ) followed by an ethanol precipitation and resuspension in 50mL of water. Polymerase chain reaction (PCR) was utilized to amplify a 794 base pair (bp) portion of the DRD2 gene. Sequences of the primers used were 50-TATAGCCCCATTCCTGCTTC-30 and 50-GCCCATGCT CTACAACACACG-30. Cycling conditions were 2min at 94°C; 35 cycles at 30sec at 94°C; 30sec at 58°C; 30sec at 68°C; followed by 10min at 68°C and held until further processing at 4°C. The total reaction volume was 20mL. Direct sequencing of the PCR product revealed an intronic A=G SNP which created a Tfi I restriction site (50-GAWTC-30) with the ‘‘A’’ allele. Following PCR, 5mL of amplified product was subjected to a 2h digestion reaction at 65°C with 2.5 units Tfi I (USB Biolabs, Boston, MA) in a total reaction volume of 20mL. Half of the reaction volume was used in agarose gel electrophoresis against a DNA size ladder (Promega, Madison, WI) and genotypes were

determined based on fragment size. Steers were then randomly assigned to one of four treatments to one of the four treatments mentioned above.

Quantification of ergot alkaloids

A total of 1546 kgs of endophyte-infected (Kentucky 31) or endophyte-free (Kentucky 32) tall fescue seed was utilized for the study; 773 kgs of each variety. Prior to the study, quantities of ergovaline and its epimer ergovalinine in fescue seed were determined using HPLC with fluorescence detection as described in Aiken et al. (2009) with modifications described by Koontz et al. (2012). Kentucky 31 contained 2.94mg of ergovaline plus ergovalinine per kg DM (1.85 and 1.09 mg/kg, respectively) and Kentucky 32 tested with a total of 0 mg/kg of alkaloids.

Additionally, both seed varieties tested negative for the presence of the alkaloid ergotamine and its epimer ergotaminine. Seed was ground using a Wiley Mill to 5mm prior to being included in the diet to provide a minimum of 0.011mg total alkaloids (ergovaline + ergovalinine) per kg BW per day to induce signs of fescue toxicosis (Klotz, 2015).

Quantification of isoflavones

To administer isoflavones for two of the treatment groups, oral boluses of isoflavones were procured by grinding the product Promensil® (PharmaCare Inc.), an over-the-counter isoflavone supplement isolated from red clover. Quantification of isoflavones in Promensil®, including biochanin A, formononetin, genistein and daidzein, was performed similarly to those used by Aiken et al. (2016), using LC-MS for detection.

Briefly, isoflavone extracts were prepared by adding 7mL of 85% methanol in 0.5% acetic acid to ground samples in 50-mL conical polypropylene tubes. Samples were vortexed briefly and sonicated for 30 min at ambient temperature. Three milliliters of deionized water was added to each sample prior to being vortexed and centrifuged for 8 min at 2200 × *g*. The supernatant was filtered through a 0.45µm GHP membrane syringe filter. Extracts were diluted and flavone added as internal standard. One portion of each sample was analyzed as-extracted and a second portion was heated at 85°C for 5 h to hydrolyze isoflavone malonyl-glucosides to the corresponding isoflavone glucosides. Concentrations of biochanin a-malonyl-glucoside and formononetin-malonyl-glucoside were determined by difference between hydrolyzed and un-hydrolyzed portions. Isoflavone extracts were analyzed by LC-MS on a Waters Acquity UPLC coupled to a Waters Synapt G2 (q-ToF) high resolution mass spectrometer. Chromatographic separation was obtained using a Waters BEH C18 UPLC column (1.7µm, 2.1mm x 150mm). The mobile phase employed a mixture of water containing 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B) in a linear gradient from 20% B to 80% B at a flow rate of 0.35mL/min. The high resolution mass spectrometer was operated in positive ion electrospray mode with a resolving power of ~14,000 and scanned from 100 to 1000Da in 0.3s. Leucine enkephalin was used to provide a lock mass (m/z 554.2615). Quantification of isoflavones was performed using QuanLynx software with a linear calibration curve and internal standard method. Extracted ion chromatograms with a mass window of 0.020Da around the accurate mass of each

analyte were used to calculate peak areas. A total of 38.17mg isoflavones per gram of Promensil® tablet was determined using this method.

A sample of red clover from various University of Tennessee pastures was procured and tested for presence of isoflavones, as a comparison against the Promensil® tablet. Additionally, the protein supplement included in the diet was also tested for isoflavone content using the previously described methods, and did not contain any isoflavones.

Sampling

Steers were acclimated and fed *ad libitum* in a GrowSafe feed intake measurement system (GrowSafe Systems Ltd. Alberta, Canada). The acclimation ration consisted of 10% protein supplement, 10% cracked corn, and 80% corn silage (all on an as-fed basis). The acclimation period began 10 d prior to the initiation of the trial, during which time steers were fitted with EID tags.

At day 0 of the trial, ground tall fescue seed was included in the diet to provide a minimum of 0.011mg ergovaline plus ergovalinine per kg body weight per day. During the 21-day study, steers were dosed orally each day with 24.7g of the ground isoflavone product via a 28.4g bolus (Torpac Inc., Fairfield, NJ). Within the 24.7g of isoflavone product, a total of 943mg of isoflavones were provided each day to treatments receiving isoflavones. Feed intake was continually monitored throughout the study utilizing the GrowSafe feeding system. On d 0, rectal temperatures, body weight, approximately 9mL of blood via coccygeal venipuncture (Corvac, Sherwood Medical., St. Louis, MO), and approximately 100mL of rumen content were collected via gastric tubing (Guan et

al., 2008). Rectal temperatures and body weights were measured on d 7, 14 and 21. A final sample of blood and rumen content was also collected on d 21. Rumen samples were stored at -80°C until further processing. Blood samples were cooled and centrifuged at 2,000 × *g* and 4°C for 20 min. Serum was separated and stored at -80°C until further processing and analysis.

Bacterial DNA extraction, PCR, sequencing and sequence processing

The procedure of the DNA extraction method is similar to that described by Yu and Morrison (2004). After the chemical/physical cell lysis and isopropanol precipitation of nucleic acids, metagenomics DNA was purified with Rnase and proteinase K treatment, followed by the use of QIAamp columns from the Qiagen DNA Stool Mini Kit (Qiagen). Genomic DNA concentration was determined using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE), and verified using PicoGreen. Extractions were stored at -20˚C until sequencing library preparation. Bacterial 16S rRNA genes were PCR-amplified with dual-barcoded primers targeting the V4 region, as per the protocol of Kozich et al. (2013). Amplicons were sequenced with an Illumina MiSeq using the 250-bp paired-end kit (v.2). Sequences were denoised, taxonomically classified using Greengenes (v. 13_8) as the reference database, and clustered into 97%-similarity operational taxonomic units (OTUs) with the mothur software package (v. 1.39.5) (Schloss et al. 2009), following the recommended procedure (https://www.mothur.org/wiki/MiSeq_SOP; accessed November 2017).

VFA Analysis

A subsample of rumen contents was aliquoted from each rumen content sample for VFA analysis using HPLC, similar to those used by Harlow et al. (2017b). Samples were analyzed for concentrations of formate, acetate, propionate, butyrate, valerate, isovalerate/methylbutyrate (IVMB) using a Summit HPLC (Dionex; Sunnyvale, CA, USA) equipped with an anion exchange column (Aminex HP-87H; Bio-Rad, Hercules, CA, USA) and UV detector. The eluting compounds were separated isocratically with an aqueous sulfuric acid solution (5mM). The parameters included: injection volume 0.1mL, flow rate 0.4mL/min, and column temperature 50°C.

Blood Serum Metabolites

Serum samples were analyzed for serum urea nitrogen (SUN), glucose, and non-esterified fatty acids (NEFA), prolactin, insulin-like growth factor 1 (IGF-1) and insulin. Samples were analyzed using a 96-cell EPOCH 2 microplate reader (BioTek Instruments, Winooski, VT) with commercially available kits for NEFA (Wako Chemicals USA, Inc., Richmond, VA; sensitivity of 0.01 mmol/L), glucose (Thermo Electron Corp., Waltham, MA; sensitivity of 0.3 mg/dL) and SUN (Thermo Electron Corp.; sensitivity of 2.0 mg/dL). Intra- and inter-assay coefficients of variation were 1.89% and 1.13% for SUN, 6.59% and 5.12% for serum glucose, and 5.41% and 4.63% for serum NEFA, respectively. Serum prolactin concentrations were derived using the radioimmunoassay protocol established by Bernard et al. (1993) with intra- and inter-assay coefficients of variation of 4.88% and 4.79%, respectively. Serum insulin concentrations were derived using a radioimmunoassay protocol (Porcine Insulin RIA Kit PI-12K; Linco Research Inc., St.

Charles, MO) with intra- and inter-assay coefficients of variation of 5.3% and 6.27%. Serum IGF-1 was determined by commercially available ELISA kit (R&D Systems, Minneapolis, MN) with intra- and inter-assay coefficients of variation of 8.54% and 12.5%, respectively.

Real time location system set up

Animal movement within the pen was monitored by inclusion of real time location system (RTLS) technology (SmartBow, MKW Electronics, Weibern, Austria). Each pen within the facility was mapped along a two-dimensional grid that included physical structures, such as waterer and feed trough, as well as the pen perimeter. Steers were outfitted with a radio frequency identification tag in the right ear. Each tag continuously transmitted a unique signal that communicates with sensors located throughout the pen and triangulated the steer's position via reception of at least 3 sensors. Spatio-temporal data was aggregated in hourly intervals at the individual level, then in 24 hour intervals for comparison of behavior between treatments.

Statistical Analyses

Physiological data were analyzed using the MIXED procedure of SAS 9.4 (SAS Inst. Inc., Cary, NC) to determine the main effects of seed, isoflavone treatment and their two-way interaction on dry matter intake, average daily gain, volatile fatty acid concentrations, rectal temperatures, and serum metabolites. Data were assessed for normality using PROC UNIVARIATE procedure. Serum prolactin levels and rectal temperatures were non-normally distributed and subsequently log-transformed to

establish normality. All other variables were approximately normally distributed, and thus were analyzed in their raw state. As animals were randomly stratified by genotype prior to the beginning of the study, the random effects of genotype, genotype × seed and genotype × seed × treatment were included. Least square means were compared using Fisher's LSD. Effects were considered significant at a P-value of < 0.05 with tendencies declared at P-values between 0.05 and 0.10. To determine correlations between glucose and insulin concentrations, as well as behavioral data and dry matter intake and average daily gain, the PROC CORR procedure was used. Analysis of rectal temperatures also included day as a main effect with subsequent interactions with seed type and isoflavone treatment.

Behavioral data was assessed for normality using the PROC UNIVARIATE procedure before analysis. Data reported less than 16 h of the day was discarded, and data with at least 16 h of the day but less than 24 h of each day was extrapolated to account for missing periods. Data needed to meet a minimum threshold of 1 for analysis. Distance traveled each day, min in vicinity of the feed bunk, min in the perimeter of the waterer, total water bouts, and min in the feed bunk were, squared, square rooted, or log transformed for normality. Overall data were compared on a weekly basis using the PROC MIXED procedure of SAS, with fixed effects of seed type, treatment, and their interaction. Accounting for the random effects of genotype, genotype × seed and genotype × seed × treatment were included.

For rumen bacterial communities, analysis was conducted in the R environment. Alpha diversity was estimated with the Shannon index on raw OTU abundance tables.

The significance of diversity differences was tested with an ANOVA. To estimate beta diversity across samples, OTUs were excluded if occurring in fewer than 10% of the samples with a count of less than three and computed Bray-Curtis indices. Beta diversity, emphasizing differences across samples, was visualized using non-metric multidimensional (NMDS) ordination. Variation in community structure was assessed with permutational multivariate analyses of variance (PERMANOVA) with treatment group as the main fixed factor and using 4,999 permutations for significance testing.

Results

Ruminal bacterial populations, VFA concentrations and pH

After stringent sequence processing, a total of 365,195 high quality reads were obtained and averaged 9,242 ± 465 per sample, which is a consistent sequencing analysis depth for ruminal samples (Myer et al., 2015). Number of observed OTUs totaled 4,033 and averaged 1,008 ± 60 per sample. Shannon's Diversity Index by treatment group was not statistically significant ($P > 0.05$, Figure 10). Non-metric multidimensional scaling (NMDS) was utilized to analyze beta diversity (Figures 11, 12) where clusters of samples represent similarity of 16s rRNA bacterial genus by group based on rank. At the genus level, endophyte-infected seed and endophyte-free seed were significantly different from one another, endophyte-free and endophyte-infected seed with isoflavone treatment was significantly different, and a significant shift occurred between endophyte-free seed and endophyte free seed with isoflavone treatment ($R^2 = 0.12, P < 0.05$). Significant shifts at the genus level between groups were dominated by *Gammaproteobacteria* and *Succinivibrionaceae* (Figure 13). When individual populations

within the bacterial communities were analyzed, differences were observed at day 21 in twenty-eight different genus classifications, either by seed, treatment, or seed × treatment interaction (Table 5). Relative proportions of genera grouped according to treatments are depicted in Figure 13. Of these, thirteen taxa were reduced with supplementation of isoflavones in the diet, eight were reduced with consumption of endophyte-free tall fescue seed, and five were increased with consumption of endophyte-free tall fescue seed relative to endophyte-infected tall fescue seed ($P < 0.05$).

Ruminal concentrations of formate, acetate, propionate, butyrate, valerate and isovalerate/methylbutyrate (IVMB) were quantified (Table 6). There were no significant differences by seed, isoflavone treatment, or their interaction for acetate, propionate, butyrate, formate, IVMB, valerate or the acetate:propionate ratio. In addition, isoflavone treatment, seed or their interaction were not different for mean rumen pH levels ($P > 0.05$), which ranged from 6.95 - 7.25.

Serum metabolites: glucose, NEFA, SUN, insulin, prolactin and IGF-1

Glucose concentrations (Table 7) did not differ by seed type ($P = 0.43$) but were significantly reduced by isoflavone treatment ($P = 0.03$). Serum insulin concentrations were not altered by seed, or isoflavone treatment ($P > 0.05$) nor were any significant interactions observed ($P > 0.05$, Table 7). There was no correlation between insulin and glucose ($P > 0.05$).

Steers consuming endophyte-infected tall fescue had a tendency for lower NEFA concentrations compared to those receiving endophyte-free tall fescue ($P = 0.07$, Table 7). With respect to seed type, SUN had a tendency to be different between steers

consuming endophyte-infected tall fescue seed and those consuming endophyte-free tall fescue seed (*P = 0.08*). There was no effect of isoflavone treatment (*P = 0.57*), or seed × treatment (*P = 0.78*) on serum urea nitrogen. IGF-1 concentrations were not significantly affected by seed type, but had a tendency to be reduced with isoflavone treatment (*P = 0.08*).

Serum prolactin concentrations were significantly reduced in steers consuming endophyte-infected tall fescue (*P = 0.02*, Table 7). There was not a significant effect of treatment with isoflavones (*P = 0.34)*, nor was a seed × treatment interaction observed (*P = 0.78).*

Dry matter intake, body weight, average daily gain, feed efficiency and rectal temperatures

Throughout the 21-day study, steers gained an average of 13.63 kg, averaging 290 kg ± 40 kg BW at the completion of the study. Body weight was not significant by seed type or treatment, or their interactions (*P > 0.1*). When measuring average daily gain (ADG), there was an effect of seed type, where steers consuming endophyte-infected seed had significantly less ADG than those on endophyte-free seed (*P = 0.03*, Figure 14), which is consistent with literature regarding growth performance. Steers gained an average of 0.081 (± 0.21) kgs per day on the endophyte-infected seed alone, 0.489 (± 0.21) kg per day on endophyte infected seed and Promensil®, 0.832 (± 0.21) kg per day on endophyte free seed alone, and 0.739 (± 0.21) kgs per day on endophyte free seed and Promensil®. There was no effect of treatment (*P* = 0.32), or seed × treatment (*P* =

0.12) on average daily gain. With respect to dry matter intake, there was no effect of seed, treatment or seed × treatment interaction (P > 0.05, Figure 15).

Rectal temperatures were reduced over the 21-day treatment period (*P < 0.0001,* Figure 16), however there was no significant difference among seed type, isoflavone treatment or seed × treatment. Temperature averages were greatest at d 7.

Animal behavioral patterns

Distance traveled was recorded as average steps taken per day. There were no significant differences by fescue seed type, isoflavone treatment or their interaction observed in this study (Table 9). Overall number of feedbouts tended to be increased among steers consuming endophyte free tall fescue seed compared to those consuming endophyte-infected tall fescue seed (*P = 0.09).* Minutes in the feedbunk differed significantly by seed type (*P = 0.03)* where steers consuming endophyte-infected tall fescue seed, regardless of isoflavone treatment, spent approximately 45 min more in the feedbunk than steers consuming endophyte-free tall fescue seed.

With respect to activity around the automatic waterers, no significant differences between isoflavone treatment or fescue seed type were observed (*P > 0.05*), where steers had an average of 9.5 min each day at the waterer.

Discussion

Characteristically, consumption of endophyte-infected tall fescue produces a marked reduction in both feed intake and average daily gain of growing cattle, which can be significantly improved with the inclusion of clover (Burns et al., 1973; Lusby et al.,

1990). In the present study, average daily gain was significantly reduced when animals were consuming endophyte-infected tall fescue seed (Figure 14). Dry matter intake was not significantly reduced when animals consumed endophyte-free tall fescue seed and isoflavones (Figure 15). It is noteworthy that the present study observed no decreases in dry matter intake with endophyte-infected tall fescue seed, as it has previously been identified to decrease (Hannah et al., 1990; Aldrich et al., 1993). Lauriault et al. (1990) established that supplementary thiamin may alleviate fescue toxicosis stress, with respect to intake. In the same study, Lauriault observed ingestive behaviors with endophyte-infected tall fescue compared to endophyte free tall fescue, but did not indicate significant differences in grazing time nor intake. The present study indicated a tendency for higher amounts of feed bouts and significantly higher min spent in the feed bunk for animals consuming endophyte-infected tall fescue compared to animals consuming endophyte free tall fescue seed. This was inconsistent with increased dry matter intake, and no correlation existed between dry matter intake and feed bouts or min in the feed bunk ($P > 0.05$). Feeding behaviors, such as feeding time or aggression have rarely been characterized with incidences of fescue toxicosis and determining more quantitative patterns of fescue toxicosis behaviors will provide insight to overall animal performance.

Symptoms of fescue toxicosis include reduced serum prolactin concentrations and subsequent failure to shed out the winter hair coat or even regrow the winter coat (Hurley et al., 1980; Hoveland et al., 1983; Goetsch et al., 1987; Porter and Thompson, 1992; Aiken et al., 2011). Campbell et al. (2014) determined a single-nucleotide polymorphism at the DRD2 receptor indicating reduced susceptibility to fescue toxicosis that was

correlated with serum prolactin levels. In comparison to steers that consumed the endophyte-free tall fescue, steers consuming endophyte-infected tall fescue had significantly reduced prolactin levels, providing evidence that the animals were likely experiencing at least a moderate level of fescue toxicosis (Table 7).

Glucose concentrations may be reduced by endophyte level, as reported by Jackson et al. (2015), but this was not observed in the present study. Conversely to the study conducted by Jackson et al., steers consuming endophyte-infected tall fescue seed had numerically higher glucose concentrations. When steers received a bolus of isoflavones regardless of seed type, significantly reduced glucose concentrations were observed ($P = 0.03$, Table 7).

The ambient temperature at the time of the study, an average of 27.8°C during the day and 17.2°C at night, may not have been extreme enough to induce more pronounced symptoms of fescue toxicosis. Overall, the average daily gains of steers receiving only E+ seed were reduced compared with animals receiving E- seed, which is consistent with fescue toxicosis. The isoflavone dosage of 943mg per day may not have been sufficient to ameliorate all symptoms of fescue toxicosis. Alternatively, isoflavones might not mitigate all of the symptoms of fescue toxicosis. Isoflavones dilate arteries via nitric oxide synthase (Wu et al., 2010). In this way, isoflavones appear to act antagonistically to ergot alkaloids (Aiken et al., 2016). However, the ability of isoflavones to improve serum prolactin and other effects of ergotism have not been documented. Blood vessel diameters and blood flow measurements were not made in this study, and ADG improved

numerically when isoflavones were given to ergot-challenged animals. These results are consistent with the idea that isoflavones mitigate the effects of fescue toxicosis.

In the study conducted by Aiken et al. (2016), the quantity of Promensil® product utilized was based on the amount required to inhibit the growth and ammonia production of several hyper-ammonia producing bacteria using an *in vitro* model with goats and cattle (Flythe and Kagan, 2010; Flythe et al., 2013b; Harlow et al., 2017a). The dosing used in the aforementioned study conducted by Aiken et al. (2016) aimed to provide a minimum of 30ppm of the isoflavone biochanin A in the rumen. As the purpose of the present study was to determine mitigation of fescue toxicosis, not reduction of ammonia, the dose was not relevant with 30ppm of biochanin A per liter of rumen used previously. Subclinical symptoms of fescue toxicosis were evident in the current study and the dosage used began to reduce some, but not all, of the symptoms. Based on the current findings, future studies may benefit from increasing the dosage to increase effectiveness of the treatment.

It is noteworthy that in previous studies the isoflavones were effective in reducing the number of *Clostridium* species, and this was also consistent in the current study in animals that were supplemented with isoflavone product. Rather than causing overall bacterial shifts due to supplementation with or without the isoflavones, finer bacterial OTUs were altered through both seed and treatment (Table 5). Recent studies conducted by Harlow et al. targeted specific amylolytic or cellulolytic bacterial species and their sensitivities to biochanin A (2017; Harlow, 2018). Three cellulolytic bacteria (*Fibrobacter succinogenes S85, Ruminococcus flavefaciens 8 and Ruminococcus albus 8*) were reduced with biochanin A, and four amylolytic bacteria *(Strep. bovis JB1, Strep. bovis*

HC5, Lactobacillus reuteri, Selenemonas ruminatium) were inhibited with biochanin A. Compared to the present study, these conducted by Harlow et al. (2018) utilized only the isoflavone biochanin A and observed shifts in the bacteria. The present study identified nine taxa that were reduced due to isoflavone additive, all of which were not described in the previous studies (Table 5). Gram-negative bacteria Gammaprotebacteria and Succinivibrionaceae were not identified among those inhibited by biochanin A in previous studies, but were two of the main shifts in the present study. These ruminal bacteria shifts may be influenced by other isoflavones, as well as by seed type. This may provide future insight to using additional culturable strains of microorganisms to determine the influence of isoflavones on them in the future. Using the isoflavone dosage from this study elucidated several rumen microbiological shifts that may provide beneficial targeting in the future, as the bacterial family Succinivibrionaceae and class Gammaproteobacteria, both known for acetate production. Improving volatile fatty acid production through a higher dosage of isoflavones should be considered in future studies to promote growth performance.

The isoflavone tablet utilized in this treatment was fortified to have a greater concentration of the isoflavone biochanin A compared to most red clover stands. Due to the potential differences between the isoflavone concentrations in the product and isoflavone concentrations in red clover pastures, samples were collected across the state of Tennessee and tested. These samples were analyzed using the previously mentioned LC-MS methodology for content of the four major isoflavones and several glucosides (Table 8). When compared to the traditional red clover stands, the tablet provided

23,191µg per tablet (tablet size ~600 mg). The present study supplemented 24.7 grams of tablet per day over the course of the 21 day study, for an estimated 943mg isoflavones per bolus each day. Across a sample of red clover stands in Tennessee (*n* = 5), 20,227µg isoflavones per gram of red clover was determined. Cattle entering a stocker or backgrounding operation may consume 2-3% of their body weight each day in dry matter (NRC, 2000). Given most Tennessee and southeastern pastures have mixed forages, red clover stands may occupy 25-35% of each pasture. Cattle weighing 200-300kg may consume at most 45-60 grams of isoflavones per day, depending on forage maturity, intake, and composition of pasture. At d 21 of the present study, steers weighed an average of 290 kg and were provided a dose of 3.29mg isoflavones per kg body weight. Thus, for the present study the isoflavone amount supplemented was substantially less than average possible consumption rates on pasture. As both physiological and microbial changes were observed at a fraction of the isoflavones found in expected forage consumption in the present study and by Aiken et al. (2016), this could provide insight to future experiments.

The combined physiological, biochemical, behavioral and microbiological results from the present study indicate more direct influences of fescue toxicosis on the animal's whole physiology than have previously been identified. In an effort to combat the detrimental effects of fescue toxicosis, the use of isoflavones at the dose used in the present study may not be a viable alternative as a top-dressed supplement to cattle grazing endophyte-infected tall fescue pastures throughout the Southeastern United State or similar agronomic climates. Thus further research discerning beneficial

isoflavone dosage, and specific species of red clovers that may provide increased concentrations of isoflavones, for mitigation of tall fescue toxicosis should be conducted.

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Appendix C

Table 5. Effects of isoflavones and fescue seed type on relative abundance of significant bacterial taxa

Classification	Percentage of total sequences[1,2]				Effect	SEM	P-value[2]
	Fescue Seed[3]						
	E+		E-				
	Treatment[4]						
	C	P	C	P			
Acholeplasma	0.00571^{b}	0.00883^{ab}	0.0216^{a}	0.00192^{b}	Seed × Treatment	0.0005	0.0265
Anaeroplasma	0.0269^{ab}	0.0422^{ab}	0.0563^{a}	0.0124^{b}	Seed × Treatment	0.0001	0.0268
Anaerostipes	0.0195^{ab}	0.0492^{a}	0.0144^{b}	0.0370^{ab}	Treatment	0.0001	0.0362
Bacillales	0.0279^{a}	0.00231^{b}	0.00411^{b}	0.00931^{b}	Seed × Treatment	0.0001	0.0026
Bacillus_unclassified	0.000469^{a}	3.13^{ab}	0.0301^{ab}	0.0155^{b}	Seed	0.0001	0.0462
Bacteriodetes_unclassified	5.25^{a}	4.48^{b}	5.54^{a}	4.79^{b}	Treatment	0.0044	0.0216
Blautia	0.0255^{a}	0.00243^{b}	0.00571^{b}	0.00213^{b}	Treatment	0.0001	0.0101
Campylobacter	0.00592^{b}	0.00411^{b}	0.0211^{a}	0.0183^{a}	Seed	0.0001	0.0005
Clostridium	0.883^{a}	0.212^{b}	0.377^{ab}	0.189^{b}	Treatment	0.0019	0.0261
Coprococcus	0.343^{a}	0.161^{ab}	0.311^{ab}	0.102^{b}	Treatment	0.0008	0.0245
Gammaproteobacteria_unclassified	0.444^{ab}	0.527^{a}	0.184^{b}	0.276^{ab}	Seed	0.0011	0.0314
Lachnospiraceae	1.60^{ab}	1.27^{ab}	1.81^{a}	1.17^{b}	Treatment	0.0021	0.0227
Lactobacillus	0.157^{ab}	0.277^{a}	0.0735^{b}	0.0941^{b}	Seed	0.0005	0.0152
Mogibactericeae	0.121^{a}	0.0962^{b}	0.133^{a}	0.0888^{b}	Treatment	0.0001	0.0315
Oceanobacillus	0.0395^{a}	0.0287^{ab}	0.00661^{c}	0.0157^{bc}	Seed	0.0001	0.0045
Olsenella	0.0157^{ab}	0.0476^{a}	0.00882^{b}	0.0329^{ab}	Treatment	0.0001	0.0301
Oscillospira	1.024^{ab}	0.831^{b}	1.51^{a}	1.18^{ab}	Seed	0.0019	0.0417
PL11B10	0.0118^{b}	0.00984^{b}	0.0568^{a}	0.00362^{b}	Seed × Treatment	0.0001	0.0153
Prevotella	29.7^{a}	26.6^{ab}	24.5^{b}	28.9^{a}	Seed × Treatment	0.0138	0.0095
Proteobacteria	0.183^{b}	1.99^{b}	8.66^{a}	6.05^{ab}	Seed	0.0201	0.0101
Ruminobacter	0.246^{b}	0.00882^{b}	1.03^{a}	0.121^{b}	Treatment	0.0021	0.0102
Succinimonas	0.0528^{b}	0.0675^{b}	0.522^{a}	0.272^{ab}	Seed	0.0009	0.0006
Succinivibrionaceae_unclassified	11.7^{ab}	16.7^{a}	2.97^{b}	5.27^{ab}	Seed	0.0406	0.0108
TG5	0.0256^{a}	0.00893^{b}	0.0274^{a}	0.00812^{b}	Treatment	0.0001	0.0135
Veillonellaceae	0.289^{b}	0.252^{b}	0.415^{a}	0.393^{a}	Seed	0.0004	0.0323
YS2	2.19^{b}	1.88^{b}	3.25^{a}	1.78^{b}	Treatment	0.0036	0.0184

[1] Data shown as LSMeans (*n*=10/treatment group)

[2] Within a row, means among treatment groups are considered significant at $P < 0.05$, and trending at $P < 0.1$

[3] Endophyte-infected (E+) tall fescue seed (0.011mg ergovaline/kg BW/day or endophyte free (E-) tall fescue seed (0mg/kg BW/day ergot alkaloids) mixed in total ration, which was fed *ad libitum*

[4] Isoflavones administered daily at 0 or 943mg per animal per day

Table 6. Effects of isoflavones and fescue seed type on rumen volatile fatty acid concentrations in beef steers

VFA[1]	Treatment[3]				SEM	P-value		
	Control		Isoflavones			Seed	Treatment	Seed × Treatment
	Fescue Seed[4]							
	E+	E-	E+	E-				
Formate	0.34	0.75	0.62	1.11	0.22	0.28	0.11	0.86
Acetate	25.4	24.4	24.5	24.6	2.92	0.88	0.80	0.84
Propionate	15.0	15.2	16.0	14.5	1.82	0.76	0.67	0.93
A:P[2]	1.76	1.73	1.57	1.62	0.10	0.92	0.23	0.69
Butyrate	5.33	4.72	4.33	4.44	0.60	0.69	0.31	0.55
IVMB	1.14	0.98	0.87	0.99	0.23	0.87	0.44	0.41
Valerate	0.06	0.15	0.07	0.44	0.13	0.21	0.30	0.34

[1]Concentrations in mmol/L
[2]Acetate: Propionate ratio
[3] Isoflavones administered daily at 0 or 943mg per animal per day
[4] Endophyte-infected (E+) tall fescue seed (0.011mg ergovaline/kg BW/day or endophyte free (E-) tall fescue seed (0mg/kg BW/day ergot alkaloids) mixed in total ration, which was fed *ad libitum*

Table 7. Effects of isoflavones and fescue seed type on serum metabolites in beef steers

Measurement	Treatment				SEM	P-value		
	Control		Isoflavones					
	Fescue Seed[4]							
	E+	E-	E+	E-		Seed	Treatment	Seed × Treatment
Insulin[1]	0.63	0.47	0.59	0.66	0.13	0.73	0.59	0.42
Glucose[2]	134.8[a]	114.12[ab]	92.42[b]	92.38[b]	15.82	0.43	0.03	0.38
NEFA[3]	81.84	104.79	80.96	122.31	17.23	0.07	0.63	0.59
SUN[2]	4.32	5.06	4.46	5.46	0.48	0.08	0.57	0.78
IGF-1[1]	171.40	207.34	111.19	145.16	30.24	0.39	0.08	0.86
Prolactin[1]	1.32[b]	15.67[a]	2.22[b]	20.65[a]	1.44	0.02	0.34	0.75

[a,b] Means with different superscripts differ ($P \leq 0.05$)
[1] Concentration in ng/mL
[2] Concentration in mg/dL
[3] Concentration in mmol/L
[4] Isoflavones administered daily at 0 or 943 mg per animal per day
[5] Endophyte-infected (E+) tall fescue seed 0.011mg ergovaline/kg BW/day or endophyte free (E-) tall fescue seed (0mg/kg BW/day ergot alkaloids) mixed in total ration, which was fed *ad libitum*

Table 8. Isoflavone quantities in red clover stands across Tennessee, and in Promensil® product

Sample Type[1]	Isoflavone[2]									
	Biochanin A	Formononetin	Genistein	Daidzein	BG[a]	FG[b]	GG[c]	DG[d]	BMG[e*]	FMG[f*]
Red Clover	1701	3819	42	20	462	1268	365	309	3421	10367
Red Clover	1258	3346	37	18	606	1223	356	265	5238	9745
Red Clover	889	2361	25	1	576	1192	423	159	4957	9883
Red Clover	1242	3067	31	9	524	1217	559	282	3871	8667
Red Clover	906	2158	25	9	489	1132	342	479	3541	8256
Promensil®[3]	7346	5997	449	169	3658	5572	0	0	0	0

[1]Red Clover samples were collected from different locations across University of Tennessee research centers

[2] Measured in µg per gram

[3]Measured in µg per tablet (~600mg per tablet)

[a]Biochanin A glucoside (sissotrin)

[b]F-glu [b]Formononetin glucoside (ononin)

[c]Genistein glucoside (genistin)

[d]D-glu [d]Daidzein glucoside (daidzin)

[e]Biochanin A malonyl glucoside

[f]Formononetin malonyl glucoside

*Corrected for hydrolysis

Table 9. Effects of isoflavones and fescue seed type on feeding behavior of beef steers

Behavior Metric	Treatment[2]				SEM	P-value		
	Control		Isoflavones					
	Fescue Seed[3]							
	E+	E-	E+	E-		Seed	Treatment	Seed × Treatment
Distance Traveled[1]	5230.9	5495.8	5617.6	5258.1	1980.5	0.53	0.47	0.92
No. Feed Bouts	272.06	196.44	277.10	212.78	23.63	0.09	0.66	0.8
No. Water Bouts	10.02	7.6	10.17	9.73	4.45	0.53	0.54	0.
Min in Feed Bunk	108.67[a]	62.71[ab]	104.97[a]	55.97[b]	1.12	0.03	0.42	0.60

[a-b] Means with different superscripts differ ($P < 0.05$)

[1]Indicates average movement in steps per day

[2] Isoflavones administered daily at 0 or 943mg per animal per day

[3] Endophyte-infected (E+) tall fescue seed (0.011mg ergovaline/kg BW/day or endophyte free (E-) tall fescue seed (0mg/kg BW/day ergot alkaloids) mixed in total ration, which was fed *ad libitum*

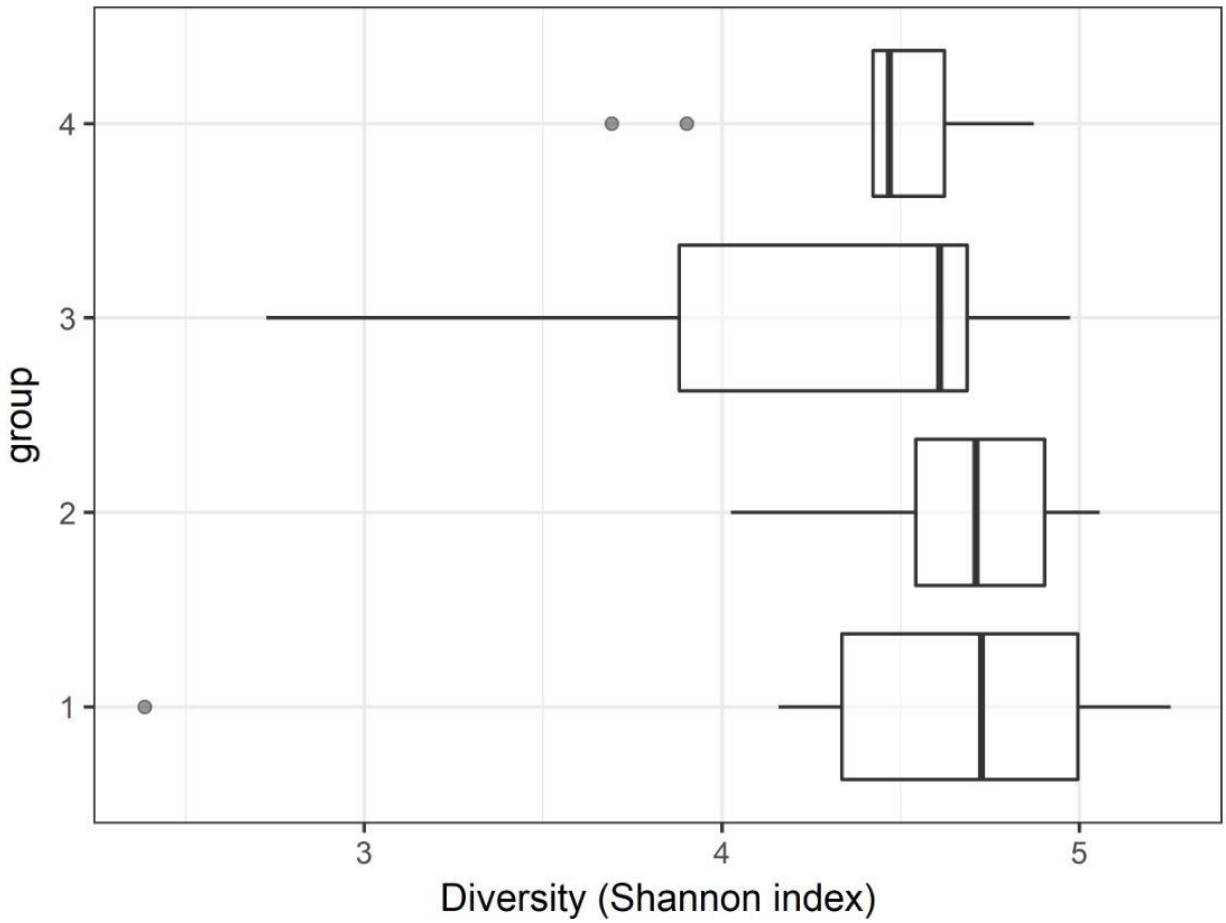

Figure 10. Shannon's Diversity Index box plot of bacterial species diversity across groups on Day 21. Group 1: Endophyte-infected tall fescue seed. Group 2: Endophyte-free tall fescue seed. Group 3: Endophyte-infected tall fescue seed with isoflavones. Group 4: Endophyte-free tall fescue seed with isoflavones.

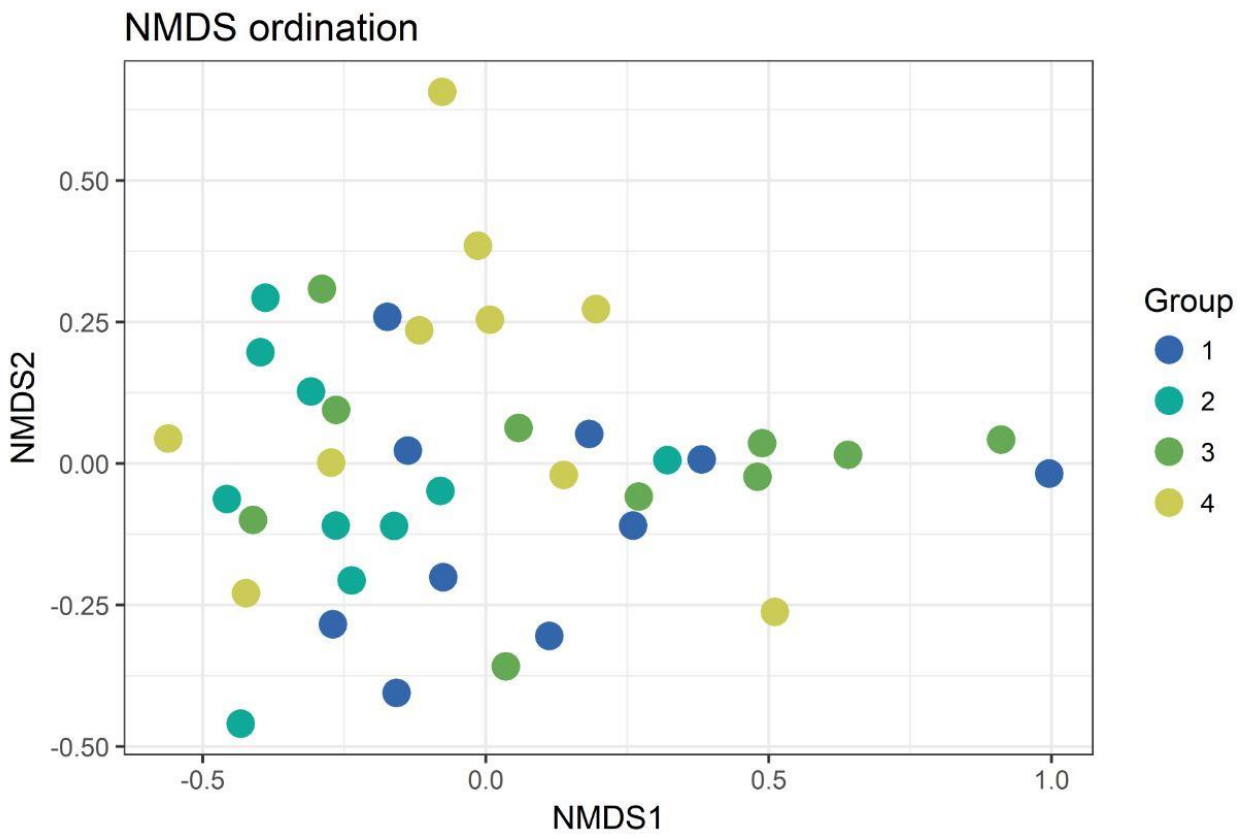

Figure 11. NMDS ordination plot grouped by treatment. Group 1: Endophyte-infected tall fescue seed. Group 2: Endophyte-free tall fescue seed. Group 3: Endophyte-infected tall fescue seed with isoflavones. Group 4: Endophyte-free tall fescue seed with isoflavones.

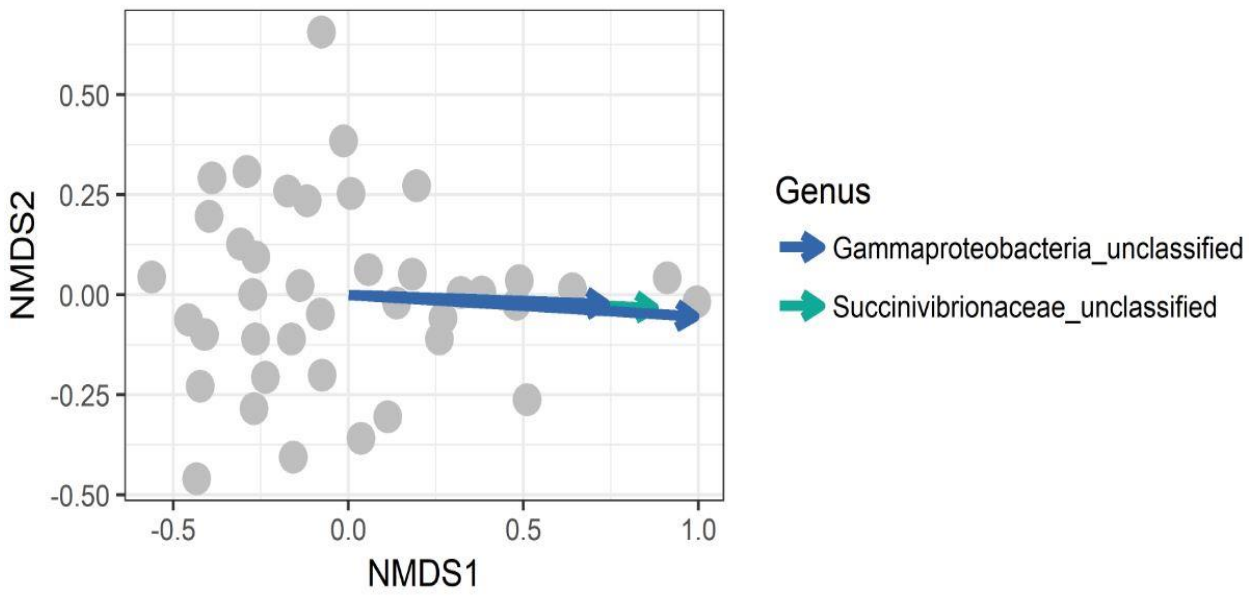

Figure 12. NMDS ordination plot of two significant taxa differing across all four treatment groups. Both *Gammaprotebacteria* and *Succinivibrionaceae* were significant at the genus level in influencing bacterial shifts

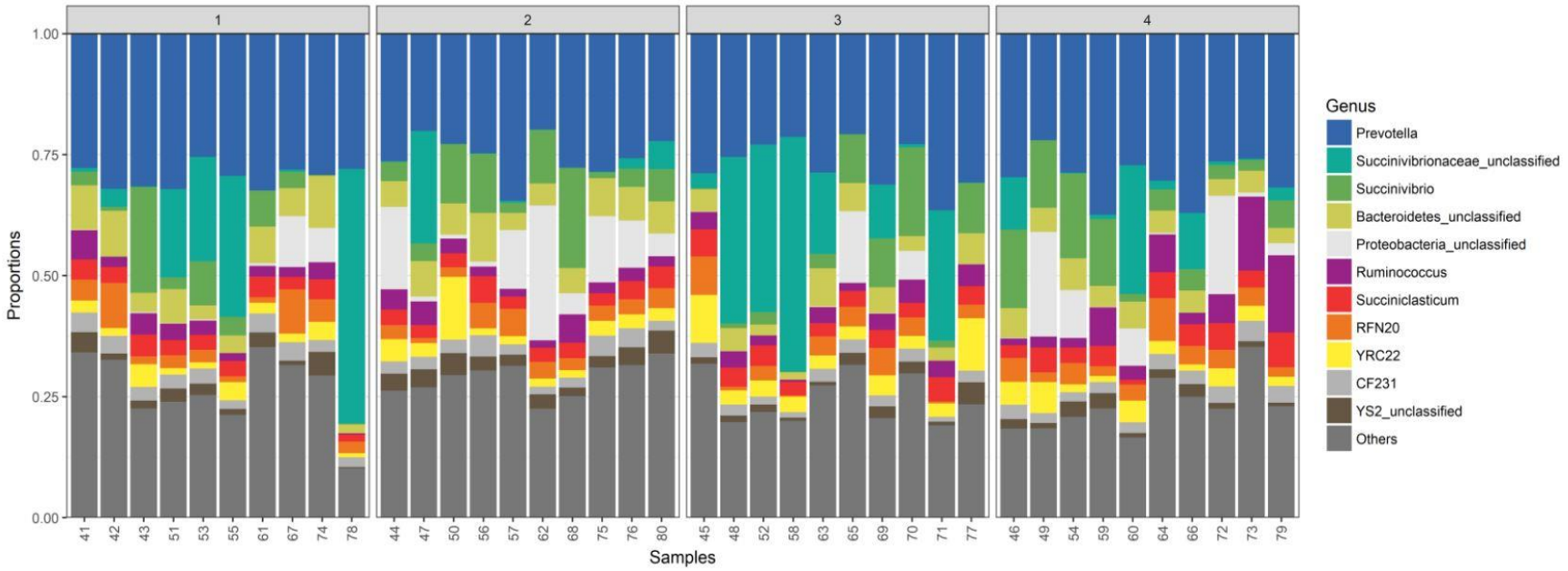

Figure 13. Taxonomic profiles of the relative proportions of bacterial communities by genus, grouped by treatment. Group 1: Endophyte-infected tall fescue seed. Group 2: Endophyte-free tall fescue seed. Group 3: Endophyte-infected tall fescue seed with isoflavones. Group 4: Endophyte-free tall fescue seed with isoflavones.

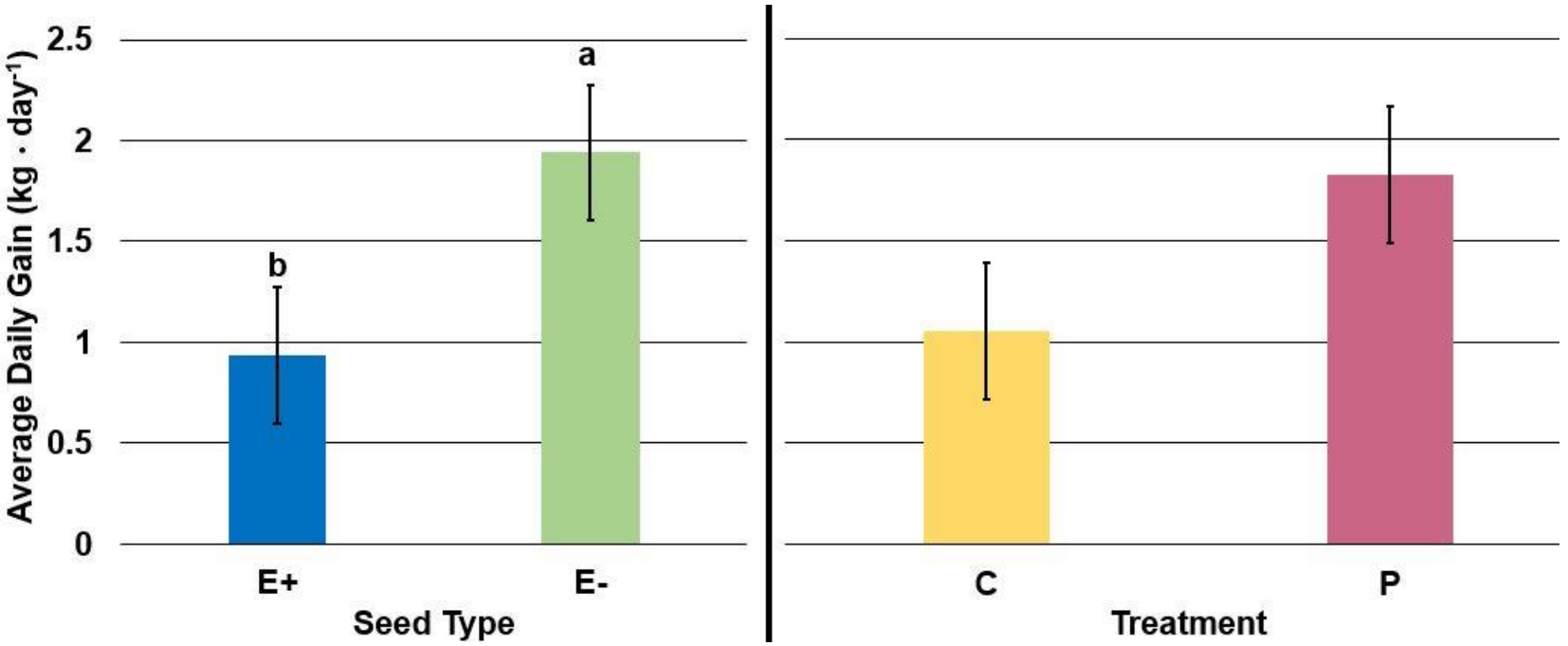

Figure 14. Average daily gain (kgs) by seed type and isoflavone treatment. Seed type abbreviated as endophyte-infected seed (E+) and endophyte free seed (E-). Treatment type abbreviated as a control (C) or with isoflavones (P). Differences in means are designated by means separation letters ($P < 0.05$). Error bars represent SEM.

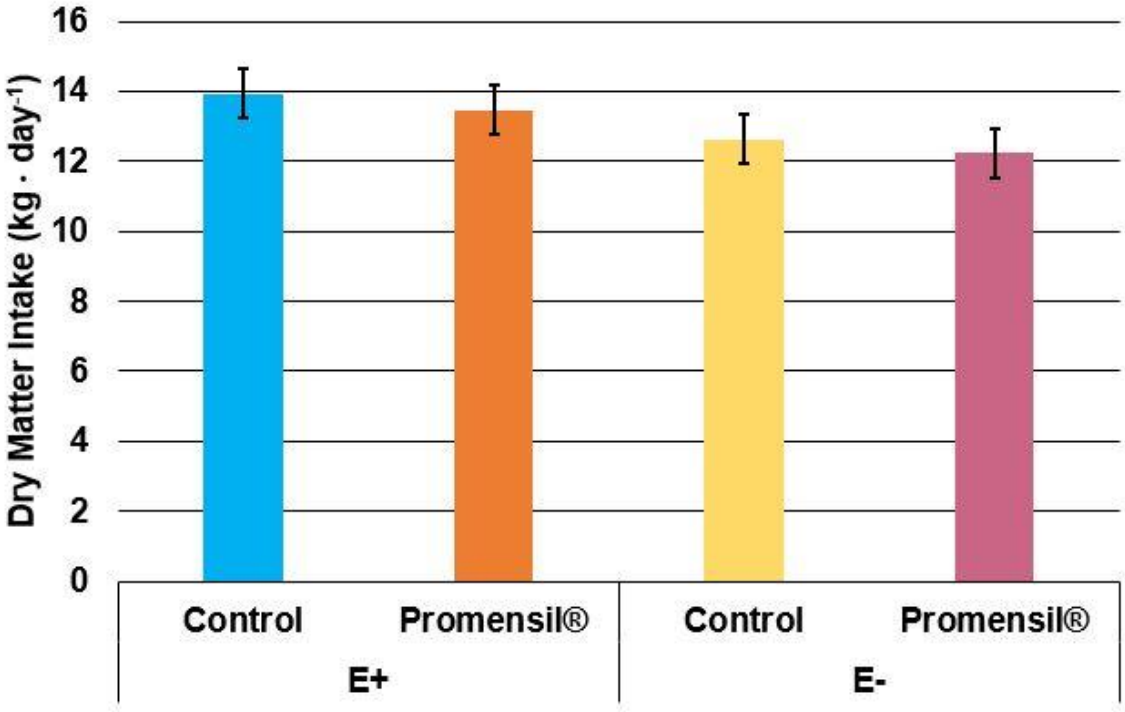

Figure 15. Average dry matter intake (kgs) by seed and treatment across treatment period. Treatments abbreviated as endophyte-infected seed (E+) as a control (C) or with isoflavones (Promensil®), and endophyte free seed (E-) as a control (C) or with isoflavones (Promensil®). Error bars represent SEM.

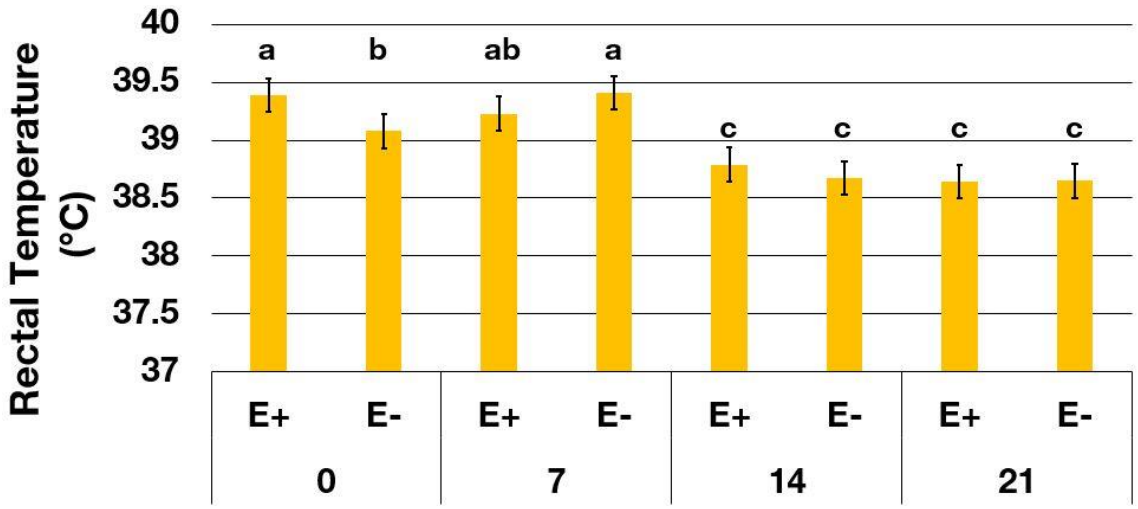

Figure 16. Rectal temperatures (°C) across treatment days by seed type, endophyte-infected (E+) or endophyte free (E-) tall fescue seed. A day effect was observed ($P < 0.001$) where days 14 and 21 had significantly lower rectal temperatures compared to days 0 and 7. Differences are noted by means separation letters. Error bars represent SEM.

CONCLUSIONS

Previous research has not examined the effects of isoflavones on beef cattle experiencing tall fescue toxicosis, but has determined improvements in ruminal fiber fermentation (Harlow, 2018), reduced ammonia production (Flythe et al., 2013a), improved vasodilation (Aiken et al., 2016). As clovers contain isoflavones and are commonly mixed in pastures in the Southeastern United States, especially Tennessee, understanding the full effects of these isoflavones on beef cattle production will provide benefits to producers.

Improving rumen function and efficiency is paramount to the livestock industry. As cow-calf and stocker operations heavily utilize forages for growth and reproduction, promoting rumen fiber degradation can increase livestock performance. Based on previous data, and in agreement with data presented in Chapter 2, isoflavones improved fiber degradation when fermented with tall fescue seed and rumen fluid over a 48 h period. However, isoflavones also increased crude protein degradation in a seed × treatment two-way interaction. There were no significant differences in volatile fatty acids nor ruminal pH. At the dose of used in Chapter 2, isoflavones did not improve overall rumen fermentative capacity, and further examinations of specific isoflavones and dosing should be conducted to improve fiber fermentation and overall rumen fermentation.

As with the dose in Chapter 2, the isoflavone dose used in Chapter 3 was provided lower than previous research and likely consumption rates with red clover and tall fescue pastures. Steers consuming endophyte-infected tall fescue seed experienced significantly reduced average daily gain and serum prolactin concentrations than steers consuming endophyte free tall fescue seed, providing evidence the study induced tall

fescue toxicosis. However significant differences of growth performance, including average daily gain, dry matter intake and rectal temperatures were not affected by treatment with isoflavones. Interestingly, isoflavones treatment resulted in lower serum glucose concentrations than the control treatment, which is a novel find that may indicate benefits of isoflavones in the diet. In an effort to simulate a grazing environment, higher doses of isoflavones should be provided over an increased period of time (greater than three weeks). Serum metabolites, including NEFA, glucose and insulin concentrations, as well as ruminal pH and volatile fatty acid concentrations should be monitored throughout the study to indicate nutritional stress on the cattle in more long-term studies. While several feeding behavior traits were examined in Chapter 3, there has been little research regarding animals experiencing fescue toxicosis. Feeding aggression and behavior has not been well characterized with cattle experiencing fescue toxicosis, and may indicate further management strategies to alleviate tall fescue toxicosis stress.

Understanding the mechanism of isoflavones on promoting growth of cattle and mitigating symptoms of tall fescue toxicosis will ultimately provide producers with improved pasture and nutritional recommendations. As concentrations of isoflavones vary by maturity in clover tissue, determining ideal grazing management or supplementation strategies including red clover will be beneficial.

VITA

Emily Anne Melchior was born in Baltimore, MD on March 28th, 1994. Emily grew up in Ellenton, FL where she was an active participant in FFA and worked at her local veterinarian's office before graduating from Braden River High School in 2012. Emily received her Bachelor's of Science in Animal Science from Berry College in Mount Berry, GA in May of 2016. Upon graduation from Berry, she pursued an internship with the University of Georgia Cooperative Extension Service. She began her Masters in Animal Science at the University of Tennessee, Knoxville under the guidance of Dr. Phillip Myer, in August 2016. Beginning in August of 2018, Emily began pursuit of her Doctor of Philosophy in Animal and Range Sciences at New Mexico State University in Las Cruces, NM.