FROM METABOLITES AND MICROBES TO GRAZING BEEF WELLBEING AND PRODUCTIVITY: A SYSTEMS BIOLOGY APPROACH TO BOVINE FESCUE TOXICOSIS

by

RYAN SCOTT MOTE

(Under the Direction of Nikolay M. Filipov)

ABSTRACT

Tall fescue, *Lolium arundinaceum*, is the predominant Southeastern US pasture grass, covering 14 million hectares and commonly infected with the endophytic fungus, *Epichloë coenophiala*. *E coenophiala* produces bioactive secondary metabolites, some (i.e., ergot alkaloids) are toxic to livestock, leading to fescue toxicosis (FT). The development of FT has been attributed ergot alkaloids but evidence suggests downstream processes may play a role. To investigate this, we employed systems biology approaches across four studies to evaluate differences in the microbiota, metabolome, and multi-compartment microbiota-metabolome interaction (throughout the tall fescue plant and animal) between Angus steers grazing a novel, non-toxic (Max-Q) or toxic (E+) tall fescue across different seasons and/or environmental conditions through 16S (bacteria) and ITS2 (fungi) sequencing, high-resolution metabolomics, and bioinformatics methods. E+ grazing altered the bovine plasma/urine metabolomes (tryptophan/lipid metabolism) and fecal microbiota (increased *Ruminococcaceae* and *Lachnospiraceae*) under thermoneutral conditions. Heat stress altered the bovine microbiota/metabolome response to E+, with the E+ microbiota susceptible to harsh environmental conditions. Three bacterial operational taxonomic units (OTUs) associated with

plasma/urinary metabolites and pathophysiological endpoints (weight gains) were identified. *E. coenophiala* altered plant phyllosphere bacterial/fungal microbiota and metabolome, with most E+ effects being plant-specific. While E+ grazing had mixed effects on rumen bacteria, it decreased most ruminal fungi. E+ infection and/or grazing perturbed amino acid and Vitamin B6 metabolism in all biological matrices (plant, rumen liquid, plasma, urine). Targeted network analysis revealed numerous microbial OTUs were significantly associated with *E. coenophiala* in the plant and rumen, the only matrices where it was detected, with *E. coenophiala* being more integral to plant network. Integrative interactomics showed similar overall structure to Max-Q and E+ networks, with fecal fungi most important in both networks but aligned taxa being different. Urinary L-metanephrine, L-dopachrome, and pyridoxal were identified as accessible biomarkers of FT dysbiosis in multiple compartments. Multiple Vitamin B family (folate, Vitamin B6, etc.) members were significantly perturbed by E+ and in the E+ integrome networks. Overall, these studies provide an outline of the FT integrome and allow future studies to take a directed approach at the development of molecularly driven management strategies and/or novel FT therapeutics.

INDEX WORDS: fescue toxicosis, ergot alkaloids, *Epichloë coenophiala*, tall fescue, metabolomics, microbiome, *Bos taurus*

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DEDICATION

I would like to dedicate this work to my parents, Ernest (Scott) and Susan Mote, and my fiancée Mackenzie, who have sacrificed greatly for me to pursue this dream. Thank you for being my rock throughout this process. Thank you to my dogs Ezra and Sandy, who have sacrificed walks so that I could work in the evenings. Finally, to my brother, sister, grandparents and other family and friends, none of this would be possible without your dedication and support. I will never forget what I have been given throughout this process and am forever grateful to everyone for allowing me to pursue this dream.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

BACKGROUND

Tall fescue

Tall fescue, *Lolium arundinaceum*, is a cool season perennial grass that is well adapted to the Southeastern United States and covers approximately 14 million hectares of land within the Fescue Belt (Ball et al., 1991). Greater than 20% of the pastures within the Fescue Belt are tall fescue, and it was recently estimated that 12 million beef cattle are present within this land mass (Brussard and Aiken, 2012). Of those, about two-thirds will likely be exposed to wild-type tall fescue at some point in their lifetime (Ball et al., 2002). The adoption of tall fescue as a forage grass was driven by compelling agronomic attributes, such as persistence under drought and grazing stresses, resistance to herbivores, nematodes, and insects, and greater potential for nutrient uptake (Clay and Schardl, 2002). Ultimately, the agronomic attributes that give wild-type tall fescue a competitive advantage over other forage cultivars have been ascribed to plant infection with the endophytic fungus *Epichloë coenophiala*.

E. coenophiala was originally identified as *E. typhina* (Bacon et al., 1977) before becoming *Acremonium coenophialum*, with the nomenclature change describing the anamorphic state of *Epichloë* species. In 1996, the endophyte was further classified as *Neotyphodium coenophialum* following maximum parsimony and neighbor-joining analysis using the nuclear small ribosomal subunit (18S rDNA; Glenn et al., 1996); subsequent rule changes for fungal

nomenclature resulted in the current classification of *Epichloë coenophiala* (Leuchtmann et al., 2014).

E. coenophiala grows systemically above ground in tall fescue and is transmitted vertically through seed heads (Schardl et al., 2013). Interestingly, the agronomic benefits of *E. coenophiala* infection are heritable, with both the plant and endophyte genotype influencing plant offspring persistence (Missaoui and Hill, 2015). Bioactive secondary metabolites derived from the endophyte are considered responsible for the increased persistence of *E. coenophiala*-infected tall fescue. Four major classes of metabolites are produced by *E. coenophiala*: 1) ergot alkaloids; 2) indole-diterpene alkaloids; 3) loline alkaloids; and 4) peramines (Siegel et al., 1990), with the ergot alkaloids, lolines, and peramine being most abundant (Young et al., 2014). While loline alkaloids and peramine contribute to plant insect resistance (Bush et al., 1997; Johnson et al., 1985; Rowan et al., 1994), the ergot alkaloids and the indole-diterpene alkaloid lolitrem-B are detrimental to grazing livestock. Within the United States, the metabolites of major economic and clinical concern are the ergot alkaloids, as they result in a costly disease culminating in livestock production deficits named fescue toxicosis (FT), and are the reason wild-type *E. coenophiala*-infected tall fescue is referred to as toxic (E+; Klotz, 2015).

Kentucky-31 was the first commercially available tall fescue cultivar, but numerous other varieties are now available. An extensive, but not exhaustive, list of the top performing tall fescue cultivars compiled by Grady Miller of North Carolina State University is presented as Table S1.1 in Appendix A and can be found here (https://content.ces.ncsu.edu/2017-top-performing-tall-fescue-kentucky-bluegrass-and-fine-fescue-cultivars). Among the cultivars not in the table is Jesup (GA-5) tall fescue. This variety, which was released in 1995, is available without an endophyte or with an ‘anti-fescue toxicosis’ endophyte, Jesup MaxQ (GA-5 MaxQ)

endophyte AR542, which was generated by cooperative efforts between AgResearch in New Zealand and the University of Georgia and is commonly referred to as Max-Q (Bouton et al., 2002; Hancock and Andrae, 2012).

Continued increased interest to find a front-end approach to mitigate the detrimental impact of fescue toxicosis has driven the development of novel endophytes that do not produce detrimental-to-the-animal secondary metabolites, i.e., "non-toxic" endophytes (Hancock and Andrae, 2012). Non-toxic endophyte varieties, such as KYFA9301 infected with novel endophyte (AR584; Klotz et al., 2013) and MaxQ, are commercially available. The efforts towards developing additional novel endophytes that provide the agronomic attributes, but not the animal toxicosis, of *E. coenophiala* are ongoing. Although novel endophytes are useful, replacing *E. coenophiala*-infected pastures with novel endophyte-infected pastures is not an ideal approach (Clay, 1990); such approaches are costly, i.e., for most production operations, labor costs, risk for soil erosion, and impairment of pasture sustainability would prove economically detrimental, as pastures would have significant delay before becoming profitable (Gunter and Beck, 2004; Hoveland, 1993; Parish et al., 2003; Pimentel and Burgess, 2013). Considering that novel endophyte-infected tall fescue pastures are unlikely to become the norm, a renewed focus has emerged on understanding the effects *E. coenophiala* infection and exposure have on the tall fescue plant and grazing livestock, respectively.

Ergot Alkaloids

Ergot alkaloid nomenclature and biosynthesis

The profile of ergot alkaloids within tall fescue is highly variable but consists of two major classes: 1) lysergic acid derivatives and 2) ergopeptine alkaloids. All ergot alkaloids share a D-lysergic acid backbone, with further classification derived from functional groups bonded to

the primary 8th carbon (Berde and Stürmer, 1978). The lysergic acid derivatives have a carboxamide functional group, with the varying chemical moieties bound to the carboxamide group used for classification. The ergopeptine alkaloids, synonymously called ergopeptides, have a tripeptide moiety bound to carbon 8 of the lysergic acid ring. The constituents of this tripeptide moiety are used for molecular nomenclature. For example, the most prevalent ergopeptine alkaloid derived from *E. coenophiala*-infected tall fescue, ergovaline, has L-alanine, L-proline, and L-valine, whereas ergotamine, a main *Claviceps purpurea* alkaloid, has L-alanine, L-proline, and L-phenylalanine as tripeptide moiety constituents (Young et al., 2014).

Most of the biochemical pathways underlying the biosynthesis of ergopeptine alkaloids have been elucidated from *C. purpurea* but likely translate to *E. coenophiala*-produced ergot alkaloids. The first step in ergot alkaloid biosynthesis is prenylation at the fourth carbon position of the tryptophan ring via electrophilic substitution, which is mediated by dimethylallyl tryptophan (DMAT) synthase, an enzyme encoded by the *dmaW* gene, which has been implicated as a key regulatory step for ergot alkaloid biosynthesis (Floss et al., 1974; Krupinski et al., 1976; Panaccione et al., 2001). This reaction produces dimethylallyl tryptophan from the parent tryptophan and dimethylallyl disphosphate compounds (Gebler and Poulter, 1992). Then, serial redox reactions generate the intermediates chanoclavine, agroclavine, and elymoclavine before conversion to D-lysergic acid by the *clo4* encoded cytochrome P450 (Floss, 1976; Haarmann et al., 2006). D-lysergic amide is then converted into lysergyl amides or ergopeptine alkaloids (Floss, 1976).

Ergopeptine formation occurs by addition of the tripeptide moiety to the active D-lysergic acid by non-ribosomal peptide synthetases (Walzel et al., 1997), which are large multimodular enzymes that utilize a non-ribosomal, thiotemplate mechanism for synthesis of the tripeptide

moiety (Marahiel et al., 1997; Walzel et al., 1997). Generally, these peptide synthetases have one module that activates and combines one amino or carboxylic acid substrate, through peptide bond formation, to an upstream module (Marahiel et al., 1997). In the case of ergopeptines, lysergic acid is bound to lysergol peptide synthase LpsB (LPS2), which is subsequently added to a specific amino acid bound to LspA (LPS1; Correia et al., 2003; Riederer et al., 1996). Subsequent amino acids are then added before being heterocyclized and released from LPS1 as lysergyl acid lactam (Walzel et al., 1997). The final ergopeptine is formed when the released lysergyl acid lactam is heterocyclized again (Quigley and Floss, 1981).

Ergot alkaloid profile in tall fescue

In *E. coenophiala*-infected tall fescue, ergovaline is the major ergopeptine alkaloid, with one study finding that it accounts for 80-100% of the ergopeptine content in forage grasses and around 50% in seed heads (TePaske et al., 1993) . In the context of fescue toxicosis, ergovaline has historically been the most studied ergot alkaloid. Of the other ergot alkaloids, ergosine and ergotamine were also found in forage, representing 0-12% and 0-6% of the ergopeptines analyzed, respectively (TePaske et al., 1993). Ergosine, ergotamine, and ergocryptine have been found in seed heads, representing 15-50% of the ergopeptine profile (TePaske et al., 1993; Yates and Powell, 1988). Ergovaline, ergotamine, ergocornine, ergocryptine, and ergocrystine are all vasoactive, but ergovaline and ergotamine are the most potent when duration and intensity of vascular effects are considered (Foote et al., 2011; Klotz et al., 2012; Klotz et al., 2007; Klotz et al., 2010; Pesqueira et al., 2014).

Studies quantifying levels of ergot alkaloids usually report levels of extracted ergovaline and total ergot alkaloid concentrations, whereas specific levels of other ergot alkaloids are less frequently reported. Two studies have found lysergic acid levels in Kentucky 31+ tall fescue

between 600 – 800 μg kg^{-1} plant material and at 24.2 μg kg^{-1} of tall fescue straw using HPLC coupled with fluorescence detection (Helander et al., 2016; Lodge-Ivey et al., 2006). Another study reported ergonovine levels in tall fescue between 500 and 200 μg kg^{-1}, with levels of ergonovine and other ergot alkaloids measured exhibiting temperature-dependent fluctuations (Salminen et al., 2005).

Ergot alkaloid levels in tall fescue are influenced by environmental factors, such as nitrogen fertilization, precipitation, and ambient temperature. Rogers et al. (2011) found that, in plots of Kentucky 31+ tall fescue located in Missouri, Georgia, and South Carolina, ergovaline concentrations were lowest during the spring (~0 – 200 μg kg^{-1} dry matter [DM]), slightly increased in the summer (~300 - 400, μg kg^{-1} DM), and then spiked in the fall (~500 – 1000 μg kg^{-1} DM). Total ergot alkaloid concentrations, however, exhibited a different pattern. In Georgia, total ergot alkaloid levels were around 1400 μg kg^{-1} DM in April, before a drop below 500 μg kg^{-1} DM in June through August, and a subsequent return to greater than 1000 μg kg^{-1} DM in September and October. The location in South Carolina followed a similar trend, whereas the plots in Missouri had 500 μg kg^{-1} DM in April, which increased to around 1500 μg kg^{-1} DM in May through June, and dropped to 600 μg kg^{-1} DM in August before returning to a level around 2000 μg kg^{-1} DM for the duration of the study. In support, elevated temperatures were associated with increased ergot alkaloid concentrations in a mixed species community of plants that included tall fescue with a 40% infection rate but not with endophyte infection incidence (McCulley et al., 2014). These data indicate the kinetics of ergot alkaloid production, but not necessary the viability of the endophyte, are susceptible to environmental influences.

Ergot alkaloid metabolism in ruminants

The pharmacokinetics of bromocryptine, a synthetic ergot alkaloid, have been extensively studied, but the toxicokinetics of the natural alkaloids of tall fescue are not well characterized. Evidence suggests intense first-pass metabolism of bromocryptine, but the extent of this for the naturally occurring alkaloids is unknown (Berde and Stürmer, 1978). The absorption, distribution, metabolism, and excretion properties of ergot alkaloids in ruminants (e.g., cattle) have been studied to an extent. Ergot alkaloids and/or their derivatives (i.e., lysergic acid) have been detected in the serum (Moubarak et al., 1996), urine (Hill et al., 2000a; Stuedemann et al., 1998), bile (Stuedemann et al., 1998), fluids of the rumen and abomasum (Hill et al., 2001), subcutaneous fat (Realini et al., 2005), and liver and kidney tissues (Zbib et al., 2015). The amount of ergot alkaloids detected decreases through sequential sampling along the ruminant's GI tract, with 50 – 60% being recovered from abomasal contents and only 5% in the feces after a bolus challenge (Westendorf et al., 1993). Further, as much as 93 - 96% of ergot alkaloids (i.e., total ergot alkaloids not speciated alkaloids) are reportedly excreted in the urine, when targeting the lysergic acid ring for quantification (Stuedemann et al., 1998). *In vivo* analysis revealed lysergic acid and lysergol were actively transported across ruminal tissues, with ergonovine, ergotamine, and ergocryptine transported across omasal tissue to a minimal extent in comparison (Hill et al., 2001). One subsequent study reported that lysergic acid was the only alkaloid transported across ruminal and omasal tissues in parabiotic chambers, with subsequent *in vivo* analysis showing only lysergic acid, not ergovaline, was found in rumen fluid and urine indicating lysergic acid may play a role in FT etiology (Ayers et al., 2009).

The cytochrome P450 3A family is important in the detoxification of ergot alkaloids, as shown with rodent liver microsomes (Moubarak and Rosenkrans, 2000). However a recent study

suggests that ergot alkaloids (i.e., bromocryptine, dihydroergotamine, and ergonovine) are also capable of inhibiting CYP450 enzymes, potentially contributing to their hepatotoxicity (Morse et al., 2016). Overall, these data indicate that ergot alkaloids undergo complex metabolism that is not yet fully characterized, especially in field studies.

Adrenergic, dopaminergic, and serotonergic effects of ergot alkaloids

The pharmacophore of ergot alkaloids (i.e., the lysergic acid ring) shares structural similarities with dopamine (DA), norepinephrine (NE), and serotonin (5HT). This similarity allows ergot alkaloids to interact with monoamine receptors systemically, eliciting serotonergic, dopaminergic, and adrenergic activities that can influence numerous important physiological functions (Berde and Stürmer, 1978).

Ergot alkaloid's dopaminergic activity was demonstrated when their agonist-like interactions with the D_2 receptors on anterior pituitary lactotrophs, which inhibit the synthesis and release of prolactin, were reported (Larson et al., 1995; Larson et al., 1994). Supporting evidence showed that metoclopramide, a D_2 receptor antagonist, administration to toxic tall fescue grazing steers increases circulating prolactin levels, time spent grazing, and average daily weight gains (ADG), with the latter two potentially being attributed to activities on D_2 receptors in the gut (Lipham et al., 1989). Metoclopramide, has been used in human medicine to increase the rate of gastric emptying (DiPalma, 1990), but the interactions of ergot alkaloids with gastrointestinal dopamine receptors and their effects on gastric emptying have not been directly studied to date. Nonetheless, peripherally, dopamine is produced by the gastrointestinal tract, spleen, and pancreas; it is estimated that around 46% of the total body dopamine is not converted to norepinephrine, indicating a large amount of non-neuronal dopamine is represented in the dopaminergic paracrine system in the mucosa of the gastrointestinal tract (Tonini et al., 2004)

and elsewhere in the periphery. Further, D_2 receptors are located both pre- and post-junctionally and exert a negative modulatory effect on the release of acetylcholine from intrinsic cholinergic nerve terminals (Tonini et al., 2004). Domperidone, a peripheral D_2 antagonist, and metoclopramide have both been identified as having prokinetic activities in the gut through enhancement of acetylcholine release from the intrinsic cholinergic motor neurons. The potential effects of ergovaline and ergotamine on enteric D_2 receptors, and subsequently alterations in cholinergic activity, is supported by one study that reported partial antagonism of the effects of ergopeptines on rumen motility after administration of the muscarinic cholinergic receptor antagonist atropine (Poole et al., 2009). The potential dopaminergic action of ergot alkaloids in the gut warrants further, detailed investigation. This was previously suggested (Oliver, 1997) as metoclopramide and domperidone restore certain FT gut-related pathophysiological signs, indicating a potential role for interactions between peripheral monoaminergic receptors and ergot alkaloids. For example, if ergot alkaloids interact with D_2 receptors in the gut, this interaction would inhibit acetylcholine release blocking cholinergic smooth muscle stimulation, which could be one mechanism through which ergot alkaloids influence feeding behavior (i.e., altering gut motility). This is notably highlighted because metoclopramide is a dopamine D_2 receptor and serotonin $5HT_3$ receptor antagonist with an agonist behavior on the $5HT_4$ subtype (Freeman et al., 1992). So, metoclopramide's inhibition of ergot alkaloid binding to enteric D_2 receptors and concomitant $5HT_3$ antagonism and $5HT_4$ agonism would stop dopamine-induced suppression of acetylcholine release in the gut while further blocking other factors related to gut motility (e.g., vasoactive intestinal peptide). This could be one potential mechanism through which ergot alkaloids influence gut motility and downstream feeding behavior.

Ergot alkaloids also have extensive serotonergic and adrenergic activities through interactions with multiple serotonin (5-hydroxytryptamine; 5HT) receptor subtypes and α1- and α2-adrenergic receptors (Berde and Stürmer, 1978; Klotz et al., 2016; Klotz et al., 2012). The vasoactivity of ergot alkaloids has been attributed to their serotonergic and adrenergic activities within the vascular smooth muscle. Contractility bioassays initially identified the presence of the $5HT_{2A}$ receptor in the bovine lateral saphenous vein and was shown to play a role in ergot alkaloid-induced vasoconstriction (Klotz et al., 2007). Subsequent work showed that dietary exposure to ergot alkaloids decreased the contractility of the mesenteric vascular bed, and noted that reduced blood flow in the mesenteric vasculature may contribute to decreased nutrient absorption for cattle grazing toxic tall fescue (Egert et al., 2014a). Pharmacological assessment of 5HT receptor subtypes within the bovine midgut found that agonists of the $5HT_{1B}$ and $5HT_{2B}$ are vasorelaxants in the mesenteric veins, while all four blood vessel types sampled had vasoconstrictive responses to $5HT_{2A}$ receptors agonists (Snider et al., 2018). Recovery from changes in vasoactivity and vasoconstriction of steers that consumed E+ fescue chronically is possible, but it takes 35 – 63 days of ergot alkaloid free diet (Klotz et al., 2016; Klotz et al., 2012). That said, Cowan et al. (2019) recently found that the acute pharmacological effects in the vasculature are reversible, indicating a complex relationship between the E+ ergot alkaloids and the bovine vasculature. In the gut, interactions between ergot alkaloids and enteric serotonin receptor subtypes are highlighted because, although metoclopramide is a dopamine D_2 receptor, it also is a $5HT_3$ receptor antagonist with agonist behavior on the $5HT_4$ subtype (Freeman et al., 1992). The ability of ergot alkaloids to interact with multiple receptors systemically, and the effects these interactions may have on physiological homeostasis, may also be modulated by

ergot alkaloid interactions with the trace amine associated receptor 1 (TAAR1; Bunzow et al., 2001; Lindemann and Hoener, 2005).

Trace amines are a group of endogenous amines (e.g., spermine, phenethylamine, tyramine, *p*-octopamine, etc.) derived from decarboxylation of the parent amino acid by aromatic L-amino acid decarboxylase (Berry, 2004) and are monoamine modulators (Miller, 2011). TAARs and their mRNA are ubiquitous and are notably found in the cardiovascular system, CNS, and GI tract (Sotnikova et al., 2009). Functionally, TAAR1 and dopamine D2 receptors form heterodimers, and data suggest TAAR1 can potentially modulate dopaminergic signaling (Espinoza et al., 2011). Further, TAAR1 activation has vascular effects in the mesenteric bed and the periphery, and lysergic acid, the pharmacophore and main ergot alkaloids metabolite, activates TAAR1 and dose-dependently decreases dopamine firing in the ventral tegmental area of adult rats (Bunzow et al., 2001; De Gregorio et al., 2016; Lindemann and Hoener, 2005). To take this a step further, TAAR1 receptors had genes highly enriched in somatostatin producing enteric D cells in a transgenic mouse model (Adriaenssens et al., 2015) and TAAR1 activation has been shown to have incretin-like effects in a human cell line, including increased production of glucagon-like peptide 1(GLP-1) and peptide tyrosine tyrosine (peptide YY; PYY). So, considering the lysergic acid backbone of all ergot alkaloids, it is plausible that lysergic acid, whether or not its originating source was the lysergic acid amides or ergopeptines, could interact with TAAR1 and perturb homeostatic monoaminergic and hormonal signaling in both the CNS and periphery.

Overall, it is likely a complex relationship exists between ergot alkaloids, multiple monoamine receptors, and downstream physiological processes. Recent evidence suggests indirect and/or downstream effects of the ergot alkaloid-monoamine receptors interaction may

also contribute to fescue toxicosis pathophysiology. To begin to understand how even small local perturbations can have large-scale systemic consequences, we propose evaluating the monoamine-ergot alkaloid receptor relationship under the purview of downstream effects.

Biomarkers of ergot alkaloid exposure and effect

Biomarkers are reproducible, quantifiable biological constructs that result from biological activities and can be used to identify different medical or disease states (Strimbu and Tavel, 2010). Biomarkers need to have high reproducibility across studies or trials while being able to encapsulate the entirety of what it is they are being used for. Generally speaking, there are two types of common biomarkers: 1) biomarkers of exposure and 2) biomarkers of effect (Mayeux, 2004). Biomarkers of exposure are biological indicators that exposure to a particular substance or compound of interest has occurred, whereas biomarkers of effect indicate that, generally, a pathological effect or disease state is present within a given system. While many biomarkers for fescue toxicosis have been proposed, evidence suggests most of the proposed biomarkers are biomarkers of exposure and are not of effect. For example, it has previously been shown that if ergot alkaloids cross gastric tissues into systemic circulation, they can interact with dopamine receptor subtype 2 (DRD2) receptors on anterior pituitary lactotrophs to decrease serum prolactin (Hurley et al., 1980b). Decreases in serum prolactin have been proposed as a biomarker for ergot alkaloid exposure, but prolactin levels are known to be influenced by photoperiod (Karg and Schams, 1974), acute ambient temperature changes (Smith et al., 1977), and different forms of stress (Bryant et al., 1970; Yayou et al., 2010), which could all lead to reproducibility problems. Detection of total ergot alkaloids in the urine is an accurate, less variable method to assess ergot alkaloid exposure when compared to previous methods, such as decreased serum prolactin (Hill et al., 2000b; Stuedemann et al., 1998). This is because ninety-four percent of ergot alkaloid

excretion occurs in the urine, appearing as early as 12 hours post-exposure, and concentrations are exposure level- and duration-dependent (Hill et al., 2000b). While urinary alkaloids are of great utility as a sensitive and reproducible biomarker of ergot alkaloid exposure and correlate with reduction in average daily weight gains (ADG; Hill et al., 2000b), this approach does not speciate individual ergot alkaloids or their metabolites. Speciating ergot alkaloids and metabolites, in plasma, urine, or rumen fluid, might help identify how ruminants metabolize ergot alkaloids and other metabolic processes that may be affected (Hill et al., 2000b; Hill et al., 2001), which is where technological advances, i.e., multi-'omics, will be beneficial.

Adverse effects of toxic tall fescue grazing and ergot alkaloids in livestock

Decreased feed intake and weight gains

Despite notable stand persistence, which sparked initial interest in tall fescue as a pasture cultivar, reports regarding the detrimental impact of tall fescue grazing in livestock began as early as the 1940's (Cunningham, 1948; Cunningham, 1949; Jacobson et al., 1963). While cattle grazing tall fescue exhibit numerous signs, such as fescue foot, thermoregulatory impairment, and decreased feed intake, the most economically costly are lowered weight gains and reproductive insufficiencies (Paterson et al., 1995).

Decreased muscle accretion and weight gains are common findings in steers grazing E+ fescue. This is a major concern in the beef industry, as these effects can go unnoticed despite their financial impacts (Paterson et al., 1995). Weight gains are decreased, in part, by lower feed intake and shortened grazing periods; these signs are exacerbated under hot and humid environmental conditions (i.e., heat stress; Paterson et al., 1995). The molecular mechanism(s) that lead to decreased feed intake are currently unknown but subject to intense investigation. Considering the dopaminergic and serotonergic activities of ergot alkaloids, it is plausible that

one mechanism could be through alterations of gut motility. Strickland et al. (1993) suggested that interaction between ergopeptine alkaloids and enteric receptors could potentially influence gut motility and feed intake. Though the authors noted the interactions between ergot alkaloids and enteric DRD2 receptors, it is now known possible enteric serotonergic activities of ergot alkaloids are another potential source of altered gut motility (Snider et al., 2018; Trotta et al., 2018). In support, increases in the baseline tonus of the rumen/reticulum and increased amplitude of reticular contractions follow intravenous infusion of ergovaline, the major ergopeptine in tall fescue (McLeay and Smith, 2006). Of note, increased rumen fill that could not be explained by increased dry matter intake, indicate ruminal passage rates may be decreased (Foote et al., 2013; Koontz et al., 2012; Koontz et al., 2013; Koontz et al., 2015) with no changes in energy balance (e.g., energy intake, O_2 consumption, CO_2 production, heat production, etc.) or feed digestibility (Koontz et al., 2015). Moreover, voluntary dry matter intake can be inhibited by increases in rumen fill and restricted flow of digesta to the lower gastrointestinal tract (Allen, 1996), indicating that, even in the absence of E+ effects on digestibility and basal metabolic rates, changes in gastrointestinal physiology post ergot alkaloid exposure, among other specific metabolic changes (i.e., certain metabolic pathways), could contribute to decreased muscle accretion/weight gains associated with fescue toxicosis through molecular changes. Notably, recent work showed ergot alkaloids are heavily degraded in the rumen (Ayers et al., 2009), indicating that other indirect effects of ergot alkaloids and/or their metabolites may also play a role in fescue toxicosis development.

Toxic tall fescue influences livestock thermoregulatory capacity

Evaporation, convection, conduction, and radiation are the four major heat loss pathways in cattle. Evaporative heat loss occurs through cutaneous or respiratory evaporation in hot and

humid environmental conditions, leading to passive diffusion of heat (Leonard, 1986). Generally, higher temperatures cause blood temperature to rise, resulting in evaporative heat loss through exhalation or sweat. Approximately 1.7 mL of sweat results in a loss of 1 kcal of heat, which is why, under harsh environmental conditions, evaporation is an efficient mechanism for thermoregulation (Buono and Sjoholm, 1988).

Convection, the transfer of heat to surrounding air or water, is most efficient under conditions where temperatures of the medium to which heat is being passed, are lower than that of the body. Therefore, convective heat loss and ambient temperatures have an inverse relationship, so as ambient temperatures rise above that of the skin, animals will absorb heat from their environment (Maia et al., 2005). Factors contributing to body heat loss by convection is the medium density, velocity (e.g., wind speed), and thermal gradient (Bligh and Johnson, 1973).

The transfer of heat between the animal and an inanimate object (i.e., cooling beds) is conduction. Conduction efficiency is mediated by thermal gradient, conductance capacity, and surface area contact (Schmidt-Nielsen and Schmidt-Nielsen, 1952).

Radiation is the final path for heat loss in cattle. Any object above absolute zero releases infrared energy (i.e., heat) and the transfer of heat flows from a higher to a lower temperature through radiant exchange (McCafferty et al., 2018). In cattle, coat or skin colors influence radiant heat exchange, i.e., the darker the coat, the more radiant heat is absorbed (Hutchinson and Brown, 1969). In this regard, it is important to note that Black Angus is a major breed of beef cattle that dominates the grazing herds of the Fescue Belt, either as purebred or, more commonly, as crossbreds where Angus breed features are heavily present. In the USA as a whole, at least 60% of the beef herd has some Angus influence in its genetics (Drouillard, 2018).

The effects of fescue toxicosis on thermoregulation are, in part, caused by ergot alkaloid interactions with vasoactive monoamine receptors. Ergot alkaloid binding to serotonergic and adrenergic receptors leads to vasoconstriction in the periphery and the pulmonary vasculature (Oliver, 2005; Osborn et al., 1992). Coupling vasoconstriction with decreased nitric oxide, a vasodilator, levels, as demonstrated by decreased arginine and nitric oxide synthesis (Al-Tamimi et al., 2007; Oliver et al., 2001a; Oliver et al., 2001b), results in decreased heat loss by lowering heat transfer to the skin or respiratory tract that can subsequently be transferred through convection or evaporation. Overall, the influences of toxic tall fescue on heat loss mechanisms (i.e., impaired thermoregulation) results in increased core body temperature in animals exposed to harsh environmental conditions and E+ fescue (Hemken et al., 1981; Settivari et al., 2006; Spiers et al., 2005). In support, ergot alkaloids/E+ fescue consumption has been shown in numerous beef studies to increase respiration rates and rectal temperatures. Notably, these responses are exaggerated under heat stress conditions, with increased respiration rates reflecting one mechanism where animals on E+ fescue attempt to increase the amount of heat loss when faced with increased core body temperatures (Al-Tamimi et al., 2007; Spiers et al., 2012b). Finally, decreases in infrared-measured surface temperatures in the periphery, under thermoneutral conditions, have also been associated with fescue toxicosis (Osborn et al., 1992; Spiers et al., 2005).

Endocrine and neuroendocrine system

The growth hormone-axis is an important physiological axis of hormones consisting of growth hormone releasing hormone (GHRH), growth hormone (GH), and insulin-like growth factor 1 (IGF-1). Alterations of the growth hormone-axis can lead to muscle hypertrophy and atrophy by influencing catabolism or anabolism through IGF-1/Akt/mammalian target of

rapamycin (mTOR) and IGF-1/Akt/Forkhead box O (FOXO) pathways, respectively (Latres et al., 2005; Schiaffino and Mammucari, 2011; Stitt et al., 2004). GH induces generation of IGF-1, IGF binding protein 3 (IGFBP-3), and the acid labile subunit (ALS), which together bind about 80% of circulating IGF-1 in a ternary complex (Laron, 2001). In the bovine, GH secretion is pulsatile, but whether pulsatility is irregular (Lee et al., 1991; Plouzek and Trenkle, 1991) or regular (Breier et al., 1986; Kasuya et al., 2012) is up for debate. Nonetheless, the regulation of GH secretion in cattle seems to be influenced by feeding regimen, age, and environmental factors (e.g., lighting regimen; Breier et al., 1986; Kasuya et al., 2008; Plouzek and Trenkle, 1991). The upstream regulators somatostatin and GHRH also influence GH pulsatility (Kasuya, 2016).

Igono et al. (1988) has shown decreased GH concentrations under hot/humid environmental conditions (temperature humidity index; THI $\geq$ 70). Decreased GH leads to lowered calorigenesis and heat production which may play a role in regulation of internal temperature homeostasis and thermal equilibrium in high environmental temperatures (Aggarwal and Upadhyay, 2013). In this regard, it has been suggested GH may be involved in heat production via stimulation of thyroid activity; however, thyroxine (T4) disappearance has shown an inverse relationship with environmental temperatures (Johnson and Yousef, 1966). E+ fescue grazing consumption has no effect on either triiodothyronine (T3) or T4 levels in ruminants, but T3 levels in Holstein calves are inversely related to environmental temperatures (Hurley et al., 1980a). Finally, in yearling Angus steers, Thompson et al. (1987) reported that grazing high toxic endophyte-infected (~44%) versus low-endophyte (~16%) infected tall fescue significantly increased GH levels (mean levels of 7.9 and 6.2 ng/mL, respectively). The effects of E+ on GH are not uniform as, for example, no significant effect on GH secretion were reported in another grazing study (Lipham et al., 1989). Levels of ergot alkaloids and/or environmental conditions

might concomitantly modulate the effects on the growth axis as, in a controlled study, GH levels increase within 30 minutes after intravenous (i.v.) injections of ergotamine tartrate (ET) and ergonovine maleate (EM), with i.v. ET and EM levels calibrated to consumed ergovaline (EV) levels in grazing cattle (i.e., cattle consuming 2-3% BW; EV levels at 0.1-6 μg/g), and return to pretreatment levels 60 minutes and 75 minutes for EM and ET, respectively (Browning and Leite-Browning, 1997).

In Angus steers, Wu et al. (2010) reported that nutritional status plays an important role in serum IGF-1 response to GH stimulation, with increased nutritional intake stimulating increased serum IGF-1 concentrations (basal levels around 100 ng/mL prior to GH injection), increased IGF-1 mRNA expression and translational efficiency, and the same responses for both IGFBP-3 and ALS. The effects of nutritional status may be important considerations as consuming E+ fescue decreases food intake, especially under harsh environmental conditions (Spiers et al., 2012a).

Plasma IGF-1 concentrations decrease in summer months (Somal and Aggarwal, 2014) and with negative energy balance (Bousqet et al., 2004). Reduced serum IGF-1 concentrations in cattle were reported (Browning, 2003) and heifers consuming E+ tall fescue seed over the course of 100 days had reduced serum IGF-1 concentrations without an influence on serum IGF-2 (Rorie et al., 1998). Basal levels of GH were slightly lower (<1ng/mL) in E+ grazing steers, in comparison with endophyte-free grazing steers, but basal serum IGF-1 levels were significantly lower, with this difference persisting up to 24 hours after lipopolysaccharide (LPS) challenge (Filipov et al., 1999). Further, strong positive correlations between serum IGF-1 and both weaning and post-grazing weights, alongside a weaker correlation between IGF-1 and average daily gains were reported for steers grazing E+ and non-toxic tall fescue varieties for 105 days

(Rosenkrans and Ezell, 2015); however, raw IGF-1 values were collected only at weaning, not after the grazing period (Rosenkrans and Ezell, 2015). Finally, bolus injection with ergotamine decreases plasma insulin concentration, while increasing plasma glucagon concentrations within 1 hour post-injection (Browning et al., 2000). Ergot alkaloids effect on plasma cortisol levels are variable, with some studies showing increased levels and others showing decreased levels (Coufal-Majewski et al., 2016; Zbib et al., 2015).

As already reviewed in the biomarkers section, one frequently reported effect of toxic fescue grazing is decreased circulating prolactin. Steers grazing high endophyte versus low endophyte tall fescue exhibited markedly different profiles of their pituitary genomes (Li et al., 2017). Among the major differences, marked decreases in the D_2 receptor and prolactin transcripts, as well as decreases in the galanin, vasoactive intestinal peptide, and proopiomelanocortin and proprotein convertase subtilisin/kexin type 1transcripts in the high endophyte grazing steers were reported (Li et al., 2016; Li et al., 2017). These data indicate E+ tall fescue grazing can alter expression of important prolactin and adrenocorticotropic hormone-regulating genes (Alexander and Sander, 1994; Cimini, 1996), which warrant further investigation. However, the robustness of the relationship between decreased circulating prolactin and the pathogenesis of fescue toxicosis is currently unclear (Diaz et al., 2018; Egert et al., 2014b; Thompson and Stuedemann, 1993).

A comprehensive study profiled the effects of E+ fescue on dopamine, serotonin, and their metabolites in the anterior pituitary, hypothalamus, and pineal gland (Porter et al., 1990). Increased 3,4-dihydroxyphenylacetic acid (DOPAC), a dopamine metabolite, and 5-hydroxyindoleacetic acid (5-HIAA), a serotonin metabolite, in the pituitary. Alongside increased pineal 5-hydroxytryptophan (5HTP), a precursor for 5HT, and decreased 5-methoxyindoleacetic

acid, a 5-HIAA metabolite, were major consequences of E+ grazing. In a subsequent study, the same group also found lowered plasma melatonin levels in high endophyte-infected tall fescue grazing cattle, when compared to low endophyte-infected tall fescue (Porter et al., 1993). Finally, cattle consuming E+ fescue have large variations in circulating norepinephrine and epinephrine levels, which may associated with behavioral alterations, i.e., increased nervousness and excitability (Patterson et al., 2011; Schmidt et al., 1982).

Overall, E+ fescue and ergot alkaloids have multiple influences on the (neuro)endocrine system, with direct and indirect effects on both circulating hormones and neurotransmitter metabolism in the hypothalamus, pituitary gland, and pineal gland.

Blood and circulating metabolites effects

For a comprehensive review on the overarching physiological changes associated with fescue toxicosis, the author would direct readers to Strickland et al. (2009). Briefly, most cellular components of the blood are not affected by toxic tall fescue grazing, but one study has found decreased erythrocyte size and hemoglobin content (Oliver et al., 2000). One consistent finding in studies analyzing the effects of toxic tall fescue on blood parameters is decreased circulating cholesterol and triglyceride levels (Oliver et al., 2000; Zbib et al., 2015). Interestingly, bolus injection with ergotamine decreases plasma insulin, while increasing plasma glucagon concentrations within 1 hour post-injection (Browning et al., 2000). The impact of ergot alkaloids on plasma cortisol, another metabolism regulating hormone, levels are variable (Coufal-Majewski et al., 2016; Zbib et al., 2015). Tall fescue affects circulating metabolites and hormones and both intra- and extra-hepatic enzyme activities (Zbib et al., 2015), indicating global metabolic effects. Jackson et al. (2015) analyzed specific blood analytes in steers that grazed either high or low endophyte-infected tall fescue pastures and found that some parameters

were altered initially (e.g., reduced triglycerides and increased bilirubin), some had persistent alterations (e.g., reduced cholesterol), and others were only altered after chronic exposure (e.g., creatinine kinase).

Immune system effects

Reports on the responses of the immune system to E+ fescue consumption and/or ergot alkaloid treatment are mixed, but, generally, indicate overactivation of the innate immune system and some deficits in adaptive (humoral) immunity. Among these effects in cattle are decreases in genes related to T-lymphocyte lineage, IgG levels, mononuclear phagocytic activity, leukocyte and eosinophil counts, and the lymphocyte to neutrophil ratio (Dew et al., 1990; Filipov et al., 1999; Saker et al., 1998; Saker et al., 2001; Settivari et al., 2006). In support, rats exhibited an increase in T-suppressor cell percentage (Dew et al., 1990) and cattle have increased clotting and acute phase protein release (Oliver et al., 2000) when fed ergot alkaloids.

Pasture studies have found that copper deficiencies in E+ grazing cattle may contribute to reduced serum expression of the major histocompatibility complex II (MHC II), as copper supplementation restores MHC II expression levels (Saker et al., 1998). In response to the endotoxin, steers on E+ fescue had greater serum TNF-α and cortisol levels than steers on endophyte-free tall fescue (Filipov et al., 1999). Further, chronic ergot alkaloid exposure via E+ was needed for this increase in inflammation and hypothalamus-pituitary-adrenal (axis) alterations, as acute pretreatment with ergotamine prior to LPS challenge decreased the response to LPS (Filipov et al., 2000). In response to concanavalin and sheep red blood cell challenge, steers fed an ergot alkaloid-containing diet had normal or increased humoral responses (Rice et al., 1997). Dew et al. (1990) showed a 42% increase in T suppressor cells in mice fed a toxic tall fescue diet; however, this study found a decreased humoral immune response after

immunological challenge. Overall, immune system effects of tall fescue or ergot alkaloids appear to be context-, i.e., environmental conditions, additional challenges, dependent.

The microbiome

The impact of E+ fescue on the immune system, (neuro)endocrine functions, and metabolic homeostasis indicate that fescue toxicosis may, in part, be mediated by microbial dysbiosis. This is because ruminant microbiota is crucial for forage digestion and also influences nutrient utilization, metabolic homeostasis, immune and endocrine responses, as well as cellular signaling within the host (Hooper et al., 2012; Levy et al., 2015; Nicholson et al., 2012; Round and Mazmanian, 2009; Sonnenburg and Backhed, 2016; Tremaroli and Backhed, 2012; Zeevi et al., 2016). Next is a review of the microbiome and how it might play a role in fescue toxicosis.

Scientific advances allowing the study of the microbiome

The development of the Human Genome Project spurred advances in next-generation sequencing (NGS) technologies. Where capillary (i.e., Sanger) sequencing had the ability to sequence small sections of genomes, newly developed sequencing platforms have allowed for detailed analysis of large genomic datasets (Buermans and den Dunnen, 2014). Subsequent development of sequencing platforms markedly increased the impact NGS-based investigations on areas ranging from cancer biology to the microbiome in both humans and animals (Buermans and den Dunnen, 2014; Serrati et al., 2016).

Interrogation of the relationship between microbiota homeostasis and beef cattle productivity is not a new idea, as studies attempting to understand the impact of specific bacteria on feed efficiency and animal health were initiated in the 1950's (Hungate et al., 1952). Recent molecular biology developments have broadened our understanding of the complexity of both the rumen and lower GI microbiota (McCann et al., 2014). The inability to culture many of the

bacteria residing along the GI tract accelerated adoption of culture-independent techniques, such as 16S or 18S ribosomal sequencing, for ecological studies of rumen bacterial communities. Further, the utility of using the 16S-23S internal transcribed spacer (ITS) region as a highly variable region for the identification of resident fungi allows reliable profiling of important rumen fungal communities (Schoch et al., 2012). The 16S or ITS regions are used for identification of bacterial and fungal species, respectively, because these regions are variable enough to allow establishment phylogenetic dispersion between species, while having conserved portions that allow proper primer design and identification (Marchesi et al., 1998; Schoch et al., 2012). In addition, the ubiquitous nature of these ribosomal sequences allows researchers the ability to profile the microbiota without having to utilize multiple primers for PCR amplification of DNA sequences (Woese, 1987; Woese and Fox, 1977; Zuckerkandl and Pauling, 1965).

The plant microbiota

The plant microbiota are complex, and consist of microbiota associated with the rhizosphere (root), phyllosphere (the above-ground plant), and the endosphere (all endophytes of the plant), which all support plant development and have complex plant-microbe interactions (Brader et al., 2017; Hardoim et al., 2015; Perez-Jaramillo et al., 2018; Vorholt, 2012). The root microbiota are dynamic, and are generally horizontally transmitted from the soil; however, recent evidence also suggests that bacteria and endophytes can be vertically transmitted through seed heads (Hardoim et al., 2012; Liu et al., 2012), as is the case with *E. coenophiala* (Schardl et al., 2013). So, the development and structure of the rhizosphere microbiota is likely influenced by both plant and environmental factors.

Phyllosphere microbiota is influenced by many factors as well, one being the endosphere. However, the phyllosphere microbiota respond to the soil environment and multiple plant and

environmental factors (Vorholt, 2012; Wallace et al., 2018; Zarraonaindia et al., 2015). Soil factors that influence the plant microbiota, namely the rhizosphere, include salinity, pH, type, moisture, structure and organic matter/plant exudates of the soil (Fierer, 2017). Climate, parasitic, and other external environmental factors are among the factors influencing the phyllosphere (Hardoim et al., 2015). Similar to the animal, the plant microbiota has both core and satellite microbial communities, with the core serving as the main structure and the satellite (i.e., low abundant microbes) serving to prevent unwanted microbial invasions (Mallon et al., 2015).

Plant microbiome contributes to plant physiological homeostasis. For example, some plant microbiota promote plant growth by producing phytohormones that modulate levels of endogenous, growth-related hormones or by secreting enzymes (i.e., 1-aminocyclopropane-1-carboxylate [ACC] deaminase) that can reduce plant stress levels (Compant et al., 2019). Plant microbiota can also protect against certain plant pathogens (Berg and Koskella, 2018; Hopkins et al., 2017). That said, not all plant microbes are beneficial and some can cause disease states by production of metabolites that are detrimental to the host (Compant et al., 2019).

Effects of dietary components (e.g., plants and their microbiota/metabolites) on livestock microbiota have been the subject of recent investigation (Jiao et al., 2017; Zeineldin et al., 2018), i.e., understanding how diet influences animal productivity. The coevolution of the microbiota/metabolome between plant and grazing animals, although of critical importance, has not been investigated in detail. Although not in agricultural animals, a bi-directional relationship between ecosystem (e.g., plants) and humans has been indirectly suggested (Flandroy et al., 2018) such that the plants provide nutrition to the human and, in turn, some of the nutrients excreted by humans can be utilized by the plants. The idea here is similar, in that the relationship

between the plant-endophyte-microbiota influences the grazing animal microbiota/physiology, and host changes derived from plant/endophyte exposure influence what the plant itself is being exposed to, allowing for potentially advantageous growing conditions.

Overall, the plant microbiota is diverse, has significant impact on plant viability, and understanding and/or manipulating it can lead to plant-derived solutions for diseases like fescue toxicosis.

The rumen microbiota: development, composition and metabolic functions

Rumen development

At birth, the rumen is undeveloped and ruminant animals' function as monogastrics until it develops. In the young ruminant, milk-based diets are digested in the abomasum. Rumen development is a physiological challenge and results in a drastic shift in the nutrients that are delivered to the intestines, liver, and peripheral tissues (Jiao et al., 2015; Li et al., 2012). Microbial colonization is the final step in rumen development, and ingested microbes colonize and become established in the young rumen by 1-3 months of age (Li et al., 2012). However, reports suggest that many strict anaerobes, which are predominant in the mature rumen, are present as early as 1-2 days after birth (Fonty et al., 1987; Morvan et al., 1994). Further, most types of ruminal bacteria are present in 14 day old calves (Li et al., 2012). Ciliate protozoa are passed from animal to animal and can be found within 14 days of birth, unless calves are isolated from other animals (Becker and Hsiung, 1929; Eadie, 1962). In calves, methanogenic archaea were detected at the day of birth and/or reported as present 2-4 days after birth (Fonty et al., 1987; Guzman et al., 2015; Morvan et al., 1994). The establishment of anaerobic fungi is more complex, as they have been reported 8-10 days after birth in lambs, before becoming largely undetectable, only to reappear in adulthood (Fonty et al., 1987; Stewart et al., 1988). Further,

fungi that are characteristic of the mature rumen were also found before feeding of forage-based diets (Orpin and Joblin, 1997).

The rumen's complexity, and its primary role in forage digestion, makes it an ideal habitat for a complex microbiota. The microbiota includes a wide range of bacteria, fungi, and archaea. Each set of microorganisms serves specific function(s) in the process of forage breakdown and utilization (Hungate, 1966a, b, c).

Ruminal bacteria

Bacterial profile in the rumen is diverse, which is due, in part, to the complexity of ruminant feedstuffs and dietary selection pressures. Bacteria make up the largest portion of ruminal microorganisms, with a cellular density up to 10^{10} bacterial cells per gram of rumen content and a bacterial biomass of 14-18 mg dry weight per mL (Russell, 2002). Of these bacteria, about 75% are bound to feed particles and the other 25% are free-floating bacteria (Russell, 2002). Although the bacteria of the rumen are anaerobic, some bacteria are facultative anaerobes and consume oxygen present from the feeding process (Stewart et al., 1988).

The complexity of breaking down feedstuffs calls for an equally complex rumen microbial population. The predominant ruminal bacteria include: *Ruminococcaceae*, *Fibrobacter succinogenes*, *Butyrivibrio* sp., *Prevotella* sp., *Selenomonas ruminantium*, *Streptococcus bovis*, *Megasphaera elsenii*, *Ruminobacter amylophilus*, *Anaerovibrio lipolytica*, *Succinimonas amylolytica*, *Succinivibrio dextrinosolvens*, obligate amino acid fermenting bacteria (*Peptostreptococcus anaerobius*, *Clostridium sicklandii*, etc.), and *Spirochetes* (Hungate, 1966b; Russell, 2002). Each of these bacteria are important, as they have unique biochemical properties that allow for proper digestion of diverse feed components. For example, some bacteria utilize cellulose and hemicellulose, while others can utilize starches, sugars, and branched chain volatile

fatty acids (VFAs), resulting in the production of a variety of VFAs (Hungate, 1966b; Russell, 2002). The metabolic profile of each bacterium is essentially unique. For example, some produce all VFAs (acetate, butyrate, propionate, etc.) or a combination of VFAs, while others produce branched chain VFAs, ethanol, and methane (Hungate, 1966b; Russell, 2002).

The metabolic profile of the rumen is important. For instance, VFAs are readily absorbed across the rumen epithelium. Further, VFA ratios can influence downstream physiological functioning. Finally, the bacterial profile of the rumen and the end metabolic products are being investigated for their potential to monitor and improve ruminant feed efficiency. Thus, bacterial shifts associated with ruminant nutrient utilization and energy production are lowered microbial richness and diversity, which correlate with increased ruminal short chain fatty acid (e.g., propionate and butyrate) production and decreased methanogenesis (Shabat et al., 2016).

Ruminal fungi

The fungal profile of the rumen is less well defined. This is because of the difficulty in quantification due to the complex fungal lifecycle (i.e., transition between zoospore, mycelium, rhizoids, and sporangium); however, estimates have placed the fungal content to be, at most, 8% of the total rumen biomass when using chitin, an important fungal cell wall component, as the method of identification (Russell, 2002). In the rumen, fungi digest cellulose and mycelium penetration of feed particles breaks the fibers apart, allowing for increased surface area and better degradation. The ability of fungi to breakdown fibrous particles of feedstuffs (i.e., tall fescue), while nutritionally beneficial, could also increase release of endophyte-derived, plant-associated metabolites (i.e., ergot alkaloids). Interestingly, there is also an inverse relationship between ruminal fungi and ruminal bacteria, likely because a bacteriocin-like substance, a fungal inhibitor, is produced by certain rumen bacteria (Dehority and Tirabasso, 2000). Typical ruminal

fungi include *Neocallimastix frontalis*, *N. patriciarum*, *N. frontalis*, *N. hurleyensis*, *Piromyces communis*, *Caecomyces communis*, *C. equi*, *Orpinomyces joyonii*, and *Anaeromyces* (Russell, 2002). The nutritional requirement of ruminal fungi, most specifically the zoospores, is not complex, as they are attracted to simple sugars and can differentiate these sugars via catabolic regulatory systems. Fungal preference for fiber has interested researchers in their cellulases (e.g., xylanases, β-glucosidase, arabinosidase, etc.), but the actual cellulose degrading enzymes are yet to be defined (Akin and Borneman, 1990; Krause et al., 2003; Ribeiro et al., 2016).

While anaerobic fungi are important constituents of the ruminant microbiome, there are no currently published data assessing the survival of aerobic endophytes in the rumen; however, it was reported that anaerobic fungi within the GI tract of cattle have, within their life cycle, one stage that provides increased tolerance to an aerobic environment (Davies et al., 1993). Nonetheless, it is unlikely that the aerobic microorganisms that reside as endophytes within tall fescue will survive and/or colonize the rumen of animals exposed through the diet. On the other hand, the rumen might be subjected to endophyte constituents, including metabolites of these plant (diet)-derived aerobic microbiota.

Ruminal archaea

The archaea *Methanobrevibacter ruminantium* is a major methane-producing bacterium that is common in the rumen (Wanapat et al., 2015). Methane is a main end-product of ruminal fermentation. However, methane production is not ideal for two reasons: 1) cattle-produced methane is a major source of greenhouse gas emissions and 2) methane production results in a loss of feed energy, with some estimates placing energy losses up to 12% of gross energy from feeds (Wanapat et al., 2015). Thus, shifting the fermentation process away from methane build

up is a major, ongoing research effort (Tapio et al., 2017).

Ruminal protozoa

The rumen also hosts a diverse population of ciliate protozoa, which are larger microorganisms and have a density up to 10^7 cells per mL (Hungate, 1966c; Russell, 2002). Despite protozoal density being lower than the bacterial population, due to their size, protozoa can account for about one-half of the ruminal mass and have an inverse relationship with ruminal bacterial population numbers (Hungate, 1966c; Russell, 2002). While abundant, the role protozoa play in digestion of dietary components is unclear.

Two classes of ciliate protozoa exist in the rumen: 1) holotrichs and 2) entodinomorphs. Holotrichs have cilia located across the entire body, whereas entodinomorphs have them in select locations, such as the oral cavity and the dorsal regions (Russell, 2002). Holotrichs utilize sugars as a substrate and are prevalent in animals consuming fresh forages (Russell, 2002). Entodinomorphs utilize starches and plant materials, and can usually be found close to feed particles (Russell, 2002). The protozoal cilia are important for movement and bringing feed, bacteria and small protozoa to the oral cavity. Generally, ruminal protozoa populations are relatively stable and consists of large and/or smaller protozoa. The larger protozoa can cannibalize smaller protozoa, but adaptation can save protozoa of the smaller size from cannibalism. Interestingly, bacteria can be attached to the outside surface of protozoa. For example, methanogens are known to be attached to protozoa, utilizing H_2 derived from protozoal hydrogenosomes (Hungate, 1966c; Russell, 2002). While bacteria are important for protozoal survival in the rumen, difficulty in creating uncontaminated cultures leaves the question of whether they are necessary currently unanswered. Further, identification of protozoa for

taxonomic purposes proves difficult, as morphological traits are not always present, but the utility of 18S rDNA or rRNA has not been tested for protozoal identification purposes.

Dietary and environmental influences on the ruminant microbiota

As stated above, it is now well known that diet significantly influences the ruminant microbiota (Zeineldin et al., 2018). Thus, the rumen microbiota is sensitive to types of dietary carbohydrates and protein, as well as to rumen fermentation profiles (Belanche et al., 2012). Subacute ruminal acidosis can cause ecological shifts, indicating rumen pH fluctuations might play a role in diet-induced microbiota changes (Mao et al., 2013). While currently documented evidence suggests major influence on the rumen microbiota from diet and internal physiological factors (e.g., ruminal fermentation and pH), less is known about how extrinsic stressors influence rumen ecology.

It is known that there is a peripheral dynamic microbiota that is influenced by diet and a core microbiota that is stable across ruminant hosts (Henderson et al., 2015). Recent work has delved into the possible negative impact of harsh environmental conditions on enteric physiology and ruminant microbiota. For example, Koch et al. (2019) demonstrated in dairy cattle that heat stress can detrimentally influence gut permeability, which can also extend to gut microbiota and physiology. Further, heat stress is associated with more lactate and less acetate-producing bacterial species in dairy cattle, with corresponding ruminal metabolites and pH moving in conjunction with these bacteria (Zhao et al., 2019). Finally, heat stress increased plasma and milk cortisol levels, decreased fecal bacterial diversity, and increased plasma cytokines, suggesting alterations within the gut-brain axis of dairy cattle (Chen et al., 2018). Overall, given the changing climate, even though data are mostly from dairy, not beef cattle, understanding the

impact of harsh environmental conditions on gut physiology and the microbiota that contribute to animal productivity in the beef cow will be imperative moving forward.

The microbiota and fescue toxicosis

Limited studies examining the effects of *E. coenophiala* on the plant and/or bovine microbiota exist. Slaughter et al. (2019) studied the effects of plant and endophyte genotype on soil fungi; they found that plant-endophyte genetics alter presence of arbuscular mycorrhizal fungi and extraradical hyphae length in the soil, indicating plant and endophyte genotype selection could alter soil characteristics. This finding adds another layer to consider for breeding programs. Although in the beachgrass *Ammophila breviligulata* and not in tall fescue, addition *E. coenophiala* as an endophyte along with different precipitation regimens altered soil bacterial and fungal diversity (Bell-Dereske et al., 2017). *E. coenophiala* infection also alter soil characteristics, such as carbon and nitrogen cycling mechanisms (Guo et al., 2015; Guo et al., 2016), but, apparently, endophyte presence does not significantly affect soil symbionts (Slaughter and McCulley, 2016). Overall, these results indicate a complex relationship between the aboveground and belowground microbial colonies in endophyte infected plants; through detailed evaluations of the microbial and metabolic profiles in the rhizosphere, phyllosphere, and endosphere we can begin to understand the complex fescue plant-*E. coenophiala* endophyte relationship.

From an animal microbiota perspective, The fungus *Aspergillus terreus* was consistently found along the enteric tract in steers that exhibited signs of fescue toxicosis (e.g., fescue foot), indicating enteric fungi may play a role in fescue toxicosis etiology or be useful as a biomarker (Futrell et al., 1974). Clavine alkaloids, which are ergot alkaloid precursors, appear to have antibiotic-like properties (Eich et al., 1984; Eich et al., 1985). Previous work has also shown that

ewes inoculated with an antibiotic resistant *Escherichia coli O157:H7* and fed a high endophyte-infected, versus low endophyte-infected seed shed a greater number of antibiotic resistance genes (Looper et al., 2007). Recently, Harlow et al. (2017) investigated rumen bacteria that may degrade the ergopeptine alkaloids, by evaluating hyper-ammonia producing bacteria (HAB) and tryptophan-utilizing bacteria in vitro; they found that all tested HAB (e.g., *Clostridium spp.*) degraded ergovaline, with varying degrees of effect. Another recent study (Melchior et al., 2018) assessed the effects of tall fescue seed and red clover isoflavones on global rumen microbial populations in vitro and found limited effects of tall fescue seed on volatile fatty acids. Interestingly, significant effects on bacteria aligned within the *Ruminococcaceae*, *Coriobacteriaceae*, and *Erysipelotrichaceae* families (Melchior et al., 2018). Of note, Alrashedi (2017) found increased ruminal *Firmicutes* and decreased fecal *Bacteroidetes* in ewes grazing high and medium endophyte-infected tall fescue pastures, with ruminal *Prevotella* and fecal *Coriobacteriaceae* operational taxonomic units (OTUs) associating with body weight changes. Finally, Koester et al. (2020) found significantly decreased bacterial and fungal fecal diversity and richness in Angus cattle that had low tolerance to toxic tall fescue exposure. In this study, the *Neocallimastigaceae* family was increased in the feces of high tolerant steers and the genus *Thelebolus* was increased in the feces of lower tolerance steers. Interestingly, the authors also found that farm-to-farm variability existed in the fecal microbiota of Angus cattle, indicating that putative fecal biomarkers should be systematically evaluated to ensure cross-study validation. Overall, these data indicate that ergot alkaloids/toxic tall fescue significantly alter gut physiology and the microbiota along the enteric tract. More *in vivo* analyses need to be performed to understand the importance of the microbiota in fescue toxicosis adaptation/development and

whether any of the adverse effects along the enteric tract could be targeted therapeutically.

Metabolomics as a method to identify toxic responses

Major approaches

Metabolomics is the study of the entire chemical fingerprint in a given matrix. It can be used to determine how dietary, environmental, or any other variable influences metabolic homeostasis and mechanisms of disease pathogenesis, potentially leading to identification of novel biomarkers of exposure or effect (Li et al., 2013; Li et al., 2014; Lin et al., 2014). Two major metabolomic approaches are utilized, untargeted and targeted. The first, untargeted metabolomics, allows putative annotation of metabolic features to MS/MS spectral databases, such as the human metabolome database (HMDB; Wishart et al., 2018), Metlin (Guijas et al., 2018; Schrimpe-Rutledge et al., 2016), or the toxin exposome database (T3DB; Wishart et al., 2015). Untargeted metabolomics allows relative quantification, usually defined as the metabolic peak feature intensity. After identifying putative metabolites of interest using untargeted metabolomics, validation of the retention time and MS/MS fragmentation data with reference standards is necessary for exact metabolite identification/confirmation (Schrimpe-Rutledge et al., 2016).

High-resolution metabolomics (HRM), a form of untargeted metabolomics that uses high-resolution mass spectrometry, has been used to identify molecular signatures in numerous biological matrices associated with different disease states. For example, HRM has been used to identify manganese-induced perturbations in human neuroblastoma cells (Fernandes et al., 2017), nicotine-induced metabolic perturbations in amniotic fluid (Fischer et al., 2017), plasma metabolomics profiling in maintenance hemodialysis (Liu et al., 2017), and urinary biomarker identification in idiopathic Parkinson's disease (Luan et al., 2015). While the development of

metabolomics technologies has been primarily driven by human medicine, HRM is increasingly applied to animal agriculture.

HRM in production animals has the potential to be a powerful tool considering the capabilities for phenotypic assessment of responses to dietary and environmental variables. For instance, metabolomics has been used as a predictive tool for assessing feed efficiency (Karisa et al., 2014), susceptibility to diseases (Hailemariam et al., 2014; LeBlanc et al., 2005; Sun et al., 2014), residual feed intake (Abarghuei et al., 2014), carcass traits (Karisa et al., 2013), and fertility and milk production (Chapinal et al., 2012) in farm animals. In dairy cattle, metabolic signatures associated with milk fever (Sun et al., 2014), clinical ketosis (Li et al., 2014), and periparturient diseases (Hailemariam et al., 2014) were profiled with HRM. However, disease/condition-specific metabolomics-based approaches have not been heavily utilized in beef cattle thus far. HRM provides researchers the ability to delineate molecular signatures, and potential mechanisms, associated with disease states of interest; moreover, recent bioinformatics developments allow for the integration of metabolomics with other ‘omics datasets, allowing for deeper insights into mechanisms associated with phenotypes of interest at multiple biological levels.

Multi-‘omics integration using systems biology-scale tools

The development of different high-throughput ‘omics technologies (i.e., genomics, transcriptomics, proteomics, metabolomics, etc.) has changed the landscape of molecular investigations of different disease states. Each of these methods has had a great impact across the scientific spectrum, but the newfound interest in integrating multi-‘omics data sets is one imperative step for thoroughly evaluating systemic global responses in complex disease states or exposures (i.e., toxic tall fescue and/or ergot alkaloid exposure). Recent statistical and

bioinformatics developments are beginning to integrate, for example, metabolomics and microbiome datasets and have highlighted role for the microbiota in the circulating metabolome and the contribution of the enteric microbiota to disease states (Chong and Xia, 2017; Noecker et al., 2016; Shaffer et al., 2017). The development of sparse partial least squares regression algorithms is one new way that allows for simultaneous dimension reduction and investigation of multi-collinearity with greater predictive accuracy and variable selection performance than partial least squares regression (Chun and Keles, 2010). This development has revolutionized the field of multi-'omics integration and led to the development of xMWAS (Uppal et al., 2018). The xMWAS algorithm can use, among other regression methods, sparse partial least squares regression to select variables in up to four separate data frames prior to execution of correlation analysis with significance testing and differential network, markedly increasing the volume of data able to be integrated. The development of these algorithms allows researchers to gain insights at multiple biological levels and, potentially, within different compartments in a single study, providing a complete picture of the global changes occurring throughout an entire organism. Although there is still much progress to be made, these bioinformatic developments are important initial steps towards harnessing the true power of high-volume 'omics-based data sets.

Outstanding questions and research objectives

The complex, systemic impact of toxic tall fescue grazing and consequent ergot alkaloid exposure, alongside the seasonal variability and other influences on animal responses, drives the need for high-throughput analytical, molecular biology, and integrative bioinformatics techniques to outline global responses that occur throughout the development of fescue toxicosis. Also, more work needs to be done to assess and speciate ergot alkaloids and their metabolites, as

ergot alkaloids other than ergovaline (i.e., lysergic acid, ergotamine, ergonovine, ergocryptine, etc.) may play a larger direct, or indirect, role in fescue toxicosis etiology than previously attributed. Finally, considering that ergot alkaloids are likely heavily degraded in the rumen and parent ergot alkaloids do not readily cross gastric tissues, it is imperative that we understand how toxic tall fescue grazing influences gut microbiota and host physiology, i.e., what are the drivers of the disease pathogenesis versus those responses that are adaptive to external stressors.

Considering the utility of HRM, alongside the impact of the microbiota on forage digestion and utilization, it is postulated that HRM and NGS profiling of the microbiota can provide efficient identification of important microbial and metabolic shifts that significantly contribute to the development of fescue toxicosis and/or can be used as molecular biomarkers of pathophysiological responses. Further, using these techniques to analyze responses in toxic tall fescue-grazing cattle during different seasons, and under fluctuating environmental conditions (i.e., high versus low temperatures and humidity), will provide necessary insights into potential seasonal and environmentally-dependent molecular signatures of fescue toxicosis and how these may be modulated by the microbiota or systemic metabolism. Finally, increasing agricultural demand and consumer demand for pasture-based finishing production systems drives the need for utilizing the predominant Southeastern US forage grass, and mitigating production losses associated with fescue toxicosis will help create a more sustainable agricultural supply chain moving forward. It is the author's belief that using top-down strategic approaches will help provide novel, valuable insights to meet these goals.

The first hypothesis of the studies described herein is that *toxic tall fescue grazing will result in swift metabolic and microbial shifts in the beef steer that will contribute to the decreased animal production, herein defined as lower weight gains, associated with fescue*

toxicosis. The second hypothesis is that, *while toxic tall fescue grazing will result in global metabolic and microbial perturbations, hot and humid environmental conditions will modulate these responses in a manner that results in shifting important nutrients towards meeting the demands of the additional external stressors and away from weight gain*. Finally, the third hypothesis for the work provided herein is that *E. coenophiala infection significantly alters the tall fescue microbiota and metabolome and the grazing beef steer multi-compartment microbiota metabolome. The E+ endophyte and ergot alkaloids will alter the structure of the plant and animal microbiota:metabolome relationship, and significantly associated variables will point towards potential biomarkers of effect for FT.* Finally, *certain microbial/metabolic shifts in E. coenophiala-infected tall fescue will induce and associate with pathological shifts in the grazing bovine, while some effects of the toxic endophyte will overlapping between the plant and animal*.

The overall hypothesis of this project is:

Toxic tall fescue would result in rapid, lasting perturbations in the microbiota and metabolomes of grazing beef steers, and external environmental stressors would exaggerate these perturbations. Additionally, the nature of the microbiome: metabolome interaction and the way it correlates with key fescue toxicosis signs will be time-, matrix-, and environment-dependent..

The overall study objectives of this project is:

Outline the effects of *E. coenophiala* infection on the plant phyllosphere metabolome and microbiomes, as well as the effects of toxic tall fescue grazing on the bovine microbiota along the enteric tract and the multi-compartment metabolome. The final goal is to integrate these data sets to outline the complex network of interactions that occur throughout the plant-endophyte-animal system that could contribute to fescue toxicosis development and/or pathophysiology, potentially providing insights into potential biomarkers or therapeutic areas of interest.

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CHAPTER 2

METABOLOMICS OF FESCUE TOXICOSIS IN GRAZING BEEF STEERS

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ABSTRACT

Fescue toxicosis (FT) results from consumption of tall fescue (Lolium arundinaceum) infected with an endophyte (*Epichloë coenophiala*) that produces ergot alkaloids (EA), which are considered key etiological agents of FT. Decreased weight gains, hormonal imbalance, circulating cholesterol disruption, and decreased volatile fatty acid absorption suggest toxic (E+) fescue-induced metabolic perturbations. Employing untargeted high-resolution metabolomics (HRM) to analyze E+ grazing-induced plasma and urine metabolome changes, fescue-naïve Angus steers were placed on E+ or non-toxic (Max-Q) fescue pastures and plasma and urine were sampled before, 1, 2, 14, and 28 days after pasture assignment. Plasma and urine catecholamines and urinary EA concentrations were also measured. In E+ steers, urinary EA appeared early and peaked at 14 days. 13,090 urinary and 20,908 plasma HRM features were detected; the most significant effects were observed earlier (2 days) in the urine and later (≥14 days) in the plasma. Alongside EA metabolite detection, tryptophan and lipid metabolism disruption were among the main consequences of E+ consumption. The E+ grazing-associated metabolic pathways and signatures described herein may accelerate development of novel early FT detection and treatment strategies.

Keywords: fescue toxicosis, *Epichloë coenophiala,* ergot alkaloids, grazing beef cattle, untargeted metabolomics

INTRODUCTION

Tall fescue, *Lolium arundinaceum*, is a valuable forage grass that is frequently infected with the endophytic fungus *Epichloë coenophiala*, previously known as *Neotyphodium coenophiala* (Young et al., 2014). The relationship between the plant and endophyte is viewed as mutualistic as the fungus produces secondary metabolites (i.e., ergot alkaloids) that protect

against extrinsic plant fitness factors and increase stand persistence (Clay, 1990b, 1993; Hill et al., 1990; Read and Camp, 1986). Thus, endophyte-infected tall fescue is a sustainable grass forage option that outcompetes other plants exposed to similar environmental conditions (Clay, 1990a, b). Today, endophyte-infected tall fescue covers approximately 14 million hectares of pasture land across the Southeastern United States (Young et al., 2014).

Despite the benefits of this mutualistic relationship, the wild-type endophyte is referred to as toxic as consumption of endophyte-infected tall fescue causes the development of fescue toxicosis (FT), a disease that is an estimated one-billion-dollar detriment to the United States beef industry (Craig et al., 2015; Matthews and Haley, 2015; Strickland et al., 2011). Drivers of these economic losses include lowered weight gains and reduced fertility (Bacon, 1995; Craig et al., 2015; Edite Bezerra da Rocha et al., 2014; Thompson and Stuedemann, 1993). The major ergot alkaloid produced by the toxic endophyte is ergovaline, comprising 80-100% of the ergopeptides produced; however, other ergot alkaloids are produced by the toxic endophyte and contribute to additive toxicity (Gadberry et al., 2003; Guerre, 2015).

Non-toxic endophyte-containing fescue varieties are available (Hancock and Andrae, 2012; Hopkins and Alison, 2006; Klotz et al., 2013), but replacement of endophyte-infected tall fescue is not a popular option for remediation (Clay, 1990b; Young et al., 2014). The non-toxic endophyte produce no ergot alkaloids; however, the stand persistence and yield benefits are still provided without the decreased steer performance seen with the toxic endophyte, with contributions from the production of peramine, terpendole, and N-acetylnorloline (Bouton et al., 2001; Young et al., 2014; Young et al., 2013) Despite this, switching to novel non-toxic endophytes requires a time lag before the land becomes profitable again, can cause soil erosion

and impairs pasture sustainability (Bouton et al., 2002; Gunter and Beck, 2004; Hancock and Andrae, 2012; Hoveland, 1993; Parish et al., 2003; Pimentel and Burgess, 2013).

Several contributing mechanisms to FT have been identified. Vasoconstrictive responses (Klotz et al., 2008; Oliver et al., 1993; Oliver et al., 1998; Rhodes et al., 1991) inducing perturbed nutrient absorption and metabolism (Egert et al., 2014a; Pesqueira et al., 2014) occur concurrently with altered circulating prolactin and cholesterol levels and intra- and extrahepatic enzyme activity changes may play a role in the etiology of FT (Bacon, 1995; Rosenkrans and Ezell, 2015; Settivari et al., 2008; Thompson and Stuedemann, 1993; Zbib et al., 2014, 2015). Foote et al. (2013) suggested impaired weight gain might be attributed to a decrease in volatile fatty acid absorption or production caused by increased concentration of ergot alkaloids in the rumen. In ruminants, volatile fatty acids are a major energy source, and decreased volatile fatty acid absorption could lead to a decreased metabolic rate and, ultimately, decreased food intake and weight gains (Dijkstra et al., 1993) if chronic exposure occurs (Foote et al., 2014).

Detection of ergot alkaloids in the urine has been shown as an accurate, less variable ergot alkaloid exposure diagnostic tool when compared to previous methods (Hill et al., 2000; Stuedemann et al., 1998), as ninety-four percent of ergot alkaloid excretion occurs in the urine, appearing as early as 12 hours post-exposure, and concentrations are exposure level- and duration-dependent (Hill et al., 2000). Speciating ergot alkaloids and metabolites, in plasma and urine, might help identify how ruminants metabolize ergot alkaloids, and how other metabolic processes may be affected (Hill et al., 2000; Hill et al., 2001).

Ergot alkaloids share structural similarity with dopamine (DA), norepinephrine (NE), and serotonin (5-HT) (Klotz and Nicol, 2016), thus having dopaminergic, adrenergic, and serotonergic activities (Berde and Stürmer, 1978; Müller-Schweinitzer and Weidmann, 1978;

Pertz and Eich, 1999) that influence numerous physiological functions (Dyer, 1993; Dyer, 2000; Klotz, 2015; Larson et al., 1995; Larson et al., 1994; Oliver, 1997, 2005; Oliver and Abney, 1989; Oliver et al., 1993; Vanhoutte and Luscher, 1986). Thus, ergot alkaloids can potentially decrease appetite and food intake through modulation of the serotonergic tone in the hypothalamus (Rossi-Fanelli and Cangiano, 1991). Sheep plasma DA levels increase (Henson et al., 1987) and plasma 3,4-dihydroxyphenyl acetic acid (DOPAC; a dopamine metabolite) levels in mares decrease (Youngblood et al., 2004) in response to toxic fescue consumption. Plasma levels of 5-HT, NE, homovanillic acid (HVA; another DA metabolite), and epinephrine in response to toxic fescue consumption are not well characterized for any agricultural species.

Metabolomics can be used to determine how toxicants affect crucial metabolic pathways and mechanisms of disease pathogenesis, potentially leading to novel exposure or effect biomarker identification (Li et al., 2013; Li et al., 2014; Lin et al., 2014). In dairy cattle, metabolomics has been used to profile metabolic signatures associated with milk fever (Sun et al., 2014), clinical ketosis (Li et al., 2014), and periparturient diseases (Hailemariam et al., 2014). In beef cattle, disease/condition-specific metabolomics-based approaches have not been utilized thus far.

High-resolution metabolomics (HRM) uses liquid chromatography (LC) coupled to ultra-high resolution mass spectrometry (MS) and advanced data extraction methods to detect thousands of metabolic features quickly (Walker et al., 2016). Samples are analyzed in triplicate to enhance the reliability of detection of low abundance chemicals, and data extraction methods utilize adaptive processing LCMS (apLCMS) (Yu et al., 2009) and automated re-extraction, statistical filtering and data merger to enhance data quality (Uppal et al., 2013). To overcome a bottleneck metabolite identification in this information-rich data, statistical tests and

bioinformatics methods are used to select mass spectral features associated with endpoints of interest (e.g., consequences of ergot alkaloid exposure), and computer algorithms are then used to test for pathway enrichment (Dunn et al., 2012; Li et al., 2013).

In the present study, HRM was utilized to analyze the plasma and urine metabolomes of beef cattle after grazing toxic or non-toxic endophyte-infected tall fescue to test for metabolic differences associated with ergot alkaloid exposure. Total urinary ergot alkaloids and targeted analyses of select catecholamines and their metabolites were also measured. Post HRM processing, metabolite annotation, network analysis, and pathway analysis were conducted using reference metabolite databases and bioinformatics processing (Deo et al., 2010; Li et al., 2013; Ma et al., 2007) for an in-depth look into the metabolic pathways that are perturbed after grazing toxic tall fescue.

MATERIALS AND METHODS

Animals and treatments

The study was conducted in the fall of 2013 (Sept. 16 – Oct. 14). All sample collection was completed in the morning, between 8-11AM. The pastures were located at the J. Phil Campbell Natural Resources Conservation Center of the University of Georgia (Watkinsville, GA), and temperature (T) and relative humidity measurements were collected using an on-site weather station. Fescue-naïve Angus steers (n=8; weighing 199.5 ± 10.5 kg (mean ± SEM) and 200.6 ± 10.5 kg for E+ and Max-Q groups, respectively; 4 steers per fescue treatment; 2 paddocks per treatment with 2 steers per fescue paddock) were randomized and assigned to Fall-saved 0.8 hectare vegetative fescue pastures that were sown in the Fall of 2004 with either a novel non-toxic endophyte (Jesup MaxQ strain AR542 containing less than 1 ppb ergot alkaloid; Max-Q) that does not decrease animal performance (Hancock and Andrae, 2012; Hopkins and

Alison, 2006) or a toxic endophyte (Jesup with wild-type endophyte; E+). The steers were maintained on their respective pastures for 28 days. At baseline (time 0), 1, 2, 14, and 28 days post pasture assignment, plasma and urine were collected and processed for HRM (Patel et al., 2015; Soltow et al., 2013), urinary ergot alkaloid analysis (Hill et al., 2000), and plasma and urine catecholamine analysis (Smith et al., 2013; Youngblood et al., 2004). Additional details of these analyses are provided below.

Determining the effects of toxic fescue on weight gain was not an objective of this study. Nevertheless, the steers were weighed prior to treatment assignment and then again approximately a month after completion of the study prior to their sale; at both times, the two groups' weights were not different. Thus, the mean steer weight prior to the study (provided above) was not different ($P > 0.8$) between the E+ and Max-Q steers. Similarly, steers' BW one month after termination of the study (279.6 [NT] and 283.1 [Toxic] kg, respectively) was not different between groups ($P > 0.9$).

The study was conducted in the fall of 2013 (Sept. 16 – Oct. 14). All sample collection was completed in the morning, between 8-11AM. Temperature (T) and relative humidity measurements were collected using an on-site weather station at J. Phil Campbell Natural Resources Conservation Center of the University of Georgia (Watkinsville, GA). All animal handling and sample collection methods were approved in advance by the Institutional Animal Care and Use Committee (IACUC) of the University of Georgia.

Sample collection and processing

Blood samples were taken from the jugular vein and placed in K_3 EDTA vacutainer tubes (Becton Dickenson and CO, Franklin Lakes, NJ) on ice until plasma was harvested. Voided urine samples were taken by urine collection in sterile cups and placement on ice in 15-mL conical

centrifuge tubes (Fisher Scientific, Waltham, MA). Upon arrival to the lab, urine samples were immediately centrifuged (300 x *g* for 10 min at 4 ˚C), aliquoted and stored at -80 ˚C until further analysis. Plasma was collected after centrifuging the blood (3,500 x *g* for 30 min at 4 ˚C) and stored at -80 ˚C until further analysis.

Metabolomics sample processing and data analysis

Metabolomics sample processing

Plasma and urine samples (1 mL aliquots) were processed as previously described for human plasma and urine (Patel et al., 2015). Briefly, randomized samples were treated with 2 volumes of ice-cold acetonitrile containing an internal standard of a mixture of 14 stable isotopes, incubated on ice for 30 min, and centrifuged (14,050 x *g* for 10 min) to remove any precipitated proteins. The isotopes used for the internal standard were: [$^{13}C_6$]-D-glucose, [^{15}N]-indole, [2-^{15}N]-L-lysine dihydrochloride, [$^{13}C_5$]-L-glutamic acid, [$^{13}C_7$]-benzoic acid, [3,4-$^{13}C_2$]-cholesterol, [^{15}N]-L-tyrosine, [trimethyl-$^{13}C_3$]-caffeine, [$^{15}N_2$]-uracil, [3,3-$^{13}C_2$]-cystine, [1,2-$^{13}C_2$]-palmitic acid, [^{15}N,$^{13}C_5$]-L-methionine, [^{15}N]-choline chloride, and 2'-deoxyguanosine-$^{15}N_2$,$^{13}C_{10}$-5'-monophosphate. Samples were kept in a refrigerated autosampler prior to a 10-µL injection. Urine samples were analyzed using a Thermo Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer (Thermo Fisher, San Diego, CA) set to collect data from a mass to charge ratio (*m/z*) 85 to 850; plasma samples were analyzed using a Thermo linear triple quadropole (LTQ) Velos Orbitrap mass spectrometer (Thermo Fisher) set to collect data from *m/*z 85 to 2000. Both mass spectrometers were coupled with either anion-exchange (AE) or reverse phase (C18) liquid chromatography. All samples were run in triplicates utilizing both forms of liquid chromatography, with a pooled reference sample, which was calibrated to the NIST SRM 1950, run every 21st sample (Phinney et al., 2013). The chromatography was

performed with a temporal acetonitrile gradient for 10 min. A flow rate of 0.35 ml/min was used for the first 6 min and 0.5 ml/min for the remaining 4 min. The first 2-min period consisted of 5% A (water with 2% formic acid), 60% water, 35% acetonitrile, followed by a 4-min linear gradient to 5% A, 0% water, 95% acetonitrile. The final 4-min period was maintained at 5% A, 95% acetonitrile, with flow rate set at 0.35 mL/min for the first six min before changing to 0.50 mL/min for the final 4 min. Data were stored as raw spectral files and converted to computable document format files using Xcalibur file converter software (Thermo Fisher). Peak detection, noise filtering, *m/z* and retention time alignment, feature quantification, and quality filter were performed using xMSanalyzer v2.0.7 (Uppal et al., 2013) with apLCMS v6.1.3 (Yu et al., 2009). Data were extracted as *m/z* features, defined by *m/z*, retention time, and integrated ion intensities. The average of the three technical replicates was used for subsequent bioinformatics analysis. Features that had at least 70% signal in either experimental condition were used to identify differentially expressed metabolites between treatment groups and collection times (Patel et al., 2015).

Plasma and urine metabolite annotation

After averaging the integrated ion intensities for each *m/z* feature for each animal, statistically significant differences were determined with a two-sided t-test ($P < 0.05$). Venn Diagrams were generated using the Bioinformatics and Evolutionary Genomics online processor (http://bioinformatics.psb.ugent.be/webtools/Venn/), entering the *m/z* ratios for the significantly ($P < 0.05$) different metabolites into the processor. Annotation was completed using the Scripps Center for Metabolomics Metlin batch processing software (Metlin; https://metlin.scripps.edu/index.php) by taking the significantly different *m/z* features and querying the Metlin batch processor with a conservative Δ5 ppm tolerance.

Metabolomics data processing

Using *R* language version 3.2.2. (R Core Team, 2015), principle component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were completed using the mixOmics *R* package (Le Cao et al., 2015), with the Reshape package (Wickham, 2007) for data frame shaping; feature peak intensities from each LC column were used as analysis variables for both biological matrices across all time points and within a specific time point post pasture assignment. Volcano plots were generated using metabolomics feature peak intensities in the metabolomics *R* package (De Livera and Bowne, 2014) for each time point, biological matrix, and column; the metabolites that were significantly different ($P < 0.05$) were extracted and analyzed as described in Section 2.3.2. Heat maps were generated using the gplots library (Warnes et al., 2015), with peak intensities of significantly ($P < 0.05$) different metabolites used for the analysis.

Network and pathway analysis

Network analysis was completed using the MetabNet *R* package (Uppal, 2015) coupled with Cytoscape v3.2.1 network visualization software (Shannon et al., 2003), where a complete Pearson correlation-based network analysis was performed for each biological matrix, with correlation stringencies ($|r|$) set at ($|r| > 0.5$) and false discovery rate (FDR) at FDR = 0.05. The target list of metabolites used for network analysis was rich with tryptophan, tyrosine, and ergot alkaloid metabolites. Pathway analysis was completed by taking the putatively identified metabolites of interest and querying the following databases: Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg/), Metlin, the Human Metabolome Database (HMDB; http://www.hmdb.ca), and the Lipid Metabolites and Pathways Strategy database (Lipid MAPS; http://www.lipidmaps.org). From this information, pathways that were

differentially regulated, i.e., multiple significantly different metabolites within the same pathway in the plasma and/or urine metabolomes, were constructed.

Total plant and urinary ergot alkaloids analysis

Individual tillers were sampled from pastures on 28 September, 2013. Sampling was performed by selecting a tiller from each of 100 locations within the pastures, cutting the tiller at the soil surface, and transporting the samples to the laboratory. Endophyte presence was analyzed from a 3-mm cross section of the stem base of each tiller using a commercial immunoblot test kit (Agrinostics Ltd. Co., Watkinsville, GA, Cat.# ENDO797-3). In addition, ergot alkaloid presence or absence was determined by testing each tiller from a second tiller cross section using a commercial ELISA test kit (Agrinostics Ltd. Co., Cat.#ENDO899-2t). The remaining tall fescue tissue was frozen, freeze-dried, ground to pass a 1-mm screen, and analyzed for total alkaloid using a commercial ELISA kit (Agrinostics Ltd. Co., Cat# END0899-96p). The detection limits for the two alkaloid test kits are 0-25 and 0-2000 μg/kg, respectively. Mean endophyte presence in the E+ pastures was 95% and all plant tillers with endophyte tested positive for ergot alkaloids. Mean endophyte presence in the Max-Q pastures was 92% with no ergot alkaloids present in any tillers. The ergot alkaloid concentration in the whole tissue samples was 1024 and 0 μg/kg for the E+ and Max-Q pastures, respectively.

Total urinary ergot alkaloids concentrations were determined at 1, 2, 14, and 28 days post-pasture assignment by enzyme-linked immunosorbent assay (ELISA; Agrinostics Ltd. Co., Watkinsville, GA) as previously described (Hill et al., 2000). The ELISA uses a lysergic acid-moiety specific antibody (Hill and Agee, 1994; Hill et al., 1994) for detection. Urine samples were serially diluted (1:2 to 1:16) before analysis and lysergic acid was used as the standard. The

urine was also analyzed for creatinine to normalize the urinary ergot alkaloids prior to statistical analysis (Murray, 1989); values are presented as ng of ergot alkaloid per mg of creatinine.

Plasma and urine catecholamine sample processing and analysis

Sample processing

Sample processing for catecholamine extraction was adapted from (Youngblood et al., 2004) for plasma and (Smith et al., 2013) for urine. Briefly, for plasma, 50 mg acid activated alumina, 500 µL sample, 500 µL 0.2 M Tris buffer (pH 9.0), and 20 µL of internal standard (epinine, 2 ng) were incubated for 15 min at room temperature on a rocker. The samples were centrifuged at 500 x *g* for one min at 23 ˚C, supernatant was removed, and 1 mL Na_2EDTA (1.1g/L) was added to wash the sample. The sample was vortexed and washed twice more (3 washes total). The supernatant was then removed, 200 µL 0.2 N $HClO_4$ was added, the sample was vortexed for 15 sec, spun as before, and final supernatant was collected and stored at -80 ˚C until analysis. For the urine ((Smith et al., 2013), 50 mg acid activated alumina, 1 mL 0.05 N HCl with 5 mM $Na_2S_2O_5$, 100 µL urine, 20 µL internal standard, and 1 mL 0.2 M Tris (pH 9.0) were mixed, and the pH was adjusted to 8.5. The mixture was vortexed and incubated at room temperature for 5 min on a rocker. The samples were centrifuged as the plasma samples, the supernatant was removed, 1 mL 18.2 mΩ water was added, and the sample was vortexed and centrifuged as before; this was repeated one more time. Then, supernatant was removed, and 200 µL of 0.1 N $HCLO_4$ with 0.1 mM $Na_2S_2O_5$ was added, the sample was vortexed for 15 sec, and spun as before. For both plasma and urine, the processed samples were transferred to a microcentrifuge tube, spun at 13,200 x *g* for 3 min at 4 ˚C, and the supernatant was then either set up for injection into the HPLC or stored at -80 ˚C until analysis.

HPLC-ECD and data analysis

A mixed catecholamine standard containing 2 ng (20 μL) each of dopamine (DA), L-3,4-dihydroxyphenylalanine (L-DOPA), 3,4-dihydroxyphenylacetic acid (DOPAC), norepinephrine (NE), epinephrine (E), homovanillic acid (HVA), and two internal standards consisting of 3,4-dihydroxybenzylamine (DHBA) and epinine (EPI), was prepared in with 0.2 N $HCLO_4$ and injected onto the column (SUPELCOSIL™ LC-18-DB HPLC Column 5 μm particle size, L × I.D. 25 cm × 10 mm; Supelco Inc., Bellefonte, PA). The mobile phase (pH 3.65) consisted of monobasic sodium phosphate, octyl sodium sulfate, EDTA, methanol, H_2O, and trimethylamine (Youngblood et al., 2004).

A Waters e2695 HPLC using Empower Pro software and a Waters 2465 electrochemical detector was used for all analysis, as in (Youngblood et al., 2004), with the flow rate set at 1 mL/min and the detector set at 850 mV. Analyte peaks in standards and samples were integrated and quantitated based on epinine recovery (plasma) and epinine recovery and creatinine normalization (urine).

Statistical analysis of non-metabolomics data

The urinary alkaloids and plasma/urine catecholamines were analyzed by Sigma Plot v12.5 (Systat Software Inc., San Jose, CA). Urinary alkaloids, DA, NE, E, DOPAC, and HVA values were analyzed by two-way ANOVA, with period and fescue treatment set as the two independent variables. If significant ($P < 0.05$) main effects or interactions were present, Student-Newman-Kuels *post-hoc* was used to separate significant differences.

RESULTS

Environmental conditions

For the duration of the study, the average daily T was 19.5 ◦C (range: 16.6 -23.6) and the average temperature-humidity index (THI; Kibler, 1964) was 66.2 (range: 62.1-72.7); these two averages for the five sampling dates were, respectively, 20.4 ◦C and 67.4. Except for day 0, when the T and THI were 23.6 and 72.7, respectively, steers were not subjected to environmental conditions associated with even mild heat stress (i.e. the THI was ≤ 72; Armstrong, 1994; Figure S1).

Ergot alkaloids: quantitation by ELISA and detection using HRM

Total urinary ergot alkaloids in E+ steers increased markedly ($P < 0.05$) from day one to day two and, again, from day two to day 14 before leveling off at 28 days (Figure 2.1). Urinary ergot alkaloids in Max-Q steers were very low and undetectable (range = 0.00 – 6.26 ng/mg creatinine) in most cases, except low, but detectable, levels in two steers (8.85 and 9.97 ng/mg creatinine) on day 14.

Via HRM, we were able to identify multiple putative ergot alkaloids in both plasma and urine. In the plasma, the number of putative ergot alkaloids identified having a basic ergoline ring structure was consistent throughout the grazing duration, except after 2 days of grazing where there is a decrease (Table 2.3A). Total number of putatively identified ergot alkaloids in the urine peaked 2 days after exposure, was stable after 14 days, and decreased after 28 days (Table 2.3B). The majority of the putative urinary ergot alkaloids were ergoline derivatives/lysergic acid amides, with two ergopeptides appearing after 14 days of grazing (Table 2.4). The number of putative ergopeptides in the plasma increased with time spent on pasture, with a total of 5 unique ergopeptides identified after 14 and 28 days of grazing

(Table 2.4).

Plasma and urine catecholamines

As a whole, plasma and urine catecholamines were variable. No significant treatment differences were observed for any of the plasma catecholamines and, as a whole, circulating catecholamines were not affected by toxic fescue grazing to a major extent or in a consistent direction; there were some numerical trends, i.e., plasma DA ($P = 0.16$; data not shown) and E ($P = 0.14$; data not shown) levels were numerically higher in E+ steers after 28 days of grazing. Urinary DA, E, and NE levels fluctuated significantly in both treatment groups, but the timing of the fluctuation differed. As a result, DA, E, and NE in the urine were lower in E+ steers after 2 days of grazing ($P < 0.05$; Figure 2.2A, 2.2B and 2.2C); urinary DA and E significantly ($P < 0.05$) and NE numerically ($P = 0.11$) increased in E+ steers after 28 days of grazing (Figure 2.2A and 2.2B). There was also an overall decrease in urinary DOPAC levels ($P < 0.05$), which was driven by decreases in E+ steers at 1, 2, and 14 days (Figure 2.2D and 2.2D inset).

Metabolomics of toxic fescue grazing: feature collection results

After HRM processing, a total of 6,478 and 6,612 unique features (i.e., compound with unique *m/z* and retention time) were detected in the urine on the AE column and C18 columns, respectively (Table 2.1). Of these, 457 (AE) and 634 (C18) features were significantly different between the treatment groups (Table 2; $P < 0.05$). In the plasma, a total of 9,403 (AE) and 11,505 (C18) unique features were detected (Table 1) with 1,179 (AE) and 1,415 (C18) of them being significantly ($P < 0.05$) different between the two groups (Table 2.2).

The largest number of significantly different metabolites in the urine occurred after 2 days of grazing; in the plasma, the largest number of significantly different features was seen at 14 and 28 days (Table 2.2). Some significantly different *m/z* features overlapped between

different time points, and Venn Diagrams were generated to evaluate significant treatment differences over the course of the study (Figure 2.3). The greatest overlap was seen between 14- and 28-day plasma samples (36 total) and between 1- and 2-day urine samples (23 total; Figure 2.3). Also, several *m/z* features overlapped across all four time points (5 plasma and 11 urine; Figure 2.3).

Further in-depth analysis of metabolomics data

Volcano plots, PLS-DA, and heat maps are common statistical analysis methods used to analyze statistical variations and trends in metabolomics studies. Volcano plots were generated for visualization of metabolites that were significantly different between treatments. As illustrated in Figure 2.4A (AE) and 2.4B (C18) for the 28-day plasma and in Figure 2.4C (AE) and 2.4D (C18) for the 2-day urine, there were multiple significantly different metabolites with large fold changes ($P < 0.05$; fold change ± 2), but there were also metabolites that were highly significantly different ($P \leq 0.01$) with smaller fold differences ($P < 0.05$; fold change < 2). All significantly different metabolites ($P < 0.05$) from all time points, irrespective of fold change, were extracted and taken through the metabolite annotation step.

From PLS-DA, steers grazing Max-Q and E+ tall fescue formed distinct clusters (C18 data: Figure 2.5; AE data: not shown). When PLS-DA was performed including all time points for plasma (C18 data; Figure 2.5A) and urine (C18 data; Figure 2.5C), the Max-Q and E+ treatments separated into two distinct clusters of non-overlapping nature. Similar separation was observed for the AE column (data not shown). Much like the combined data, there was marked treatment separation in the PLS-DA plots for 28-day plasma (Figure 2.5B) and 2-day urine (Figure 2.5D) C18 data; similar results were found within every sampling date and for AE

column data (data not shown). Overall, these data point to treatment-related changes in the plasma and urine metabolomes of steers that appear early and persist.

After the PLS-DA analysis, heat maps were generated to analyze further the differences in the metabolomes between the E+ and Max-Q treatment groups and to determine if there is any indication of pasture (paddock) effect within treatment. The 28-day plasma AE and 2-day urine AE heat maps for significantly different *m/*z features (Figure 2.6A and 2.6B, respectively) are presented as examples, but heat maps for other time points/columns are similar. Overall, the heat maps for significantly different ($P < 0.05$) *m/*z features demonstrate clear metabolome differences between the Max-Q and E+ steers. On the other hand, the impact of pasture within grazing treatment was minimal (i.e., differences in color intensity on the heat maps between the two pastures within a treatment; Figure 2.6A and 2.6B).

Putative metabolites of interest

Initially focusing on significantly different putative metabolites that overlapped between 1 and 2 days, between 14 and 28 days, and among all time points in the two sample matrices, tyrosine and tryptophan metabolites (Table 2.5) were identified as two major metabolite groups affected by E+ grazing. In the urine, significant increases of certain eicosanoids, i.e., 12-hydroxyeicosatetreanoic acid, were observed after 14 and 28 days of E+ grazing. In addition, urinary trace amines, i.e., phenylethanolamine and octopamine, were other putative metabolites that were significantly increased at each time point, with temporal increases occurring in E+ steers. Cortolone-3-glucoronide and tetrahydroaldosterone-3-glucaronide were increased after 1, 2, and 28 days of E+ grazing, whereas chlormadinone acetate, a progesterone antagonist, was increased in the urine at 2 days.

In the plasma, among the putative metabolites significantly impacted by E+ consumption were many glycerophospholipids, namely phosphatides and lecithins, along with several glycolipids, i.e., phosphatidylinositol, phosphatidylethanolamine, and 3-beta-D-galactosyl-sn-glycerol (Table 2.5). Most plasma glycerophospholipids increased by toxic fescue grazing, whereas some of the phosphatides decreased. Of note, arachidonyl serotonin (AA-5-HT), a lipid signaling molecule, increased in E+ steers at all sampling dates. Finally, metabolites involved in tryptophan and tyrosine metabolism were found to be significantly different (metabolite-specific increase or decrease) across all time points, but plasma and urine metabolite profiles were different (Table 2.5).

Network analysis

Targeted networks were generated based on tryptophan, tyrosine, and ergot alkaloid metabolites that were previously identified as impacted in E+ steers in Section 3.6. In the plasma (Figure 2.7), two clusters formed around associations between ergot alkaloids, serotonin, and indole metabolites, with the center of the main cluster being dihydroergocryptine, 5-methoxytryptamine, and indolylmethylthiohydroximate. Separate clusters associated with methylergonovine and 5-hydroxyindoleacetylglycine (Figure 2.7) indicate secondary metabolic processes are influenced based on the link between these two metabolites, the constituents of their clusters, and metabolites residing in the main cluster. Also, three separate clusters, apart from the main alkaloid cluster, indicate associated effects on the anchors [tyramine, dihydroergotamine, 5-hydroxyindoleacetylglycine] and constituents of these clusters without associations to the main cluster (Figure 2.7).

In the urine (Figure S4), one main metabolite cluster centered around 5-hydroxy-6-methoxyindole and 5-hydroxyindoleacetaldehyde, with a slight cluster separation around

tetrahydroaldosterone-3-glucuronide and cortolone-3-glucuronide. This indicates that tryptophan and steroid hormone metabolisms are affected in the urine, and several metabolites can be seen correlating with this perturbation. Finally, N(1)-acetyl-N(2)-formyl-5-methoxykynuramine (AFMK), yellow node in Figure 9, was multi-linked to the metabolites within the main cluster, indicating a secondary metabolic process-type relationship.

Pathway analysis

The tryptophan metabolic pathway was highly perturbed in E+ steers in both the plasma and urine, apparently impacted differentially (Figure 2.8). Four putatively identified metabolites in the plasma are directly involved in tryptophan metabolism, with a significant decrease of metabolism following the pathway from serotonin through melatonin end products (Figure 9A; i.e., 5-hydroxyindolacteyl-glycine), and a significant increase in metabolism of serotonin to 5-methoxytryptamine. The indole pathway of tryptophan metabolism was effected, with significantly increased levels of tryptamine and indole-3-thiohydroximate, indicating increased tryptophan metabolism into tryptamine and its derivatives (Figure 2.8A). Tryptophan biosynthesis from the shikimate pathway had one increased putative metabolite, 3-dehydroshikimate, a precursor to anthranilate, which is an important precursor in the biosynthesis of both tryptophan and tyrosine (Figure 2.8A).

In the urine (Figure 2.8B), decreased levels of serotonin breakdown products and increased levels of the melatonin metabolite AFMK in E+ steers point toward increased metabolism of melatonin (Figure 2.8B).

Finally, tyrosine metabolism was affected in E+ steers across all time points, but the direction of the effects was inconsistent; this applies to both the plasma and urine (data not shown).

DISCUSSION

Untargeted metabolic profiling of the plasma and urine of beef cattle had not previously been conducted. Utilizing HRM, we detected and quantified on average 6,500 unique features per column (AE and C18) in the urine and about 10,500 per column in the plasma. Using two different columns allowed for better metabolite separation, as metabolites interact differently with the stationary phases of these two columns. Here, using untargeted HRM of bovine plasma we detected a slightly lower number of unique features (per column) than reported data for human plasma analyzed similarly (Burgess et al., 2015; Frediani et al., 2014; Patel et al., 2015). However, compared with other untargeted metabolomics processing methods for human and rodent plasma and urine (Chen et al., 2012; Feng et al., 2016; Ohta et al., 2009; Wikoff et al., 2009), our HRM method detected more unique features in the bovine plasma and urine. Overall, these data indicate the robustness of HRM for metabolic profiling and its utility to analyze bovine plasma and urine metabolomes.

Ergot alkaloid levels in plants is influenced by numerous abiotic factors (Guerre, 2015), and E+ ergot alkaloid levels here were slightly lower than previous summer studies (Hill et al., 2000). This is expected, however, as warming increases E+ alkaloid content (McCulley et al., 2014). Total urinary ergot alkaloid concentration increased after 1 day of grazing, peaked on day 14, and remained high until day 28. The trend in urinary alkaloid excretion was consistent with previous studies (Ayers et al., 2009; Hill et al., 2000; Stuedemann et al., 1998), indicating animal's response to toxic fescue grazing from this experiment is typical. While direct toxicity based on ergot alkaloid levels in the grass, using the alkaloid excretion versus weight gains curve provided in (Hill et al., 2000), even the highest detected level in E+ herein would only slightly decrease the average daily gain. This indicates that exposure levels in this study were below the

previously found toxic threshold for clinical levels

HRM can provide metabolite-specific information of the ergot alkaloids and similar/related compounds in the plasma and urine. The largest number of ergot alkaloids identified in the urine were lysergic acid amides and ergoline derivatives, peaking after 2 days of grazing, and only after 14 days were ergopeptides detected in the urine, consistent with previous findings that ergot alkaloids less than 450 Da are excreted in the urine (Stuedemann et al., 1998). In the plasma, both lysergic acid amides/ergoline derivatives and ergopeptides were consistently identified throughout the grazing period, with slightly greater numbers detected after 14 and 28 days of grazing; potentially attributed to plasma and/or tissue accumulation of ergot alkaloids resulting from continuous exposure (Klotz, 2015).

Our metabolomics approach was optimized for extraction of the entire metabolome, not specifically ergot alkaloids, and this is one potential reason for the lack of identification of ergovaline in the plasma (Klotz and Nicol, 2016; Schultz et al., 2006) or lysergic acid (Lodge-Ivey et al., 2006; Schultz et al., 2006) in the urine. Another potential reason is ergovaline has been found accumulated in liver and kidney tissues, but is hard to detect in plasma as it is rapidly metabolized (Ayers et al., 2009; De Lorme et al., 2007). Besides using a broad, non-ergot alkaloid focused extraction method, some ergot alkaloid metabolites might not have been identified because of the lack of matching ergot alkaloid metabolites with proper MS/MS spectra in existing reference metabolomics databases. Nevertheless, we putatively identified previously unreported ergot alkaloids and ergot alkaloid metabolites in the plasma and urine of E+ steers. The detection of hydrogenated and hydroxylated ergot alkaloids is potentially indicative of detoxification attempts by E+ animals, as increased polarity and hydrophilicity facilitate ergot alkaloid excretion and, potentially, toxicity (Mulac et al., 2011); increased alkaloid metabolite

numbers in the plasma after 14 and 28 days of grazing potentially indicate lowered rates of excretion (Berde and Stürmer, 1978). Interestingly, pergolide sulfone is a secondary metabolite of pergolide produced by microorganisms (Smith et al., 1983), and increased urinary pergolide sulfone may indicate increased microbiome metabolic activity in the rumen of E+ steers. Using HRM to identify novel ergot alkaloids, their structures and biotransformation, followed by verification with targeted approaches is fundamental to our greater understanding of ergot alkaloids in general and, in particular, fescue toxicosis.

No significant differences were seen in plasma catecholamine levels at any time point. However, the trend for increased plasma DA levels after 28 days of grazing is in line with previous findings in sheep (Henson et al., 1987). The urine catecholamines DA and E followed biphasic patterns. Interestingly, pergolide, a D-2 receptor agonist, has biphasic effects on sleep and wakefulness patterns (Monti et al., 1988), which are dysregulated in E+ animals (Perumbakkan et al., 2007). Here, the secondary metabolite of pergolide, pergolide sulfone, was detected in the urine of E+ steers. Of note, in mares fed E+ seed-based diets, decreased plasma DOPAC levels were reported (Youngblood et al., 2004); however, DOPAC levels were not affected in the plasma, but were decreased in the urine in E+ steers, with more prominent effects occurring in the first two weeks of grazing.

Tryptophan metabolism was a major E+ target, being affected in both plasma and urine. One portion of this pathway, tryptophan metabolism through serotonin, was effected in opposite directions in the plasma and urine. For example, while melatonin was not directly measured, metabolites derived from it were seen increased in the urine (i.e., AFMK) and decreased in the plasma. A previous study has reported decreased plasma melatonin in cattle grazing high endophyte versus low endophyte tall fescue, which would be consistent with the findings here

(Porter et al., 1993). AFMK is the product of the reaction between melatonin and reactive oxygen species (ROS; de Almeida et al., 2003; Hardeland et al., 1995; Tan et al., 2000), and previous reports found decreased intra- and extrahepatic antioxidant defense mechanisms (Lakritz et al., 2002; Settivari et al., 2008; Zbib et al., 2014, 2015). Thus, increased AFMK may indicate an early compensatory mechanism utilizing melatonin for the mitigation of E+-induced oxidative stress.

One putative metabolite that was impacted in E+ steers and is of potential importance to the pathophysiology of FT is AA-5-HT. AA-5-HT, a lipid signaling molecule, is a selective fatty acid amide hydrolase (FAAH) inhibitor that was significantly increased in the plasma of E+ steers throughout the study. AA-5-HT-induced FAAH inhibition increases four-fold when oxidized by CYP450's (Siller et al., 2014). AA-5-HT is present in the GI tract of guinea pigs, rabbits, and mice (Verhoeckx et al., 2011), and it was recently shown in both bovine and human brain and thymus tissues, indicating that it becomes systemic (Siller et al., 2014). AA-5-HT is sensitive to dietary fat (Verhoeckx et al., 2011) and decreased uptake of volatile fatty acids (Dijkstra et al., 1993; Foote et al., 2013; Foote et al., 2014), along with significant increases in putative plasma glycerophospholipid metabolites seen herein, may play a role in decreased nutrient utilization, resulting in lowered weight gains which is a finding consistently reported in chronic E+ grazing (Oliver, 1997; Strickland et al., 2009). It is hypothesized that AA-5-HT increases in tissues with higher levels of serotonin (Verhoeckx et al., 2011); increased serotonin in the central nervous system (Porter, 1995) and increased serum tryptophan levels (Oliver et al., 2000b) are all results of E+ grazing, which is consistent with the increases in AA-5-HT.

As stated, AA-5-HT is a selective FAAH inhibitor and FAAH predominantly cleaves one of the two major endocannabinoids, anandamide (Kathuria et al., 2003). AA-5-HT-induced

increased levels of anandamide caused by inhibition of FAAH, in the GI tract, decreases gut motility (Bashashati et al., 2012; Capasso et al., 2005), consistent with gut hypomotility found in FAAH knockout mice (Cravatt et al., 2001). Of note, Egert et al. (2014b) recently showed that intravenous or intraruminal injections of an ergovaline and ergovalinine mixture did not reduce rumen motility. Alternatively, the impact of AA-5-HT on food intake we hypothesize is independent of its effects on gut motility as plasma anandamide is also associated with better feed efficiency, at least in feedlot steers (Artegoitia et al., 2016). Further carefully designed studies are necessary to investigate the effects of AA-5-HT and E+ consumption in more detail.

Many putative glycerophospholipids in the plasma were significantly different across all time points, indicating significant changes in lipid metabolism caused by E+ grazing; a greater number of glycerophospholipids were affected after 14 and 28 days of grazing. Accumulation of ergot alkaloids in adipose tissue (Realini et al., 2005) could potentially be responsible for the likely disruption of lipid metabolism associated with E+ fescue grazing, as it has been suggested recently (Klotz, 2015). Glycerol is an important energy source in ruminants and is a precursor for volatile fatty acids (Nafikov and Beitz, 2007); the increased levels of putative glycerol metabolites (i.e., phosphatidylinositol and 3-beta-D-galactosyl-sn-glycerol) identified in the plasma could indicate perturbed glycerol utilization. Further, acetate, a major acetyl-CoA source, and glucose are both main carbon sources for fatty acid synthesis in ruminants (Ballard and Hanson, 1967). Glucose is also the main substrate in intramuscular adipose tissue (Smith and Crouse, 1984), and increased plasma phospholipid levels could be one potential utilization route of these important energy sources. Glycerophospholipids are also major structural lipids and are integral, alongside cholesterol, for membrane structures (Cooper, 2000). Lowered cholesterol has been reported in E+ grazing beef cattle (Oliver et al., 2000a) and this, coupled with an increase in

plasma phospholipids and their precursors, could be an indication of decreased membrane integrity, including of muscle cells.

Interestingly, phosphocholine was significantly increased in the plasma after 14 and 28 days of E+ grazing. Phosphocholine is one precursor, along with phosphatidylcholine, and constituent of the platelet activating factor (PAF; McIntyre et al., 1999; Snyder, 1990). Also, phosphocholine, phosphorylcholine, and phosphatidylethanolamine, are intermediates in the synthesis of phosphatidylcholine (DeLong et al., 1999). This significant increase of phosphatidylcholine and PAF precursors involved in the most common PAF biosynthetic pathway could be partially responsible for/reflective of early FT signs, such as smooth muscle cell hyperplasia, endothelial cell damage (Strickland et al., 2009), and enhanced platelet aggregation (Oliver, 1997, 2005), when considered alongside elevated von Willebrand factor, angiotensin converting enzyme (Oliver, 1997), and endothelin (Strickland et al., 1994).

Other putative metabolites of interest affected by E+ grazing include cortolone-3-glucoronide, tetrahydroaldosterone-3-glucoronide, and 12-hydroxyeicosatetreanoic acid (12-HETE). Increased urinary tetrahydroaldosterone-3-glucoronide has been proposed as a predictive urinary biomarker for primary aldosteronism (Abdelhamid et al., 2003). In support, Piper (1988) reported increased basal levels of aldosterone in E+-exposed sheep. 12-HETE has been used as a urinary biomarker of hypertension and platelet activation (Sterz et al., 2012; Yeung and Holinstat, 2011), and has vasoconstrictive properties in the kidneys, where production increases in response to angiotensin II (Ma et al., 1991; Yiu et al., 2003), which is likely increased in FT as (Oliver, 1997, 2005) reported increased levels of angiotensin converting enzyme in E+ animals.

The plasma and urine networks indicate significant primary and secondary interactions between E+ grazing and target metabolites, shown by primary and secondary clustering in the

networks. Most overlapping features in the plasma networks were putatively identified as glycerophospholipids, tryptophan metabolites, and steroid hormone metabolites. Overlapping features in the urine networks contained tryptophan, steroids (i.e., chlormadinone acetate) and unidentified metabolites.

Overall, data from this study validate HRM as a novel, robust method for metabolic profiling of bovine plasma and urine, with the plasma metabolome being more appropriate for determining global effects of E+ fescue and ergot alkaloid exposure in this case. The urine metabolome might be more useful for early detection of biomarker metabolites of exposure to and effects of ergot alkaloids that point to pathways and processes that are affected. The implementation of HRM allows for sensitive detection of both large and small metabolic changes in cattle plasma and urine, which can provide detailed evidence of molecular changes associated with pathologies specific to cattle and allow for targeted treatments designed for their alleviation. The ability to analyze both plasma and urine using HRM is important, as key metabolic changes may be detectable only in a specific biological matrix. Data presented here provide evidence that E+ grazing causes important metabolic perturbations, namely of tryptophan, tyrosine, and glycerophospholipid metabolism. The data also provide putative metabolite-based explanation(s) for complications seen in fescue toxicosis, such as decreased food intake and weight gains. AA-5-HT in the plasma, and tetrahydroaldosterone-3-glucoronide and 12-HETE in the urine are metabolites of possible use as biomarkers, but require further investigation. Studies of the nature of the one described here are imperative for potential exploration of new treatment and diagnostic strategies for FT.

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Table 2.1. Total number of unique m/z features detected in bovine plasma and urine of Angus steers grazing non-toxic or toxic tall fescue for up to 28 days when analyzed using anion exchange (AE) or reverse phase (C18) liquid chromatography coupled with apLCMS are presented.

	Unique Features Detected			
Biological Matrix	Plasma		Urine	
LC Column	AE	C18	AE	C18
Unique Features	9,403	11,505	6,478	6,612

Table 2.2. Number of significantly different (P < 0.05) m/z features between the a novel, non-toxic (Max-Q) or toxic (E+) groups in bovine plasma and urine of Angus steers grazing non-toxic or wild-type tall fescue for up to 28 days when analyzed using AE or C18 liquid chromatography coupled with apLCMS after 1, 2, 14, and 28 days of grazing.

Matrix	Plasma								Urine							
LC Column	AE				C18				AE				C18			
Days on Pasture	1	2	14	28	1	2	14	28	1	2	14	28	1	2	14	28
Max-Q vs E+ (P<0.05)	267	192	400	320	315	189	388	523	73	234	91	59	138	264	166	66

Table 2.3. Number of putative ergot alkaloid metabolites detected using metabolomics (AE and C18 columns combined) in the (A) plasma and (B) urine of Angus steers grazing toxic tall fescue for 28 days after 1, 2, 14, and 28 days and the total number of ergot alkaloids identified from across all time points.

3A	Plasma				
Days on Pasture	1 Day	2 Days	14 Days	28 Days	Total
Ergoline Derivatives and Lysergic Acid Amides	4	0	3	3	10
Ergopeptides	2	1	5	5	13

3B	Urine				
Days on Pasture	1 Day	2 Days	14 Days	28 Days	Total
Ergoline Derivatives and Lysergic Acid Amides	1	5	2	1	9
Ergopeptides	0	0	2	0	2

Table 2.4. Names of ergot alkaloid metabolites identified after Metlin querying, separated by biological matrix, detection column, and grouped by their basic ring structure.

Putative Ergot Alkaloids Identified via Metlin				
Biological Matrix	Plasma		Urine	
LC Column of Detection	Anion Exchange	Reverse Phase	Anion Exchange	Reverse Phase
Ergoline Derivatives and Lysergic Acid Amides	Ergine	Ergoline-1-carboxaldehyde, 8-(hydroxymethyl)-10-methoxy-6-methyl-, (8b)-	Nicergoline	Pergolide sulfone
	Dihydroisolysergic acid II	Ergonovine	Ergoline-8-methanol, 10-methoxy-1,6-dimethyl	
	N-Despropylpergolide	Methylergonovine	Ergonovine	
	Ergonovine	Pergolide sulfone	Methysergide	
Ergopeptides	Dihydroergocornine	Dihydro-α-ergocryptine		Ergosine
	α-Ergocryptine	Dihydro-β-ergocryptine		
	Ergotamine	α-Ergocryptine		
		Dihydroergocornine		
		Dihydroergotamine		
		Ergocornine		8'-Hydroxydihydroergotamine
		8',10'-Dihydroxydihydroergotamine		
		8'-Hydroxydihydroergotamine		

Table 2.5. Selected putatively identified metabolites, detected using HRM (AE and C18 columns combined), in the plasma and/or urine of Angus steers that were significantly impacted during the 28-day toxic fescue grazing (all sample dates [1, 2, 14, and 28 days] combined). The group or pathway that the metabolites belong to and their respective KEGG or Metlin IDs are also included.

Group/Pathway	Metabolite Name	KEGG ID (or Metlin)	Biological Matrix	Direction of Change
Glycerophospholipid	Phosphocholine (Phosphorylcholine)	C00588	Plasma	Increased
	Phosphatidylinositol	C00626	Plasma	Increased
	Phosphatidylethanolamine		Plasma	Increased
Glycerophospholipid & Galactose Metabolism	3-beta-D-Galactosyl-sn-glycerol	C03692	Plasma	Increased
Trace Amines	Phenylethanolamine	59 (Metlin)	Urine	Increased
	Octopamine	C04427	Urine	Increased
Tryptophan Metabolism	5-methoxytryptamine	C05659	Plasma	Increased
	5-hydroxyindolacetylglycine	C05832	Plasma	Decreased
	Tryptamine	C00398	Plasma	Increased
	Indole-3-thiohydroximate	C16516	Plasma	Increased
	3-dehydroshikimate	C02637	Plasma	Increased
	AFMK	C05642	Urine	Increased
	5-methoxyindoleacetate	C05660	Urine	Decreased
	5-hydroxyindolacetaldehyde	C05634	Urine	Decreased
Tyrosine Metabolism	Tyramine	C00483	Plasma, Urine	Both
Fatty Acyls	Arachidonyl serotonin (AA-5-HT)	63067 (Metlin)	Plasma	Increased
Sterol Lipids	Cortolone-3-glucuronide	61643 (Metlin)	Urine	Increased
	Tetrahydroaldosterone-3-glucuronide	61672 (Metlin)	Urine	Increased
Eicosanoids	12-hydroxyeicosatetreanoic acid (12-HETE)	75018 (Metlin)	Urine	Increased
Steroid Hormone	Chlormadinone acetate	C12729	Urine	Increased

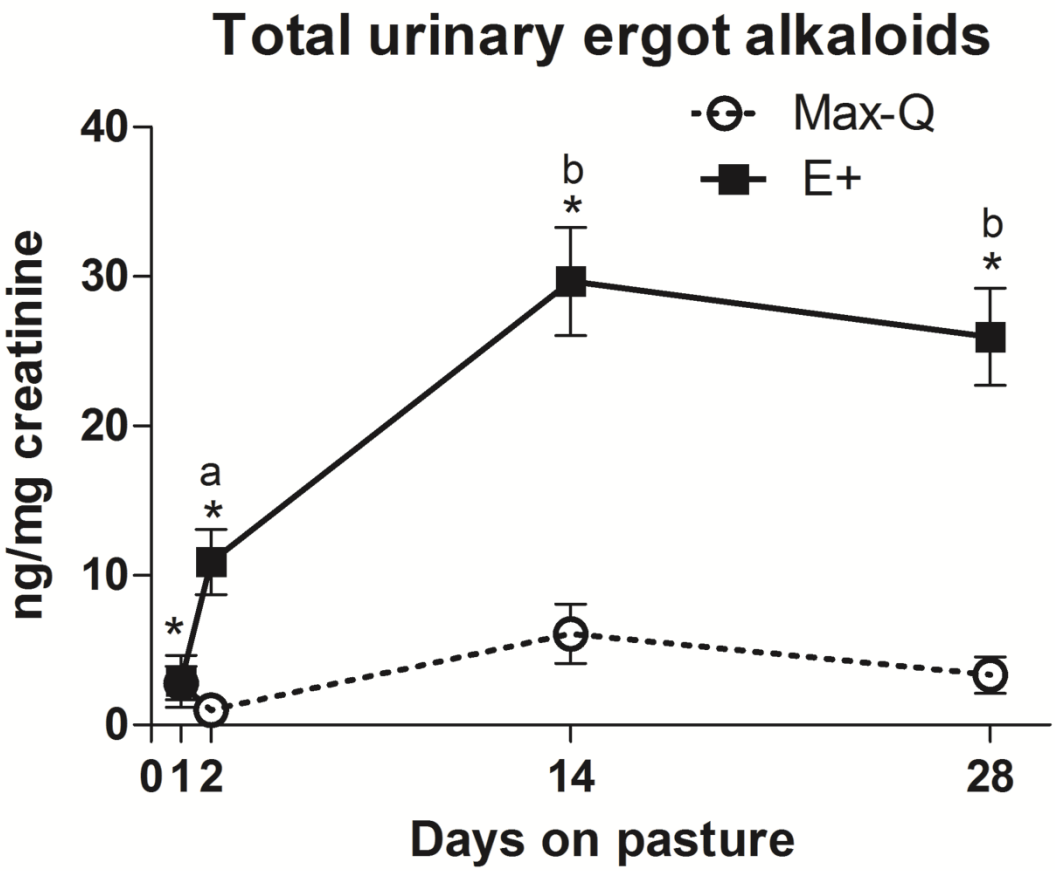

Figure 2.1. Urinary ergot alkaloid concentration detected via ELISA after 1, 2, 14, and 28 days on pasture for steers grazing non-toxic endophyte-infected tall fescue (Max-Q; n=4) versus steers grazing wild-type endophyte-infected tall fescue (E+; n=4). * indicates significant differences between treatment groups within a time point, [a,b] indicate differences within fescue treatment due to duration of grazing ($P < 0.05$). Data are $\bar{x} \pm$ SEM.

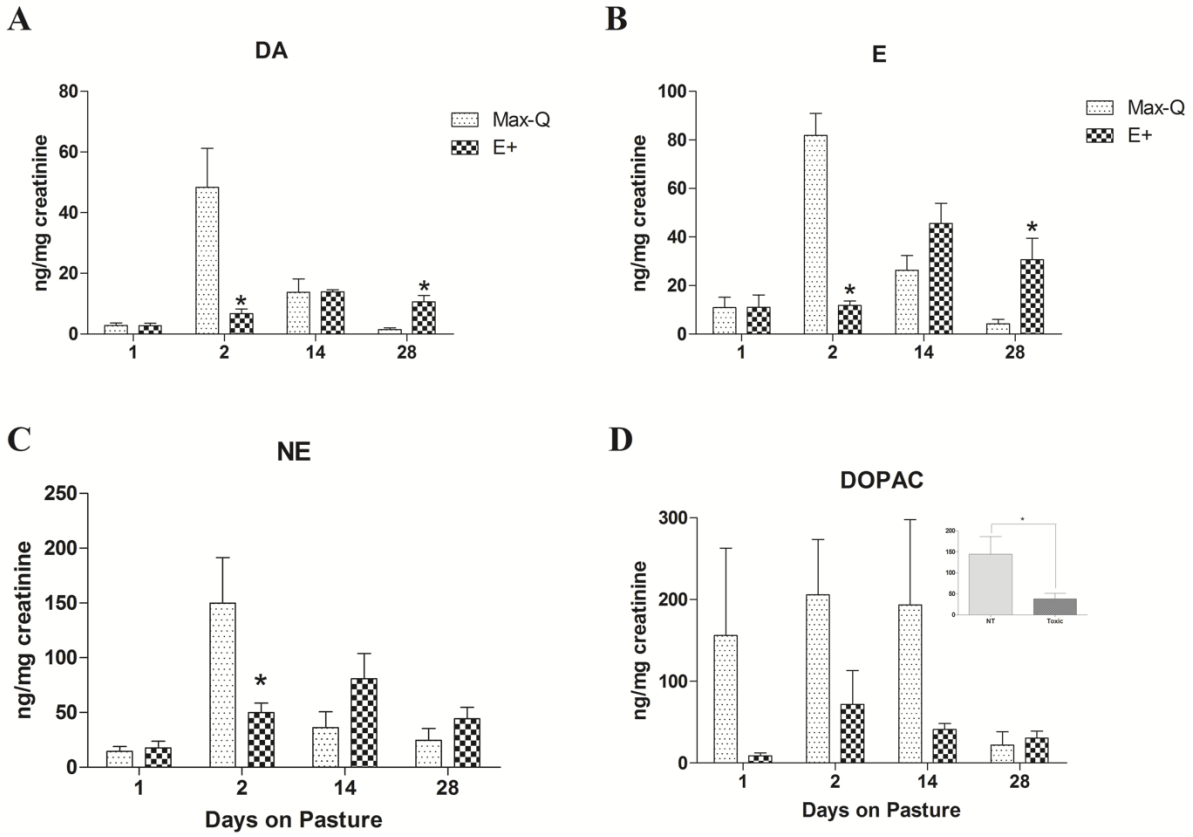

Figure 2.2. Internal standard- (epinine) and creatinine-normalized urinary (A) dopamine (DA), (B) epinephrine (E), (C) norepinephrine (NE), and (D) 3,4-dyhydroxyphenylaceticacid (DOPAC) levels (ng/mg) after 1, 2, 14, and 28 days of steers grazing non-toxic endophyte-infected tall fescue (Max-Q; n=4) versus steers grazing wild-type endophyte-infected tall fescue (E+; n=4). Data are presented as $\bar{x} \pm$ SEM. * Indicates significant differences ($P < 0.05$).

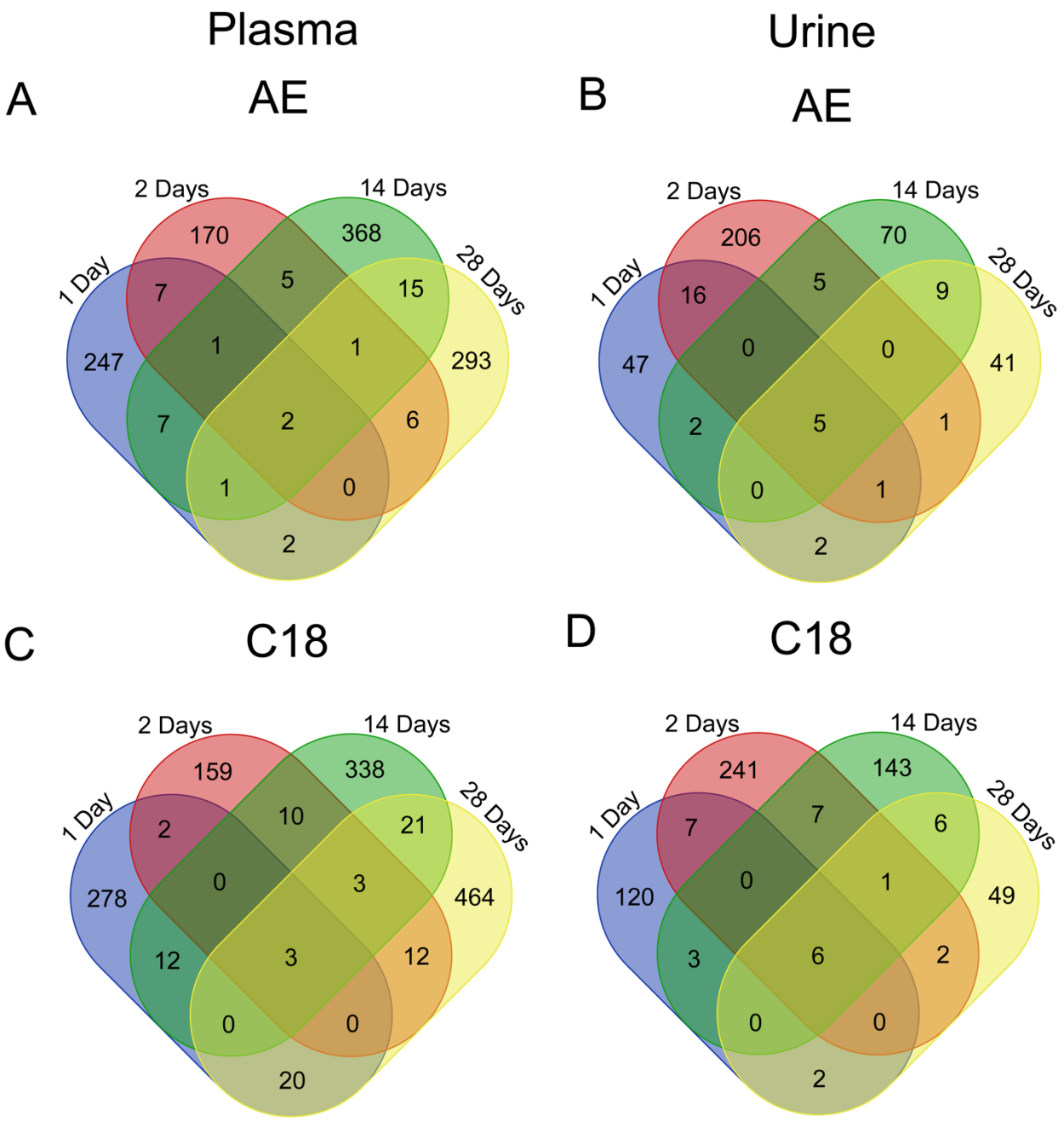

Figure 2.3. Venn Diagrams presenting the significantly different ($P < 0.05$) m/z features for all sample time points for bovine plasma analyzed with (A) AE and (C) C18 liquid chromatography and bovine urine analyzed with (B) AE and (D) C18 liquid chromatography.

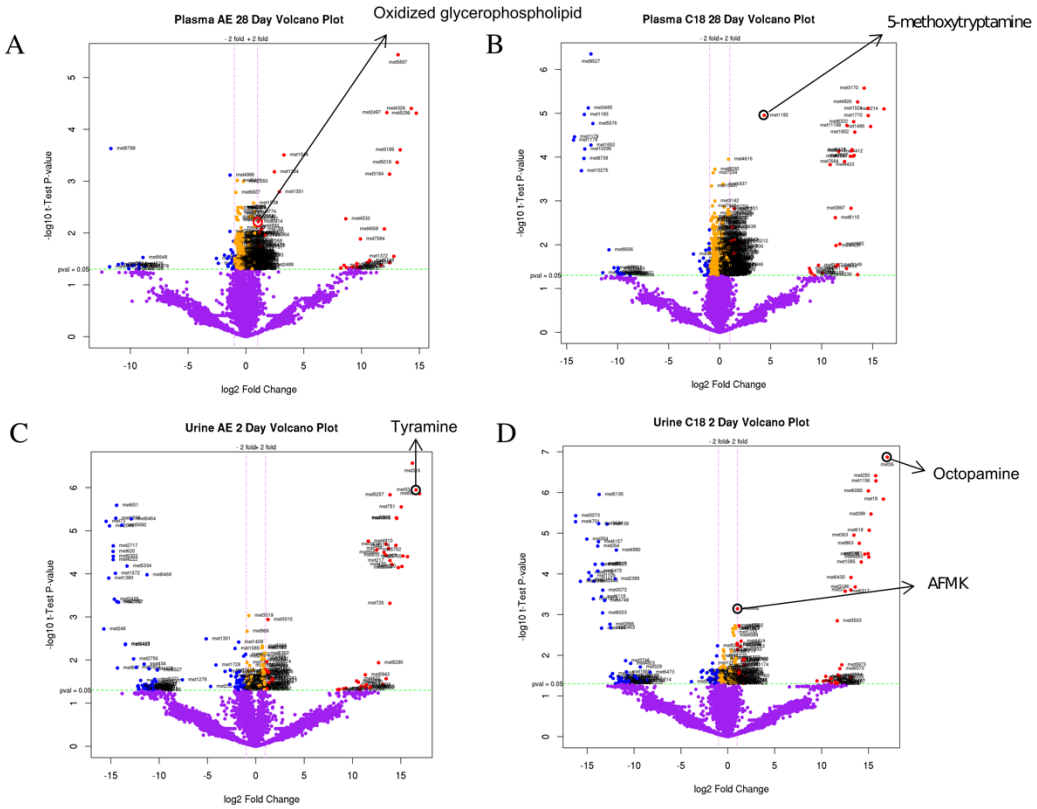

Figure 2.4. Volcano plots representing HRM-derived unique features within bovine plasma after 28 days of grazing analyzed with (A) AE and (B) C18 liquid chromatography and bovine urine after 2 days of grazing analyzed with (C) AE and (D) C18 liquid chromatography.

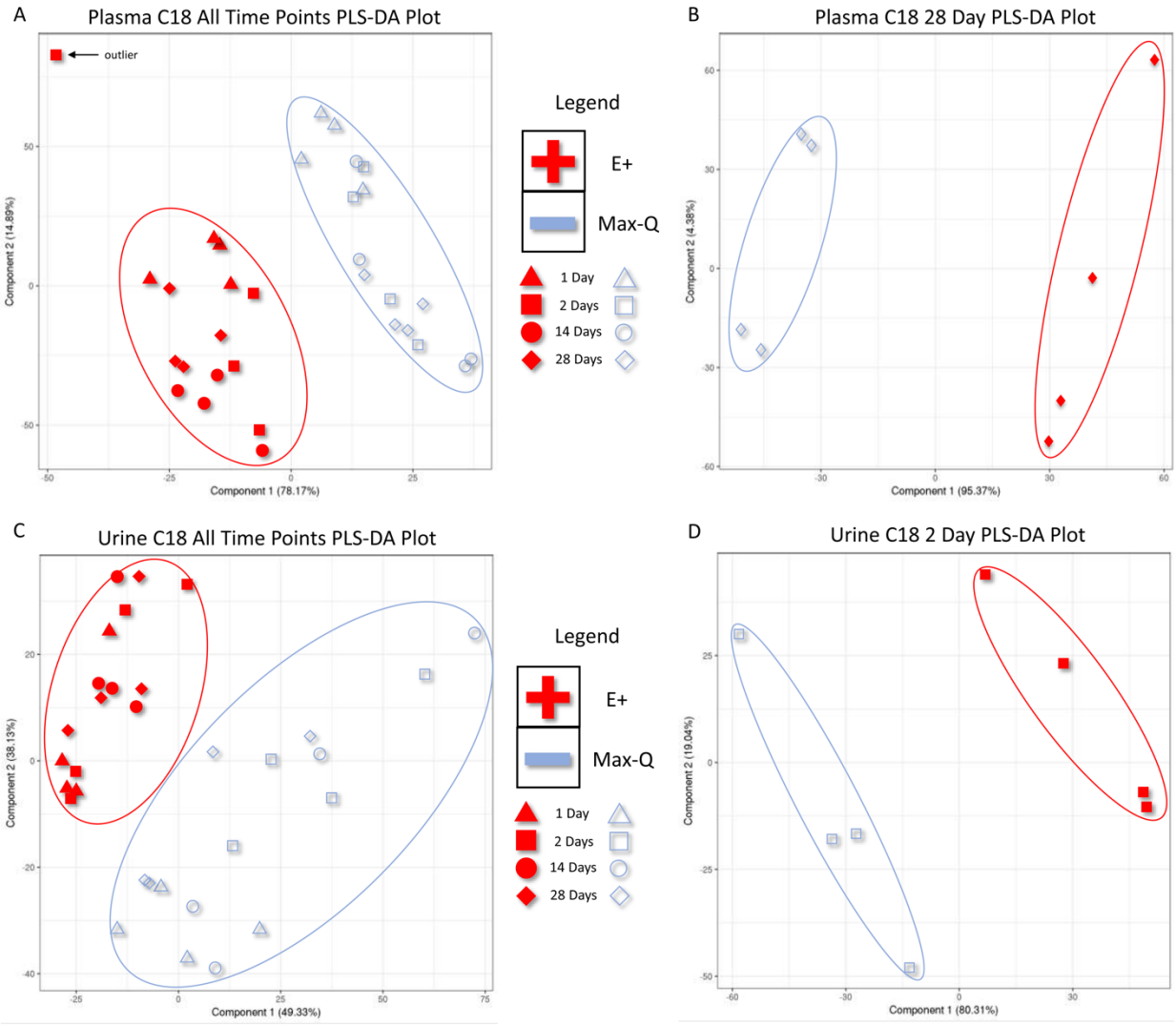

Figure 2.5. PLS-DA plots (C18 column) for bovine plasma (**A**) containing all time points and (**B**) after 28 days of grazing; PLS-DA plots (C18) for bovine urine (**C**) containing all time points and (**D**) after 2 days of grazing.

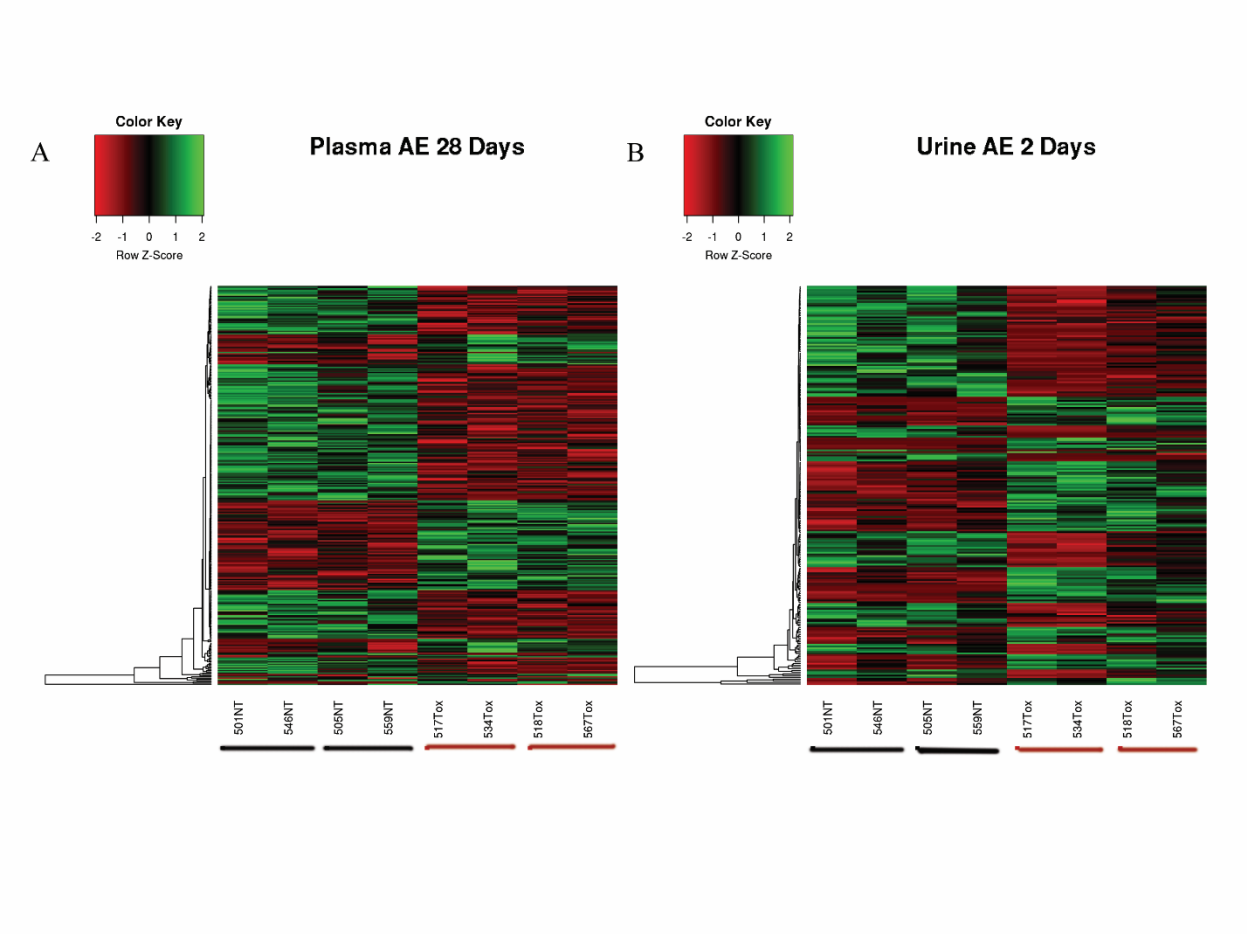

Figure 2.6. Heat maps representing apLCMS unique features (AE column) that were significantly different ($P < 0.05$) between the NT and Toxic groups for (A) bovine plasma after 28 days of grazing and (D) bovine urine after 2 days of grazing. Actual steer number and treatment (NT vs Toxic) is indicated below the map. Neighboring animals (n=2) within the heat maps are from the same paddock (2 per paddock) (indicated by black and red underlines for the NT and Toxic treatment groups, respectively).

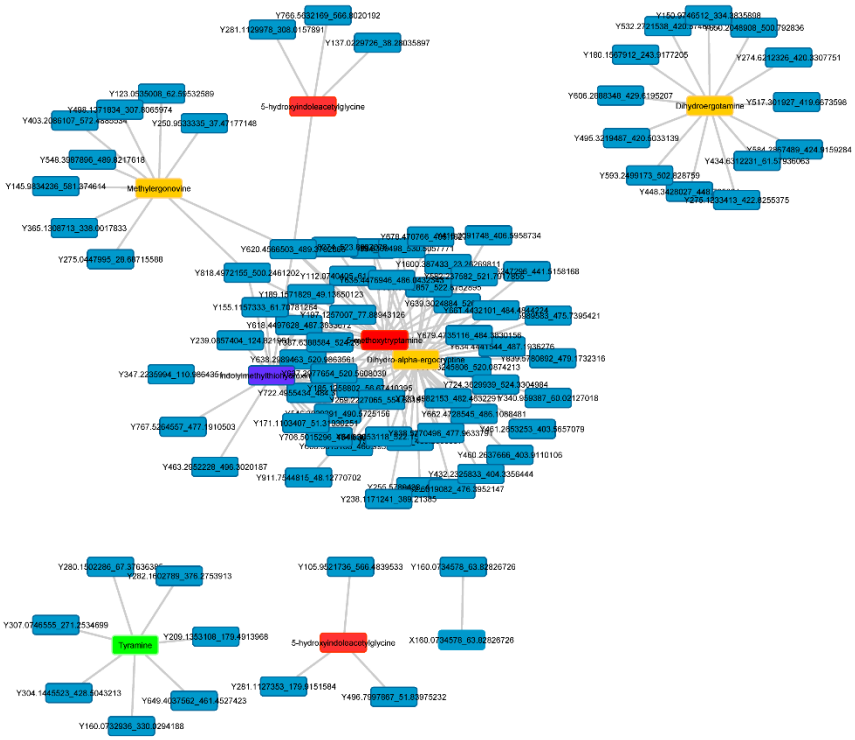

Figure 2.7. Targeted complete Pearson correlation-based network ($|r| > 0.5$; FDR = 0.05) for bovine plasma metabolome of Angus steers grazing wild-type tall fescue for 28 days (C18 column data). Numbers indicate the *m/z* and retention times of putative metabolites. Key anchor metabolite names are included. Red = serotonin metabolite, purple = indole metabolite, green = tyrosine metabolite, orange = ergot alkaloid metabolite.

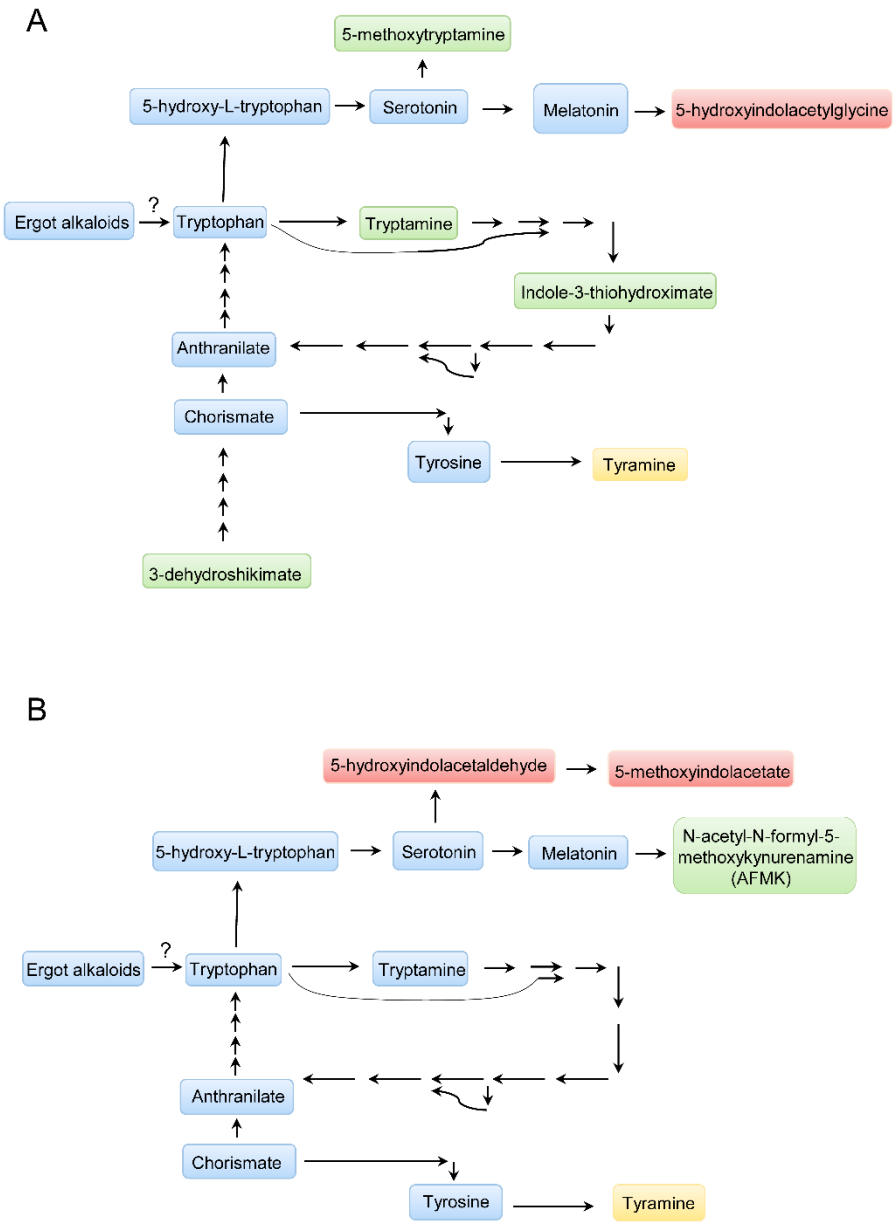

Figure 2.8. Putative significantly different tryptophan pathway metabolites in the (**A**) plasma and (**B**) urine metabolomes of Angus steers grazing non-toxic and wild-type tall fescue. Red boxes = significant decreases, green boxes = significant increases, and blue boxes = significant increases or decreases that are time-dependent caused by wild-type fescue grazing. Number of arrows indicate individual metabolic steps. '?' indicates potential source of tryptophan.

CHAPTER 3

ANGUS STEER FECAL MICROBIOTA RESPONSE TO TOXIC TALL FESCUE GRAZING

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ABSTRACT

Tall fescue, the predominant Southeastern United States cool season forage grass, frequently becomes infected with an ergot alkaloid-producing toxic endophyte, *Epichloë coenophiala*. Consumption of endophyte-infected fescue results in fescue toxicosis (FT), a condition that lowers beef cow productivity. Limited data on the influence of ergot alkaloids on rumen fermentation profiles or ruminal bacteria that could degrade the ergot alkaloids are available, but how FT influences the grazing bovine fecal microbiota, or what role fecal microbiota might play in FT etiology, and associated production losses, has yet to be investigated. Here, we used 16S rRNA gene sequencing of fecal samples from weaned Angus steers grazing toxic (n = 6; E+) or non-toxic (n = 6; Max-Q) tall fescue before, 1, 2, 14, and 28 days post-pasture assignment. Bacteria in the *Firmicutes* and *Bacteroidetes* phyla comprised 90% of the Max-Q and E+ steer fecal microbiota throughout the trial. Early decreases in the *Erysipelotrichaceae* family and delayed increases of the *Ruminococcaceae* and *Lachnospiraceae* families were among the major effects of E+ grazing. E+ also increased the abundances within the *Planctomycetes*, *Chloroflexi*, and *Proteobacteria* phyla and the *Clostridiaceae* family. Multiple operational taxonomic units classified to the *Ruminococcaceae* and *Lachnospiraceae* were correlated negatively with weight gains (lower in E+) and positively with respiration rates (increased by E+). These data provide insights into how E+ grazing alters the Angus steer microbiota and the relationship of fecal microbiota dynamics with FT.

IMPORTANCE

Consumption of toxic endophyte-infected (E+) tall fescue has an estimated annual $1 billion negative impact on the U.S. beef industry, with one driver of these costs being lowered weight gains. As global agricultural demand continues to grow, mitigating production losses

resultant from grazing the predominant Southeastern United States forage grass is of great value. Our investigation of the effects of E+ grazing on the fecal microbiota furthers our understanding of bovine fescue toxicosis in a real-world grazing production setting and provides a starting point for identifying easy-to-access fecal bacteria that could serve as potential biomarkers of animal productivity and/or FT severity for tall fescue grazing livestock.

Keywords: Fescue toxicosis, *Epichloë coenophiala*, beef cattle, microbiome, tall fescue, ergot alkaloids

INTRODUCTION

Culture-independent next-generation sequencing (NGS)-based microbiota studies (e.g., 16S rRNA gene) in food-producing animals, like ruminants, are on the rise. The influence of the bovine microbiota on host energy status and metabolism has been studied (Hungate, 1966a, b, c; McCann et al., 2014), and recent NGS studies have linked enteric microbiota shifts to animal performance e.g., weight gains, in multiple species (Dill-McFarland et al., 2017; Guan et al., 2008; Jewell et al., 2015; Oikonomou et al., 2013; Sun et al., 2016). Notably, in beef cattle, the influence of feed additives and/or diet on the rumen and fecal microbial communities has been evaluated (Carberry et al., 2012; Petri et al., 2013; Rice et al., 2012; Thomas et al., 2017) and ubiquitous and foodborne pathogenic bacteria in their fecal matter were identified (Dowd et al., 2008). Overall, the various dietary contributions to shifts in the bovine microbiome and subsequent changes in animal performance metrics are a major research focus; however, potential changes in the resident microbial community and the impact of these changes on animal performance in grazing beef, including tall fescue grazing, are not yet studied.

Tall fescue, *Lolium arundinaceum*, is the predominant Southeastern United States forage grass covering approximately 14 million hectares, and is commonly infected with the endophytic

fungus *Epichloë coenophiala* (Young et al., 2014). The wild-type fungus produces multiple metabolites. Some metabolites increase stand persistence through protection against extrinsic plant fitness factors. However, other fungal metabolites, namely ergot alkaloids, are detrimental to grazing livestock and are key etiological agents of fescue toxicosis (FT; Clay, 1990a, b, 1993; Hill et al., 1990), a disease with an estimated $1 billion negative impact on the U.S. beef industry, when adjusted for inflation (Craig et al., 2015; Matthews and Haley, 2015). FT is a complex, multi-system disease manifested with metabolic dysregulation (Mote et al., 2017), decreased volatile fatty acid (VFA) absorption (Foote et al., 2013; Foote et al., 2014), and immune/inflammatory alterations (Oliver et al., 2001a; Oliver et al., 2001b; Strickland et al., 2009). Other important signs of FT in grazing beef include increased respiration rates (Al-Haidary et al., 2001; Browning and Leite-Browning, 1997), thermoregulatory impairments (i.e., decreased ability to reguate core body temperatures; Aldrich et al., 1993; Strickland et al., 2009) and lowered weight gains (Klotz, 2015).

Clavine alkaloids, a major alkaloid type produced by *E. coenophiala,* and other ergot alkaloid precursors have previously been shown to exhibit antibiotic-like properties (Eich et al., 1984; Eich et al., 1985), indicating that they may contribute to changes in the microbial populations of animals grazing toxic (E+) tall fescue. Although some previous studies report on how specific ruminal bacteria and/or fermentation profiles shift after exposure to E+ fescue or ergot alkaloids (De Lorme et al., 2007; Harlow et al., 2017; Schumann et al., 2008), understanding the relationship between toxic tall fescue grazing and the fecal microbiota is important, as beef cattle fecal microbiota is dynamic and shifts in response to dietary and/or management strategy changes (Bessegatto et al., 2017; Rice et al., 2012). This is particularly important for FT, as rotational grazing is a common management practice to minimize the

impact of E+ grazing on animal productivity. Further, a previous study has found around 30% of operational taxonomic units (OTUs) from 16S rRNA gene sequencing were shared between the rumen and hindgut microbiota (de Oliveira et al., 2013), indicating that some shifts in rumen populations may be detectable in the lower GI tract. Therefore, understanding how E+ grazing influences the fecal microbiota could provide an easier means of assessing global (rumen and hindgut) microbiota changes that could either contribute to, or, be associated with decreased animal productivity. Finally, these studies can be used in the future as a starting point to ascertain different management practices or therapeutic approaches that mitigate the effects of E+ fescue grazing on the fecal microbiota, or, to use the microbiota to diminish the adverse effects of the toxic fescue on the fescue grazing beef.

Here, we sought to provide an initial characterization of the beef cattle fecal microbiota and to evaluate changes in the fecal microbiota community composition and dynamics that result from grazing toxic tall fescue. To accomplish this, we employed a next-generation sequencing-based analysis of the fecal microbiota of Angus steers by targeting the V4 region of the 16S rRNA gene. Fecal samples were collected from animals grazing tall fescue infected with either a toxic endophyte (E+) or a novel, non-toxic endophyte (Max-Q) across a 28-day grazing trial. Fecal microbiota changes due to E+ grazing were identified and correlated to animal performance and physiological responses. Taken together, these data describe the hindgut microbiota of beef cattle grazing toxic, ergot alkaloid-producing endophyte-infected tall fescue.

MATERIALS AND METHODS

Animals, pastures, and environmental conditions

All animal handling and sampling procedures were preapproved by the University of Georgia's Institutional Animal Care and Use Committee. Post-weaning Angus steers (n = 12)

were blocked by weight and randomly assigned to non-toxic (n = 6; weight: 332.8 ± 11.77 kg [$\bar{x}$ ± SEM]; Max-Q; Jesup MaxQ with endophyte AR542; 3 paddocks; 2 steers per paddock) or toxic (n = 6; weight: 349.3 ± 19.34 kg [$\bar{x}$ ± SEM]; E+; Jesup with wild-type endophyte; 3 paddocks; 2 steers per paddock) tall fescue treatments. Pastures, sown in fall 2004, have been described previously (Mote et al., 2017). Individual tillers were sampled on April 11th, 2016 from 100 locations within the pastures, cutting the tiller at the soil surface, and transporting the samples to the laboratory on ice. Plant tissue was frozen, freeze-dried, ground to pass a 1-mm screen, and analyzed for total ergot alkaloid using a commercial ELISA (Agrinostics Ltd., Cat. # END0899-96p). Steers were kept on the same pasture (0.8 ha) throughout the grazing trial. Temperature and humidity measurements were recorded for 24 hours daily and across sampling times (8:00 - 11:00 AM) on sampling dates using an on-site weather station.

Statistical power calculations

The power calculations performed utilized previously published average daily weight gain data where the same two forage types (Max-Q and Wild-type Jesup tall fescue) were used, and the overall average daily gains were used to estimate the potential treatment difference. The number of groups was set at two with six individuals per group, and the resultant power was 1.00. The power calculations for the correlational analysis (post pasture placement) with a set correlation coefficient at ($|r| = 0.4$) and an alpha of 0.05 resulted in calculated power of 0.493. Finally, for the t-test comparison of an arcsine normalized relative abundances of operational taxonomic units (OTUs), the difference in means and standard deviation was estimated based on the significantly different OTUs throughout this study; the power calculation was 0.347 based on an alpha of 0.05. These calculations assured that the study is sufficiently powered.

Urinary ergot alkaloid analysis

Total urinary ergot alkaloid concentrations were determined before and 1, 2, 14, and 28 days after pasture placement via ELISA (Agrinostics Ltd., Watkinsville, GA) as previously described (Hill et al., 2000; Mote et al., 2017; Stuedemann et al., 1998). Lysergic acid was used as the standard and serially diluted (1:2 to 1:16) urine samples were used for analysis. Urinary ergot alkaloid levels were creatinine normalized as in (Murray, 1989) prior to statistical analysis.

Sample collection and processing

Steer body weights were recorded before, 14 and 28 days post pasture assignment with a digital scale. Fresh fecal samples were collected by hand using new gloves for every collection, and stored on ice for transport before being stored at -80ºC until DNA extraction. Respiration rates (RR) were monitored by counting full flank respiration movements for 60 seconds, twice per animal, and calculating the average, similar to (Al-Haidary et al., 2001; Browning and Leite-Browning, 1997; Koontz et al., 2012). Rectal temperatures (RT) were taken with a handheld DeltaTrak® (Pleasanton, CA) digital thermometer once a stable reading for 15 seconds was acquired. Fecal samples, RR, and RT were taken before (Pre), 1, 2, 14, and 28 days post pasture assignment.

DNA extraction

Genomic DNA was extracted from fecal samples using a mechanical disruption and phenol extraction protocol established in (Stevenson and Weimer, 2007) with the 25:24:1 phenol:chloroform:isoamyl alcohol modification used in (Dill-McFarland et al., 2017). All DNA samples were resuspended in TE buffer and quantified using a Qubit® Fluorometer (Invitrogen, San Diego, CA). For each set of DNA extractions, a negative control using the TE extraction

buffer was performed alongside each extraction and was taken through the amplification and sequencing protocol described below.

DNA amplification and sequencing

Samples were diluted to 1 ng/μL for amplification, and universal bacterial primers for the 16S rRNA gene variable region V4, as previously described (Kozich et al., 2013), were used in the amplification reactions. For each amplification, a negative PCR control containing the extraction buffer and PCR primers was used. A total of 5 μL of diluted DNA, 0.5μL of 10 μM forward primer (5'-GTGCCAGCMGCCGCGGTAA-3'), 0.5 μL of 10 μM reverse primer (5'-GGACTACHVGGGTWTCTAAT-3'), 6.5 μL water, and 12.5 μL KAPA 2x HiFi Master Mix (Kapa Biosystems, Wilmington, MA) were used in each 25-μl reaction with the cycling conditions as follows: initial denaturation at 95ºC for 3 min; 25 cycles of 95ºC for 30 sec, 55ºC for 30 sec, 72ºC for 30 sec; with the final extension set at 72ºC for 5 min. Water was used for PCR negative control. PCR products were purified with a 1% w/v low-melt agarose gel and recovered using a Zymoclean 96-well DNA recovery kit (Zymo Research, Irvine, CA). If bands were present in either the DNA extraction or PCR amplification negative controls, the sample were re-extracted and amplified until no visible band was present in the gel. Samples were then quantified using the Qubit® Fluorometer and equimolar pooled into the final library. The pooled library and 10% PhiX control DNA was sequenced on an Illumina MiSeq sequencing platform using the 2x250 bp paired end MiSeq v2 sequencing kit (Illumina, San Diego, CA) using custom primers (Kozich et al., 2013). The sequenced control and sample DNA were taken through quality filtering and normalization procedures described below.

Fecal 16S rRNA gene sequence processing and bioinformatics analysis

Raw sequence files were obtained in fastq format from the sequencer and processed using mothur v.1.38.1 (Schloss et al., 2009) as in (de Oliveira et al., 2013). In brief, quality filters were applied to remove sequences with a quality score of less than 35, homopolymers longer than 8 base pairs, and greater than 2 different bases to the primer. Next, unique sequences were aligned to the SILVA version 119 reference alignment database (Pruesse et al., 2007) and chimeras were removed using *chimera.uchime* (http://drive5.com/uchime). Only bacterial sequences were retained and classified using the Greengenes database v13.8 (http://greengenes.secondgenome.com; DeSantis et al., 2006). Rarefaction curves and Good's coverage were calculated in mothur. The OTU table was normalized for sequence depth in mothur using the "*normalize.shared*" function prior to statistical analysis. For all OTU or taxa level statistical tests, taxa abundance threshold was > 0.1% and had to be present in >50% of samples in the respective analysis. Community diversity was estimated using Chao1 richness (Chao, 1984) and the inverse of Simpson's diversity index (Simpson, 1949); alpha diversity metrics were tested for E+ grazing effects using the non-parametric Kruskal-Wallis test by ranks. A non-parametric permutational analysis of variance (PERMANOVA) was used to test for E+ effects on the entire microbial community, with fescue treatment and time spent grazing set as the two factors. Principal component analysis and partial least squares discriminant analysis (PLS-DA) and PLS-DA loadings were performed using the mixOmics *R* package (Le Cao et al., 2015; R Development Core Team, 2016) and plots were recreated in Adobe Illustrator (Adobe Systems; San Jose, CA). PCA and PLS-DA were performed on the post-sequencing depth normalized OTU table, the data were mean centered, and Total Sum Scale normalized prior to analysis. For the PLS-DA, the internal nearZeroVar function was used to flag and remove

predictors (i.e., OTUs) that had low to zero variance, which is recommended for sparse datasets, such as 16S rRNA gene sequencing studies. Linear discriminant analysis effect size (LEfSe) was performed within the Huttnehower lab's galaxy instance using the relative abundance table as input (https://huttenhower.sph.harvard.edu/galaxy/; Segata et al., 2011), with Kruskall-Wallis ($P < 0.05$), Pairwise Wilcoxon ($P < 0.05$), and logarithmic LDA score (> 2.0). Comparison of differentially abundant OTUs was performed in *R* (R Development Core Team, 2016), with Bonferroni correction for multiple comparisons ($\alpha < 0.05$).

All included OTUs in the analyses were determined at a 97% sequence identity, allowing genus-level resolution (Roesch et al., 2007), using the furthest neighbor (most conservative) clustering algorithm. Visualization of OTUs, at all levels, was completed using Krona Tools 2.7 (Ondov et al., 2011) and are provided as .html file in the Supplemental Data. Network analysis was performed using the CoNet app within Cytoscape as in (Faust and Raes, 2016) with Pearson's correlation, Spearman's correlation, and the Bray-Curtis and Kullback-Leibler dissimilarity parameters with the thresholds set so the 1,000 top- and bottom-scoring edges are kept in the network. Relationships between parent-child taxa were excluded within the algorithm to prevent overabundance of correlations based on similar lineages. Differential network analysis was performed within Cytoscape (Shannon et al., 2003), and removal of all nodes and edges shared between Max-Q and E+ networks resulted in the final E+ steers' network presented herein.

Statistical analysis of non-microbiome data

Statistical analyses of weight gains, RR, RT, and urinary ergot alkaloids were done with Sigma Plot v12.5 (Systat Software, Inc., San Jose, CA) using two-way analysis of variance (ANOVA) with days of sampling and fescue treatment set as the two independent variables. If

significant ($P < 0.05$) effects based on treatment or days spent grazing were observed, the Holm-Sidak *post-hoc* analysis was applied to separate significant differences. Graphs were generated with GraphPad Prism 5 (La Jolla, CA).

Accession number(s)

All DNA sequences are publicly available in the NCBI Sequence Read Archive and are accessible under BioProject PRJNA540841.

RESULTS

Environmental conditions, pathophysiological responses, and urinary ergot alkaloids

This study was conducted in spring 2016 (April 6th – May 4th) at the J. Phil Campbell Natural Resources Conservation Center of the University of Georgia (Watkinsville, GA). The average 24-hour temperature was 17.8°C (range 8.3 – 23.3°C) and the average 24-hour temperature-humidity index (THI) was 63.1 (range 49.3 – 72.6). Pasture total ergot alkaloid levels on April 11th were 2,357.7 ± 19.70 ppb and 164.3 ± 11.30 ppb for E+ and Max-Q pastures, respectively. Both cumulative and average daily weight gains were significantly lower ($P < 0.01$) in toxic fescue-grazing (E+) steers at the end of the grazing trial (Fig. 3.1). Respiration rates (RR) were significantly higher ($P < 0.05$) after 14 and 28 days of E+ grazing, and rectal temperatures (RT) tended ($P = 0.096$) to be elevated in E+ steers after 14 days of grazing (data not shown). Total urinary ergot alkaloids were significantly elevated ($P < 0.05$) in the E+ steers throughout the 28-day grazing period, reaching their maxima after 14 days of grazing (Fig. 3.2). Max-Q steers, while having measurable alkaloid levels prior to placement on the non-toxic Max-Q pastures (i.e., Day 0), had markedly lower urinary alkaloids than the E+ steers throughout the 28-day grazing trial (Fig. 3.2).

Fecal 16S rRNA gene sequencing results

Following Illumina sequencing, the 60 fecal samples produced 3,199,179 raw reads. After sequence quality filtering using the mothur pipeline, which includes removal of short sequences, preclustering, and chimera removal, a total of 1,694,182 high-quality sequences were obtained; the mean number of sequences per sample was 28,235 ± 10,927 ($\bar{x}$ ± SD; range: 12,038-73,982). Operational taxonomic unit (OTU) determination resulted in 3,809 unique bacterial OTUs across all samples, with an average of 1,047 ± 200 OTUs per sample ($\bar{x}$ ± SD; range: 491-1,462). Taxonomically, 95% of all sequences were classified into 21 different phyla and 74% were further classified to at least the family level when queried against the Greengenes database.

Sequencing depth and coverage

A rarefaction analysis for the bovine fecal microbiota by days on pasture is presented as Fig. S1. Although the collector's curves did not appear, upon visual inspection, to reach an asymptote when rarefied at a minimum of 2,000 sequences per sample (Fig. S1), the calculated Good's coverage indicated that the sampling depth captured most of the species diversity, with an average coverage of 99.00 ± 0.38%, irrespective of fescue cultivar, for the entirety of the grazing trial (i.e., Pre, Day 1, 2, 14 and 28; data not shown).

Alpha-diversity metrics

When comparing alpha-diversity metrics, both diversity (Fig. S2A) and richness (Fig. S2B) were constant throughout the 28-day grazing period, with a trend for an increase in diversity and a slight decrease in richness after 14 days of grazing. A non-parametric permutational analysis of variance (PERMANOVA, permutations = 10,000) found a significant main effect of time ($P < 0.001$) without a significant fescue treatment ($P = 0.156$) or treatment and time interaction ($P > 0.9$) effect on the fecal bacterial communities using the Bray-Curtis

(abundance and presence/absence) dissimilarity metric. Also, a significant main effect of time ($P < 0.001$) and a trend towards a fescue treatment effect ($P = 0.095$), with no interaction ($P > 0.9$), when using the Jaccard (presence/absence) dissimilarity metric was found. Further, there was a trend for a main effect of fescue treatment on the diversity of the fecal microbial community (Inverse Simpson's Diversity $P = 0.051$), with no significant effect based on time ($P = 0.21$) or the treatment and time interaction ($P = 0.89$). There were no effects of fescue cultivar treatment ($P > 0.5$), time ($P > 0.4$), or treatment by time interaction ($P > 0.8$) on Chao1 richness. These data indicate that E+ grazing has a tendency to change the overall fecal microbial communities, and that this effect is mainly on species evenness, not the total number of species.

Overall fecal microbial community composition of the Angus beef cattle

Independent of fescue cultivar, *Firmicutes* and *Bacteroidetes* were the two predominant phyla in the Angus steers' fecal samples before and throughout the grazing period, with combined sequences in these two phyla accounting for 90% (range: 53 – 61% and 27 – 37%, respectively) of the entire microbial community in both E+ and Max-Q steers (File S1). The remaining classified (e.g., *Verrucomicrobia*, *Actinobacteria*, *Tenericutes*, etc.) and unclassified bacterial phyla accounted for < 4% of the total sequences (File S3.1). Overall, the Max-Q and E+ fecal microbiota at the phylum level was relatively stable, with no major differences between the two treatments. However, significant compositional differences between the Max-Q and E+ fecal microbiota were observed at the order, family, and genus levels (Fig. S3.3A, S3.3B, and S3.3C).

Throughout the grazing trial, bacteria in the *Ruminococcaceae* and *Lachnospiraceae* families accounted for, respectively, 44% and 23% of all *Firmicutes* sequences. An additional 16% of the sequences were unclassified at the family level and all other families accounted for less than 3% of the total number of sequences within this phylum (File S3.1). For the

Bacteroidetes phylum, 41% of the sequences were unclassified at the family level, with 14% of these being classified into the order *Bacteroidales* (File S3.1). The most abundant classified families included the *Paraprevotellaceae* (21%), *Rikenellaceae* (14%), *Bacteroidaceae* (10%), and the candidate family *RF16* (4%; File S1). All other classified families made up less than 3% of the *Bacteroidetes* population.

The family *Coriobacteriaceae* accounted for 53% of all *Actinobacteria* sequences within the Max-Q steers and 84% in E+ steers; another major *Actinobacteria* family, the *Corynebacteriaceae*, constituted 45% and 9% of all *Actinobacteria* sequences within Max-Q and E+ steers after 1 day of grazing, respectively (File S3.1). Max-Q steers had consistently higher *Corynebacteriaceae* and lower *Coriobacteriaceae* levels throughout the grazing period than the E+ steers (File S3.1). By the end of the study, the *Coriobacteriaceae* dominated the *Actinobacteria* phylum, with 93% (Max-Q) and 97% (E+) of all *Actinobacteria* sequences aligning to the *Coriobacteriaceae* family (File S3.1).

Prior to pasture (E+ or Max-Q) placement, the *Streptococcaceae* family made >80% of all *Bacilli* within the *Firmicutes*; however, after placement on their respective pastures, steers from both Max-Q and E+ pastures had sharp decreases in the *Streptococcaceae* (down to 20%) accompanied by increases in the *Planococcaceae* and *Turicibacteraceae* families. These two families were more robustly represented in Max-Q (~60% of *Bacilli*) than in E+ (~30% of *Bacilli*) steers (File S3.1). Also, the *Paraprevotellaceae* family was greater than 10% of the total *Bacteroidetes* sequences and more abundant in E+ steers than Max-Q steers after 2, 14, and 28 days of grazing; most of the changes within the *Paraprevotellaceae* during the grazing period occurred for the candidate genera *CF21* and *YRC22* (File S3.1). There was also a sharp decrease

in an OTU classified as belonging to *Ruminococcus bromii* in both E+ and Max-Q steers post tall fescue pasture placement, suggesting fescue cultivar-independent effect (File S3.1).

Multivariate analyses to interrogate E+ grazing-related fecal microbiota shifts

Principal component analysis (PCA) was conducted to interrogate sources of variability within the bovine fecal microbiota resulting from time spent grazing and from the E+ endophyte. The two principal components accounted for approximately 21% of the overall variance, with the first component being the major contributor (Fig. S3.4A). Although the 95% confidence intervals overlap between the Pre, Max-Q and E+ data, PCA demonstrated distinct clustering and separation of Pre samples from Max-Q and E+, with this separation becoming greater as the grazing trial progressed (Fig. S3.4A). Importantly, while the Max-Q and E+ treatments clustered similarly along the first two components, they separated along the third principal component, most notably on Days 14 and 28 of the grazing trial (Fig. S3.4B).

Partial least squares discriminant analysis (PLS-DA) was also performed using the same normalized sequence count dataset throughout the grazing trial (Fig. 3.3A) and individually for the 1, 2, 14, and 28-day sampling dates (Fig. 3.3B). For the overall PLS-DA, three components were used to maximize the amount of cumulative variance explained (88.1%) by the analysis. The Pre, Max-Q and E+ steers all formed distinctly separated clusters, with the Max-Q and E+ clusters being more similar, yet remaining separated across the third component (Fig. 3.3A). Within sampling dates post-pasture assignment, steers grazing Max-Q and E+ tall fescue formed two distinct clusters that separated primarily across the first principal component, with the second component contributing to clustering only in the 14-day E+ (Fig. 3.3B) and 28-day Max-Q (Fig. 3.3B) steers. The distinct PLS-DA clustering and separation between the Pre, Max-Q and E+ steers is indicative of rapid changes of the Angus steers' fecal microbiota of that occur after 1

day of tall fescue grazing, with the Max-Q and E+ steers developing and maintaining distinct fecal microbiota profiles.

Sampling date-specific PLS-DA loadings plots were generated to assess the top 50 loading weights (i.e., OTUs; Fig. S3.5). The loadings of the first component were used, as the samples separated mainly across the first component. The families *Ruminococcaceae* and *Lachnospiraceae* (all dates), *Bacteroidaceae* and *Mogibacteriaceae* (1, 2 and 14 days), *Erysipelotrichaceae* and *Coriobacteriaceae* (1, 2 and 28 days), and *Prevotellaceae* and *Clostridiaceae* (1, 14 and 28 days) were the main contributors to the explained variance (Table S1). The candidate family *S24-7* (1 and 2 days) and the *Lactobacillaceae* family (14 and 28 days) were also important to the loading weights (Table S3.1). Finally, several unclassified families were also drivers of the treatment separation seen in the PLS-DA (Table S3.1).

Linear discriminant analysis of effect size (LEfSe) identified numerous bacteria with significantly different abundances between Pre, Max-Q and E+ steers (Fig. 3.4). The abundances of the phyla *Planctomycetes*, *Lentisphaerae*, *Elusimicrobia*, *Chloroflexi*, and *Proteobacteria* were increased in E+ steers (Fig. 3.4). Further, a number of genera within the *Lachnospiraceae*, *Ruminococcaceae*, and *Erysipelotrichaceae* families were also increased in E+ steers (Fig. 3.4). Moreover, we found that overall abundance of bacteria in the *Actinobacteria* phylum was greater in Max-Q steers, but the *Coriobacteriaceae* family within this phylum was more abundant in E+ steers (Fig. 3.4). This suggests that suborder bacteria abundance may be affected specifically, even if the cumulative abundance of all sequences within the phyla are affected differently. Although treatment differences became even more apparent at lower taxonomic levels (i.e., order, family, and genus; Fig. S3A, S3B, and S3C), the LEfSe analysis also identified significant differences at the phylum level for some of the lower abundance phyla (Fig. 3.4).

Specific microbiome (OTUs) changes after E+ grazing

A total of 25, 41, 55, and 37 OTUs were significantly ($P < 0.05$) different between Max-Q and E+ after 1, 2, 14, and 28 days of grazing, respectively. Of these, 1 OTU overlapped between days 1 and 2, 1 OTU overlapped between days 2 and 14, and 4 OTUs overlapped between days 14 and 28 (Fig. S3.5). The significantly different OTUs that overlapped between sampling dates include those classified to the families *Erysipelotrichaceae* (between 1 and 2 days), *Bacteroidaceae* (1 and 14 days), *Coriobacteriaceae* (1 and 28 days), *Mogibacteriaceae* (2 and 14 days), and *Lactobacillaceae* and *Prevotellaceae* (14 and 28 days). *Lachnospiraceae*, *Ruminococcaceae*, plus four unclassified families overlapped across all sampling dates (Fig. 3.5).

To check for potential paddock effects within fescue cultivar, heat maps were generated from the arcsine normalized relative abundance data used for OTU comparisons by fescue treatment (Fig. S3.6). Overall, the heat maps for significantly ($P < 0.05$) different OTUs demonstrate OTU differences between Max-Q and E+ steers that are consistent across the three paddocks within treatment, indicating no apparent paddock effects (Fig. S3.6).

We then sought to determine if there were specific OTUs associated with the effects of E+ grazing on weight gain and obtain some preliminary information to that effect. We found that the majority of OTUs that differed between the most and least affected E+ steers belonged to the order *Clostridiales* (Fig. S3.7). For example, OTUs from the *Lachnospiraceae* genera *Butyrivibrio* and *Clostridium* had higher sequence counts in weight-gaining E+ steers, whereas the genus *Blautia* and two OTUs belonging to the genus *Ruminococcus* were increased in the most susceptible steers (Fig. S3.7). *Clostridium* genus OTUs were all increased in the susceptible steers. Of those OTUs not in the order *Clostridiales*, the genus *Akkermansia* in the family

Verrucomicrobiaceae were increased the most in susceptible steers, while the *Coriobacteriaceae* genus *Atopobium* had higher sequence counts in E+ resistant steers (Fig. S3.7).

Correlation analysis between OTUs relative abundance and pathophysiological responses

The relative abundance of several OTUs, referred to herein just as OTUs, correlated negatively with average daily gains (ADG) in E+ steers; the most frequently correlating families are presented in Table 1. A total of 11, 7, 4, and 3 OTUs that correlated with ADG were classified to, respectively, *Lachnospiraceae*, *Ruminococcaceae*, *Coriobacteriaceae*, and *Erysipelotrichaceae* families (Table 3.1). *Ruminococcus* was the only genus of the *Ruminococcaceae* family that had OTUs negatively correlated with ADG (4 OTUs; Table 3.1). The *Olsenella*, *Atopobium*, and *Adlercreutzia* genera, all within the *Coriobacteriaceae* family, had OTUs negatively correlating with weight gain (Table 3.1). Finally, the *Erysipelotrichaceae* family had three negatively correlating OTUs classified at the genus level, with two and one OTUs classified to the, respectively, *Bulleidia* and the candidate *p-75-a5* genera (Table 3.1).

Of the OTUs that positively correlated with respiration rates (RR), a total of 11, 6, 4, and 3 OTUs classified into, respectively, the families *Ruminococcaceae*, *Lachnospiraceae*, *Victivallaceae,* and candidate family *S24-7* (Table 3.1). The classified genera within the *Ruminococcaceae* included the *Clostridium* (2 OTUs), *Oscillospira* (1 OTU), and *Ruminococcus* (1 OTU). Further, *Dorea*, *Coprococcus*, and *Blautia* were the classified *Lachnospiraceae* genera, all with 1 OTU positively correlating with RR (Table 3.1). Most OTU relative abundances that positively correlated with RR within the *Ruminococcaceae* and *Lachnospiraceae* were increased in E+ steers.

For rectal temperature, the top four most frequent correlates had a total of 18, 4, 2, and 2 OTUs classified to the families *Lachnospiraceae*, *Ruminococcaceae*, *Coriobacteriaceae*, and

Paraprevotellaceae, respectively (Table S3.2). The genera within the family *Lachnospiraceae* included *Butyrivibrio*, *Roseburia*, *Coprococcus*, and *Dorea*, with *Butyrivibrio* having two OTUs while all other genera had one (Table S3.2). One *Coriobacteriaceae* OTU was classified as *Enterococcus casseliflavus*, and the two *Paraprevotellaceae* OTUs were classified into the candidate genera *CF231* and *YRC22* (Table S3.2).

Co-occurrence network inference analysis

Using the CoNet app within Cytoscape, we conducted a differential network analysis that resulted in one large, highly intraconnected and positively correlated cluster of OTUs within E+ steers. The anchors, defined here as the most highly connected nodes, were from OTUs within the phyla *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria* (Fig. 3.6). Members of the families *Lachnospiraceae*, *Ruminococcaceae*, *Prevotellaceae*, and *Paraprevotellaceae* were the most prevalent families within the E+ network (Fig. 3.6). The other highly connected families in the E+ network included the *Clostridiaceae*, *Mogibacteriaceae*, *Coriobacteriaceae*, *Succinovibrionaceae* and *Staphylococcaceae*. These data indicate that E+ grazing results in a distinct pattern of bacterial families and genera that are highly associated with one another.

DISCUSSION

Here, we describe the fecal microbiota of Angus steers grazing tall fescue, thereby increasing our current understanding of potential mechanisms underlying one of the costliest diseases to the United States beef industry, fescue toxicosis (Klotz, 2015; Klotz and Smith, 2015). Lowered weight gains are an economic driver of production losses caused by ergot alkaloids found in E+ tall fescue and are a common finding in FT studies (Filipov et al., 1999; Hoveland, 1993; Klotz, 2015). Similarly, we found that after a 28-day grazing trial, E+ steers had significantly lower cumulative and average daily weight gains than steers grazing the non-toxic

Max-Q cultivar. Urinary ergot alkaloid levels followed similar trends, as in previous studies (Ayers et al., 2009; Hill et al., 2000; Stuedemann et al., 1998), but excreted levels were substantially higher than a fall study of similar duration (Mote et al., 2017), supporting reported seasonal variability in urinary ergot alkaloid levels (McCulley et al., 2014; Rogers et al., 2011; Rottinghaus et al., 1991).

In our study, respiration rates (RR) were more sensitive to E+ grazing than rectal temperatures (RT) under spring thermoneutral conditions and were significantly higher after 14 days of E+ grazing, consistent with an acute ergot alkaloid challenge study (Browning and Leite-Browning, 1997). Effects of E+ on RT are dependent on E+ exposure duration and environmental conditions (Strickland et al., 2009), which here were thermoneutral. Thus, based on urinary ergot alkaloid levels, decreased weight gains and physiological responses (increased RR), steers in this study exhibited classic signs of FT.

Our analysis of the fecal microbiota throughout the grazing period revealed that, at the phylum level, most sequences aligned to *Firmicutes* (53-59%) and *Bacteroidetes* (33-37%). These data indicate that the grazing Angus beef cattle fecal microbiota, at this phylogenetic level, is in line with what is reported for other beef steers (Shanks et al., 2011), poultry (Pan and Yu, 2014), swine (Isaacson and Kim, 2012), and dairy cattle (de Oliveira et al., 2013). Therefore, it was not surprising that most compositional differences between the E+ and Max-Q grazing steers we found began at the suborder level.

By using dimension reduction analyses (PLS-DA and PLS-DA loadings), we were able to identify a number of OTUs that contributed to the differences in the fecal microbiota profiles of Max-Q and E+ steers at individual dates throughout the grazing trial. Interrogation of the data using these analyses also allowed identification of common bacterial families that had a

significant response to E+ grazing. Further, the OTUs identified by these analyses, and the subsequent taxonomic identification of these OTUs, supports the findings of the other analyses presented herein. Many of the OTUs and bacterial families that were identified as contributing to the differences observed between Max-Q and E+ steers in our dimension reduction analyses were also identified as being significantly influenced by E+ grazing and significantly correlated to pathophysiological signs of interest within the context of FT (e.g., lowered weight gains). This data lends support to the results of the PLS-DA in selecting features that can differentiate fescue treatments. These analyses allowed us to identify the relationships between the data for each group (i.e., Pre, Max-Q, and E+), with both Max-Q and E+ fecal microbiota profiles being distinct from their fecal microbiota prior to fescue pasture placement, yet responding to fescue treatment in an endophyte-dependent manner, as the plants used in the study are genetically identical. Further, the differential network analysis revealed a subnetwork of highly correlated OTUs, mostly belonging to families found to drive treatment separation in the PLS-DA analysis, that were specific to E+ grazing steers, indicating the toxic endophyte not only shifts the fecal microbiota profile, but that this shift also results in a highly correlated structure of bacteria that have a mutualistic relationship.

Our findings indicate that, irrespective of the type of endophyte present, tall-fescue grazing resulted in a marked shift in the fecal microbiota, reflecting the selective pressure placed by fescue grazing. For example, we found one OTU specifically affected by tall fescue grazing (discussed below), *Ruminococcus bromii*, which accounted for 35% of the *Ruminococcus* sequences prior to placement of the animals on the respective fescue pastures. Upon pasture placement and throughout the grazing trial, the sequence abundance of this OTU in the fecal microbiota of both E+ and Max-Q steers was negligible. Of note, this is a known dominant

community member of cattle on a barley diet (Klieve et al., 2007) and the steers prior to fescue pasture placement were fed mixed diets that contained some barley. The sharp decline of this OTU in the fescue grazing steers is a likely indicator that the utilization of resistant starch from the fescue plant is not dependent on this bacterium, which is a well-known degrader of resistant starches (Ze et al., 2012), and that the animals likely relied on other bacteria instead.

Most bacterial OTUs that were significantly affected by E+ grazing classified into the *Ruminococcaceae* and *Lachnospiraceae* families, which was more prominent later in the grazing trial. The relative abundances of the *Lachnospiraceae* OTUs increased up to 8-fold in E+ steers, whereas the number of significantly increased *Ruminococcaceae* OTUs in E+ steers increased with time. Both families include cellulose and hemicellulose degrading bacteria and members contributing to butyrate production (La Reau et al., 2016; La Reau and Suen, 2018; Zhang and Davies, 2016). Butyrate, an important energy source for ruminants, has been shown to influence energy expenditure (den Besten et al., 2015; den Besten et al., 2013), modulate nuclear receptor activity and interact with free fatty acid receptors via the sympathetic nervous system to increase glucagon-like peptide 1 (GLP-1) and peptide YY (PYY) secretion from enteric L cells (Larraufie et al., 2017; Lin et al., 2012; Tolhurst et al., 2012). However, the influence of butyrate on PYY production has been recently shown to be species-dependent *in vitro* (Larraufie et al., 2018). Nonetheless, if excess butyrate reaches gastric receptors, the result would be reduced gastric emptying, suppressed gut motility, and increased satiety (Conterno et al., 2011), potentially modulating host metabolism and feeding behavior in FT via the enteroendocrine system (Holzer and Farzi, 2014; Sternini et al., 2008). Although we did not directly measure butyrate levels herein, elucidating the short-chain fatty acid profile and its interactions in the hindgut of tall fescue grazing beef steers is important for future, more detailed studies.

We also found that certain E+ steers were relatively resistant to E+ grazing effects (i.e., lowering of weight gain), while others were more susceptible. There were also a select few OTUs that were differentially abundant between the "resistant" and "susceptible" steers, indicating a potentially epistatic microbiota profile as it relates to productivity. While these data are limited and preliminary, the role that gut bacteria play in susceptibility versus resistance to lowered weight gains caused by E+ grazing is worthy of future exploration as it might lead to possible microbiota-directed treatments for FT.

Among the other notable effects was an increase in the sequence abundance for the genus *Oscillospira* in E+ steers after 14 and 28 days of grazing. One previous study has associated colonic *Oscillospira* presence with lowered ADG in crossbred steers (Myer et al., 2015). *Oscillospira* is a genus commonly found in both human and bovine gut microbiota (Goodrich et al., 2014; Stevenson and Weimer, 2007), and is known to degrade host glycans in multiple species (Kohl et al., 2014), thus altering glycoprotein homeostasis. For FT, the metabolic activity of *Oscillospira* may contribute to the lowered weight gains as its increased presence in the human gut correlates with lower body weights (Konikoff and Gophna, 2016). In addition to glycan breakdown, *Oscillospira* has also been inversely associated with plasma acetate levels (Org et al., 2017), a VFA that is a main energy metabolite in ruminants.

The abundance of the family *Erysipelotrichaceae* significantly decreased after 1 and 2 days of grazing and was a major early driver of the E+ and Max-Q separation seen within the PLS-DA. Notably, in humans, members of the *Erysipelotrichaceae* have recently been associated with host lipid metabolism (Kaakoush, 2015; Martinez et al., 2013). Potential relationships between the *Erysipelotrichaceae* family and enteric leucine-rich repeat kinase 2 (LRRK-2) activity (LRRK2; Maekawa et al., 2017) and IgA production (Kaakoush, 2015) have been

suggested, indicating that this family of bacteria might be involved in both the regulation of lipid metabolism and enteric nervous and immune systems functioning. Disruption of lipid homeostasis is a feature of FT; dyslipidemia, as well as suppressed serum cholesterol and triglycerides, have all previously been associated with FT (Mote et al., 2017; Nihsen et al., 2004). The early decreases in the *Erysipelotrichaceae*, along with the previously reported decreases in enzymes associated with lipid metabolism in FT (e.g., lipase; Brown et al., 2009; Oliver, 1997), could potentially contribute to the dyslipidemia.

Significant shifts in the families *Clostridiaceae* (increases after 14 days) and *Prevotellaceae* (decreases after 14 and 28 days), as well as increases in the *Planctomycetes* family *Pirellulaceae*, the *Chloroflexi* phylum, and the *β-Proteobacteria* order *Burkholderiales* could all reflect ergot alkaloid-induced selection pressure. The *Chloroflexi*, *Pirellulaceae*, and *Burkholderiales* from the earthworm *Eisenia fetida* have members previously reported as capable of degrading ergovaline, the main ergopeptine alkaloid in E+ tall fescue (Perumbakkam et al., 2007). Also, *ex vivo* ruminal sampling of tryptophan-utilizing bacteria demonstrated that *Prevotellaceae* are not heavily involved in ergovaline degradation, while *Clostridiaceae* strains degrade the majority of ergovaline (Harlow et al., 2017). Although ergot alkaloids are quickly metabolized in the rumen (Ayers et al., 2009), the changes identified herein potentially reflect either ruminal ergot alkaloid selection pressure, as about 30% of OTUs are shared between the rumen, small intestine, and fecal samples (de Oliveira et al., 2013), or, alternatively, ergot alkaloid bioavailability in the lower GI tract.

The genus *Lactobacillus*, which had OTUs with decreased abundances as the study progressed, was one of the main PLS-DA separation contributors and is known to produce indole metabolites (Zhang and Davies, 2016). Recently, we reported that E+ grazing increased plasma

indole metabolites (Mote et al., 2017) and the results here indicate that these increases are likely not *Lactobacillus*-derived; rather, we posit that breakdown of ingested ergot alkaloids and/or microbiota-related tryptophan metabolism by other bacteria, such as ruminal *Clostridium sporogenes* and/or *Clostridium sticklandii* (Harlow et al., 2017), are likely partially responsible for the plasma indole metabolite increases we previously reported (Mote et al., 2017).

The bacterial families that positively correlated with RT were similar to the ones negatively correlated with ADG in E+ steers, with the *Lachnospiraceae* family OTUs being a major one. The enteric microbiota can influence thermogenesis by altering lipid availability and absorption (Nicholls et al., 2016) and thermoregulation is impaired in FT (Strickland et al., 2009). Although these data are preliminary, the contribution of the enteric microbiota to increased RT is unclear, yet worthy of more detailed investigation. Further, while E+ steers' RT fluctuated concomitantly with environmental conditions, it was not affected by E+ grazing to a major extent as our study was conducted under thermoneutral conditions. This suggests that the relationship between RT and E+ grazing should be investigated further under more extreme environmental conditions (e.g., high THI).

Finally, our study also determined correlations between the fecal microbiota and RR. To our knowledge, there are no current data establishing associations between fecal microbiota shifts and changes in pulmonary physiology in food-producing animals. However, there is increasing evidence of communication between the gut and lung mucosa, and respiratory microbiota influencing lung mucosa immunological homeostasis has been shown in both humans and rodents (Noverr et al., 2005; Noverr et al., 2004; Segal et al., 2013; Segal et al., 2014). The correlations between RR and fecal microbiota members were strong, and, considering the effects

of E+ grazing on RR and the potential for gut-lung mucosal crosstalk, are worthy of future investigation.

Overall, our study contributes to an increased understanding of how the fecal microbiota functions in the overall health of fescue grazing beef cattle. Our work also provides insights into the influence of E+ grazing on the Angus steer fecal microbiota and it suggests that E+ grazing has a significant, rapid impact on the fecal microbiota, predominantly at the family/genus levels. Likely, microbiota-derived metabolites will also be affected. These key changes potentially modulate energy expenditure, metabolic homeostasis, and feeding behaviors manifested in FT-related signs, such as dyslipidemia, shortened grazing periods, decreased feed intake and lowered weight gains. However, additional targeted investigations are necessary to interrogate pathophysiological changes that result from microbiota dysbiosis induced by E+ grazing and determine if/which microbiota changes are the result of, or contribute to, the development of FT.

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Table 3.1. Top classified bacterial families and genera that significantly negatively correlated with average daily gains and significantly positively correlated with respiration rates. Spearman's average correlations and P-values are presented.

Average Daily Gains				
Family	Genus	No. of OTUs	Correlation	P-value
Lachnospiraceae		11	-0.50	0.036
Ruminococcaceae		7	-0.55	0.020
	Ruminococcus	4	-0.54	0.024
Coriobacteriaceae		4	-0.56	0.021
	Olsenella	1	-0.65	0.004
	Atopobium	1	-0.57	0.013
	Adlercreutzia	1	-0.48	0.045
Erysipelotrichaceae		3	-0.53	0.025
	Bulleidia	2	-0.54	0.020
	p-75-a5	1	-0.51	0.030
Respiration Rates				
Ruminococcaceae		11	0.56	0.022
	Clostridium	2	0.58	0.024
	Oscillospira	1	0.53	0.024
	Ruminococcus	1	0.51	0.024
Lachnospiraceae		6	0.52	0.027
	Dorea	1	0.58	0.011
	Coprococcus	1	0.53	0.023
	Blautia	1	0.52	0.028
Victivallaceae		4	0.52	0.026
S24-7		3	0.58	0.018

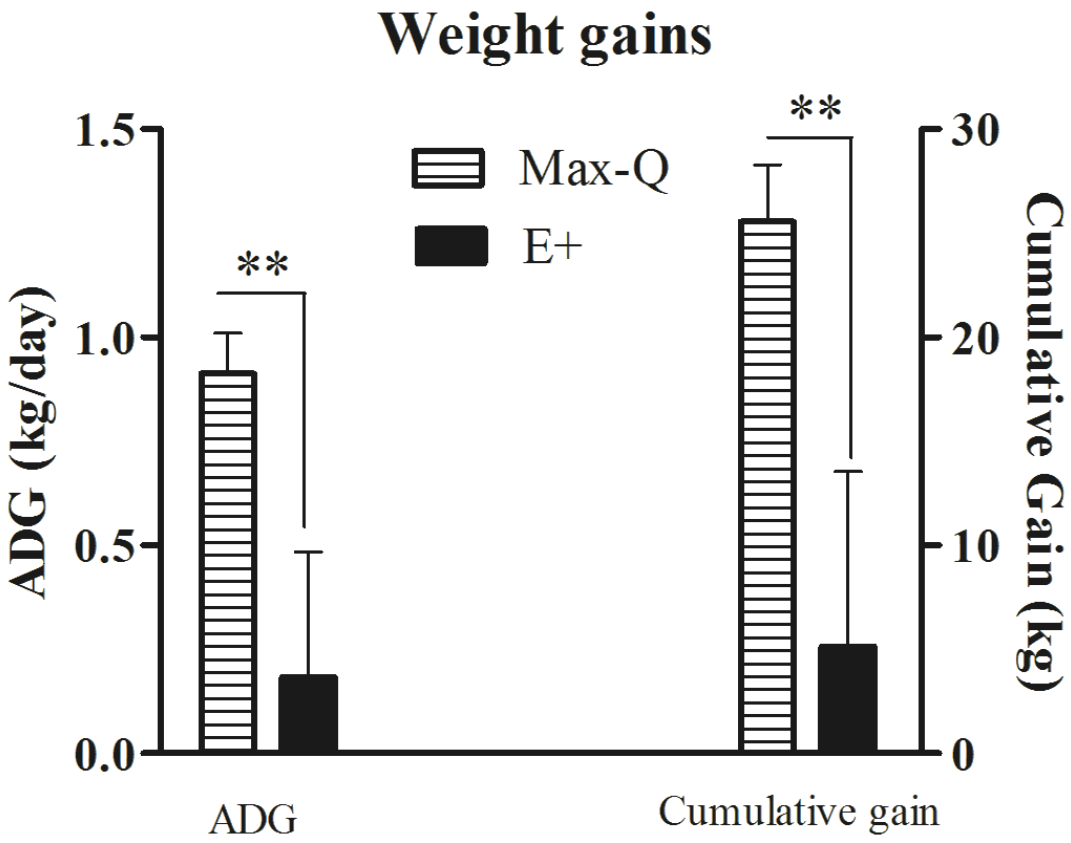

Figure 3.1. Average daily gain (ADG; kg/day) and cumulative gain (kg) in the weight of Angus steers that grazed either a non-toxic endophyte-infected (Max-Q; n = 6) or toxic endophyte-infected (E+; n = 6) tall fescue for a 28-day grazing trial. (**) indicates a significant difference between treatment groups ($P < 0.01$).

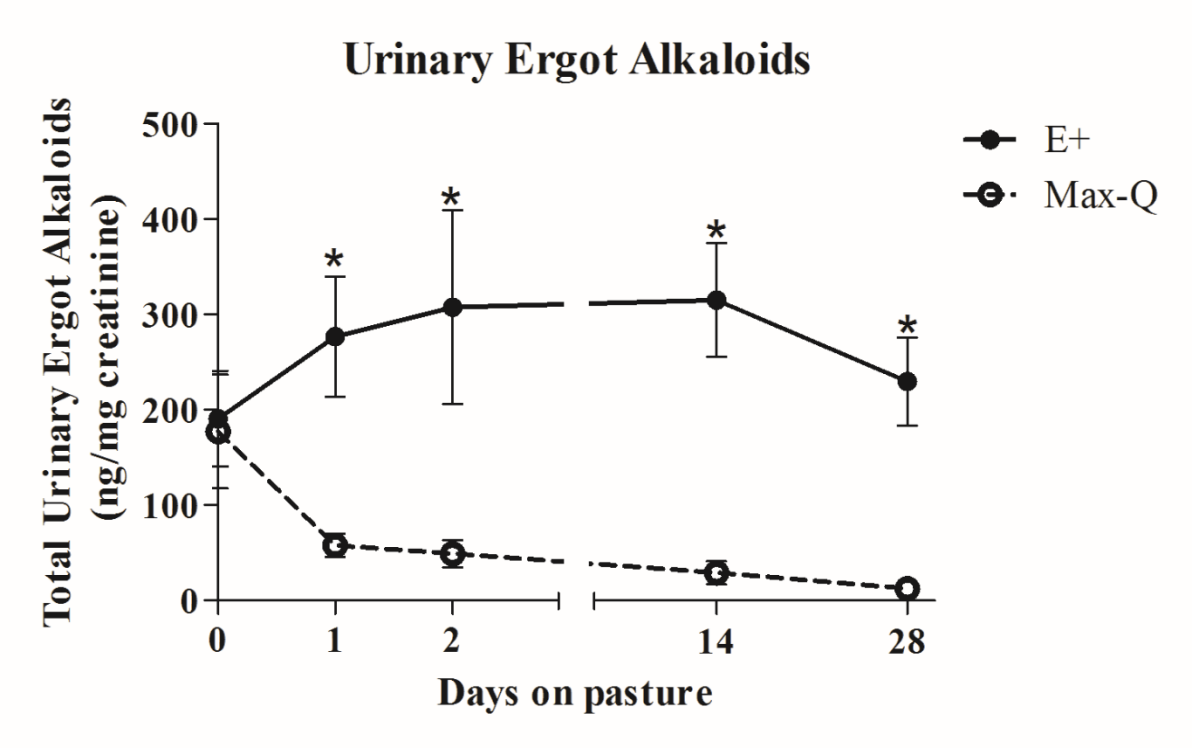

Figure 3.2. Total ergot alkaloids (EA) in the urine of Angus steers before pasture placement (Day 0) and after 1, 2, 14, and 28 days of grazing either a non-toxic endophyte-infected (Max-Q; n = 6) or toxic endophyte-infected (E+; n = 6) tall fescue. Data are presented as ng/mg creatinine. (*) indicates significant difference between treatment groups within a sampling date ($P < 0.05$).

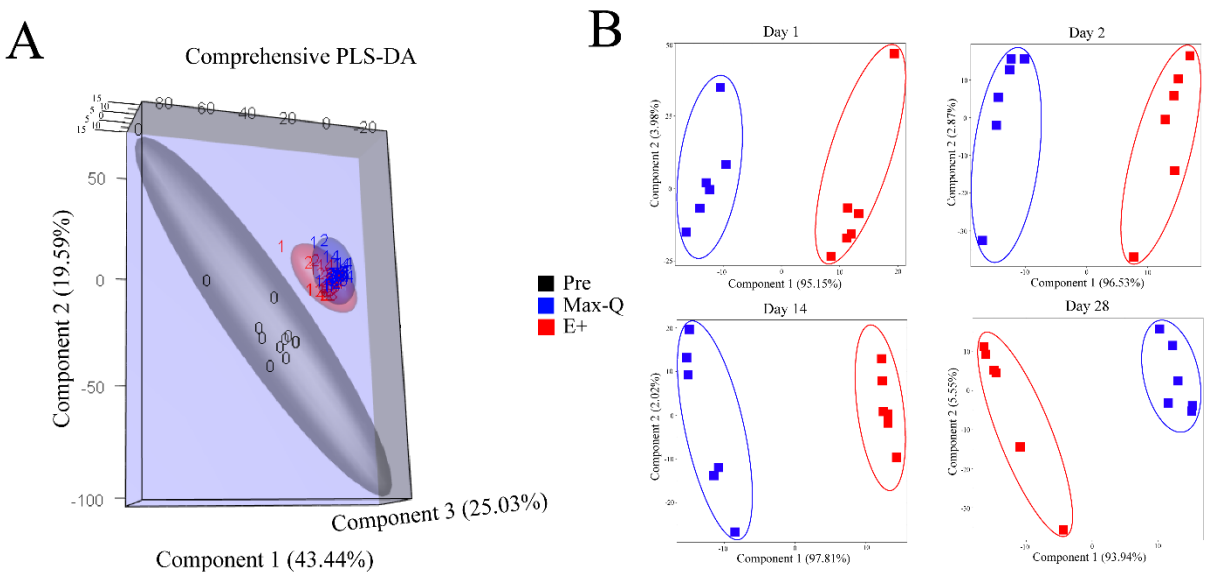

Figure 3.3. Partial least squares discriminant analysis (PLS-DA) plots of the fecal microbiota of Angus steers grazing either a non-toxic (Max-Q; n = 6) or toxic (E+; n = 6) endophyte-infected tall fescue (A) over the 28-day grazing trial or (B) 1, 2, 14, and 28 days post-pasture assignment.

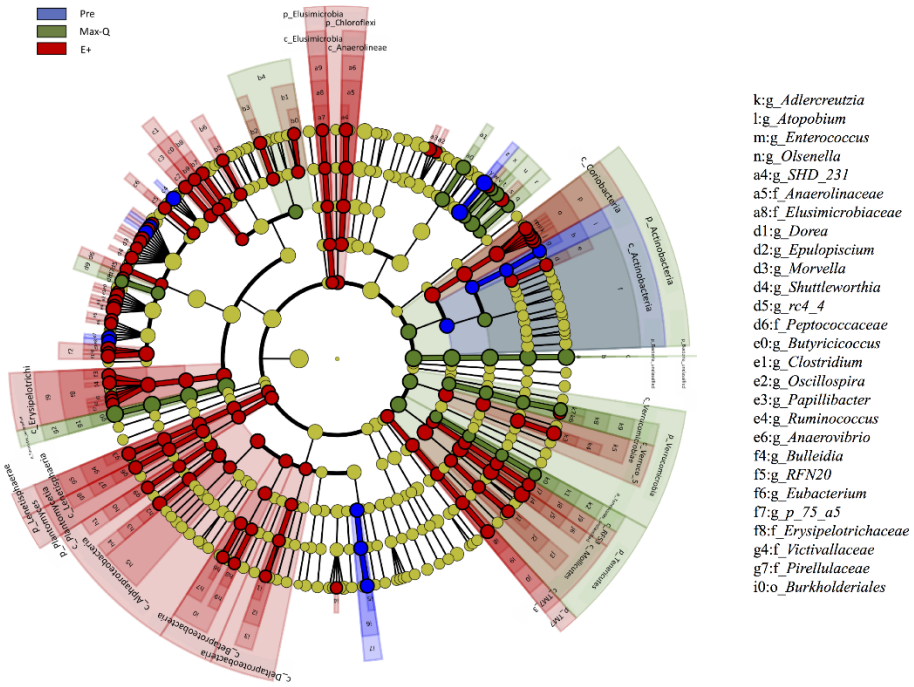

Figure 3.4. Linear discriminant analysis (LDA) effect size (LEfSe; Kruskall-Wallis [P < 0.05]; Pairwise Wilcoxon [P < 0.05]; logarithmic LDA score > 2.0) of the fecal microbiota of Angus steers before (Pre) placement or across a 28-day grazing trial after placement on either a non-toxic (Max-Q; n = 6) or toxic (E+; n = 6) endophyte-infected tall fescue. Blue, green, and red shading indicates greater abundance in Pre, Max-Q, or E+ steers, respectively. Taxonomic rank labels are provided before bacterial names: "p_; c_; o_; f_; g_" indicate phylum, class, order, family, and genus, respectively. Letters and numbers within the cladogram refer to bacterial names located in the key to the right of the cladogram.

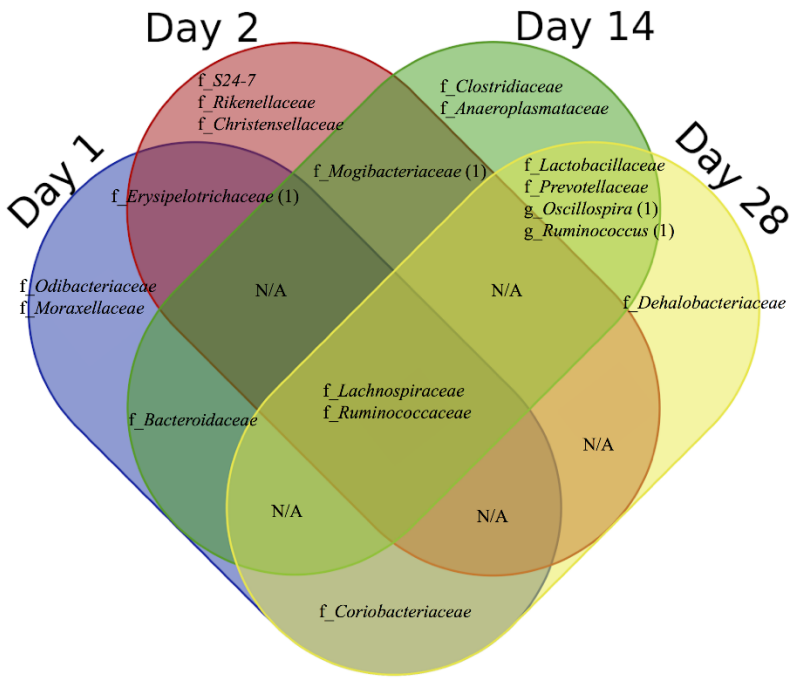

Figure 3.5. Venn diagram demonstrating classified bacterial families and genera having OTUs that were significantly different between steers grazing either a non-toxic (Max-Q; n = 6) or toxic (E+; n = 6) endophyte-infected tall fescue throughout a 28-day grazing trial. "f_" indicates bacterial families and "g_" indicates bacterial genera. The number indicated in parentheses following the family or genus name represents specific OTUs within that family or genus that overlap between sampling dates.

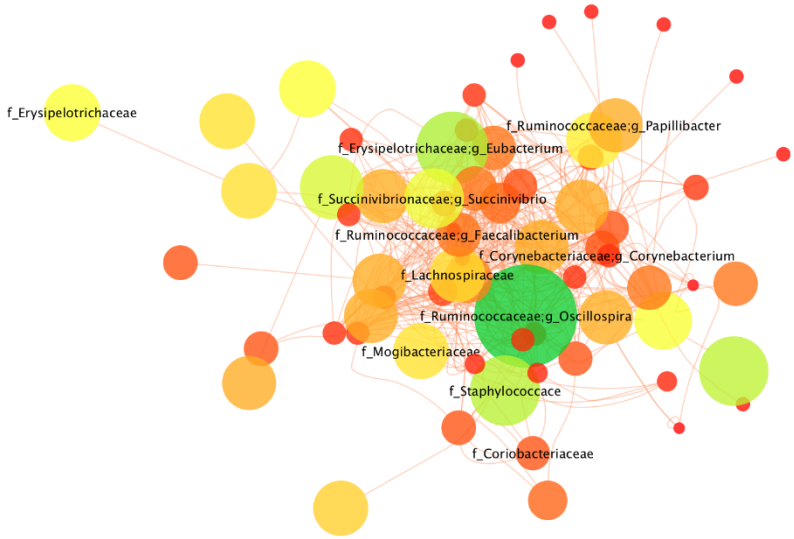

Figure 3.6. Co-occurrence network inference analysis of the fecal microbial communities of steers grazing toxic endophyte-infected tall fescue (E+; n = 6) after removal of nodes shared with the network of steers grazing a non-toxic (Max-Q) endophyte-infected tall fescue. Node size is reflective of overall OTUs abundance; node labels are the furthest phylogenetic classification of each node. Networks were generated using the CoNet app for Cytoscape.

CHAPTER 4

TOXIC TALL FESCUE GRAZING INCREASES THE SUSCEPTIBILITY OF THE ANGUS STEER FECAL MICROBIOTA AND PLASMA/URINE METABOLOME TO ENVIRONMENTAL EFFECTS

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ABSTRACT

Impaired thermoregulation and lowered average daily gains (ADG) result when livestock graze toxic endophyte (*Epichloë coenophiala*)-infected tall fescue (E+) and are hallmark signs of fescue toxicosis (FT), a disease exacerbated by increased temperature and humidity (+temperature-humidity index; +THI). We previously reported FT is associated with metabolic and microbiota perturbations under thermoneutral conditions; here, we assessed the influence of E+ grazing and +THI on the microbiota:metabolome interactions. Using high-resolution metabolomics and 16S rRNA gene sequencing, plasma/urine metabolomes and the fecal microbiota of Angus steers grazing non-toxic or E+ tall fescue were evaluated in the context of +THI. E+ grazing affected the fecal microbiota profile; +THI conditions modulated the microbiota only in E+ steers. E+ also perturbed many metabolic pathways, namely amino acid and inflammation-related metabolism; +THI affected these pathways only in E+ steers. integrative analyses revealed the E+ microbiota correlated and co-varied with the metabolomes in a THI-dependent manner. operational taxonomic units in the families *Peptococcaceae*, *Clostridiaceae*, and *Ruminococcaceae* correlated with production parameters (e.g., ADG) and with multiple plasma/urine metabolic features, providing putative FT biomarkers and/or targets for the development of FT therapeutics. Overall, this study suggests that E+ grazing increases Angus steer susceptibility to +THI and offers possible targets for FT interventions.

INTRODUCTION

As global consumer interest towards pasture-based finishing production systems for beef cattle rises, a recent study estimated that, in the United States (U.S.), the current beef production capacity could only support about 27% of the current demand (Matthew and Rachael, 2018). As such, a capacity increase of 30% would be required if there were a nationwide transition to grass-

finishing production systems (Matthew and Rachael, 2018). Considering the shift in consumer interest and the contribution of ruminant-derived methane to a warming environment, the detrimental impact of harsh environmental conditions (i.e., heat stress) on animal production efficiency, and the potential environmental impact of the necessary increase of grazing beef herds to meet future agricultural demand, there is an urgent need to optimize animal health and performance under numerous physiological stressors, including those posed by the warming environment.

Tall fescue, *Lolium arundinaceum*, covers 14 million hectares across the Southeastern U.S., and is used widely as a pasture forage in beef production systems. Wild-type tall fescue is commonly infected with the endophytic fungus *Epichloë coenophiala*, which produces secondary metabolites that improve plant vigor, thereby allowing the plant to be more tolerant of external environmental stressors (e.g., grazing, heat stress, insects) (Young et al., 2014). While the endophyte-infected tall fescue provides increased plant biomass (Guo et al., 2015), the endophyte also produces toxic metabolites (e.g., ergot alkaloids) that have been linked to decreases in grazing animal health and performance, leading to a disease called fescue toxicosis (FT), which is exacerbated in harsh climates (Stuedemann et al., 1998; Young et al., 2014). Thus, from an animal perspective, the wild-type endophyte-infected tall fescue is referred to as toxic (E+).

FT has many clinical signs, but the most economically costly, at about $1 billion per year to the U.S. beef industry, are decreased weight gain and reproductive insufficiencies (Thompson and Stuedemann, 1993). Recent studies also estimate heat stress-related average annual economic losses to the U.S. animal agricultural sector at $2.4 billion, with $369 million of this accounted for by the beef industry (St-Pierre et al., 2003). Heat stress, however, does not act

alone, and its negative effects are frequently compounded by underlying illness (Aldrich et al., 1993) and/or malnutrition (Bernabucci et al., 2010). In this regard, important FT pathophysiological changes (e.g., impaired thermoregulatory capacity) become more profound under heat stress conditions (Bhusari et al., 2007; Eisemann et al., 2014; Gadberry et al., 2003; Strickland et al., 2009). This heat stress-FT interaction further complicates the development of production-restoring therapeutic and/or management strategies (Al-Haidary et al., 2001).

Previous FT microbiota studies have focused on the effects of E+ on ruminal bacterial and fermentation profiles (Melchior et al., 2018; Schumann et al., 2008). For example, one study identified ruminal hyper ammonia-producing bacteria capable of degrading the toxic ergot alkaloids (Harlow et al., 2017), indicating that these bacteria may be involved in the extensive metabolism/detoxification of ergot alkaloids occurring in the rumen (Ayers et al., 2009). As ergot alkaloids, the key etiological agents of FT, are heavily metabolized in the rumen, this suggests that ergot alkaloids might affect animal performance indirectly, i.e., through ergot alkaloid metabolites (e.g., lysergic acid) or by targeting other downstream processes responsible for the decreased animal productivity associated with FT. In this regard, we recently found that E+ grazing alters the fecal microbiota, namely affecting the *Ruminococcaceae* and *Lachnospiraceae* bacterial families under thermoneutral conditions, while also leading to a highly correlated structure of hindgut microbiota specific to E+ grazing steers (Mote et al., 2019). Separately, we reported that E+ grazing perturbs the metabolome, under thermoneutral conditions, mainly tryptophan and tyrosine metabolism and it does so in opposing patterns in the plasma and urine (Mote et al., 2017). The influence of E+ grazing on the microbiota-metabolome interaction, or how elevated environmental temperature and humidity might affect the grazing beef microbiota, metabolome, and their interactions, have not been assessed thus far.

Therefore, the goals of this current study were to assess the influence of E+ grazing under thermoneutral and harsher environmental conditions on the fecal microbiota, plasma and urine metabolomes, and the way interact. We hypothesized that E+ grazing results in rapid and lasting perturbations of the plasma/urine metabolomes and fecal microbiota and the microbiota:metabolome interactions. As E+ steers have impaired thermoregulation, we also hypothesized that harsh environmental conditions would compound these perturbations in E+ steers, while having minimal consequential effects non-toxic tall fescue (Max-Q; Jesup Max-Q AR542).

MATERIALS AND METHODS

Animal treatments and environmental conditions

All animal handling and sample collection methods were approved in advance by the Institutional Animal Care and Use Committee of the University of Georgia (A2015 11-004). Post-weaning Angus steers (n = 12; BW = 311.3 ± 5.4 kg) were blocked by weight and randomly assigned to non-toxic (Max-Q; Jesup MaxQ AR542; 3 paddocks, 2 steers per paddock) or toxic (E+; Jesup with wild-type endophyte; 3 paddocks, 2 steers per paddock) tall fescue treatments at the J. Phil Campbell Natural Resources Conservation Center of the University of Georgia (Watkinsville, GA) in the late Spring and early Summer of 2016 (May 11, 2016 – June 6, 2016). The temperature-humidity index (THI) was used as a predictor of heat stress risk and was calculated from temperature and humidity measurements as previously described (Kibler, 1964). Briefly, THIs were calculated using the equation: $THI = 1.8 * T - \left(\frac{100-RH}{100}\right) * (T - 14.3) + 32$; where *T* is the ambient temperature in degrees centigrade and *RH* is the percent relative humidity. Samples were collected before and at 1, 2, 12 (low THI; -THI), 16 (high THI; +THI), 20 (+THI), and 26 (-THI) days post pasture assignment. Low THI (no heat stress risk) dates

were defined as THI < 72 on two successive dates, and high THI (mild-to-moderate heat stress risk) was defined as THI > 72 for two successive dates with sample collections occurring in the afternoon on the second day. Temperature and humidity measurements were recorded from 8:00 AM - 8:00 PM daily and across sampling times (8:00 - 11:00 AM for -THI dates, 12:00 - 4:00 PM for +THI dates).

Date selection for assessment of environmental influences

To assess the effects of E+ grazing and the THI on the microbiota, metabolome, and the microbiota:metabolome interactions, Day 12 (lowest -THI) and Day 20 (highest +THI) sampling dates were used as representative dates to assess potential effects of different ambient environmental conditions (i.e., -THI vs +THI) on Max-Q and/or E+ grazing steers. Further, these two dates were selected as the urinary ergot alkaloid (UEAs) levels, which have been used as an indicator of ergot alkaloid exposure (Hill et al., 2000), were significantly higher in E+ than Max-Q steers on Day 12 and Day 20. Thus, Day 12 and Day 20 were used in our microbiota, high-resolution metabolomics (HRM), and the microbiota:metabolome integrative analyses to assess E+:THI interactive effects. These analyses are described later in this study.

Urinary ergot alkaloid analysis

Total UEAs, a sensitive biomarker of exposure to E+ (Hill et al., 2000), were determined at 1, 2, 12, 16, 20 and 26 days post-pasture placement via ELISA (Agrinostics Ltd. Co., Watkinsville, GA) as previously described (Hill et al., 2000; Mote et al., 2017; Stuedemann et al., 1998).

Sample collection and processing

Steer body weights were recorded before 16, 20 and 26 days post pasture assignment with a digital scale. Fresh fecal samples were collected by hand using new gloves for every

collection. Plasma was collected via jugular blood draw and voided urine was collected in freshly cleaned cups. Plasma, urine, and fecal matter were stored on ice for transport to the lab and long-term storage at -80 °C. Fecal, plasma, and urine samples were taken before (Pre) and at 1, 2, 12, 16, 20, and 26 days post pasture assignment.

DNA extraction

Fecal genomic DNA was extracted using a mechanical disruption and phenol extraction protocol established in (Stevenson and Weimer, 2007) with a 25:24:1 phenol:chloroform:isoamyl alcohol modification (Dill-McFarland et al., 2017). All DNA samples were resuspended in TE buffer and quantified using a Qubit® Fluorometer (Invitrogen, San Diego, CA). For each set of DNA extractions, a negative control (TE extraction buffer) was included alongside each extraction and taken through the amplification and sequencing protocols described below.

DNA amplification and sequencing

Samples were diluted to 1 ng/µL for amplification, and universal bacterial primers for the 16S rRNA variable region V4 were used in the amplification reaction as described in (Mote et al., 2019). After amplification, samples were equimolar pooled into the final library, and sequenced on an Illumina MiSeq using the 2x250 bp paired end MiSeq v2 sequencing kit (Illumina, San Diego, CA) using custom primers (Kozich et al., 2013). The sequenced control and sample DNA were subjected to quality filtering and normalization procedures described next.

16S rRNA gene fecal sequence processing and bioinformatics analysis

Raw sequence files were processed using mothur v.1.38.1 (Schloss et al., 2009) as in (Mote et al., 2019). After quality filtering, unique sequences were aligned to the SILVA version 119 reference alignment database (Pruesse et al., 2007) and chimeras were removed

(http://drive5.com/uchime). Bacterial sequences were classified using the Greengenes database v13.8 ((http://greengenes.secondgenome.com) DeSantis et al., 2006). Good's coverage was calculated in mothur, and the operational taxonomic units (OTU) were normalized for sequence depth and abundance filtered (> 0.1% abundance; >50% of within-date samples) prior to statistical analysis. Community diversity was estimated using Chao1 richness (Chao, 1984) and Simpson diversity index (Simpson, 1949); alpha diversity metrics were tested for E+ grazing and time effects utilizing the non-parametric Kruskal-Wallis test by ranks. The non-parametric permutational analysis of variance (PERMANOVA) was used to test for E+ grazing effects on the entire microbial community using both Bray-Curtis (abundance) and Jaccard (presence/absence) distance matrices, with fescue treatment and time spent grazing set as the two factors alongside the treatment by time interaction term. The same analysis was performed on day 12 (-THI) and 20 (+THI) samples using fescue treatment and THI as the two factors. Sparse principal component analysis (sPCA) and partial least squares discriminant analysis (PLS-DA) were performed, with near zero variance filtering for predictors, using the mixOmics *R* package (Le Cao et al., 2015; R Development Core Team, 2016, https://www.r-project.org/) on the post-sequencing depth normalized OTU table; the sequences were log transformed, mean centered and Total Sum Scale normalized prior to these analyses (Le Cao et al., 2015). Linear discriminant analysis effect size (LEfSe) was performed within the Huttnehower lab's galaxy instance using the relative abundance table (https://huttenhower.sph.harvard.edu/galaxy/; 93), with Kruskal-Wallis ($P < 0.05$), Pairwise Wilcoxon ($P < 0.05$), and logarithmic LDA score (> 2.0). All included OTU analyses presented here were determined at a 97% sequence identity, allowing for genus-level resolution (Roesch et al., 2007), as determined using the furthest

neighbor clustering algorithm. Correlational analysis was performed using Hmisc R package (Harrell, 2018).

High-resolution metabolomics

Metabolomics sample processing was performed similar to that described in (Mote et al., 2017). Briefly, 50 µl of urine or plasma were mixed with 100 µl acetonitrile and 2.5 µl of internal standard, kept on ice (30 min) and centrifuged (10 min at 14,000 rpm). Metabolomics samples (100 µL of the supernatant) were analyzed on the Orbitrap Fusion Mass Spectrometer (Thermo Fisher), with instrument settings at 120,000 resolving power, 5 min runs, and 10 µl injection. Both reverse phase (negative mode) and hydrophilic liquid interaction chromatography (HILIC; positive mode) columns were used for each sample in triplicate. Peak detection, noise filtering, m/z and retention time alignment, feature quantification, and quality filtering were done using xMSanalyzer v2.0.7 with apLCMS v6.1.3 (Uppal et al., 2013; Yu et al., 2009), running all samples (i.e., plasma and urine) simultaneously so any unique feature (i.e., unique *m/z* and retention time) identified would be the same regardless of biological matrix. Data were extracted as HRM features, and the average of three technical replicates were $\log_2$ transformed with a +1 pseudocount and quantile normalized prior to bioinformatics analysis.

HRM data analysis

Feature intensity tables were analyzed in *R*. Circular Manhattan plots were generated using the CMplot R package (LiLin-Yin, 2018). sPCA and PLS-DA were performed using the mixOmics R package v6.3.2., with near zero variance predictor filtering (Le Cao et al., 2015). Pathway analysis was performed using *mummichog* within each date and for the entire grazing trial using the FDR corrected p-value and t-scores as input values, with respective ionization

mode and [M + H] adduct matching (Li et al., 2013); all pathways presented herein are the -log10 mummichog corrected p-value.

HRM and 16S rRNA gene data integration

For analyzing the interaction between the plasma/urine metabolomes and fecal microbiota, procrustes was performed on the Max-Q and E+ data independently (throughout the grazing trial and on Day 12 [-THI] and 20 [+THI]) with the vegan *R* package (Oksanen et al., 2018), using the normalized 16S rRNA and combined plasma and urine metabolomes prior to running the monoMDS command to generate ordination plots. After running the *procrustes* function, the *protest* function was used to perform permutational Monte Carlo simulation to estimate the significance of the microbiota:metabolome correlation. Coinertia analysis (CIA) was performed on the Max-Q and E+ data independently (throughout the grazing trial and on Day 12 and 20) using the ade4 *R* package (Dray and Dufour, 2007) to estimate the covariance between the 16S rRNA OTUs and the plasma and urine metabolic features within a fescue cultivar. The CIA was done after combining the normalized plasma and urine metabolomes into one data frame prior to performing the *dudi.PCA* function. The *cia* function was used to perform the CIA with the OTU duality diagram as the X data matrix and the duality diagram of the HRM features as Y data matrix. sPLS regression was performed using the mixOmics *R* package v6.3.2. (Le Cao et al., 2015), using two components in the canonical mode with the plasma and urine combined HRM datasets, subset to each feature present in at least 50% of samples throughout the trial as the X matrix and the normalized 16S rRNA OTU table as the Y matrix. The Max-Q and E+ datasets were then separated and analyzed independently to assess relationships specific to fescue cultivar; correlation threshold was ($|r| > 0.70$). The resulting sPLS bipartite networks were

saved in .gml format using the igraph *R* package (Csardi and Nepusz, 2006) for Cytoscape v3.6.0 visualization and differential network analysis.

Statistical analysis of non-'omics data

Statistical analyses of weight gains and urinary ergot alkaloids were done with Sigma Plot v12.5 (Systat Software, Inc., San Jose, CA) using two-way ANOVA (as a within-subjects design) with days of sampling and fescue treatment set as the two independent variables. If significant ($P < 0.05$) effects based on treatment or days spent grazing were observed, the Holm-Sidak *post-hoc* analysis was applied to separate significant differences. Graphs were generated with GraphPad Prism 5 (La Jolla, CA).

Accession number(s)

All DNA sequences are publicly available in the NCBI Sequence Read Archive and are accessible under BioProject accession number PRJNA603062.

Transparency document(s)

HRM feature intensity tables have been deposited in Metabolomics Workbench (www.metabolomicsworkbench.org).

RESULTS

Environmental conditions, animal weight gains and UEAs

THI values across sampling times were 69.9 (Day 0; Pre), 71.9 (Day 1), 70.8 (Day 2), 64.0 (Day 12; 1^{st} −THI), 74.3 (Day 16; 1^{st} +THI), 78.7 (Day 20; 2^{nd} +THI), and 74.0 (Day 26; 2^{nd} −THI); of note, rain on sampling Day 26 increased the relative humidity (used in THI calculation), but the temperature on this day was 23.64 °C, similar to day 2 (23.33 °C). E+ grazing steers had significantly ($P < 0.05$) lower average daily (0.5 ± 0.1 kg/day; ADG) and cumulative weight gains (13.9 ± 4.7 kg; CWG) compared to steers that grazed the non-toxic

endophyte-infected tall fescue (Max-Q; [0.9 ± 0.3 kg/day ADG; 23.2 ± 6.9 kg CWG]) after the 26-day grazing period. Prior to pasture assignment (Day 0), the steers had some UEAs (67.9 ± 14.4 ng/mg creatinine), likely due to fescue hay supplementation. After pasture placement, UEAs in E+ animals were significantly ($P < 0.001$) increased beginning at 2 days throughout the remainder of the study (Day 2 = 221.3 ± 53.3, Day 12 = 246.9 ± 56.3, Day 16 = 296.9 ± 29.1, Day 20 = 234.4 ± 31.1, Day 26 = 253.8 ± 38.1 ng/mg creatinine), with Max-Q steers levels precipitously dropping and becoming markedly lower as the grazing trial progressed (Day 2 = 64.4 ± 37.9, Day 12 = 19.9 ± 10.1, Day 16 = 6.5 ± 4.8, Day 20 = 6.1 ± 4.2, Day 26 = 17.9 ± 6.9 ng/mg creatinine).

Microbiota Results

16S rRNA gene sequencing

We generated 3,575,828 raw sequences, which resulted in 1,787,914 high quality sequences after filtering that clustered and aligned into 2,973 OTUs. The average number of paired sequences per sample was 21,285 (range: 7,035 – 60,852) and the average number of populated OTUs per sample was 954 (range: 493 – 1,298). The average Good's coverage for the data set was 98.7 ± 0.6, indicating adequate sequencing depth and coverage to capture most of the species diversity in the samples.

Alpha diversity metrics

Overall, both Chao1 richness and Simpson's diversity had minor fluctuations throughout the trial. The highest diversity occurred between 2 and 16 days of grazing. Sample richness was slightly more variable, with the lowest richness being after 2 and 16 days and the highest richness being after 20 days of grazing. Neither Simpson's diversity ($P = 0.11$) nor Chao1 richness ($P = 0.35$) were affected by fescue treatment. The overall microbiota profile was

significantly affected by fescue treatment and grazing time using both Jaccard (Treatment: $P < 0.01$; Time: $P < 0.001$) and Bray-Curtis (Treatment: $P < 0.01$; Time: $P < 0.001$) distance matrices. When comparing Day 12 (–THI) and Day 20 (+THI), there was a significant effect of treatment and a trend for an effect of THI for both Bray-Curtis (Treatment: $P < 0.001$; THI: $P = 0.06$) and Jaccard (Treatment: $P < 0.001$; THI: $P = 0.14$) distance matrices, without any significant effect on Inverse Simpson's diversity or Chao1 richness measurements ($P > 0.8$).

Microbial data reduction analysis

Partial least squares discriminant analysis (PLS-DA) showed that both Max-Q and E+ steer microbiota profiles shifted from the Pre steers in a distinct manner (Fig. S1A). Further, the samples later in the grazing period had shifted furthest from the Pre steers, with no significant specific shifts on −THI and +THI sampling dates. PLS-DA at each sampling date indicated the Max-Q and E+ steers formed distinct clusters, with the separation and clustering occurring along the first principal component (Fig. S4.1A). Generally, clustering along the second principal component was only seen in Max-Q steers on Day 2, 20, and 26 (Fig. S4.1A) and E+ on day 20 (Fig. S4.1A). Overall, the first component explained >90% of the overall variance, with >99% of the cumulative variance explained when the second component was added for each sampling date (Fig. S4.1A).

From the PLS-DA loadings analysis, classified families that overlapped between sampling dates and had OTUs as drivers of the Max-Q and E+ separation included those in the families *Erysipelotrichaceae*, *Lachnospiraceae*, *Ruminococcaceae* , *Rikenellaceae, Mogibacteriaceae, Coriobacteriaceae, Rikenellaceae* and *Clostridiaceae* (throughout the trial), the *Paraprevotellaceae*, *Peptostreptococcaceae*, candidate family *BS11* (early to midtrial), and

candidate *S24-7* (late in the trial). A detailed list off all bacteria separated by sampling dates can be found in Table S4.1.

Day 12 (-THI) versus Day 20 (+THI) PLS-DA, irrespective of fescue treatment, revealed two distinct clusters, with Component 1 explaining 83.58% and Component 2 explaining 15.40% of the cumulative variance for the analysis (Fig. S4.1B). The top OTU loading weights were aligned to the family *Lachnospiraceae* (6 OTUs), with all other classified families (e.g., *Ruminococcaceae*, *Coriobacteriaceae*, and *Peptostreptococcaceae*) only having 1 OTU.

Similar analyses were performed within E+ to assess the specific effects of THI on the E+ microbiota profile. When comparing Day 12 (-THI) versus Day 20 (+THI), the Day 12 and Day 20 steers formed distinct clusters, with the first component contributing 82.50% and the second component contributing 16.70% of the explained variance (Fig. S4.1B). *Ruminococcaceae* and *Lachnospiraceae* were the two most prevalently classified families that were driving the THI-based separation in E+ steers. Seventeen of the top 50 OTUs driving the separation were unclassified at the order level. *Mogibacteriaceae*, *Clostridiaceae*, *Coriobacteriaceae*, and *Porphyromonadaceae* were among other classified families driving the E+ THI-based separation.

Linear discriminant analysis of effect size (LEfSe)

All effects listed as a result of the LEfSe met the following criteria for significance: Kruskal-Wallis $P < 0.05$; Pairwise Wilcoxon $P < 0.05$; logarithmic LDA score > 2.0. Overall, Max-Q grazing significantly increased the *Lentisphaerae* and *Proteobacteria* phyla (Fig. 4.1A). For the *Actinobacteria*, Max-Q grazing significantly increased the *Coriobacteriaceae* genus *Adlercreutzia*. From the class *Bacilli* within the phylum *Firmicutes*, Max-Q increased the *Planococcaceae* family and the *Bacillus* genus. Max-Q grazing also significantly increased the

genus *Dehalobacterium*, the *Lachnospiraceae* genera *Dorea* and *Blautia*, and the *Ruminococcaceae* genera *Ruminococcus* and *Oscillospira* (Fig. 4.1A). Finally, within the *Bacteroidetes* phylum, the *Rikenellaceae* was significantly increased in Max-Q steers (Fig. 4.1A).

Overall, E+ grazing significantly increased the abundance of the *Firmicutes*, *Chloroflexi*, and *Actinobacteria* phyla. Within the *Proteobacteria*, the genera *Ruminobacter* and *Suttrella* were significantly increased in E+ steers (Fig. 4.1A). Further, E+ grazing significantly increased the candidate genus *SHD-231* (of the family *Anaerolinaceae* and phylum *Chloroflexi*; Fig. 4.1A). For the *Actinobacteria*, E+ grazing increased *Intrasporangiaceae* and the *Coriobacteriaceae* genera *Enterococcus* and *Olsenella* (Fig. 4.1A). The families *Mycoplasmataceae* and candidate *RFP12* of the *Verrucomicrobia* were significantly increased by E+ grazing. From the class *Bacilli* in the phylum *Firmicutes*, E+ grazing increased the genus *Solibacillus* of the family *Planococcaceae*. The *Clostridium* and candidate genera *SMB53* of the family *Clostridiaceae*, the genus *Coprococcus* in the family *Lachnospiraceae*, the candidate genus *rc4-4* of the family *Peptococcaceae*, and the genus *Anaerovorax* of the proposed family *Mogibacteriaceae* were all increased in E+ steers (Fig. 4.1A). Finally, within the *Bacteroidetes*, E+ grazing increased the genera *Paludibacter* and candidate *CF231* (Fig. 4.1A).

When comparing Day 12 (−THI) and Day 20 (+THI) within Max-Q steers, there were no significant effects on any fecal bacteria in response to the different THI conditions (Fig. 4.1B). However, when comparing the E+ steers, we found that the genera *Mycoplasma* and *Clostridium* of the family *Ruminococcaceae* were significantly decreased by +THI (Fig. 1C). Additionally, the *Coriobacteriaceae* genus *Olsenella*, *Streptococcaceae* genus *Streptococcus*, *Turicibacteraceae* genus *Turicibacter*, *Clostridiaceae* candidate genus *SMB53*, the genus

Butyrivibrio, and the family *Peptostreptococcaceae* were all significantly increased in E+ steers under +THI conditions (Fig. 4.1C).

Microbiota OTUs and THI/ADG correlation analysis

These correlational analyses of bacterial OTUs were performed to test if OTUs within individual families have either a positive or negative correlation with the endpoint of interest. Overall, the *Lachnospiraceae* and *Ruminococcaceae* families, alongside the proposed family *Mogibacteriaceae*, had OTUs whose relative abundances were positively correlated with ADG ($r > 0.3$; $P < 0.05$). Further, *Ruminococcaceae*, proposed *Paraprevotellaceae*, *Lachnospiraceae*, and *Bacteroidaceae* families had OTUs whose relative abundance negatively correlated with ADG. The genus *Prevotella* had one OTU that negatively correlated strongly with ADG ($r = -0.68$; $P < 0.001$). Within E+ steers, the classified families with the greatest number of negatively correlated OTUs were the *Ruminococcaceae*, *Lachnospiraceae*, *Bacteroidaceae*, *Mycoplasmataceae*, and *Clostridiaceae* (Table 4.1).

When considering all steers, five classified families had OTUs with relative abundances positively correlating ($r > 0.3$; $P < 0.001$) with THI. Within E+ steers, the same classified families, excluding the *Peptostreptococcaceae* and including the *Ruminococcaceae*, positively correlated with THI (Table 4.1). Many OTUs were classified at the genus level as well, with *Olsenella* having the highest number of OTUs.

Ruminococcaceae was the only classified family that had OTUs with relative abundances negatively correlating ($r < -0.3$; $P < 0.001$) with THI, regardless of fescue treatment. Within E+, the top classified families that negatively correlated with THI were the *Ruminococcaceae*, *Lachnospiraceae*, *Erysipelotrichaceae*, *Mogibacteriaceae*, and candidate family *RFP12* (Table

4.1). For all analyses, numerous OTUs were classified at the genus level, and these genera are provided in Table 4.1.

High-Resolution Metabolomics (HRM)

Descriptive statistics

A total of 12,030 and 12,407 HRM unique features were detected in the, respectively, plasma and urine with C18-LCMS; 16,878 and 17,484 HRM features were detected using HILIC-LCMS. Overall, a large number of HRM features were significantly influenced by E+ grazing throughout the grazing trial in both plasma and urine (Fig. S4). More specifically, a total of 1,753 (C18-LCMS) and 2,642 (HILIC-LCMS) HRM features were significantly ($P < 0.05$) affected by E+ grazing in the plasma, and 1,348 (C18-LCMS) and 3,250 (HILIC-LCMS) features were significantly ($P < 0.05$) affected by E+ grazing in the urine.

HRM data reduction analysis

HRM PLS-DA revealed results similar to the microbial analyses for both plasma and urine, in that both Max-Q and E+ steers metabolomes distinctly shifted from the metabolome composition prior to pasture placement (Pre; Fig. S3). Plasma PLS-DA loadings were metabolites primarily involved in Vitamin A (retinol), amino acid (e.g., tryptophan, tyrosine), butanoate, and arachidonic acid metabolism (throughout the trial), glutathione metabolism (early in the trial [Day 1–2]), biopterin and energy related metabolism (e.g., TCA cycle; late in the trial [Day 12–26]). The urine loadings were putative metabolites primarily involved in glycolysis and gluconeogenesis, amino acid, leukotriene and arachidonic acid, C21-steroid hormone metabolism, prostaglandin formation from arachidonate, and urea cycle/amino group metabolism (throughout the trial) and energy metabolism (e.g., fatty acid β-oxidation, etc.) and androgen/estrogen biosynthesis and metabolism (Day 16, 20, and 26).

When comparing Day 12 (-THI) and 20 (+THI) within E+ steers, we found that those HRM features that drove their separation were metabolites putatively involved in: prostaglandin formation from arachidonate, arachidonic acid metabolism, C21-steroid hormone biosynthesis and metabolism (plasma and urine) leukotriene and biopterin metabolism (plasma) and bile acid biosynthesis, aspartate and asparagine metabolism, arachidonic acid metabolism, mon-unsaturated fatty acid beta-oxidation, and Vitamin E metabolism (urine).

HRM pathway analysis

The overall top three pathways influenced by E+ grazing throughout the trial, for both plasma and urine, were tryptophan, tyrosine, and biopterin metabolism (Fig. 4.2A,B). Further, a number of amino acid metabolic pathways were significantly altered by E+ in both plasma and urine (Fig. 4.2A,B). Other significantly altered pathways in the plasma include lineolate metabolism and important energy producing pathways (e.g., TCA cycle and carnitine shuttle; Fig. 4.2A). In the urine, other metabolic pathways affected by E+ grazing include those involved in carbon fixation, drug, vitamin, lipid, and nucleic acid metabolism (Fig. 4.2B). Temporal merging of the mummichog activity networks resulted in clusters being formed around metabolites involved in amino acid and lipid metabolism, indicating these specific metabolic perturbations may be important for FT etiology (Fig. 4.2C,D).

There were a number of metabolic pathways that were significantly affected by E+ grazing on least (−THI) and most (+THI) harsh days of the grazing trial (Fig. 4.3). Overall, 24 metabolic pathways were significantly affected in both plasma and urine, whereas 36 and 7 were specifically affected in, respectively, the plasma or the urine (Fig. 4.3). Within the plasma, 30 of the resultant metabolic pathways were THI-independent, and 10 and 20 metabolic pathways were affected on Day 12 or Day 20, respectively (Fig. 4.3). In the urine, only 7 metabolic

pathways were significantly affected by E+ grazing independent of THI, with 10 and 12 being specific to Day 12 and 20, respectively (Fig. 4.3). These data indicate that more metabolic pathways in the plasma than in the urine are affected by E+ and that the +THI conditions modify this effect.

More specifically, in the plasma, tyrosine and tryptophan were the top two pathways significantly affected by E+ grazing, regardless of THI. Other highly affected metabolic pathways include chondroitin sulfate degradation, histidine metabolism, methionine and cysteine metabolism and aspartate and asparagine metabolism (Fig. 4.3). In the urine, the top altered metabolic pathways were biopterin and Vitamin B6 metabolism on both Day 12 and Day 20 (Fig. 4.3). The exhaustive list of metabolic pathways affected irrespective of THI or on either Day 12 or 20 can be seen in Fig. 4.3.

Plasma and urine sPLS networks

For both Max-Q and E+ steers, sPLS variable selection resulted in two distinct clusters, one with plasma metabolites (orange) as the anchors and one where plasma and urine metabolites are dispersed evenly throughout the network (Fig. S4). The Max-Q Day 12 (−THI) and 20 (+THI) networks were similar to those of the overall network. On the other hand, while the E+ Day 12 was similar, the E+ Day 20 network had urinary metabolites as the anchors of both clusters within the network (Fig. S4). In support, the metabolites involved in the Max-Q networks were putatively identified as involved in methionine and cysteine, tyrosine, and arginine and proline metabolism, regardless of THI. While the E+ metabolites in the overall and Day 12 networks were similar, the Day 20 E+ network had metabolites involved in multiple pathways only existent on Day 20 (e.g., pentose phosphate pathway, C21-steroid hormone biosynthesis and metabolism, and tryptophan, tyrosine, and xenobiotics metabolism) (Fig. S4).

Microbiota:metabolome integration

Procrustes analysis revealed that the overall E+ fecal microbiota and the total metabolome are overall strongly correlated (Fig. 4.4A, Monte Carlo $P < 0.05$); but this is not the case for Max-Q steers (Fig. 4.4B, Monte Carlo $P = 0.49$). The inter-'omic relationship was not apparent when the procrustes analyses was done on a subset of the data within -THI (Fig. 4.4C, Fig. 4.4D) or +THI (Fig. 4.4E, 4.4F). We found that the Monte Carlo simulation insignificance was similar for Max-Q ($P = 0.13$) and E+ ($P = 0.14$) steers under thermoneutral conditions (Day 12) or during +THI (i.e., Day 20), although the E+ ($P = 0.49$) correlation was numerically stronger than the Max-Q one ($P = 0.96$).

Coinertia analysis (CIA) indicated that OTUs classified to the *Firmicutes* had strong inter-omic covariance in both Max-Q and E+ steers, but the magnitude of this covariance is higher in E+ steers (Fig. 4.5A & B). Further, the covariance (i.e., distance from the origin) for a number of *Actinobacteria*, *Tenericutes*, and *Verrucomicrobia* OTUs was higher in E+ steers (Fig. 4.5A & B). Finally, a number of low-level phyla (i.e., < 10 OTUs; labeled as Other) had high covariance in E+ steers but not in Max-Q steers (Fig. 4.5A & B). Under thermoneutral conditions (Day 12), no significant shift between the microbiota-total metabolome covariance was observed between Max-Q (Fig. 4.5C) and E+ (Fig. 4.5D) steers. On Day 20, the Max-Q (Fig. 4.5E) CIA revealed a similar pattern to Max-Q Day 12, but the E+ (Fig. 4.5E) CIA revealed a distinct shift in the microbiota-metabolome covariance leading to a bi-modal distribution of OTUs. Notably, the magnitude of *Firmicutes* OTUs covariance was greater, but this similar differential pattern was similar for almost all phyla, i.e., most OTUs had significantly shifted from the point of origin. This indicates that the overall E+ effect on CIA was driven by greater differences during +THI.

After sPLS variable selection using the data from all dates post pasture placement, Max-Q steers had significantly fewer OTUs and metabolomics features that had strong correlation (|r| > 0.7), supporting the results of the Procrustes and CIA analyses (Fig. 4.6A and B). Considering this, differential network analysis was performed. In the resultant E+ network, most of the selected HRM features that highly correlated with fecal OTUs were plasma metabolites (blue; 486 features; Fig. 4.6C), but urinary metabolites were also part of the network (orange; 30 features; Fig. 4.6C). Further, two distinct clusters were formed and most of the urinary HRM features were in the cluster on the right; most plasma HRM features were in the left cluster (Fig. 6A). In the left cluster, the OTUs that correlated with urinary metabolites were mainly aligned in the *Ruminococcaceae* and *Lachnospiraceae*, with one OTU aligned in the candidate family *S24-7* and one unclassified at the family level (Fig. 4.6C). The plasma metabolites in the left subnetwork mapped to C21-steroid hormone biosynthesis and metabolism, and tryptophan and tyrosine metabolism (Fig. 4.6C). Similarly, in the right subnetwork, most OTUs also aligned to the *Ruminococcaceae*, *Lachnospiraceae*, and *Erysipelotrichaceae* families, with OTUs also aligning in the candidate *RFP12* and *Mogibacteriaceae* families (Fig. 4.6A). The plasma and urinary metabolites were involved in tryptophan, tyrosine, and androgen and estrogen biosynthetic metabolic pathways (Fig. 4.6A). Finally, many of the urinary and plasma HRM features were unidentified, and many OTUs were unclassified at the family level.

Interestingly, one OTU (Otu00087) aligned to *Peptococcaceae* candidate genus *rc4-4* within the sPLS network and was also significantly correlated (+r) with ADG and UEAs (-r) in E+ steers. Another OTU (Otu01305) aligned to the *Clostridiaceae* genus *Clostridium* and correlated with both ADG in E+ steers and THI (+r). Finally, a *Ruminococcaceae* OTU (Otu00042) correlated with THI and UEAs in E+ steers (+r). These three OTUs shared most of

the highly significantly correlated plasma and urinary metabolites which were involved in tyrosine and tryptophan metabolism, valine, leucine and isoleucine degradation, and C21- steroid hormone biosynthesis. Otu01305 was also peripherally connected to metabolites involved in prostaglandin formation from arachidonate (Fig. 4.6D). Notably, these three OTUs were present when sPLS and differential network analysis was performed on Day 12 and Day 20 independently. So, although the abundance of these OTUs may be influenced by environmental conditions, as indicated by positive or negative THI correlations, these OTUs may also be robust predictors of E+ effects on the fecal microbiota:metabolome interactions regardless of environmental conditions.

DISCUSSION

The data presented herein provide important insights into the nature of the pathophysiological shifts that occur while beef cattle graze E+ tall fescue pastures, and highlight the complexity of developing FT therapeutics, as both grazing and external (e.g., environmental) stressors influence the animal response to E+. In a 26-day grazing trial, we found that even relatively short-term grazing results in significant production deficits (i.e., significantly lower ADG) and rapid fluctuations in ambient environmental conditions modulate pathophysiological responses to E+ grazing. These results have significant implications for future E+ therapeutic development and grazing management strategies that seek to help alleviate the economic and environmental burden of FT. As temperatures continue to rise, improving animal health and productivity for beef cattle grazing the predominant Southeastern U.S. pasture grass under environmentally stressful conditions is of great urgency.

We recently reported that E+ grazing significantly alters the fecal microbiota in grazing beef steers under thermoneutral conditions (Mote et al., 2019). The data presented herein support

the previous study, indicating Max-Q or E+ grazing results in rapid shifts in the fecal microbiota that persist throughout the grazing trial, with the effects of E+ being differential. In both studies, *Firmicutes* were the most prominently affected phylum by E+ grazing; however, here we also found that E+ grazing results in a microbiota more susceptible to perturbations as a result of fluctuating environmental conditions. In this regard, it has already been shown that heat stress can influence the fecal microbiota in dairy cattle (Chen et al., 2018). Recent evidence directly attributed heat stress to impaired bovine gut integrity and changes the gut immune profile (Koch et al., 2019). It has been suggested that the gut permeability and immune status can influence enteric microbiota populations (Chelakkot et al., 2018; Mao et al., 2018). In our study, there were numerous bacteria that were increased in E+ steers under +THI. However, the number of *Ruminococcaceae* OTUs that were significantly increased by E+ grazing was lower under +THI conditions, indicating that E+-related *Ruminococcaceae* increases in the feces may be offset by harsh environmental conditions. Overall, these data indicate that the beef fecal microbiota is inherently dynamic, and it responds rapidly to both dietary and environmental exposures. While future studies assessing the ecological effects of E+ should further track ruminal and fecal OTUs associated with susceptibility/resistance to E+ production deficits and the environment, it may also be important to perform whole genome sequencing of the microbiome to assess whether there are characterized or novel bovine-specific small proteins that could have important regulatory or housekeeping functions for the microbiome, as was recently discovered for humans (Sberro et al., 2019).

HRM revealed that the most significantly affected metabolic pathways were the tryptophan, tyrosine, and biopterin metabolism in both the plasma and urine, supporting our previous metabolomics study with the more robust data being herein (Mote et al., 2017). When

we integrated the activity networks from sequential sampling dates, we found a highly interconnected network of metabolites anchored around amino acid metabolism. It is also noteworthy that metabolomics analyses were able to provide crucial insights into potential indirect effects on global metabolism resultant from ergot alkaloid and/or E+ exposure. Although alkaloid biosynthesis II was a pathway significantly affected in the plasma, this pathway is not related to ergot or indole-alkaloid biosynthesis. Further, tryptophan and tyrosine metabolism, among other amino acids, are involved in numerous physiological processes, including skeletal muscle anabolism. For example, tryptophan has been previously shown to stimulate the IGF-1/mTORC1/S6K1 skeletal muscle building pathway in mice (Dukes et al., 2015), with previous studies finding E+ tall fescue can reduce serum IGF-1 concentrations (Browning Jr, 2003; Filipov et al., 1999). It is well known that proteins/amino acids are important for skeletal muscle accretion in growing cattle (i.e., lean growth) (Wilkerson et al., 1993). Of note, recent investigations are beginning to elucidate the mechanisms by which amino acids regulate skeletal muscle autophagy in response to stressors, i.e. one study reported that amino acid supplementation can reduce muscle loss (autophagic) in neonatal pigs during endotoxemia (Hernandez-Garcia et al., 2016), indicating that alterations in amino acid metabolism as a result of E+ grazing could be related to decreased lean weight gain not only by decreasing skeletal muscle accretion but also by promoting skeletal muscle autophagy.

The data also showed that environmental conditions can modulate certain metabolic pathways that are significantly affected by E+ grazing. sPLS network visualization of the plasma and urine metabolomes revealed that the metabolic features that best describe the variability in these physiologically important data matrices were only sensitive to -THI and +THI fluctuations in E+ steers, indicating that the E+ steer metabolomes are more susceptible to environmental

stressors than non-toxic fescue (i.e., Max-Q) grazing steers. Notably, PLS-DA loadings analysis revealed that, in E+ steers, the main metabolites driving the separation between -THI and +THI conditions were components of inflammatory metabolic pathways (e.g., arachidonic acid metabolism and prostaglandin formation). Previous studies found that moderate heat stress induces an immune/inflammatory response in dairy cattle (Mehla et al., 2014; Min et al., 2016). As it relates to FT, steers on toxic tall fescue had greater serum TNF-α and cortisol levels than steers on endophyte-free tall fescue in response to LPS challenge (Filipov et al., 1999). Under stressful conditions, inflammation is just one factor contributing to alterations in global metabolism, and the animal will reprioritize important muscle building nutrients to meet allostatic load demand, ultimately resulting in decreased muscle accretion (Elsasser et al., 2000). These data, coupled with impaired thermoregulation in E+ animals, indicate that under short-term E+ grazing, which is relevant to rotational grazing practices common to E+ (Brink et al., 2010), mild to moderate heat stress conditions could induce inflammatory responses, resulting in perturbed metabolism and increased nutrient demand to maintain thermoneutrality.

A noteworthy finding from the integrative analyses was that the non-toxic (Max-Q) steer microbiota had little predicted covariance with the plasma and urine metabolomes, but the fecal microbiota of E+ grazing steers significantly covaried with the metabolomes overall. Moreover, a number of urinary and plasma metabolites were significantly correlated with fecal OTUs and were involved in androgen/estrogen biosynthesis, C21-steroid hormone biosynthesis and metabolism, and fatty acid activation. As it relates to FT and animal productivity, a recent study has shown that the androgen receptor signaling can influence myogenic differentiation through Wnt and TGF-β/Smad signaling (Singh et al., 2009), potentially indicating that E+ grazing could result in perturbed muscle accretion by altering the microbiota-metabolome relationship. Also,

the higher correlation and covariance of the E+ tall fescue, notably under +THI conditions, suggests decreased complexity of the host-microbe relationship. The microbiota:metabolome differences between Max-Q and E+ steers could be a result of changes in E+ gut microbial metabolism leading possibly to increased gut permeability (Zhong and Zhou, 2014) or E+ effects resulting in a heat stress-susceptible physiological and/or immunological background prior to +THI exposure. These data point to potential biomarkers, either in plasma or urine, that could be readily accessed and used to identify the presence of particular bacteria associated with animal productivity in E+ grazing steers. As shown for necrotizing enterocolitis in humans (Rusconi et al., 2017), these urinary biomarkers may be combined with other clinical signs (e.g., UEAs) to predict a microbiota associated with low productivity or beef cattle that may be amenable to either dietary or therapeutic intervention in the future.

We identified three OTUs that were unique to the E+ sPLS networks and were significantly associated with THI, UEAs, and ADG. These were *Peptococcaceae* candidate genus *rc4-4*, *Clostridiaceae* genus *Clostridium*, and one *Ruminococcaceae* OTU. *Peptococcaceae rc4-4*, which was significantly increased by E+, has been associated with increased thermogenesis in cold environments in mice (Zietak et al., 2016), and was found to be negatively associated with circulating amino acids and choline compounds and positively associated with circulating cholesterol and fatty acids (Sanguinetti et al., 2018). Although it is unlikely the sole factor, this particular bacteria could potentially contribute to altered amino acid metabolism we have found here and under thermoneutral conditions (Mote et al., 2017). The *Ruminococcaceae* and *Clostridiaceae* families are bacteria we have previously found affected by E+ grazing, and deep sequencing of these two families would help delineate which specific species/strains of bacteria may be most relevant for FT etiology. Nonetheless, these OTUs, or

perhaps more easily the urinary metabolites associated with these OTUs, can be further assessed for their utility to detect E+ microbiota presence that is indicative of decreased animal production efficiency regardless of environmental conditions.

The only study, to our knowledge, directly assessing the effects of E+ grazing on methane production found no difference in methane levels for steers grazing endophyte-free and endophyte-infected tall fescue pastures (Pavao-Zuckerman et al., 1999). Although we did not directly measure archaea, hydrogen-producing bacteria, such as the cellulolytic *Ruminococcous*, are important for methanogens and other bacteria have been associated with low- or high-methane producing microbiota (e.g., other *Ruminococcaceae*, *Lachnospiraceae*, *Prevotella*, etc. for high-producers beef heifers (; Lan and Yang, 2019)). While utilizing fecal bacteria as easily accessible biomarkers of ruminal methane production has previously been proposed (Ross et al., 2013), the data herein suggest the urinary metabolome could be indicative of specific endpoints of the fecal microbiota, such as E+ or ruminal methane production. Regardless, the influence of E+ on methane emissions has not been extensively evaluated, and the decreases in animal productivity resultant from E+ grazing will inherently result in a need to increase herd size to meet pasture-based agricultural demands (Matthew and Rachael, 2018); therefore, E+ grazing will, at the least, indirectly result in a greater contribution of E+ grazing animals to methane emissions. Ultimately, multiple studies, like the one performed herein, are beginning to highlight the utility of top-down strategic approaches (Suravajhala et al., 2016) for improving animal health and welfare.

CONCLUSIONS

Overall, these data demonstrate that E+ grazing contributes to decreases in animal productivity through significant alterations in the microbiota and global metabolism. Further, E+

grazing results in greater susceptibility to environmental stressors such as high heat and humidity, which are common in the Southeastern US and will become a greater burden on animal health and productivity as the climate continues to warm. As shown by our summary figure, E+ grazing resulted in significant metabolic and microbiota perturbations, while increasing the susceptibility of these important physiological parameters to +THI conditions (Fig. 4.7). As a result of this work, we were able to use bioinformatics techniques to identify important microbiota-metabolome relationships that could provide important, easily-accessible biomarkers for E+ producers (Fig. 4.7). The increased susceptibility of E+ steers to +THI conditions indicate that, even for rotational grazing production settings, monitoring both environmental and seasonal responses will be important for developing efficacious microbial and/or metabolic-targeted FT interventions in the future. The data herein suggest that E+ grazing results in significant shifts in amino acid metabolism in the plasma, which could contribute to decreased skeletal muscle accretion by altering available amino acids or perturbing basal levels of skeletal muscle autophagy. Finally, the changes in the microbiota-metabolome interaction could be used as biomarkers and as targets for management and/or therapeutic interventions. As a warming climate and future scientific policy will drive the need for increased production efficiency, studies like the one presented here are informative will help in meeting future agricultural demand and in reducing the environmental impact of the livestock industry by identifying potential production-restoring markers and/or therapeutic targets under varying environmental conditions.

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Table 4.1. Top classified families and genera correlating with average daily gains (ADG), urinary ergot alkaloids (UEAs) and temperature humidity index (THI) in steers grazing toxic (n = 6; E+) tall fescue for a 26-day grazing trial. The number of OTUs within a family/genus, average Spearman correlation coefficients (|r| > 0.30), and average P-values (P < 0.05) are presented

Family	Genus	Number of OTUs	Spearman's R	P-value
		ADG (+r)		
Lachnospiraceae		13	0.458	0.027
	Dorea	1	0.460	0.024
Ruminococcaceae		9	0.462	0.025
	Oscillospira	1	0.454	0.026
	Ruminococcus	1	0.426	0.038
Bacteroidaceae		3	0.499	0.023
	5-7N15	3	0.499	0.023
Coriobacteriaceae		3	0.471	0.028
Erysipelotrichaceae		2	0.473	0.026
	p-75-a5	1	0.412	0.045
		ADG (-r)		
Lachnospiraceae		5	-0.494	0.016
	Butyrivibrio	2	-0.451	0.029
Ruminococcaceae		3	-0.440	0.033
	Clostridium	1	-0.406	0.049
	Ruminococcus	1	-0.480	0.018
		THI (+r)		
Lachnospiraceae		24	0.417	0.019
	Butyrivibrio	3	0.394	0.018
	Blautia	1	0.441	0.007
	Dorea	1	0.550	0.001
Ruminococcaceae		12	0.395	0.023
	Ruminococcus	6	0.400	0.020
Coriobacteriaceae		9	0.417	0.019
	Olsenella	4	0.455	0.015
	Enterococcus	1	0.393	0.018
[*Mogibacteriaceae*]		7	0.446	0.016
	Mogibacterium	3	0.395	0.020
Clostridiaceae		3	0.453	0.011
	Clostridium	2	0.399	0.016
	SMB53	1	0.600	0.0003
		THI (-r)		
Ruminococcaceae		15	-0.470	0.010
	Oscillospira	2	-0.386	0.020
	Papillibacter	1	-0.362	0.030
Lachnospiraceae		12	-0.423	0.016
	Clostridium	2	-0.425	0.009
[*Mogibacteriaceae*]		2	-0.382	0.024

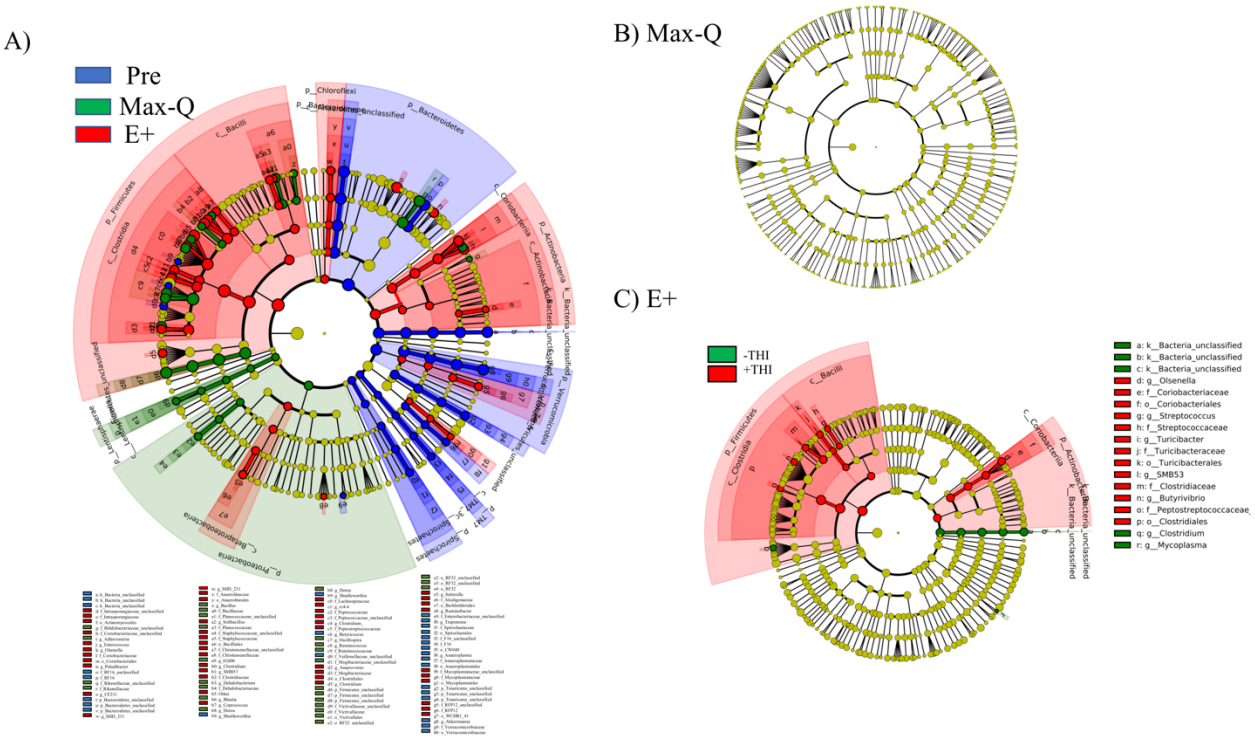

Figure 4.1. Linear discriminant analysis (LDA) effect size (LEfSe; Kruskall-Wallis [P < 0.05]; Pairwise Wilcoxon [P < 0.05]; logarithmic LDA score > 2.0) of the fecal microbiota of **(A)** Angus steers before (Pre) placement or across a 26-day grazing trial after placement on either a non-toxic (Max-Q; n = 6) or toxic (E+; n = 6) endophyte-infected tall fescue; **(B)** steers grazing Max-Q tall fescue on Day 12 (- temperature humidity index [-THI]) versus Day 20 (+THI); **(C)** steers grazing E+ tall fescue on Day 12 (-THI) versus Day 20 (+THI). **(A)** Blue, green, and red shading indicates greater abundance in Pre, Max-Q, or E+ steers, respectively **(C)** Green and red indicates greater abundance in E+ steers on Day 12 (-THI) and 20 (+THI), respectively. Taxonomic rank labels are provided before bacterial names: "p_; c_; o_; f_; g_" indicate phylum, class, order, family, and genus, respectively. Letters and numbers within the cladogram refer to bacterial names located in the key for panel **(A)** below the cladogram and panel **(C)** to the right of the cladogram.

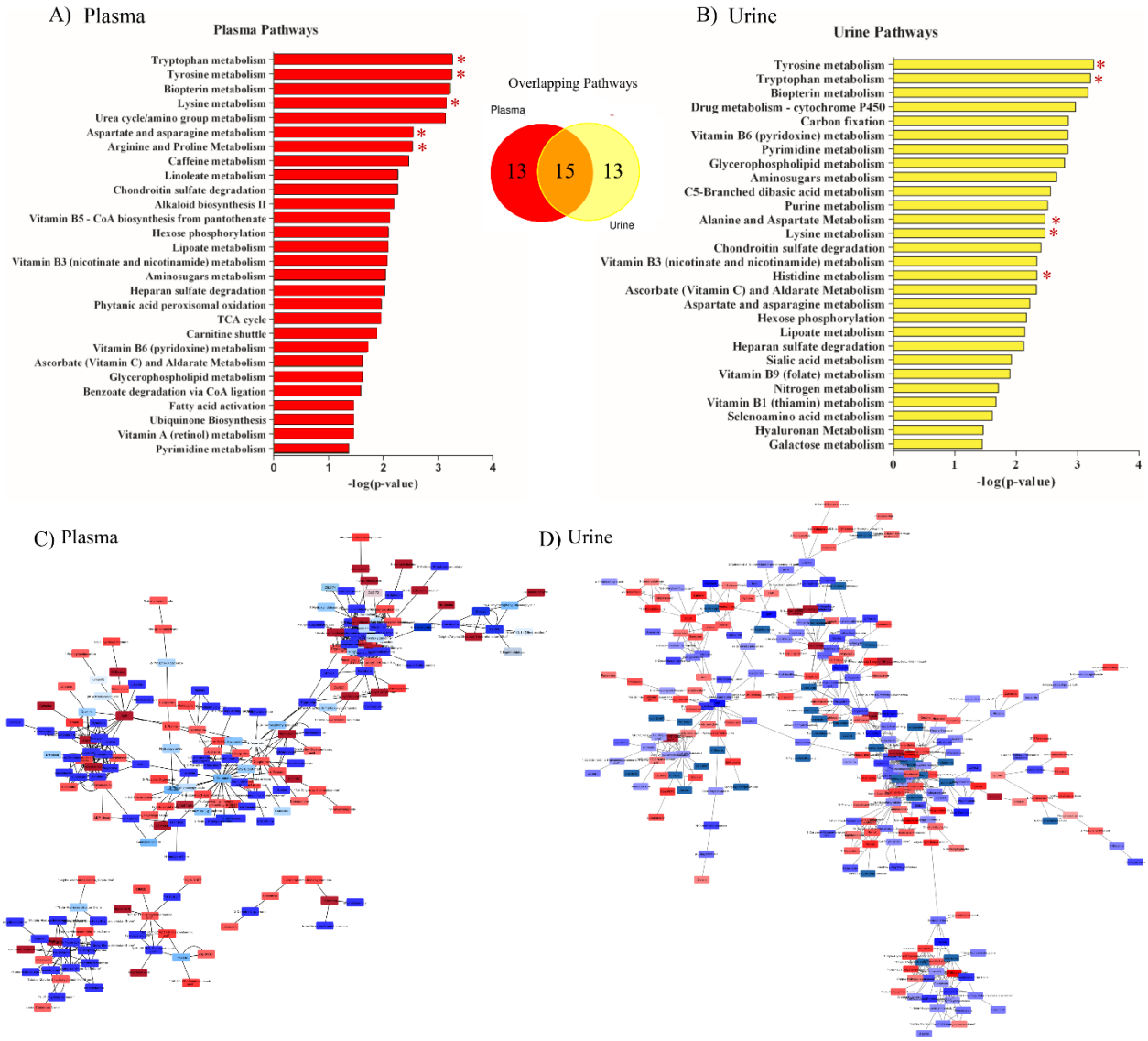

Figure 4.2. Pathway analysis performed on the **(A)** plasma (red) and **(B)** urine (yellow) high-resolution metabolomics features using the mummichog python program that indicates putative metabolic pathways significantly ($P < 0.05$) affected by toxic tall fescue (E+) grazing in beef steers throughout the 26-day grazing trial ($n = 6$). The x-axis indicates the negative log of the FDR corrected p-value for each metabolic pathway indicated on the y-axis. The Venn Diagram details the number of metabolic pathways that were significantly affected by E+ grazing in both the plasma (red) and urine (yellow), or distinctly in the plasma or urine. Red asterisk indicated production (i.e., weight gain)-related metabolic pathways.

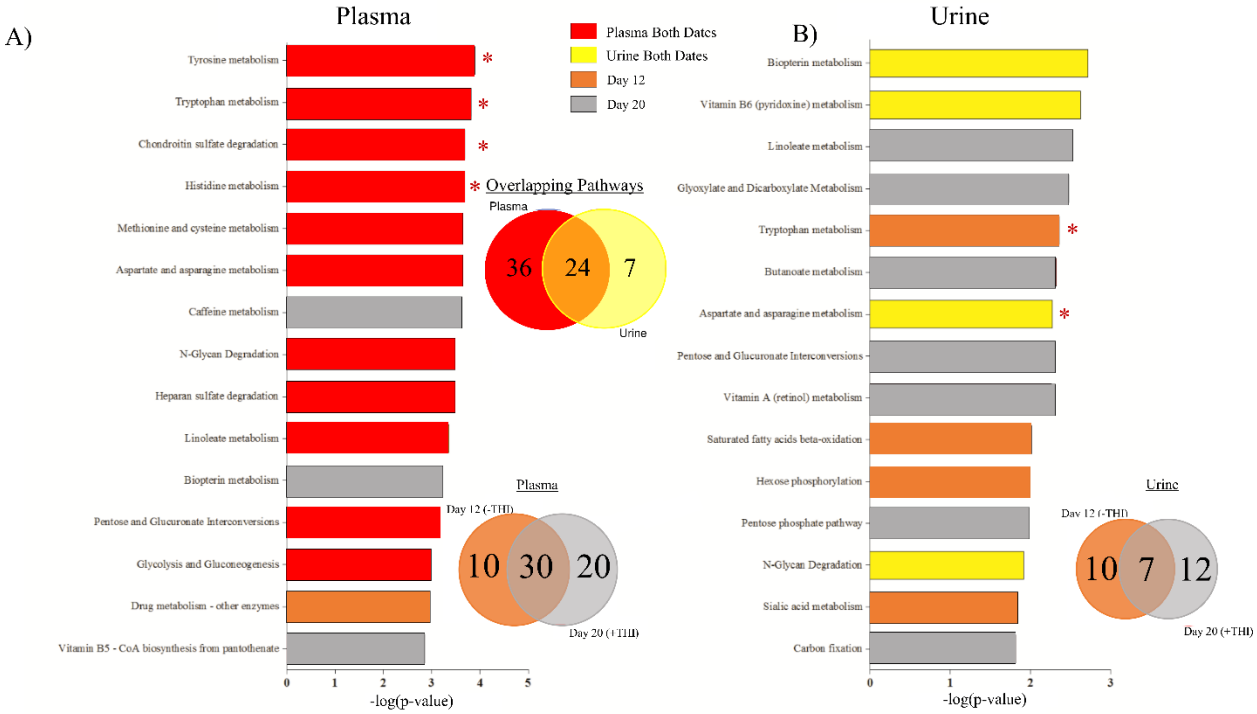

Figure 4.3. Top 15 pathways from pathway analysis performed on the **(A)** plasma and **(B)** urine high-resolution metabolomics features using the mummichog python program within Day 12 (-THI) and 20 (+THI), with putative pathways that are significantly affected by toxic tall fescue (E+) grazing solely on Day 12 (-THI; orange), only on Day 20 (+THI; gray), or on both dates (plasma = red; urine = yellow). The Venn diagrams detail the number of putative metabolic pathways that were affected in the plasma and/or urine (top) or in the plasma on Day 12 and/or Day 20 and (bottom left) or in the urine on Day 12 and/or Day 20 (bottom right).

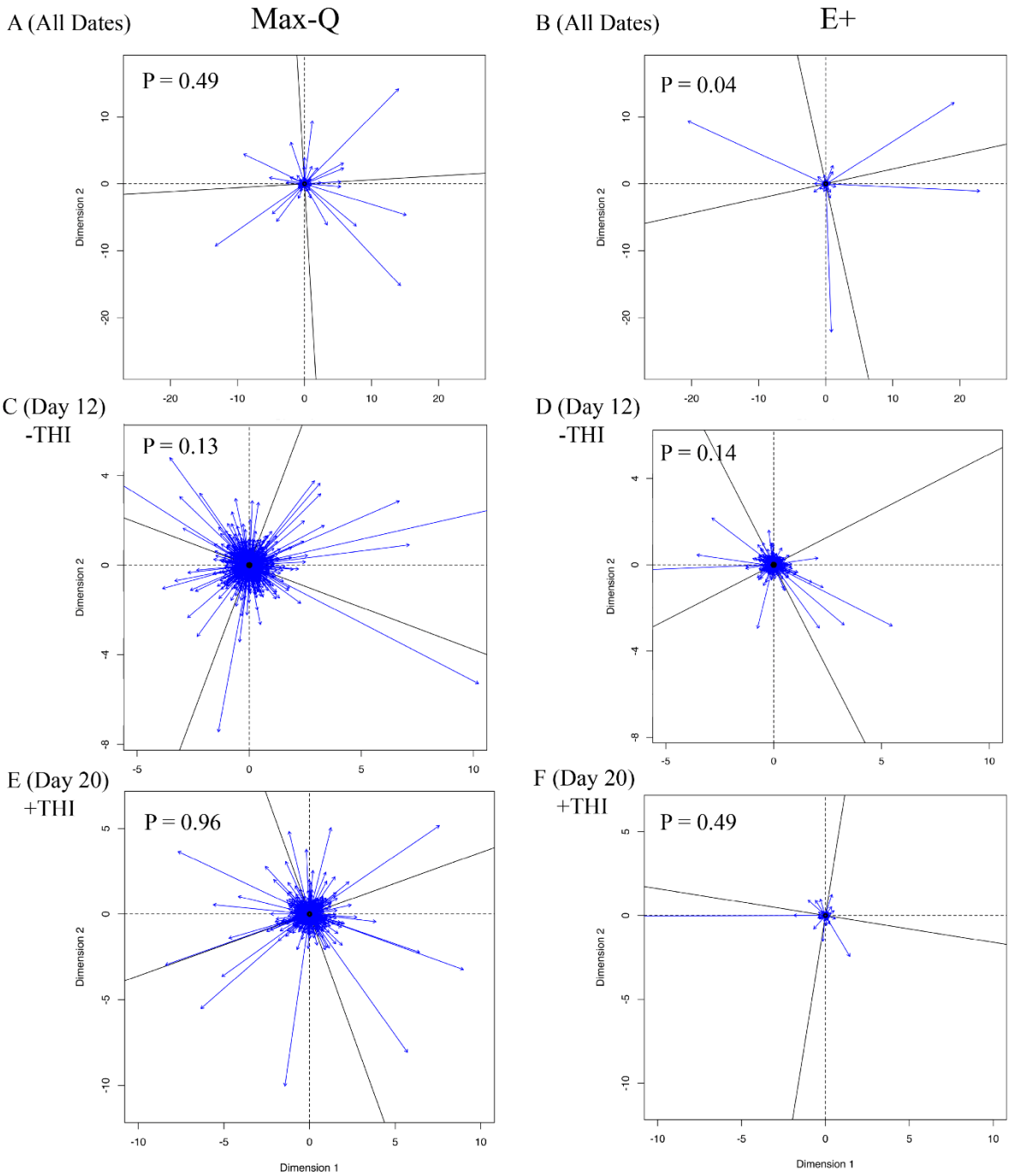

Figure 4.4. Procrustes analysis performed using the *vegan* R package for steers grazing either a (**A**; Max-Q; n = 6) novel, non-toxic or (**B**; E+; n = 6) toxic endophyte-infected tall fescue over the course of a 26-day grazing trial; Max-Q steers on Day 12 (**C**; -THI) and Day 20 (**E**; +THI) and E+ steers on Day 12 (**D**; -THI) and Day 20 (**F**; +THI). The significance values were estimated using the *protest* function to perform permutational procrustes to test for significance, with the P values reported in the upper left hand side of each respective plot. The greater vector length is indicative of greater inter-'omic variability.

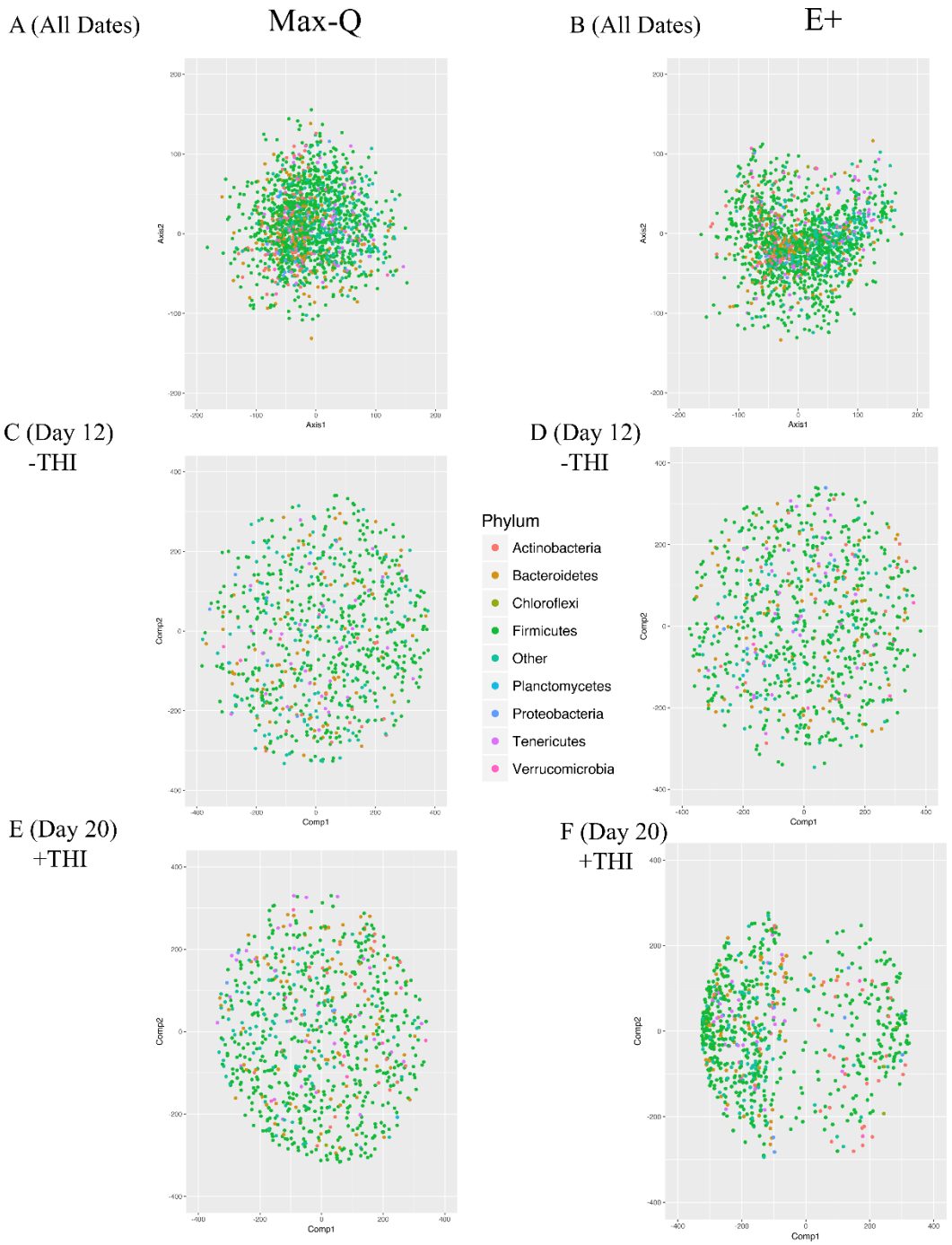

Figure 4.5. Coinertia analysis performed on OTUs for steers grazing either a (**A**; Max-Q; n = 6) novel, non-toxic or (**B**; E+; n = 6) toxic endophyte-infected tall fescue over the course of a 26-day grazing trial; Max-Q steers on Day 12 (**C**; lowest -THI) and Day 20 (**E**; highest +THI) and E+ steers on Day 12 (**D**; -THI) and Day 20 (**F**; +THI). Each point is plotted based on the OTUs predicted covariance with the plasma and urine metabolomes (i.e., greater distance from the point of origin indicates a greater covariance) of the respective group of steers. Red = *Actinobacteria*, brown = *Bacteroidetes*, green = *Firmicutes*, teal = other (unclassified, *Planctomycetes*, *Chloroflexi*, and other low abundance phyla), blue = *Proteobacteria*, purple = *Tenericutes*, and pink = *Verrucomicrobia*.

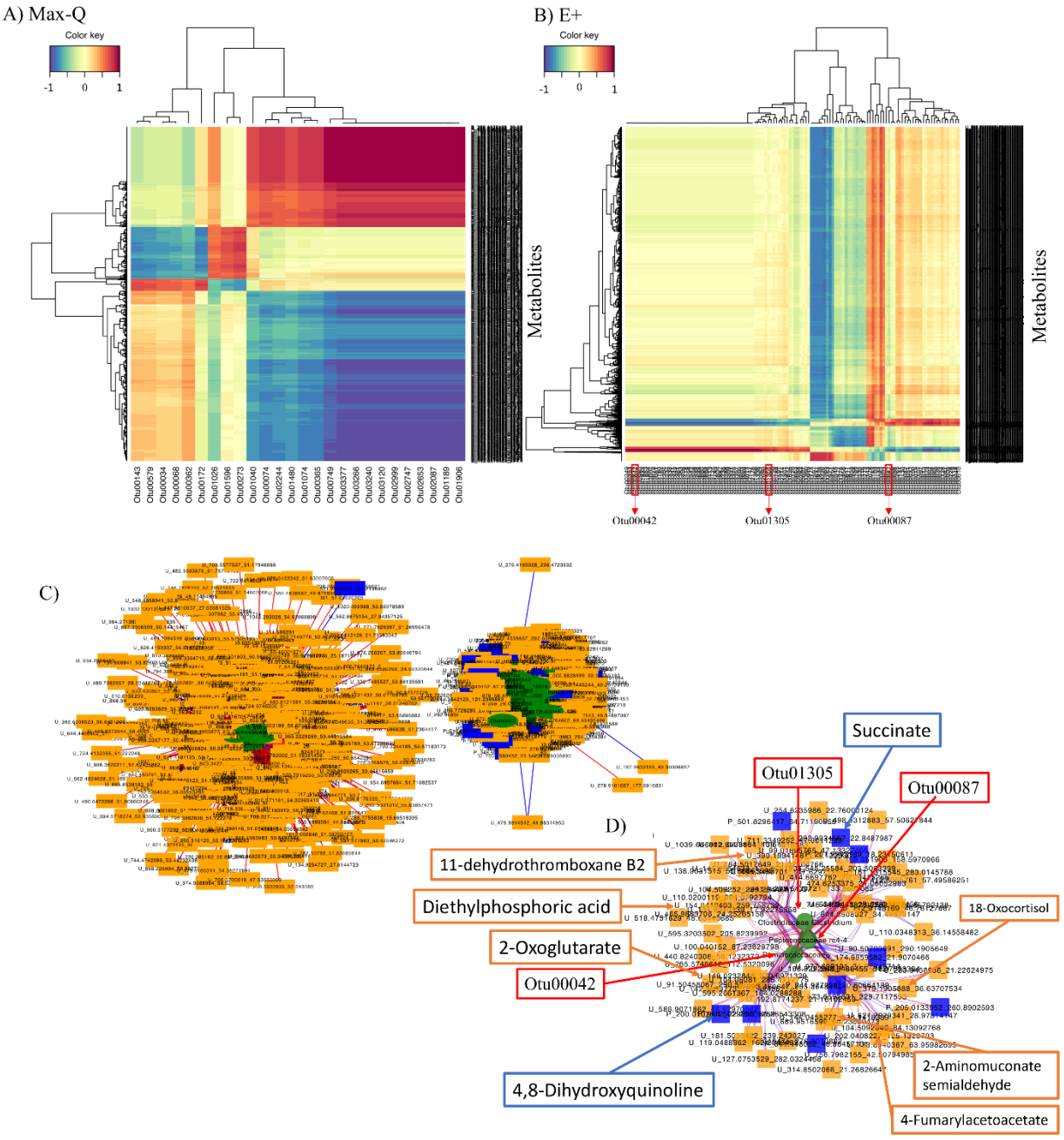

Figure 4.6. Bipartite network of fecal 16S rRNA OTUs (green circles) and plasma (orange rectangles) and urine (blue rectangles) high-resolution metabolomics (HRM) features that were selected by sparse partial least squares regression (sPLS; top 100 OTUs [X matrix] and 500 plasma and urine HRM features [Y matrix]) using the *mixOmics* R package and were highly correlated ($|r| > 0.7$) **(A)** throughout the grazing trial and **(B)** a subnetwork of selected OTUs (green circles) that correlated with average daily weight gains, urinary ergot alkaloids (biomarkers of exposure and key etiological agents of FT), and THI, with the plasma (orange rectangles) and urine (blue rectangles) HRM features that were highly correlated ($|r| > 0.7$) with these OTUs in the original network. Red and blue edge indicates positive or negative correlations, respectively. Select OTUs proposed as potential E+-associated microbiota biomarkers pointed to by red arrows as the anchors of the network in panel **B**.

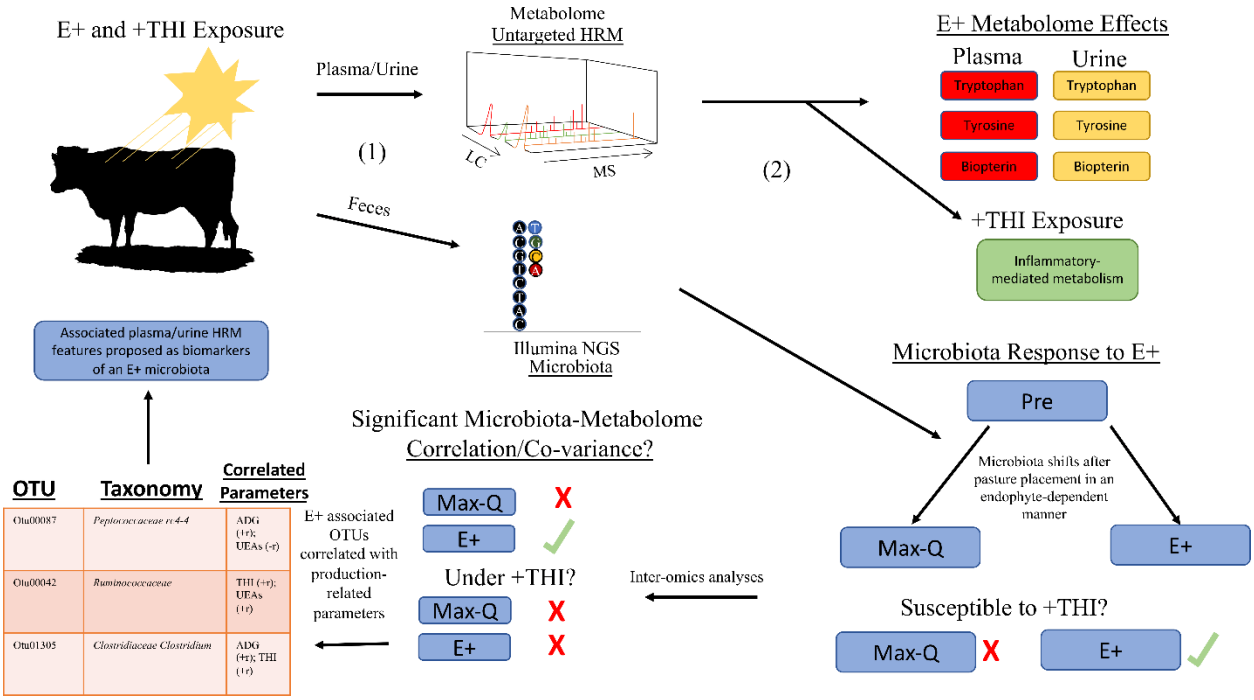

Figure 4.7. Summary figure representing the study described herein. Angus steers (n = 12) were allowed to graze either a toxic (E+; n = 6) or non-toxic (Max-Q; n = 6) tall fescue pastures throughout a 26-day grazing trial in the summer of 2016. During this trial, the samples were collected on sampling dates where there was no risk of heat stress (-THI) and where there was a mild-to-moderate risk of heat stress (+THI). Plasma and urine samples were analyzed by high-resolution metabolomics (HRM) and feces were analyzed by using next-generation sequencing techniques targeting the 16S rRNA gene. Samples were then subjected to bioinformatics analysis where we found significant perturbations of both the metabolome and the microbiota, which were modulated by +THI conditions in E+ steers alone. We then assessed the microibiota:metabolome relationship, and found that only E+ steers in thermoneutral conditions had a significant correlation/covariance between the microbiota and metabolome, but sparse partial least squares regression revealed three OTUs that were predictive of E+, regardless of environmental conditions, and were highly correlated with plasma/urine metabolites that have been proposed as biomarkers of an E+ microbiota associated with production deficits.

CHAPTER 5

FROM PLANT TO ANIMAL: AN INTEGRATIVE INTERACTOMICS-BASED APPROACH TO GRAZING BOVINE TOXICOSIS

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ABSTRACT

Fescue toxicosis is a disease complex pathophysiology resulting from livestock grazing endophyte (*Epichloë coenophiala*)-infected tall fescue, the predominant Southeastern US forage grass. While recent studies have reported on effects the endophyte has on the microbiota, global metabolism, and their interaction in the grazing animal, no study to date has investigated these effects in conjunction with *in planta* effects. Therefore, this study focused on global perturbations in the plant and animal multi-compartment microbiota-metabolome using integrative interactomics (bacterial [16S] and fungal [ITS2] sequencing and untargeted metabolomics) over the course of a 28-day trial where Angus steers grazed either non-toxic (Max-Q) or toxic (E+) tall fescue. *E. coenophiala* significantly altered the plant and animal microbiota, notably decreasing most ruminal fungi, while have mixed effects on the rumen bacteria and fecal microbiota. Significant effects on the plant rumen, plasma, and urine metabolome were prominent, with some plant to animal carryover (i.e., tryptophan and Vitamin B6 metabolic perturbations in all biological matrices). Targeted network analysis revealed a number of plant and animal operational taxonomic units (OTUs) and ruminal metabolites associated with *Epichloë* OTU and an ergopeptine it produces, ergovaline. Key network metabolites involved in ruminal steroid biosynthesis were identified. Integrative interactomics revealed similar network structures for Max-Q and E+, but with unique constituents. Notably, only the E+ had ruminal solid OTUs (bacterial) within the network, and the fecal fungal OTUs, which were most important to both networks, consisted of unique taxa (e.g., E+ *Anaeromyces*; Max-Q *Erthrobasidium*).

SIGNIFICANCE

Fescue toxicosis costs the United States beef industry between $1-2 billion annually, and the systemic pathophysiological mechanisms that lead to these economic losses are not well characterized; however, the etiological agents, ergot alkaloids, are known to have systemic biological interactions. This study provides a framework of the fescue toxicosis integrome and highlights key features that could be targeted therapeutically. While the integrome structures were similar between Max-Q and E+, the constituency of these integrative networks was different between the two fescue cultivars/grazing animals. These differences point to global, multi-compartment perturbations of the microbiota-metabolome relationship that could be targeted therapeutically. Specific OTUs and/or metabolic signatures reflective of these can be utilized as biomarkers of effect.

Keywords: fescue toxicosis, ergot alkaloids, tall fescue, microbiome, metabolomics, integrative interactomics, integrome

INTRODUCTION

The ability to integrate multi-‘omics data into single interpretable outputs, representing multi-compartment and multi-level interaction, is a significant new development (Uppal et al., 2018). While multi-‘omics have been used to identify complex adaptations to environmental exposures in relation to the human exposome (Go et al., 2014), we recently proposed such integrative interactomics-based approaches as potentially valuable for investigating diseases with complex pathophysiologies farm animal (Mote and Filipov, 2020). As numerous environmental, pandemic, and geopolitical factors increasingly affect the agricultural supply chain, utilizing such global analyses to uncover disease signatures or favorable conditions for maximum and sustainable productivity is timely and important.

Fescue toxicosis (FT) is a complex livestock disease that occurs when animals graze tall fescue, *Lolium arundinaceum*, infected with the endophyte *Epichloë coenophiala* (Leuchtmann et al., 2014; Paterson et al., 1995). While the plant-endophyte relationship is viewed as mutualistic because of beneficial agronomic attributes (Clay and Schardl, 2002), *E. coenophiala* produces ergot alkaloids, a group of secondary metabolites, thought to be key etiological agents of FT. For this reason, wild-type tall fescue is generally referred to as toxic, as it leads to significant production deficits, such as decreased weight gains (Paterson et al., 1995). The ergot alkaloids are promiscuous by nature and interact with multiple monoamine receptors (Foote et al., 2011; Foote et al., 2013; Klotz, 2015) to elicit systemic responses. They are heavily metabolized in the rumen *in vivo* and lysergic acid, not ergopeptines (e.g., ergovaline), was able to cross gastric barriers (Ayers et al., 2009).

The plant microbiota consists of the parts: the rhizosphere, the phyllosphere, and the endosphere. The phyllosphere (i.e., aboveground plant) microbiota are of particular concern in grazing diseases like FT and respond to factors in the soil, environment and other parasitic (Fierer, 2017; Hardoim et al., 2015; Vorholt, 2012b; Wallace et al., 2018; Zarraonaindia et al., 2015). The plant microbiota has both core and satellite microbial communities, with the core serving as the main structure and the satellite (i.e., low abundant microbes) serving to prevent unwanted microbial invasions (Mallon et al., 2015). Previous work showed environmental factors influence fungal ergot alkaloid production and that *E. coenophiala* contributes to parasitic resistance (Clay and Schardl, 2002). However, the extent to which the toxic endophyte influences the *in planta* microbiota and metabolic profile and to what extent these changes carry over into the animal have not been investigated thus far.

Interest in how toxic tall fescue grazing or ergot alkaloid exposure alters the animal microbiota and other physiologically important parameters is on the rise. For example, Harlow et al. (2017) evaluated the ability of hyper-ammonia producing bacteria (HAB) and tryptophan-utilizing bacteria to degrade ergovaline *in vitro*, and found that all HAB they tested (e.g., *Clostridium* spp.) were able to degrade the majority of ergovaline. Melchior et al. (2018) assessed the effects of tall fescue seed and red clover isoflavones on rumen microbial populations *in vitro* and found limited effects of tall fescue seed on volatile fatty acids (VFAs), but significant effects on bacteria aligned within the *Ruminococcaceae*, *Coriobacteriaceae*, and *Erysipelotrichaceae* families. Decreased diversity and richness associated with a lower tolerance to FT, and increased *Neocallimastigaceae* and *Thelebolus* abundance in high-tolerant and low-tolerant steers, respectively, was reported (Koester et al., 2020). We recently showed that toxic tall fescue grazing (E+) not only significantly affects specific fecal bacterial taxa but also leads to a unique hindgut microbiota structure (Mote et al., 2019). Finally, we found E+ exposure significantly altered the bovine plasma and urine metabolomes (Mote et al., 2017), and subsequently identified potential plasma and urinary biomarkers of a FT hindgut microbiota that was significantly associated with decreased animal productivity irrespective of additional external stressors (e.g., hot and humid environmental conditions; Mote et al., 2020a). Despite this, no study thus far has considered the plant-endophyte relationship *in planta* in conjunction with E+ effects on the animal's microbiota and/or metabolome across the GI tract and in the plasma and urine.

Therefore, using grazing beef steers, the goals of this study were to: (i) characterize the bacterial and fungal microbiota and metabolic differences between non-toxic (Max-Q) and toxic (E+) endophyte-infected tall fescue plant; (ii) assess changes in rumen and fecal microbial

(bacterial and fungal) communities and the rumen, plasma and urine metabolomes that result from E+ grazing; and (iii) importantly, assemble and evaluate the fescue toxicosis integrome to characterize the structure of the fescue toxicosis multi-compartment microbiota-metabolome relationship.

MATERIALS AND METHODS

Animals, pastures, and environmental conditions

Post-weaning Angus steers (n = 12) were blocked by weight and randomly assigned to non-toxic (weight: 306.6 ± 12.2 kg [$\bar{x}$ ± SEM]; Max-Q; Jesup MaxQ with endophyte AR542; 3 paddocks; 2 steers per paddock) or toxic (weight: 312.2 ± 16.0 kg [$\bar{x}$ ± SEM]; E+; Jesup with wild-type endophyte; 3 paddocks; 2 steers per paddock) tall fescue treatments on pastures described previously (Mote et al., 2017). Individual tillers were sampled from 15 random locations throughout the pastures, by cutting the tiller at the soil surface, placing it on dry ice and transporting the samples to the laboratory for storage at -80ºC. Plant tissue for pasture ergot alkaloid analysis were treated as before (Mote et al., 2019) and temperature and humidity measurements were recorded as in (Mote et al., 2019).

Sample collection and processing

Steer body weights were recorded before and 28 days post pasture assignment. Plasma, fecal, and urine samples were collected before (Pre), 2, 7, 14, and 28 days post pasture assignment as previously described (Mote et al., 2020a; Mote et al., 2019; Mote et al., 2017). Ruminal samples were collected with an ororuminal probe as previously described (Lourenco et al., 2020). Rumen liquids and solids were separated with three layers of sterile cheesecloth into 3 mL (liquids) and ~5 g (solids) aliquots and were frozen immediately on dry ice prior to transport to lab and storage at -80 ˚C.

Urinary ergot alkaloid analysis

Total urinary ergot alkaloid concentrations were determined all days of sampling after pasture placement as previously described (Hill et al., 2000; Mote et al., 2017; Stuedemann et al., 1998).

DNA extraction

Genomic DNA was extracted from all bovine matrices as previously described (Mote et al., 2020a; Mote et al., 2019). Tall fescue samples were placed in a sterile stomacher bag and stomached for 15 minutes with in TE extraction buffer. The supernatant was collected and taken through the same extraction procedures as previously described (Mote et al., 2020a; Mote et al., 2019).

DNA amplification and sequencing

Sequencing for 16S rRNA was performed as described before (Mote et al., 2020a; Mote et al., 2019). For the fungal sequencing, primers targeting the 5.8S-internal transcribed spacer 2 (ITS2) rRNA region were used as previously described (Taylor et al., 2016) with forward primer (ITS4: 5' – TATGGTAATTGTAGCCTCCGCTTATTGATATGCTTAART), index (AGWGATCCRTTGYYRAAAGTTGGCTGGCTGACT) and reverse primer (5.8S [structural RNA gene]: 3' – AGTCAGCCAGCCAACTTTYRRCAAYGGATCWCT). The following cycling (40 total) conditions were used with 30 ng of extracted DNA: initial denaturation at 95ºC for 3 min; 29 cycles of 95ºC for 30 sec, 58ºC for 30 sec, 72ºC for 30 sec; with the final extension set at 72ºC for 5 min. Controls and both 16S and ITS2 PCR products were treated as before (Mote et al., 2020a; Mote et al., 2019).

NGS sequence processing and bioinformatics analysis

Raw 16S and ITS2 sequence files were processed using mothur v.1.41.3 as previously described (Mote et al., 2020a; Mote et al., 2019), ITS2 sequences were aligned to UNITE database v04.02.2020. Sequences that were classified within the UNITE database as the genus *Neotyphodium* will be referred to as the updated genus nomenclature *Epichloë* (Leuchtmann et al., 2014) in the text. Herein, reference to *Epichloë coenophiala* will be termed: *E.* (*coenophiala*) for all references to NGS results as this is putative annotation and *E coenophiala* for all other references.

High-resolution metabolomics (HRM)

Metabolomics sample processing for urine, plasma, and rumen liquids was performed as previously described for plasma and urine (Mote et al., 2017). For tall fescue preparation, approximately 50 mg of plant material was added to vials with 100 µL acetonitrile, sonicated for 10 sec (ActiveMotif Epishear sonicator), sat on ice for 30 min prior to centrifugation (10 min at 14,000 rpm) and then placed into autosampler vials. All samples were analyzed with the Thermo Scientific linear triple quadrupole (LTQ) Orbitrap Velos with either hydrophilic liquid interaction chromatography (Waters Xbridge BEH Amide 2.5 µm, 2.1 x 100mm) or C18 chromatography (Higgins Targa C18 5µm, 2.1 x 100mm) with 10 min gradient runs and positive ionization, with instrument settings at 120,000 resolving power, 5 min runs, and 10 µL injection. Detection of metabolomics features and QC was performed as described in detail previously (Mote et al., 2017). All metabolomics annotation presented herein was performed using xMSannotator (Uppal et al., 2017) and either the Human Metabolome Database (HMDB; Wishart et al., 2018) or the T3DB toxic exposome database (T3DB; Wishart et al., 2015). All

pathway analysis was performed using mummichog and annotating the bovine samples with the bovine KEGG database and plant samples with thale cress KEGG database (Li et al., 2013).

Overlapping feature analysis

Normalized for sequencing depth OTU tables were utilized to assess overlapping OTUs. Only OTUs with greater than 10 sequences (50% presence) within a matrix and a treatment after normalization were kept in the analysis. For the metabolomics overlap, all features that were present in greater than 80% of samples within a matrix within a treatment were used. Venn Diagrams were generated using the Bioinformatics and Evolutionary Genomics online processor (http://bioinformatics.psb.ugent.be/webtools/Venn/).

Targeted network analysis

Normalized and scaled bacterial and fungal OTUs detected in >50% of samples in the E+ plant and rumen, specific to each analysis, were correlated to the *Epichloë* OTU, using the Hmisc *R* package (Harrell, 2018) and network analysis was performed on the significantly ($P < 0.05$) correlated features using the qgraph *R* package (Epskamp et al., 2012). The same approach was utilized for the rumen liquids metabolomics targeted network correlated to one metabolic feature putatively annotated as ergovaline.

Integrative interactomics analysis

xMWAS v0.552 (Uppal et al., 2018; https://kuppal.shinyapps.io/xmwas/) was used for the integrative interactomics analyses with the following parameters for the global analysis: dataX = metabolomics; dataY= 16S OTU table; dataZ = ITS2 OTU table; RSD > 1; Maximum number of variables from dataX = 500, from dataY and dataZ = 250; integration methods = sparse partial least squares regression (sPLS) in canonical mode with optimization of sPLS components with 500 [dataX], 250 [dataY], and 250 [dataZ] variables selected by sPLS; the

association analysis was set to ($|r| > 0.5$; $P < 0.05$); centrality analysis used eigenvector centrality; and graphical options were default. For the fescue xMWAS, altered parameters include: maximum number of variables from dataX = 500, from dataY and dataZ = 250, correlation threshold ($|r| > 0.3$), 1000 [dataX], 100 [dataY], and 100 [dataZ] variables selected by sPLS. The rumen analysis included the same variables as the fescue plant analysis, but the correlation threshold was set to ($|r| > 0.5$) The output files were downloaded, differential networks were imported into Cytoscape (https://cytoscape.org/) for further graphical visualization and analysis.

Targeted animal integrative interactomics analysis

Metabolites that were significantly affected by toxic tall fescue grazing and putatively annotated by mummichog using the *bos taurus* KEGG database into tryptophan metabolism, tyrosine metabolism, Vitamin B6 metabolism, steroid hormone biosynthesis and primary bile acid metabolism were used to target common metabolic pathways shown in previous studies (Mote et al., 2019; Mote et al., 2017), overlapping pathways herein, and growth-related pathways in the rumen. sPLS was performed using these 290 metabolites and the microbiota data sets from the untargeted analysis described above for both Max-Q and E+ data sets, and differential network analysis was performed to find those metabolic and microbial nodes specific to E+ steers in Cytoscape.

Statistical analysis of non-‘omics data

Statistical analyses of weight gains and urinary ergot alkaloids was performed using Sigma Plot, v 12.5 (Systat Software, Inc., San Jose, CA) as in (Mote et al., 2020a; Mote et al., 2019; Mote et al., 2017).

Transparency statement(s)

All raw sequence and metabolomics data files will be uploaded to NCBI Sequence Read Archive and Metabolomics Workbench upon acceptance.

RESULTS

Physiological results

E+ grazing significantly increased urinary ergot alkaloids and reduced cumulative and average daily weight gains (Fig S5.1), indicating the presence of fescue toxicosis and justifying downstream microbiome, metabolome, and integrative analysis.

General influence of the E+ on plant and animal bacterial microbiota profiles

Permutational analysis of variance (PERMANOVA) on the bacterial microbiota revealed significant main effects of E+ and time for both Bray-Curtis and Jaccard indices in all sample matrices (fescue plant, rumen solids, rumen liquids, feces; Table 5.1). However, the only significant treatment by time interaction was for the fescue plant bacterial profile (Table 5.1). These data indicate that the bacterial profiles of the fescue plant, rumen solids, rumen liquids and feces are all significantly affected by E+, regardless of whether considering presence/absence or abundance for the bacterial profiles.

For the bacterial alpha diversity analysis, Simpson's diversity was increased by E+ in the fescue plant and decreased by E+ in the rumen liquids, whereas only the rumen liquids had a main effect of time (Fig S5.2). There were main effects of E+ and time for Chao1 richness in the rumen liquids (increased in E+), rumen solids (increased in E+ after 14 days), and fecal matter (mixed effects; Table 1; Fig S5.2).

General influence of the E+ on plant and animal fungal microbiota profiles

PERMANOVA on the plant fungal (ITS2) profile (Table 5.1) revealed numerical trends ($P < 0.1$) towards a main effect of E+ and time for both Bray-Curtis and Jaccard matrices. Main effects of E+ and time were observed in the rumen solids, rumen liquids and feces (Table 5.1). There was also an E+:time interaction for the rumen solids and rumen liquids, with a trend ($P < 0.1$) towards significance for the fescue plant and feces (Table 5.1).

For the ITS2 alpha diversity analysis, there was a main effect of E+ in the rumen solids (increased by E+), with only a trend ($P < 0.1$) for the fescue plant (trend towards increase by E+; Table 5.1; Fig S5.2). Fescue plant and rumen liquids diversity had a main effect of time (Table 5.1; Fig S5.2). Finally, there was a main effect of time on ITS2 richness within the fescue plant (decreased over time, slight decrease by E+) and rumen liquids (decreased across the grazing trial; Table 5.1; Fig S5.2). Only matrices with significant main effects of E+ or time are shown in Figure S5.2.

Detection of OTU aligned to *E. coenophiala* in the microbiota

After quality filtering, alignment, and normalization for sequencing depth, one OTU aligned to *Epichloë* remained and was detected only in E+ plant and rumen samples, with the greatest abundance being after 14 days of grazing. This OTU was used for subsequent targeted network analysis in the E+ plant and rumen.

LEfSe results

Fescue plant

For the tall fescue bacterial communities (Figure 5.1A), the following were significantly increased in the Jesup Max-Q cultivar across the grazing trial: order (o_)*Flavobacteriales*; genus (g_)*Rudanella* of the family (f_)*Cytophagaceae*; g_*Clostridium* (f_*Clostridiaceae*);

f_*Peptostreptococcaceae*; multiple unclassified *Alphaproteobacteria*; g_*Methylobacterium*; g_*Roseomonas*; g_*Sphingomonas* and g_*Novosphingobium*; g_*Massila*; g_*Frigoribacterium*; and g_*Hymenobacter*. Bacteria that were significantly increased in E+ tall fescue included: f_*Solirubrobacteraceae* and f_*Patulibacteriaceae*, g_*Patulibacter*; g_*Bifidobacterium*; f_*Nakamurellaceae*; f_*Micromonosporaceae*; g_*Faecalibacterium*; g_*Labrys.* Within the fungal communities (Figure 5.1B), most differences were at the suborder level. Fungi more abundant in Max-Q fescue were: g_*Phaedoactyllum*; g_*Zymoseptoria* and g_*Mycosphaerella*; g_*Septoriella*; g_*Setosphaeria*; g_*Coniella*; g_*Colletotrichium*; g_*Acremonium*; g_*Tylospora*; g_*Sistotrema*; g_*Phyliozyma*; g_*Dioszegia* and g_*Bulleribasidium*. The fungi that were significantly more abundant in E+ fescue were: g_*Oomyces*; family *g_Sphaerellopsis* and g_*Pleurophoma*; f_*Montagnulaceae*; g_*Leptospora*; g_*Paraphoma*; g_*Setophoma*; g_*Setophaeosphaeria*; g_*Preussia*; g_*Tetraplosphaeria*; f_*Plectosphaereliaceae*; g_(*E. coenophiala*); and g_*Coprinellus*.

Rumen solids

When considering Pre, Max-Q, and E+ steers, the rumen solid microbiota was vastly different from before (Pre) and post pasture placement (Figure S5.3). Focusing on Max-Q and E+ samples post-pasture placement (Figure 5.2A), all constituents of the bacterial phyla (p_)*Elusimicrobia* were increased in Max-Q steers, but g_*Dehalobacterium*. p_*Spirochaetes* (notably g_*Treponema*), and g_*Prevotella* were increased in E+ steers. The rumen solid fungi (Figure 5.2B) were significantly more abundant for most taxa in Max-Q steers. Nonetheless, select fungi were significantly increased by E+ grazing. Examples are g_*Sporormiella* (of which the order *Polyporales* and other related fungi were increased in Max-Q), f_*Neocallimastigaceae*

(only one genus within this family, *Cyllamyces*, was increased in Max-Q steers), and p_*Chytridiomycota*.

Rumen liquids

Comparisons between treatment groups including before pasture placement are shown in Figure S3. Post pasture placement (Figure 5.2C), the bacteria candidates f_*F16* and o_*RF39* were increased in Max-Q steers, whereas c_*Clostridia* was significantly increased in E+ steers. Notably, the g_*Mogibacterium*, g_*Selenomonas*, g_*Moryella,* g_*Butyrivibrio*, g *Prevotella* and candidate g_*02d06* were significantly increased in E+ steers. For the rumen liquid fungal communities (Figure 5.2D), p_*Ascomycota* was increased in Max-Q steers, alongside f_*Glomerellaceae* and f_*Colletotrichum*. The g_ *Pseudorobillarda*, g_*Diaporthe*, f_*Neocallimastigaceae*, g_*Caecomyces*, o_*Agaricales* order, and f_*Lycoperdaceae* were all significantly increased in E+ rumen liquids.

Feces

For the fecal bacterial microbiota, the c_*Akkermansia*, f_*F16*, g_*Eubacterium*, and g_*Clostridium* (f_*Erysipelotrichaceae*) were increased in Max-Q steers (Figure 5.3). The c_*Clostridia,* g_*Mogibacterium*, and g_*Clostridium* (c_*Clostridia*) were all increased in E+ steers (Figure 5.3). For the fecal fungal microbiota, while numerous fungi were increased in Pre, Max-Q, and E+ steers, it is notable that the c_*Sordariomycetes* and o_*Hypocreales* were increased in E+ steers, but f_*Clavicipitaceae* and g_*Epichloë* were not (Figure 5.3).

Metabolic effects of E+ exposure

Across the grazing trial, *E. coenophiala*-infection, when compared to the novel Max-Q endophyte, affected numerous metabolic pathways in the tall fescue plant, including phenylalanine tyrosine and tryptophan metabolism, Vitamin B6 metabolism,

glycerophospholipid metabolism, and tropane, piperidine, and pyridine alkaloid biosynthesis (Figure 5.4A). E+ grazing had significant effects on steroid hormone biosynthesis, arachidonic acid metabolism, aminoacyl-tRNA biosynthesis, histidine metabolism, and Vitamin B6 metabolism in the rumen liquids of beef steers (Figure 5.4B). In the plasma, E+ grazing significantly perturbed purine metabolism, the pentose phosphate pathway, amino/nucleotide sugar metabolism, phenylalanine, tyrosine, and tryptophan metabolism, and Vitamin B6 metabolism (Figure 5.4C). In the urine, starch and sucrose metabolism, amino/nucleotide sugar metabolism, terpenoid backbone biosynthesis, and tryptophan, glycerophospholipid, and arachidonic acid metabolism were all perturbed by E+ grazing (Figure 5.4D). Interestingly, Vitamin B6 metabolism was one of few metabolic pathways affected by E+ presence/grazing across all biological matrices (Figure 5.4).

Putative ergovaline feature intensity

Using xMSannotator and the T3DB toxic exposome database, one metabolic feature (*m/z* 534.2708451, time 281.4581094; [M+H]) was putatively identified as ergovaline. Interestingly, this feature was only detected in the E+ plant and rumen liquid. In the E+ fescue, putative ergovaline was detected in multiple individual plant samples (6/18 E+ samples), with the average overall and positive sample intensities depicted in Figure 5.5A. In the rumen liquid, no detection occurred in any Max-Q nor in E+ before pasture placement. Detection in E+ steers first occurred on Day 2 and peaked on Day 14, with Day 28 levels equaling Day 7 (Figure 5.5B).

Overlapping microbial and metabolic features

Overall, no general overlap existed overlap between plant and animal bacterial OTUs, but there was significant overlap between plant and rumen fungal OTUs (Max-Q 103; E+ 115). Further, there was substantial overlap existed between bacterial (332 Max-Q; 378 E+) OTUs in

the rumen solids and rumen liquids regardless of fescue cultivar (Figure 5.6A and 5.6B). Notably, only one overlapping bacterial OTU was detected in the rumen solids, rumen liquids, and feces, and this OTU aligned to f_*Lachnospiraceae* family within the Max-Q steers (Figure 5.6A). The only bacterial OTU that overlapped between the rumen liquids and rumen solids, in both Max-Q and E+, was of o_*Clostridiales* (Figure 5.6A and 5.6B). Overlapping OTUs between the rumen solids and feces within E+ steers alone were those aligned to f_*Coriobacteriaceae*, f_*Lachnospiraceae*, and f_*Ruminococcaceae* families (Figure 5.6B), which are families we have previously found affected in the feces (Mote et al., 2019) of E+ grazing steers.

For the fungi, more OTUs overlapped between the grass and rumen liquids than grass and rumen solids in both Max-Q (Grass x Liquids = 35; Grass x Solids = 15) and E+ (Grass x Liquids = 40; Grass x Solids = 14) steers. Notably, an OTU aligned to the *Epichloë* (UNITE *Neotyphodium*) genus, alongside multiple *Phaeosphaeriaceae* OTUs, was found to overlap between all four biological matrices sampled in E+ group. Many OTUs overlapping between the fescue grass, rumen solids, and rumen liquids were cultivar specific. Those OTUs overlapping between these three biological matrices in E+ steers included *Cryptococcus aureus, Saitozyma paraflava, Phaeosphaeria, Trematеia, Erythrobasidium, Filobasidium, Bullera alba,* and *Cryptococcus dimennae* (Figure 5.6D). Further, multiple OTUs aligned to f_*Neocallimastigaceae* overlapped between the rumen solids, rumen liquids, and feces in both Max-Q and E+ steers. At the subfamily level, more group specific OTUs overlapped, i.e., in E+ g_*Vishniacozyma taibaiensis*, g_*Mucor fragilis*, g_*Lycoperdon pratense*, g_*Wallemia*, g_*Occultifur*, g_*Papiliotrema flavescens*, g_*Puccinia xanthii*, and a different g_*Cryptococcus dimennae* OTU. Max-Q specific OTUs included, among others, g_*Toxicocladosporium* and

g_*Penicillium citrinum*. A full list of overlapping bacterial and fungal OTUs can be found in File S5.1.

In both Max-Q and E+ steers, we found that the number of overlapping metabolic features between multiple biological matrices from both cultivars was similar. Notably, 533 metabolic features overlapped between all biological matrices in Max-Q steers (Figure 5.7A), whereas this number was 543 in E+ steers (Figure 5.7C). Of these metabolic features that overlapped between all biological matrices, 526 were shared between Max-Q and E+ steers, and 7 and 17 were distinct to Max-Q and E+, respectively (Figure 5.7B). Among the distinct E+ features were metabolites putatively annotated as: 11-dimethoxydecane (*m/z* 102.1042 [M+H], a common membrane constituent), urea (m/z 121.0718 [2M + H]), L-kynurenine (m/z 209.0921 [M+H], metabolite of the amino acid L-tryptophan and used in the production of niacin), and (R)-Pterosin B (*m/z* 219.1379, plant derived), plus multiple putatively unannotated metabolites.

Targeted *Epichloë* and ergovaline correlation network analysis

(*E. coenophiala*) targeted network analysis in the fescue plant revealed one highly interconnected network of 29 fungal and 31 bacterial OTUs (Figure 5.8A). Notably, the (*E. coenophiala*) OTU had the third highest centrality in the fescue network, preceded only by a *Periconia* and *Nocardioidaceae* OTU (Figure 5.8B). The classification of most OTUs in the fescue network were unique (i.e., 1 OTU per family/genus/species). The only classified taxa that had more than 1 OTU were the *Lichtheimia ramose*, *Neocallimastigaceae*, *Comamonadaceae*, *Dyadobacter*, and *Methylobacterium adhaesivum*.

For the rumen, targeted analysis was done using the (*E. coenophiala*) OTU in the rumen liquids, as it was not prominent in the rumen solids; 180 total (27 fungi [8 solids, 19 liquids], 153 bacteria [61 solids, 92 liquids]) OTUs were significantly correlated with the *Epichloë* OTU

(Figure 8C). Of these, 4 liquid and 2 solid *Orpinomyces sp*, 2 solid and 1 liquid *Cyllamyces aberensis*, and 2 *Neocallimastigaceae* OTUs were the most prominent fungi in the network. *Aspergillus cristatus* and *Piromyces sp* were the other fungi in the rumen network that had an OTU from both rumen solids and rumen liquids. From the bacteria, *Prevotellaceae Prevotella* and *Lachnospiraceae* both had 14 liquid and 13 solid OTUs in the network. Other prominent bacteria in the network included *BS11* with 7 liquid and 4 solid OTUs, *Paraprevotellaceae* candidate *CF231* with 6 liquid OTUs, *Butyrivibrio* with 6 liquid and 3 solid OTUs, and *Ruminococcaceae* with 5 liquid OTUs. Notably, the *Epichloë* OTU had median centrality within the network (Figure 5.8D).

Finally, a rumen targeted metabolite network using the putative *E. coenophiala*-derived metabolite ergovaline was performed with ($|r| > 0.4$; $P < 0.05$). 497 total metabolic features (255 c18; 242 HILIC) were significantly correlated with the putative ergovaline feature intensity profile (Figure S5.5). The metabolites in this network that significantly associated with ergovaline in the rumen liquids were primarily involved in steroid hormone biosynthesis, tryptophan metabolism, tyrosine metabolism, amino and nucleotide sugar metabolism, glucose and energy metabolism, and Vitamin B6 metabolism. Considering the large size of this network, the threshold was increased ($|r| > 0.6$; $P < 0.05$). The newly generated network (Figure 5.8E) had 43 metabolites, where ergovaline was at the center of the network and had the highest centrality measure of all nodes within the network (Figure 5.8F). Pathway analysis revealed that most annotated metabolites in the focused network were involved in steroid hormone, tryptophan, and tyrosine metabolism.

Differential interactomics

Fescue plant

Integrative interactomics analysis on the tall fescue plant revealed network modularity measures at 0.73 and E+ at 0.7 for Max-Q and E+ respectively, indicating comparable levels of network clustering. In both networks, and common throughout these results regardless of biological matrix, bacterial and fungal OTUs were the central nodes in most clusters within the networks and were surrounded by associated metabolites (Figure 5.9). The Max-Q network consisted of five communities of clustered metabolites and OTUs, whereas the E+ network consisted of seven (Figure 5.9). Of the nodes that had centrality measurements > 0.5, 71 were metabolites, 30 were bacteria, and 27 were fungi in the Max-Q network (File S5.2A); E+ network consisted of 0 metabolites, 22 bacteria, and 34 fungi (File S5.2B). That said, cluster 5 (File S5.2B) had all nodes with centrality measurements > 0.5. Only one other feature had a centrality about 0.5, an OTU classified as *Methylobacteriaceae*, in cluster 1. Notably, in the E+ tall fescue network (Figure 9; File S2B), no OTU aligning to the (*E. coenophiala*), or higher levels (e.g., *Clavicipitaceae*), was identified. Numerous OTUs were present only in the E+ tall fescue network, including those aligned to *Clostridium* (*Ruminococcaceae*), *Pseudorobillarda*, *Apenidiella*, candidate *LD1-PB3*, candidate *RF39*, *Periconia*, *Colletotrichum*, *Geodermatophilaceae*, *Rhodosporidiobolus*, *Cladosporium*, *Cryptococcus*, *Wallemia*, *Lentitheciaceae*, *Neodevriesia*, *Mogibacteriaceae* (File S5.2B). Those OTUs appearing only in the Max-Q tall fescue network included *Dinemasporium*, *Fibrobacter succinogenes*, *Paenibacillus*, *Stictidaceae*, candidate *Saprospirales*, *Pseudomonas*, *Escherichia coli*, *Xanthomonadaceae*, *Tremellales*, *Deinococcus*, *Dothideomycetes*, *Myrmecridium*, *Comamonadaceae*, *Xylariaceae*, candidate *L7A_E11*, and *Coriobacteriaceae*. Interestingly, the

steroid biosynthesis, nucleic acid and glucosinolate biosynthesis metabolic pathways appeared in both Max-Q and E+ networks when querying metabolic nodes with centrality measurements above (> 0.2). Metabolic pathways singular to E+ include ubiquinone and other terpenoid-quinone biosynthesis, sesquiterpenoid and triterpenoid biosynthesis, tyrosine metabolism, inositol phosphate metabolism, arginine and proline metabolism, brassinosteroid biosynthesis, phenylpropanoid biosynthesis, phosphatidylinositol signaling system, caffeine metabolism, and valine, leucine and isoleucine metabolism.

Rumen integrative interactomics

Rumen interactomics revealed the one main group of clusters with a single cluster unattached to the main network in both Max-Q and E+ steers (Figure 5.10). Additionally, in both Max-Q and E+ networks, fungal OTUs had the highest centrality measurements (= 1). While some fungi with high centrality measurements were present in both treatments (e.g., *Neocallimastigaceae*) others were distinct. For example, fungi specific to the Max-Q network included *Mucoraceae*, *Cystobasidium*, *Golubevia*, *Baeospora*, *Occultifur*, *Sympventuriaceae*, *Vishniacozyma*, *Hypomyces*, *Hortaea*, *Lichtheimia*, *Mycosphaerella*, *Fellomyces*, *Bjerkandera*, *Hannaella*, *Sarocladium*, *Pilidium*, *Neosetophoma*, *Rhodotorula*, *Chionosphaeraceae*, and *Naohidea*. Fungi specific to the E+ network included *Purpureocillium*, *Microdochium*, *Dinemasporium*, *Rahicladosporium*, *Delitschia*, *Thermomyces*, *Diaporthe*, *Moesziomyces*, *Devriesia*, *Aspergillus*, *Leptospora*, *Ramichloridium*, *Curvularia*, and *Fusarium*. The features with the next highest centrality measurements were bacterial OTUs and most of these were similar in Max-Q and E+ networks; however, *Pyramidobacter* and *Treponema* were unique to the E+ network and *Fusobacterium*, *Shuttleworthia*, candidate genus *TG5*, *Bibersteinia trehalosi*, and *Atopobium* were unique to the Max-Q network. Interestingly, steroid hormone biosynthesis,

primary bile acid biosynthesis, and fatty acid metabolism were some of the top pathway metabolic features with centrality measurements (>2) in both Max-Q and E+ steers. Metabolic pathways distinct to E+ network included glycosphingolipid biosynthesis, metabolism of xenobiotics by cytochrome P450, and folate biosynthesis.

Global animal integrative interactomics

Global (i.e., rumen, plasma, urine microbiota and metabolomes) xMWAS resulted in two unique networks between Max-Q and E+ samples. The Max-Q network had a total of 2837 metabolite (1638 urine, 226 plasma, and 973 rumen liquid) and 275 OTU (205 fecal and 70 rumen liquid) nodes with a modularity measure of 0.69. The E+ network had 2689 metabolite (1696 urine, 221 plasma, and 772 rumen liquid) and 296 OTU (108 fecal, 92 rumen solids, 96 rumen liquid) nodes and had a modularity measure of 0.57 (Figure 5.11). While the E+ network had fewer overall nodes (Max-Q 3112; E+ 2985), it had a greater number of connections between nodes (Max-Q 77,501; E+ 127,446). Notably, the E+ network was the only network that had nodes from the rumen solids (Figure 5.11).

The nodes with the highest centrality measurements in the E+ network were from the fecal fungal, rumen solids/liquids bacterial, and fecal bacterial features (File S5.2F; eigenvector centrality > 0.7; rest of nodes centrality was less than 0.3); in the Max-Q network, those nodes were fecal fungi, rumen liquids bacteria, and fecal bacteria (File S5.2E; centrality > 0.842; rest less than 0.3). The fecal fungal OTUs within the Max-Q and E+ networks were mostly distinct, with the E+ network having two *Neocallimastigaceae Anaeromyces*, one *Aspergillus*, one *Acremonium brachypenium*, and one *Meyerozyma* OTUs (File S5.2F). The Max-Q fecal fungal OTUs were aligned to the *Issatchenkia*, *Monographella*, *Poitrasia*, *Fibroporia*, *Erthrobasidium*, and *Myrmecridium* genera, among other unclassified OTUs (File S5.2E). Although ruminal

liquid OTUs had some overlap between the two networks (i.e., *Lachnospiraceae*, etc.), there were multiple distinct features. The OTUs distinct in the E+ network aligned to unclassified *Betaproteobacteria* and *Bacteroidetes*, one each from candidates *BS11* and *LD1-PB3*, and one *Ruminococcaceae* and *Mogibacteriaceae* (File S5.2F); whereas unique Max-Q OTUs were *Fusobacterium*, *Prevotella*, *ML615J-28*, *Leptotrichiaceae*, and candidate genus *CF231* (File S5.2E). While the only fecal bacterial OTUs unique to the E+ network were one *Erysipelotrichaceae* and one *Anaerolinaceae* OTUs (File S2F), there were multiple fecal bacterial OTUs unique in the Max-Q network, including, among others, *Ruminococcaceae*, *Rikenellaceae*, *Coriobacteriaceae*, and *Lachnospiraceae* (File S5.2E).

The rumen solid bacterial OTUs in the global E+ network were diverse, with candidate families *RF39* and *RFP12* having 3 OTUs, candidate *S24-7*, *Mogibacteriaceae*, *Lachnospiraceae*, candidate *R4-458*, and the *Prevotella* and *Oscillospira* genera all having 2 OTUs each (File S2F). Multiple other (e.g., *Clostridium* and *Treponema*) classified OTUs had only one present in the E+ network (File S5.2F).

Interestingly, we sought to identify metabolites with high centrality (> 0.2) that were different between the Max-Q and E+ global networks. Most metabolites were urinary metabolites, with rumen liquid and plasma metabolites making up a smaller portion of these important nodes. Unique to the E+ network, plasma metabolites were mainly involved in fatty acid and riboflavin metabolism, whereas the plasma metabolites in the Max-Q network were primarily involved in galactose, fructose and mannose metabolism, and amino sugar and nucleotide sugar metabolism. The rumen metabolic features unique to the E+ networks were primarily involved in steroid biosynthesis, folate biosynthesis, and metabolism of xenobiotics by cytochrome P450, where the Max-Q networks had numerous unique pathways (e.g., pentose

phosphate pathway, glycolysis, etc.). The E+-specific urinary metabolites were associated with steroid hormone biosynthesis, purine, arachidonic acid, pentose phosphate pathway, and tryptophan and tyrosine metabolism. The urinary metabolites in the Max-Q network were involved in steroid hormone biosynthesis, metabolism of xenobiotics by cytochrome P450, valine, leucine and isoleucine degradation, and glycosphingolipid biosynthesis. Although urinary steroid hormone biosynthesis as a pathway appeared in both networks, the metabolic features annotated within this pathway were unique.

The Max-Q plot had three standalone clusters apart from the main network structure. In cluster 8 (Figure 5.11A), *Curvibasidium*, *Anaeromyces*, *Thermoascus*, *Thermomyces* were main nodes surrounded by metabolites related to phenylalanine metabolism and glycosaminoglycan/steroid hormone biosynthesis (plasma); arachidonic acid, simple and complex sugar, and arginine/proline metabolism (rumen); and steroid and valine, leucine and isoleucine metabolism (urine). Cluster 10 (Figure 5.11A) consisted of three fecal ITS2 OTUs, ten rumen liquid, and fifteen fecal bacterial OTUs (File S5.2E). The rumen liquid OTUs were largely unclassified, *Lachnospiraceae*, or *Ruminococcaceae* (File S5.2E). The fecal 16S OTUs included *Prevotella*, , *Ruminococcaceae*, and *Lachnospiraceae*, among others (File S5.2E). Cluster 9 (Figure 5.11A) consisted of fecal fungal (*Phaeophyscia*, *Sphaerellopsis*, etc.) and bacteria(*Butyrivibrio*, *Ruminococcaceae*, etc.), and rumen liquid bacterial candidate family *S24-7*, *YRC22*, and *Olsenella*. Full node tables for the fescue, rumen, and global xMWAS analyses can be found in File S5.2.

Global integrative interactomics analysis was only possible on the entire data set post pasture placement, individual dates were unsuccessful even when lowering correlation threshold to ($|r| > 0.3$).

Targeted animal integrative interactomics analysis

Targeted interactomics analysis assessed the multi-compartment relationship between metabolites and microbes that were significantly affected by E+ and present solely in a targeted E+ integrome network. One cluster resulted specific to E+ that was centered on three urinary metabolic features that were highly associated ($|r| > 0.7$) with bacterial and fungal OTUs from every animal biological matrix (Figure 5.12). The three metabolites central to this network are L-metanephrine, L-dopachrome, and pyridoxal, which are involved in tyrosine and Vitamin B6 metabolism, respectively (Figure 5.12). Of those OTUs associated with all three metabolites in the network include: *Anaeroplasma*, *Prevotella ruminicola*, *Clostridium*, *Ruminococcus*, and *Prevotella* (rumen liquids); *Clostridium* (rumen solids); *Ruminococcaceae*, *Rikenellaceae* (feces); other unclassified OTUs in all matrices (Figure 5.12). Notably, one unclassified fecal bacterial OTU and one rumen liquid *Cryptococcus aspenensis* OTU were solely associated with urinary L-dopachrome in the targeted integrome network (Figure 5.12).

DISCUSSION

Herein, is the first fescue toxicosis global analysis using an integrative multi-'omics approach that includes both the plant and animal. *E. coenophiala* infection and exposure significantly alters both the plant and animal multi-compartment microbiota, metabolomes, and it leads to unique integrome structure. Further, while there is little overlap between the plant and animal microbiota, some E+-associated metabolic changes are common to all biological matrices.

E. coenophiala infection significantly altered the tall fescue phyllosphere microbiota. There were increases in numerous plant specific bacteria and fungi, g_*Epichloë* included. While it is known that the phyllosphere microbiota can be influenced by plant and environmental

factors, the plant microbiota are likely also influenced by endophytes, such as *E. coenophiala* (Vorholt, 2012a; Wallace et al., 2018; Zarraonaindia et al., 2015). Most bacteria/fungi affected by E+ in the plant and animal were distinct from one another, but it is significant that the *Epichloë* genus was increased in the E+ rumen liquids. While it is unlikely that aerobic microbes, fungi included, are able to thrive in the anaerobic ruminal environment, one study has found that anaerobic fungi within the GI tract of cattle have, within their life cycle, one stage that provides increased tolerance to an aerobic environment (Davies et al., 1993). Considering this and previous reports of anaerobic fungi being viable in bovine feces (McGranaghan et al., 1999), one possible explanation for the number of fungal OTUs that overlapped between all biological matrices (plant and animal) is that the complex life cycle and sporulation of fungi could allow them to persist in adverse environments (Davies et al., 1993; McGranaghan et al., 1999). This should be evaluated further.

Across the grazing trial E+ exposure reduced the majority of ruminal fungal taxa in both the solid and liquid fractions. Fungi digest cellulose in the rumen, and mycelium penetration of feed particles breaks the fibers apart, allowing for increased surface area and better degradation (Russell, 2002). The ability of fungi to degrade fibrous particles of feedstuffs is an important part of ruminant nutrient extraction (Russell, 2002). Increased rumen fill that could not be explained by increased dry matter intake, and may be indicative of decreased ruminal passage rates, has been reported in fescue toxicosis (Foote et al., 2013; Koontz et al., 2012; Koontz et al., 2013; Koontz et al., 2015). In the study herein, we noticed, but did not quantify, that E+ steers had greater rumen solid contents fraction. Considering the role ruminal fungi play in feed degradation, outlining the relationship between fungal shifts in response to E+ exposure and

alterations in rumen fill/passage rates should be evaluated, especially considering the role fungal microbiota may play in providing tolerance to E+ exposure (Koester et al., 2020).

While limited carryover effects from the plant to the animal were observed for the microbiota, there were several metabolic pathways that were affected by E+ in both the plant and the animal. Some (e.g., tryptophan) pathways align with our previous E+ grazing studies (Mote et al., 2020b; Mote et al., 2019; Mote et al., 2017), but we also identified other important pathways, such as Vitamin B6 metabolism, as significantly perturbed in all biological matrices. We already reported altered urinary Vitamin B6 metabolism in fescue toxicosis (Mote et al., 2020b), but herein we found it to be altered in all biological matrices sampled. Multiple forms of Vitamin B6 exist (Rosenberg, 2012) and it is an essential vitamin in humans and animals (Percudani and Peracchi, 2009). While plants have the ability to *de novo* synthesize Vitamin B6, animals must obtain it through diet (Fitzpatrick, 2011; Tambasco-Studart et al., 2005). Alterations in Vitamin B6 metabolism in the plant and rumen could alter downstream amino acid metabolism through microbial means (Mohammed et al., 2004). Also, multiple enzymes in the tryptophan metabolic pathways (e.g., kynureninase) use Vitamin B6 (pyridoxal phosphate) as an essential co-factor, and reduction of Vitamin B6 has been shown to decrease tryptophan metabolites in mice (Bender et al., 1990; da Silva et al., 2013). Notably, L-kynurenine was a metabolite we found overlapping between all biological matrices (plant and animal) that was specific to E+ samples. Vitamin B6 is also a co-factor for transaminases (Romo and Liu, 2011) that are induced by glucocorticoids, a central stress response, indicating likely widespread effects of Vitamin B6 alterations. Notably, folate (Vitamin B9) biosynthesis was a metabolic pathway that appeared only in the E+ rumen and global integrative networks. It was also previously demonstrated that thiamin (Vitamin B1) supplementation provided benefits to E+ grazing steers

(Lauriault et al., 1990). Overall, plant and animal alterations in Vitamin B6 metabolism could influence subsequent amino acid (e.g., tryptophan) metabolism that we consistently see altered in E+ grazing studies (Mote et al., 2020a; Mote et al., 2017), and the relationship between vitamins in the B family and the pathophysiology of fescue toxicosis and, perhaps, broader vitamin supplementation/monitoring as a FT therapeutic is worthy of further investigation.

Interestingly, this study putatively identified ergovaline, the most prevalent toxic fescue ergot alkaloid (Klotz, 2015; Klotz and Nicol, 2016), via untargeted metabolomics. This is novel, and a confirmation of the identity of this metabolic feature by targeted means is important. The pattern of putative ergovaline detection aligns with what would be expected from the literature. First, no ergovaline was detected in any biological matrix where the Max-Q endophyte was used, which is expected since this novel endophyte was created to not produce ergot alkaloids while still providing other agronomic benefits (Bouton et al., 2002; Gunter and Beck, 2004; Parish et al., 2003). In E+, putative ergovaline was found in the fescue plant and rumen. In the plant, one-third of samples were positive for this metabolite. Whether this is due to the untargeted metabolomics-based, non-ergot alkaloid specific (Patel et al., 2015) extraction method, varied level of endophyte infection, small sample size, or other factors is unknown. Given the small variability of stem and leaf ergovaline levels measured by targeted plant analysis (Rottinghaus et al., 1991), small sample amount in combination with the broad untargeted metabolomics extraction method is more likely responsible for the non-uniform detection.

In the rumen of E+ steers, putative ergovaline was undetected prior to placement on pastures, with levels increasing until peaking at Day 14. Urinary ergot alkaloids in this study also indicate these steers were fescue naïve prior to the study, so it was not expected to detect ergovaline on Day 0. The pattern of ergovaline in the rumen follows a similar pattern to the

urinary ergot alkaloids, a sensitive biomarker of ergot alkaloid exposure (Hill et al., 2000), until Day 14 of the grazing trial. The decrease in ruminal ergovaline on Day 28 of the grazing trial could have multiple origins. For example, our lab has previously found that urinary ergot alkaloid levels tend to plateau or decrease and differences between the E+ and Max-Q microbiota become stable after 14 days of grazing Jesup wild-type tall fescue across three different grazing trials in the Fall, Spring, and early Summer (Mote et al., 2020a; Mote et al., 2019; Mote et al., 2017). This plateau, including in the current study, across seasons may indicate an adaptive \ response to E+ after 14 days on pasture and/or that steady state metabolism has been reached. If the animal microbiota shifted and/or cytochrome P450 levels increased globally (Moubarak and Rosenkrans, 2000; Settivari et al., 2008) to adapt to ergovaline and other ergot alkaloids found in E+ accelerated metabolism and/or biotransformation into less toxic metabolites would take place and result in decreased ruminal ergovaline towards the end of the study as observed here.

Targeted network analysis in the plant and rumen revealed that most OTUs associated with the (*E. coenophiala*) OTU were distinct between the plant and the animal. The bacterial family *Lachnospiraceae*, one family with OTUs commonly effected by E+ in the grazing animal, and the *Orpinomyces* genus were the only microbes that had OTUs significantly associated with (*E. coenophiala*) in both the plant and rumen. Notably, none of the *Lachnospiraceae* or *Orpinomyces* OTUs in the plant and the animal overlapped, indicating that sub-genus differences exist between what is present in the plant and/or the animal. The only fungus that was associated ($r = -0.43$) with (*E. coenophiala*) and was increased in the plant was a *Leptospora sp* OTU. A member of the *Phaeosphaeriaceae* family, which contains economically costly plant pathogens, some relatives of the *Leptospora sp* have been identified as endophytes in monocotyledons plants (Phookamsak et al., 2014), which tall fescue is. The genera *Sphingomonas* ($r = 0.45$) and

Methylobacterium (r = 0.32) were associated with (*E. coenophiala*) but were decreased in E+ tall fescue. Although it is unclear what the relationship between these microbes and (*E. coenophiala*) is, it seems possible that they could potentially be competing for resources with *Epichloë* and/or be influenced by some *Epichloë* secondary metabolites.

Within the rumen, the majority of correlated OTUs were bacteria, but some notable fungi associated with the rumen liquid (*E. coenophiala*) OTU. For example, the *Neocallimastigaceae* family, namely the *Orpinomyces* genus, had the majority of fungal *Epichloë* associated OTUs. Two OTUs classified to *Neocallimastigaceae* had negative correlation coefficients, whereas those classified further to *Orpinomyces* had variable coefficients. Notably, two *Orpinomyces* OTUs were correlated with *Epichloë* in both the rumen solids and rumen liquids, one positively (r= 0.56) and one negatively (average r = -0.48). Interestingly, of the 15 rumen liquid *Prevotella* OTUs, 10 were negatively correlated and 5 were positively correlated; whereas of the 13 rumen solid OTUs, 3 were negatively correlated and 10 were positively correlated. Overall, for most taxa where multiple correlations were present, there was a mix of positively and negatively correlated OTUs, indicating that sub-genus targeted analysis of these fungi and bacteria may help us in understanding the complex ruminal microbiota relationship within the context of fescue toxicosis.

Targeted ergovaline network analysis revealed a highly interconnected network between rumen metabolites. The majority of metabolites in this network were involved in steroid hormone biosynthesis, tryptophan and tyrosine metabolism, and Vitamin B6 metabolism. This is interesting considering that these metabolites are not only correlated with putative ergovaline in the rumen but are components of the metabolic pathways most significantly affected by E+ grazing overall. Previously, we have found E+ altered tryptophan and tyrosine metabolism (Mote

et al., 2020a; Mote et al., 2017). So, while putative ergovaline did not have the highest centrality in the full network, it is interesting that the metabolic pathways it was associated with were the same ones identified by more robust, broader metabolomics-based methods. In the focused networks, the only pathways affected were steroid hormone and primary bile acid biosynthesis and ergovaline had the highest centrality of all metabolic features. It has been previously shown that ergot alkaloids can influence systemic hormonal homeostasis (Oliver, 2005), but these data seem to indicate ergovaline and E+ tall fescue may begin to induce hormonal imbalances presystemically, i.e., in the rumen; however, this will require further investigation.

Integrative analysis revealed the general structures of the global Max-Q and E+ networks were fairly similar (i.e., microbial nodes are the anchors with peripherally associated metabolites), but the constituents of the networks were distinct between the two treatment groups. Notably, in both networks, fecal ITS2 OTUs had the highest centrality (i.e., most important to the structure of the network) measurements, but the fungal classifications were generally distinct between the two networks. Interestingly, one of the unique genera in the E+ network was the fecal *Neocallimastigaceae Anaeromyces*. Although *Anaeromyces* was not a genus reported, it was previously found that the *Neocallimastigaceae* family was increased in steers with greater tolerance to E+ exposure, indicating this family may be important in the structure of the E+ integrome and play a role in the severity of fescue toxicosis symptoms (Koester et al., 2020). Considering E+ reduced the abundance of most fungal taxa, yet ruminal solid fungal OTUs appear in the E+ integrome only, future work on the specific influence of E+ on ruminal fungi homeostasis is needed.

Previously, urinary ergot alkaloids have been proposed as a sensitive biomarker of exposure (Hill et al., 2000; Mote et al., 2020a; Mote et al., 2019; Mote et al., 2017; Stuedemann

et al., 1998) and, potentially, a biomarker of effect (Hill et al., 2000) for E+ exposure in beef cattle. Although these provide great utility for producers and scientists alike, additional biomarkers that encapsulate the molecular mechanisms of E+-induced decreased weight gains may be more therapeutically valuable for keeping steers on E+ pastures. In our search for subsequent/supplemental biomarkers, we identified an OTU that aligned to the *Epichloë* genus only in E+ animals and was most abundant after 14 days of E+ grazing. As this specific OTU did not have track well with pasture alkaloids, urinary ergot alkaloids, or weight gains in E+ steers, its identification in the animal is interesting, likely consequential, but not a good biomarker of exposure or effect. Part of this may be related to endophyte breakdown together with plant material in the upper GI but this would need to be evaluated further.

Akin to what we found for plasma/urinary metabolites having utility as a biomarker of a decreased productivity-associated hindgut microbiota (Mote et al., 2019), targeted global integrative analyses performed herein revealed three urinary metabolic features (L-metanephrine, L-dopachrome, and pyridoxal) are descriptive of E+ and positively associated with the microbiota in multiple animal matrices. All three of these nodes were urinary metabolites, not plasma or rumen liquid, indicating urinary metabolites can be discriminatory between Max-Q and E+ steers and ideal candidates for easy-to-access biomarkers of FT. Further, urinary metanephrines have shown equal utility as plasma metanephrines as a diagnostic biomarker of pheochromocytoma (Grouzmann et al., 2010) and urinary dopachrome tautomerase protein has been suggested as a potentially sensitive biomarker of drug-induced liver injury (van Swelm et al., 2012). Considering we found urinary dopamine, epinephrine, and norepinephrine increased, and 3,4-Dihydroxyphenylacetic acid (DOPAC) decreased, in E+ steers after 28 days on pasture (Mote et al., 2017), pathways associated with these urinary catecholamines consistently

perturbed, and they are all positively associated with microbiota from multiple compartments, these urinary metabolites may be useful biomarkers for FT from a therapeutics perspective. Finally, these data provide foundational evidence that show the previous perturbations we have identified (Mote et al., 2020a; Mote et al., 2019; Mote et al., 2017) are snapshots of systemic perturbations that occur in toxic tall fescue grazing steers, and understanding the multi-level multi-compartment integrome will provide more actionable insights moving forward. Although it is unclear the relationship between urinary L-dopachrome and *Cryptococcus aspenensis*, it is interesting that L-dopachrome was the only feature associated with this fungal OTU indicating a relationship that should be evaluated further in the FT context.

In this novel study of the effects of *E. coenophiala* infection on the plant and animal microbiota, metabolome, and the multi-compartment, multi-'omics integration are presented. The data suggest the majority of the microbiota profile, and the effects of E+, are distinct between the plant and the animal. Notably, effects of the E+ endophyte on the plant and multi-compartment animal metabolome did share some similarities. Finally, we provide the first overview of the complex interactions between the bovine multi-compartment microbiota, both bacterial and fungal, and metabolome; these relationships are endophyte-specific. We found that only the E+ integrome had OTUs associated with the rumen solids, indicating these may be an important microbial point-of-origin for global E+ pathophysiology. Overall, these data support our previous work, where we have shown E+ disrupts plasma and urine metabolic and fecal microbiota homeostasis (Mote et al., 2020a; Mote et al., 2019; Mote et al., 2017). The data herein provide additional context in that E+ begins altering the microbiota/metabolome *in planta* and in the rumen of toxic fescue grazing steers and these changes significantly associate with plasma/urine metabolic and fecal microbiota changes. This suggests a complex, systemic

pathophysiological response to fescue toxicosis, and future valuations, whether it be therapeutic- or management-based intervention strategies, assessing the microbiota and metabolomes should consider the efficacy of these strategies through a systemic approach. Further, this same approach could allow for delineation of the adaptive versus pathophysiological responses in subsequent, more detailed studies.

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Table 5.1. Effects on the overall microbial profile (PERMANOVA) using abundance profiles (Bray-Curtis) or presence absence (Jaccard) and diversity in richness for the bacterial (16S) and fungal (ITS2) microbiota based on fescue treatment (Max-Q [non-toxic] vs E+ [toxic]), time spent grazing, and the treatment by time interaction in fescue cultivar, rumen solids, rumen liquids, and fecal matter. “Inc”, “dec”, “mix” indicate significant increase, decrease or mixed effects, respectively, by E+ and/or time. P values equate to: (---) > 0.1; (^) < 0.1; (*) < 0.05; (**) < 0.01; (***) < 0.001.

Biological Matrix	PERMANOVA (Bray-Curtis)		PERMANOVA (Jaccard)		Simpson's Diversity		Chao1 Richness	
	16S	ITS2	16S	ITS2	16S	ITS2	16S	ITS2
Fescue cultivar								
Treatment	*	^	**	^	*$_{(inc)}$	^$_{(inc)}$	---	---
Time	**	***	***	***	^$_{(mix)}$	***$_{(mix)}$	---	**$_{(mix)}$
Treatment:Time	***	^	**	^	---	---	---	---
Rumen Solids								
Treatment	***	***	***	***	---	***$_{(inc)}$	***$_{(inc)}$	---
Time	***	***	***	***	---	---	***$_{(dec)}$	---
Treatment:Time	---	*	---	*	---	---	---	---
Rumen Liquids								
Treatment	***	***	***	***	**$_{(dec)}$	---	***$_{(inc)}$	---
Time	***	***	***	***	***$_{(inc)}$	**$_{(inc)}$	**$_{(dec)}$	***
Treatment:Time	---	*	---	*	---	---	---	---
Feces								
Treatment	***	**	***	**	---	---	***$_{(mix)}$	^$_{(mix)}$
Time	***	**	***	**	---	*$_{(mix)}$	**$_{(mix)}$	---
Treatment:Time	---	---	---	---	---	---	---	*

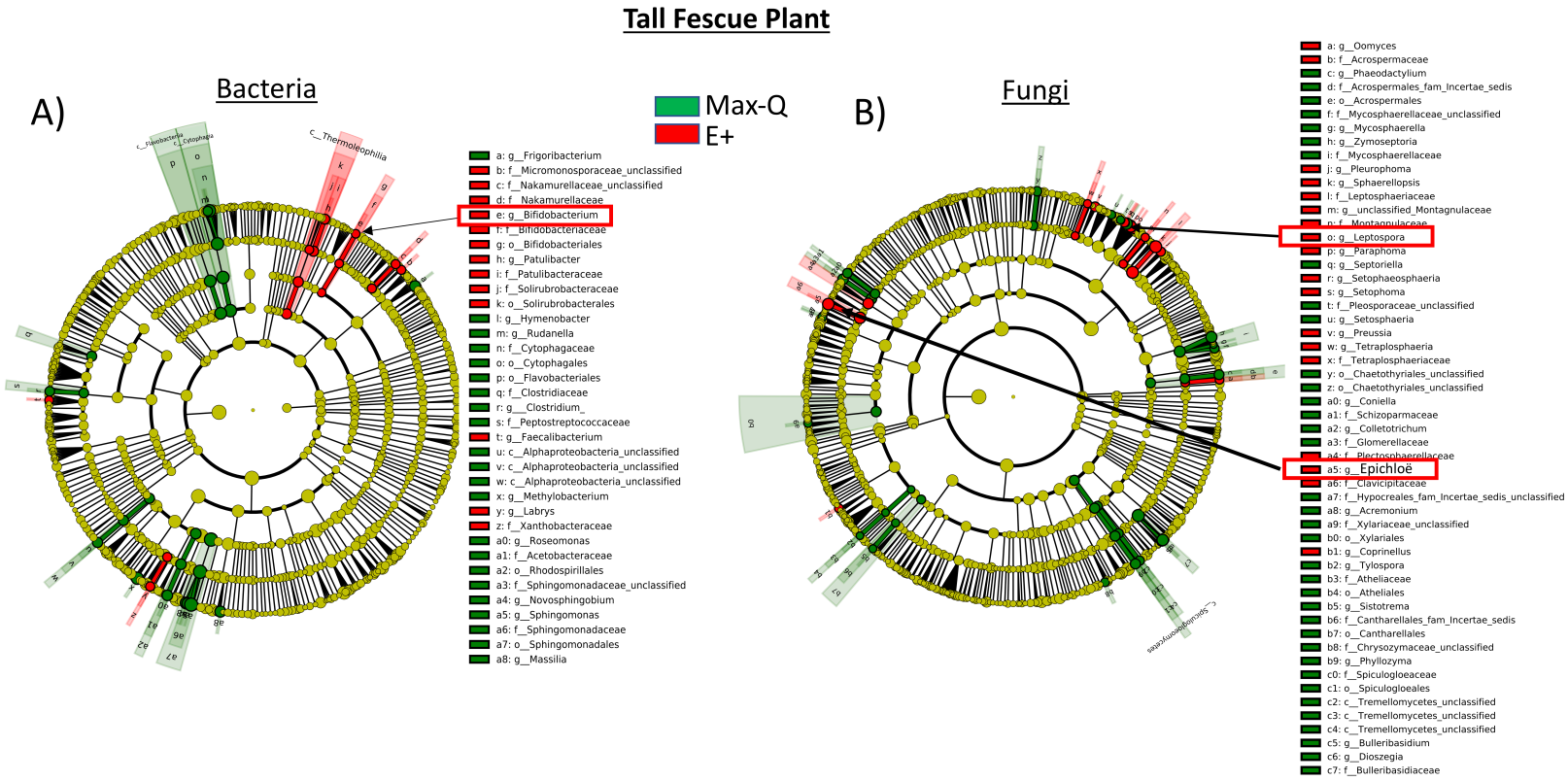

Figure 5.1. Linear discriminant analysis (LDA) effect size (LEfSe; Kruskall-Wallis [$P < 0.05$]; Pairwise Wilcoxon [$P < 0.05$]; logarithmic LDA score > 2.0) of tall fescue cultivar (A) bacterial and (B) fungal microbiota across a 28-day grazing trial where green and red indicate significant increases in the non-toxic (Max-Q) and toxic (E+) tall fescue cultivars, respectively. Taxonomic rank labels are provided before bacterial names: "p_; c_; o_; f_; g_" indicate phylum, class, order, family, and genus, respectively. Letters and numbers within the cladogram refer to respective bacterial or fungal names located in the keys to the right of each cladogram. Select taxa of interest have been highlighted by boxes and arrows point to position within cladogram.

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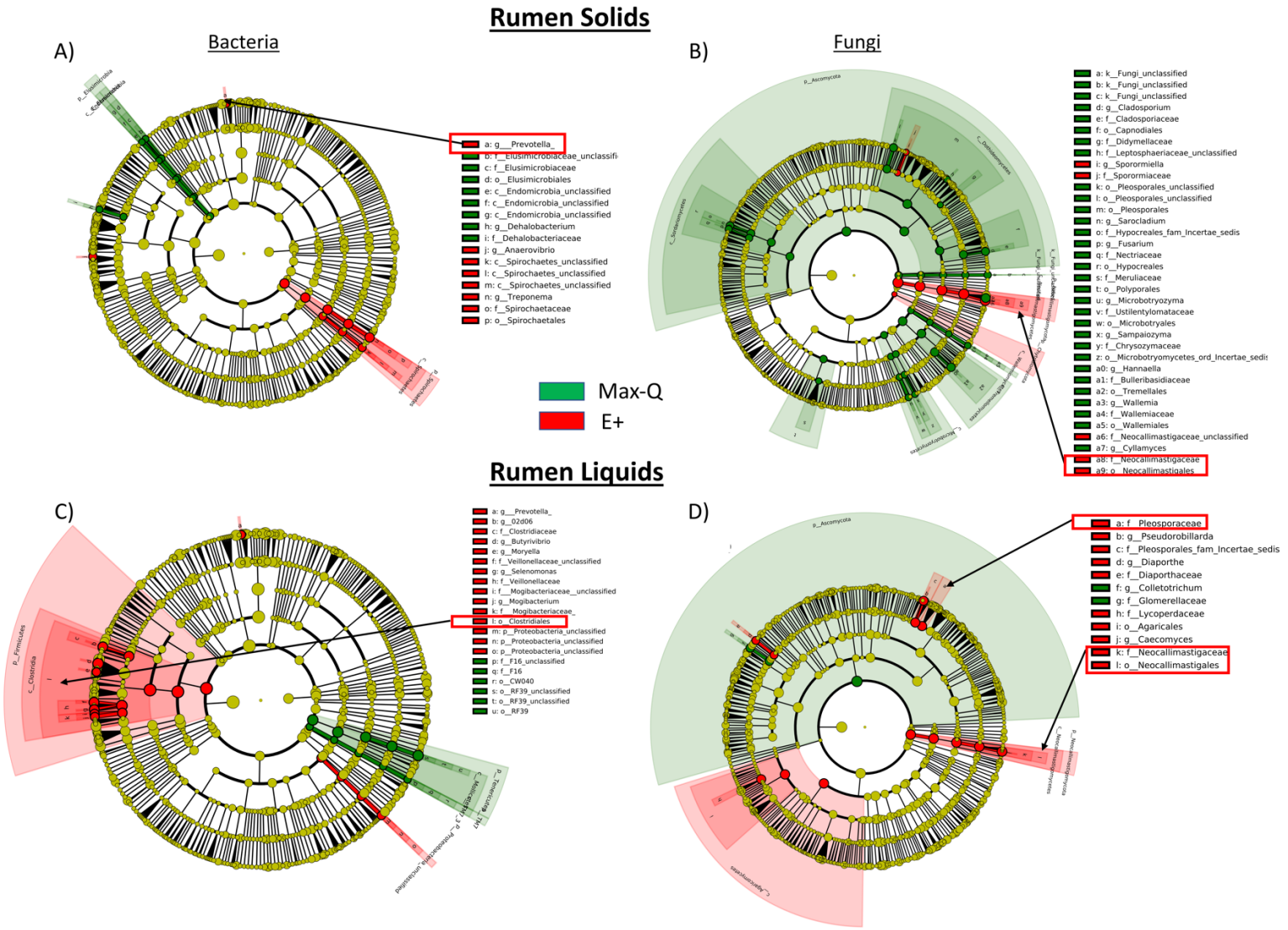

Rumen Solids
A)
Bacteria
a: g__Prevotella_
b: f__Elusimicrobiaceae_unclassifi
c: f__Elusimicrobiaceae
d: o__Elusimicrobiales
e: c__Endomicrobia_unclassified
f: c__Endomicrobia_unclassified
g: c__Endomicrobia_unclassified
h: g__Dehalobacterium
i: f__Dehalobacteriaceae
j: g__Anaerovibrio
k: c__Spirochaetes_unclassified
l: c__Spirochaetes_unclassified
m: c__Spirochaetes_unclassified
n: g__Treponema
o: f__Spirochaetaceae
p: o__Spirochaetales
Max-Q
E+
B)
Fungi
a: k__Fungi_unclassified
b: k__Fungi_unclassified
c: k__Fungi_unclassified
d: g__Cladosporium
e: f__Cladosporiaceae
f: o__Capnodiales
g: f__Didymellaceae
h: f__Leptosphaeriaceae_unclassified
i: g__Sporormiella
j: f__Sporormiaceae
k: o__Pleosporales_unclassified
l: o__Pleosporales_unclassified
m: o__Pleosporales
n: g__Sarocladium
o: f__Hypocreales_fam_Incertae_sedis
p: g__Fusarium
q: f__Nectriaceae
r: o__Hypocreales
s: f__Meruliaceae
t: o__Polyporales
u: g__Microbotryozyma
v: f__Ustilentylomataceae
w: o__Microbotryales
x: g__Sampaiozyma
y: f__Chrysozymaceae
z: o__Microbotryomycetes_ord_Incertae_sedis
a0: g__Hannaella
a1: f__Bulleribasidiaceae
a2: o__Tremellales
a3: g__Wallemia
a4: f__Wallemiaceae
a5: o__Wallemiales
a6: f__Neocallimastigaceae_unclassified
a7: g__Cyllamyces
a8: f__Neocallimastigaceae
a9: o__Neocallimastigales
Rumen Liquids
C)
a: g__Prevotella_
b: g__02d06
c: f__Clostridiaceae
d: g__Butyrivibrio
e: g__Moryella
f: f__Veillonellaceae_unclassified
g: g__Selenomonas
h: f__Veillonellaceae
i: f__Mogibacteriaceae__unclassified
j: g__Mogibacterium
k: f__Mogibacteriaceae_
l: o__Clostridiales
m: p__Proteobacteria_unclassified
n: p__Proteobacteria_unclassified
o: p__Proteobacteria_unclassified
p: f__F16_unclassified
q: f__F16
r: o__CW040
s: o__RF39_unclassified
t: o__RF39_unclassified
u: o__RF39
D)
a: f__Pleosporaceae
b: g__Pseudorobillarda
c: f__Pleosporales_fam_Incertae_sedis
d: g__Diaporthe
e: f__Diaporthaceae
f: g__Colletotrichum
g: f__Glomerellaceae
h: f__Lycoperdaceae
i: o__Agaricales
j: g__Caecomyces
k: f__Neocallimastigaceae
l: o__Neocallimastigales

Figure 5.2. Linear discriminant analysis (LDA) effect size (LEfSe; Kruskall-Wallis [P < 0.05]; Pairwise Wilcoxon [P < 0.05]; logarithmic LDA score > 2.0) of the rumen solid **(A)** bacterial and **(B)** fungal and rumen liquid **(C)** bacterial and **(D)** fungal microbiota of Angus steers across a 28-day grazing trial after placement on either a non-toxic (Max-Q; n = 6) or toxic (E+; n = 6) endophyte-infected tall fescue. Green and red shading indicates greater abundance in Max-Q or E+ steers, respectively. Taxonomic rank labels are provided before bacterial names: "p_; c_; o_; f_; g_" indicate phylum, class, order, family, and genus, respectively. Letters and numbers within the cladogram refer to respective bacterial or fungal names located in the keys to the right of each cladogram. Select taxa of interest have been highlighted by boxes and arrows point to position within cladogram.

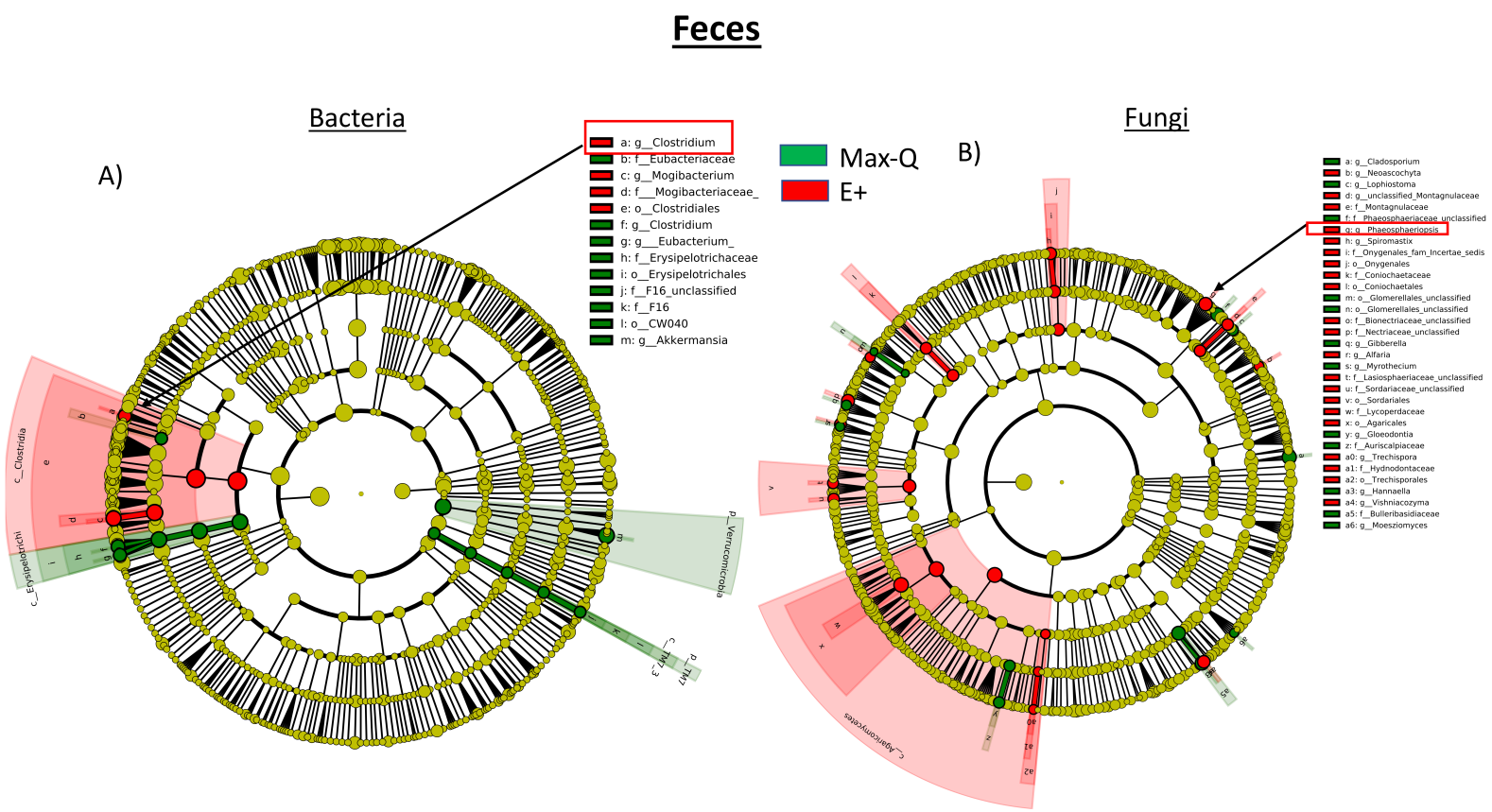


Figure 5.3. Linear discriminant analysis (LDA) effect size (LEfSe; Kruskall-Wallis [$P < 0.05$]; Pairwise Wilcoxon [$P < 0.05$]; logarithmic LDA score > 2.0) of the **(A)** bacterial and **(B)** fungal fecal microbiota of Angus steers across a 28-day grazing trial after placement on either a non-toxic (Max-Q; n = 6) or toxic (E+; n = 6) endophyte-infected tall fescue. Green and red shading indicates greater abundance in Max-Q or E+ steers, respectively. Taxonomic rank labels are provided before bacterial names: "p_; c_; o_; f_; g_" indicate phylum, class, order, family, and genus, respectively. Letters and numbers within the cladogram refer to respective bacterial or fungal names located in the keys to the right of the cladogram. Select taxa of interest have been highlighted by boxes and arrows point to position within cladogram.

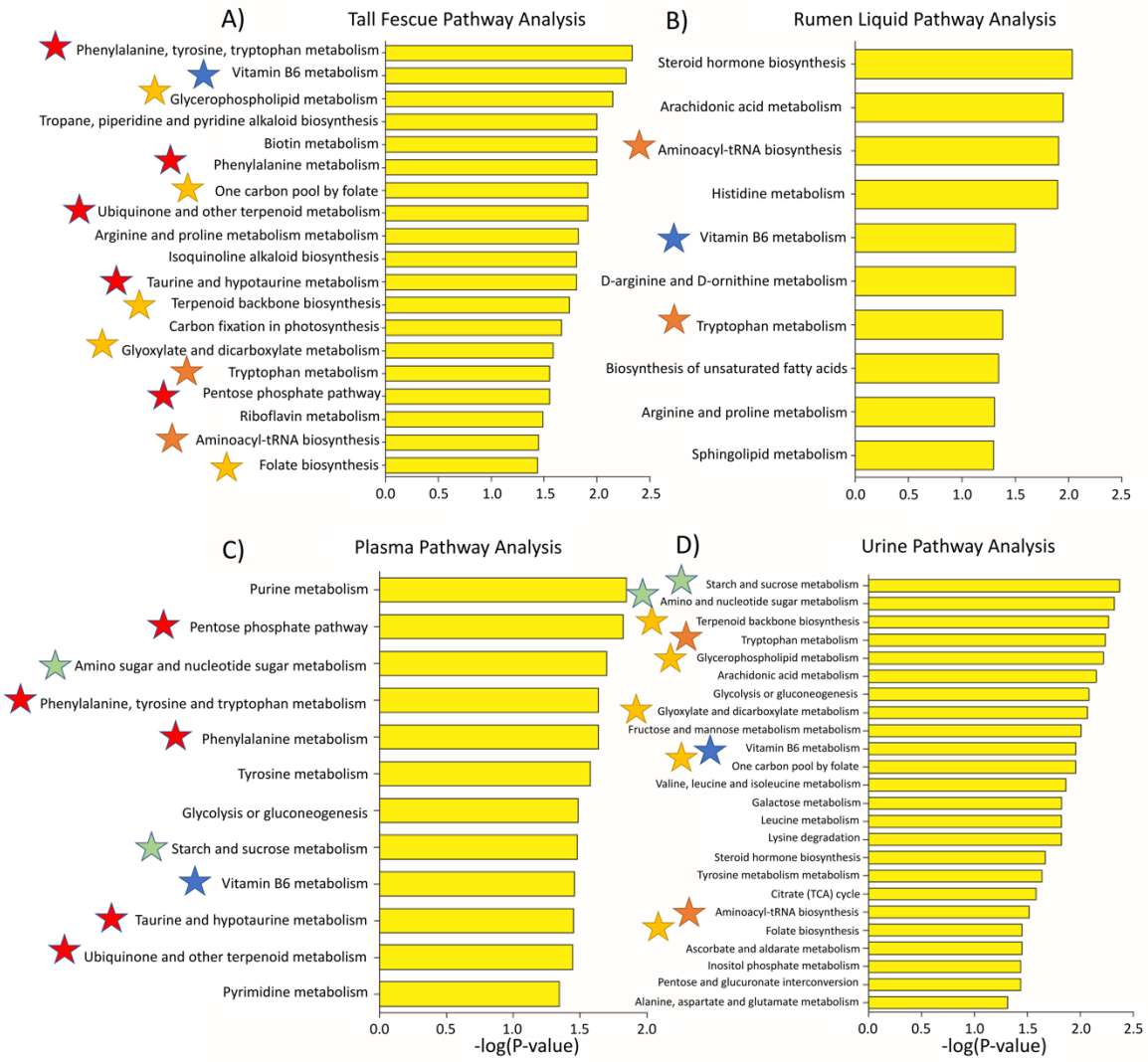


Figure 5.4. Metabolic pathway analysis performed on the **(A)** tall fescue plant, **(B)** rumen, **(C)** plasma and **(D)** urine high-resolution metabolomics features using the mummichog python program that indicates putative metabolic pathways significantly ($P < 0.05$) affected by toxic tall fescue (E+) grazing in the plant and animal throughout the 28-day grazing trial. The x-axis indicates the negative log of the FDR corrected p-value for each metabolic pathway indicated on the y-axis. Blue star indicates overlapping pathways between all biological matrices; orange is fescue grass, rumen liquid, and urine; red is fescue grass and plasma; yellow is fescue and urine.

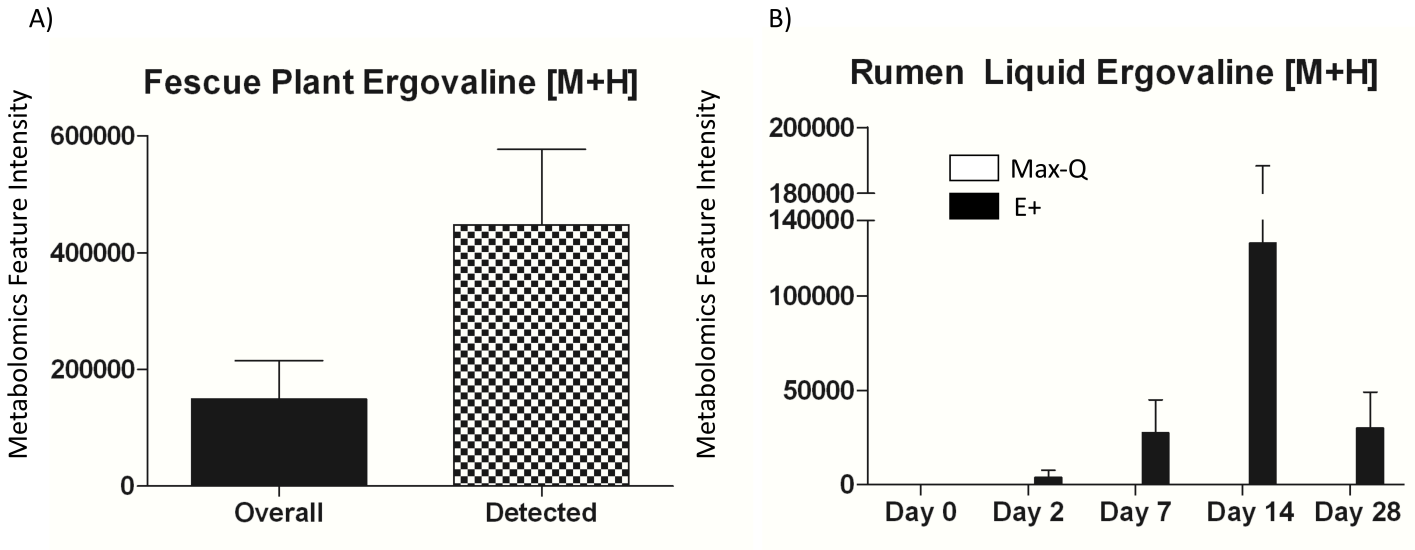


Figure 5.5. Average putative ergovaline [M+H] feature intensity in **(A)** toxic (E+; n = 18) tall fescue plant for all samples (black) and only in samples where ergovaline was detected (checkered) and **(B)** the rumen liquids of Angus steers grazing either a novel, non-toxic (Max-Q; n = 6) or toxic (E+; n = 6) tall fescue over the course of the 28-day grazing trial. Data are presented as mean ± SEM feature intensity.

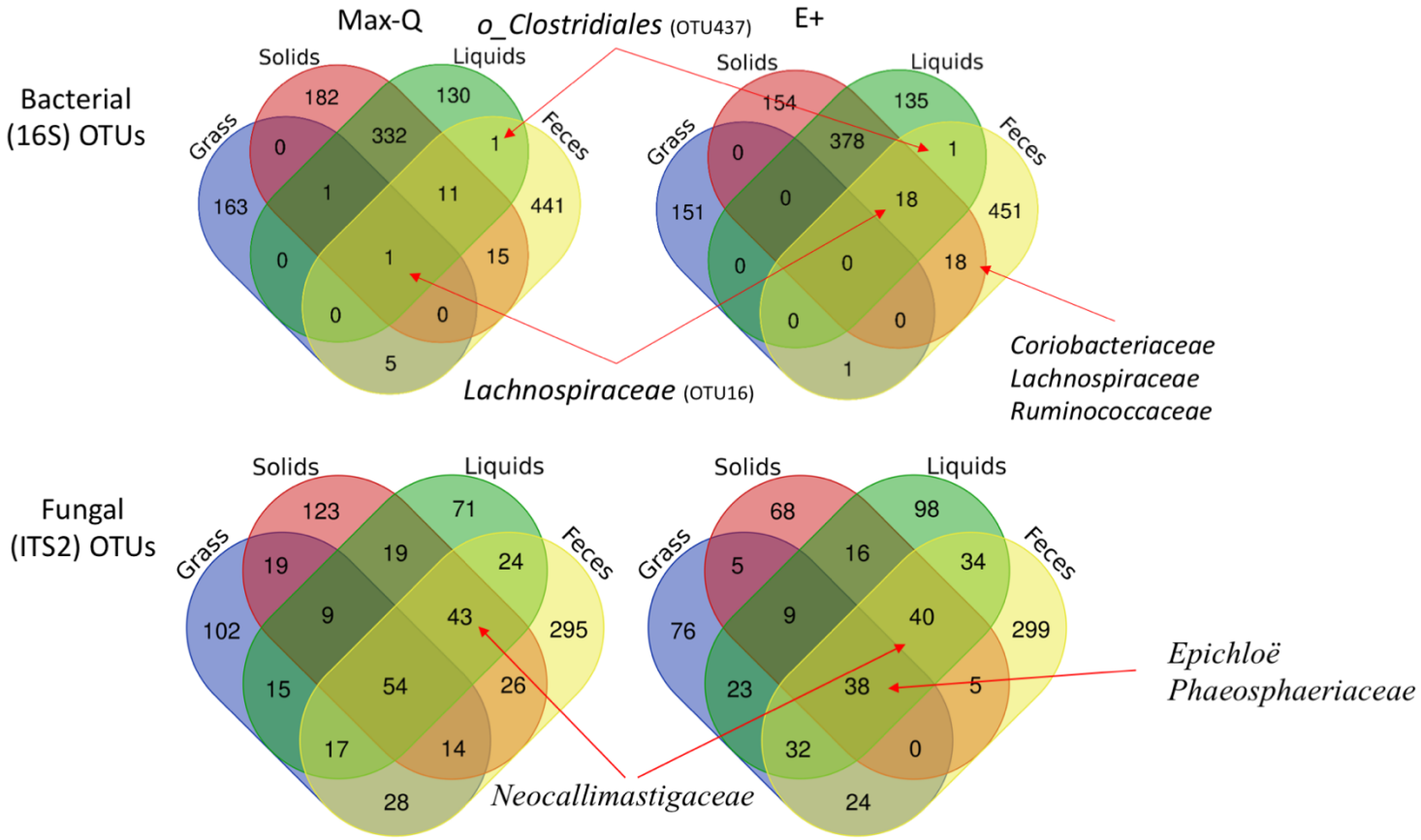


Figure 5.6. Venn diagrams representing specific bacterial (16S; **top**) and fungal (ITS2; **bottom**) OTUs that overlapped between biological matrices in steers grazing a novel, non-toxic (Max-Q; n = 6; **left**) or a toxic (E+; n = 6; **right**) tall fescue over the course of a 28 day grazing trial. Only OTUs with sequence counts (nseq > 10) were included in the analysis. Red arrows indicate where specific microbes of interest had overlapping OTUs.

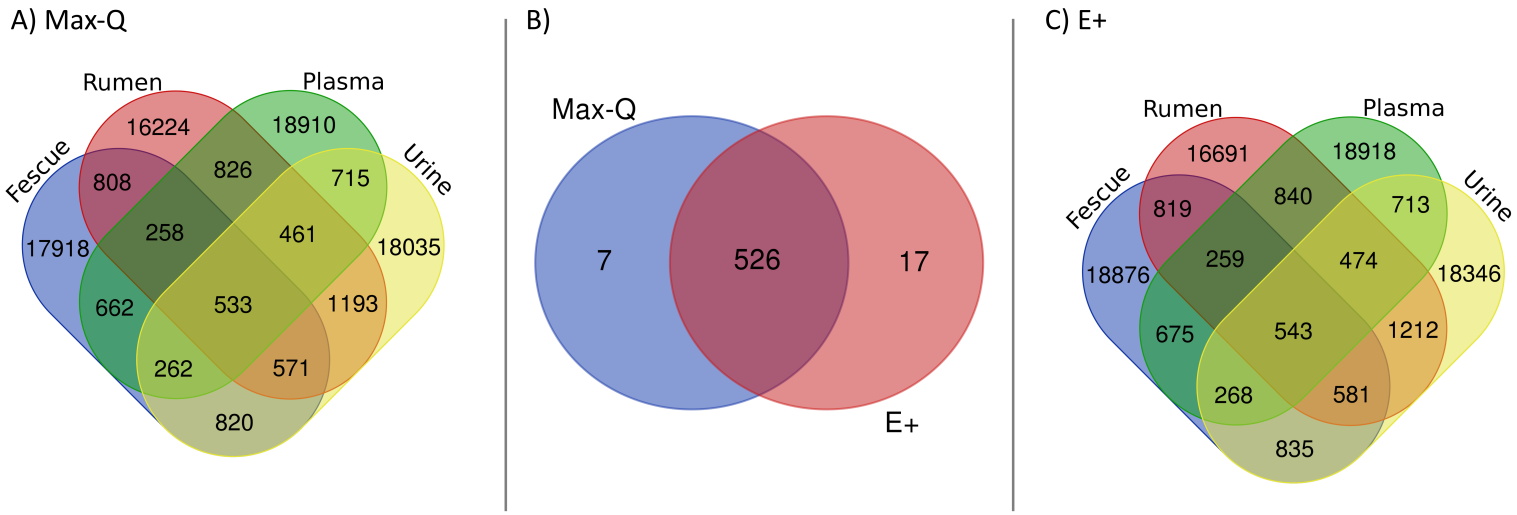


Figure 5.7. Venn diagrams representing specific metabolic features with exact mass-to-charge ratios (*m/z*'s) that overlapped between biological matrices in steers grazing **(A; left)** a novel, non-toxic (Max-Q; n = 6) or **(C; right)** a toxic (E+; n = 6) tall fescue over the course of a 28 day grazing trial with **(B; middle)** represents shared or distinct features between Max-Q and E+ that overlapped between all four biological matrices in each respective cultivar. Only metabolic features present in >80% of samples within a treatment and matrix were included in the analysis

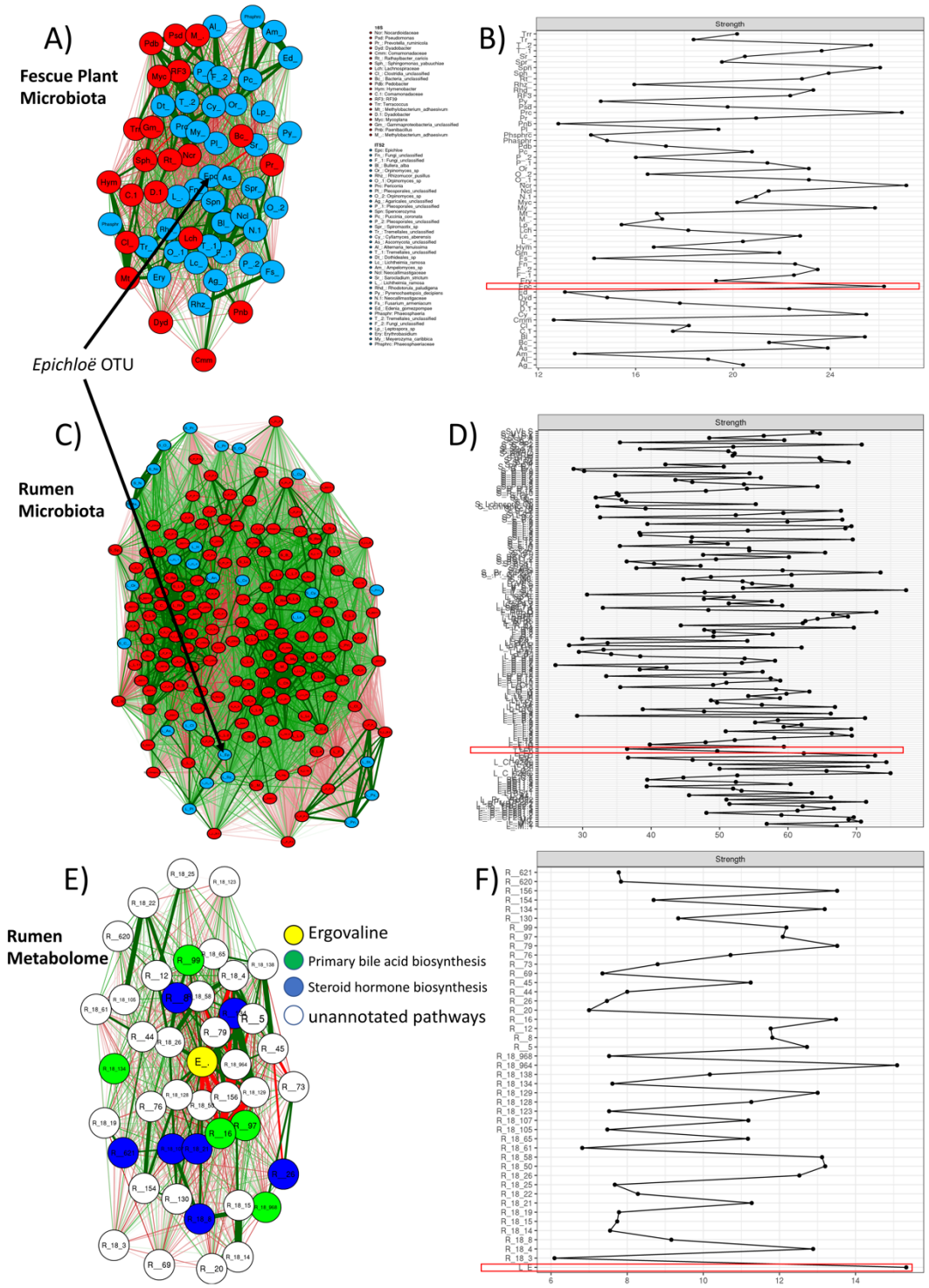


Figure 5.8. Targeted correlation-based network analysis with features significantly correlated with **(A, B)** fescue plant *Epichloë* (*E. coenophiala)* OTU, **(C, D)** rumen liquid (*E. coenophiala)* OTU, and **(E, F)** and ergovaline **(A)** network of toxic tall fescue plant (E+; $P < 0.05$) bacterial and fungal OTUs **(B)** respective centrality measurements with (*E. coenophiala)* marked in red; **(C)** network of toxic tall fescue grazing steers (E+; $P < 0.05$) ruminal bacterial and fungal OTUs

with **(D)** respective centrality measurements with *Epichloë* marked in red; **(E)** focused network of toxic tall fescue grazing steers (E+; $|r| > 0.6$; $P < 0.05$) ruminal metabolic features that significantly correlated with ruminal ergovaline and **(F)** respective centrality measurements with (ergovaline*)* marked in red. Blue and red nodes in **(A, B, C, D)** represent fungal and bacterial nodes, respectively. Yellow, green, blue, and white nodes in **(E, F)** indicate ergovaline, metabolites involved in primary bile acid biosynthesis, metabolites involved in steroid hormone biosynthesis, and metabolites from unannotated pathways, respectively. (*E. coenophiala*) presence in the network is highlighted by arrows. Green and red lines indicate positive and negative correlations, respectively.

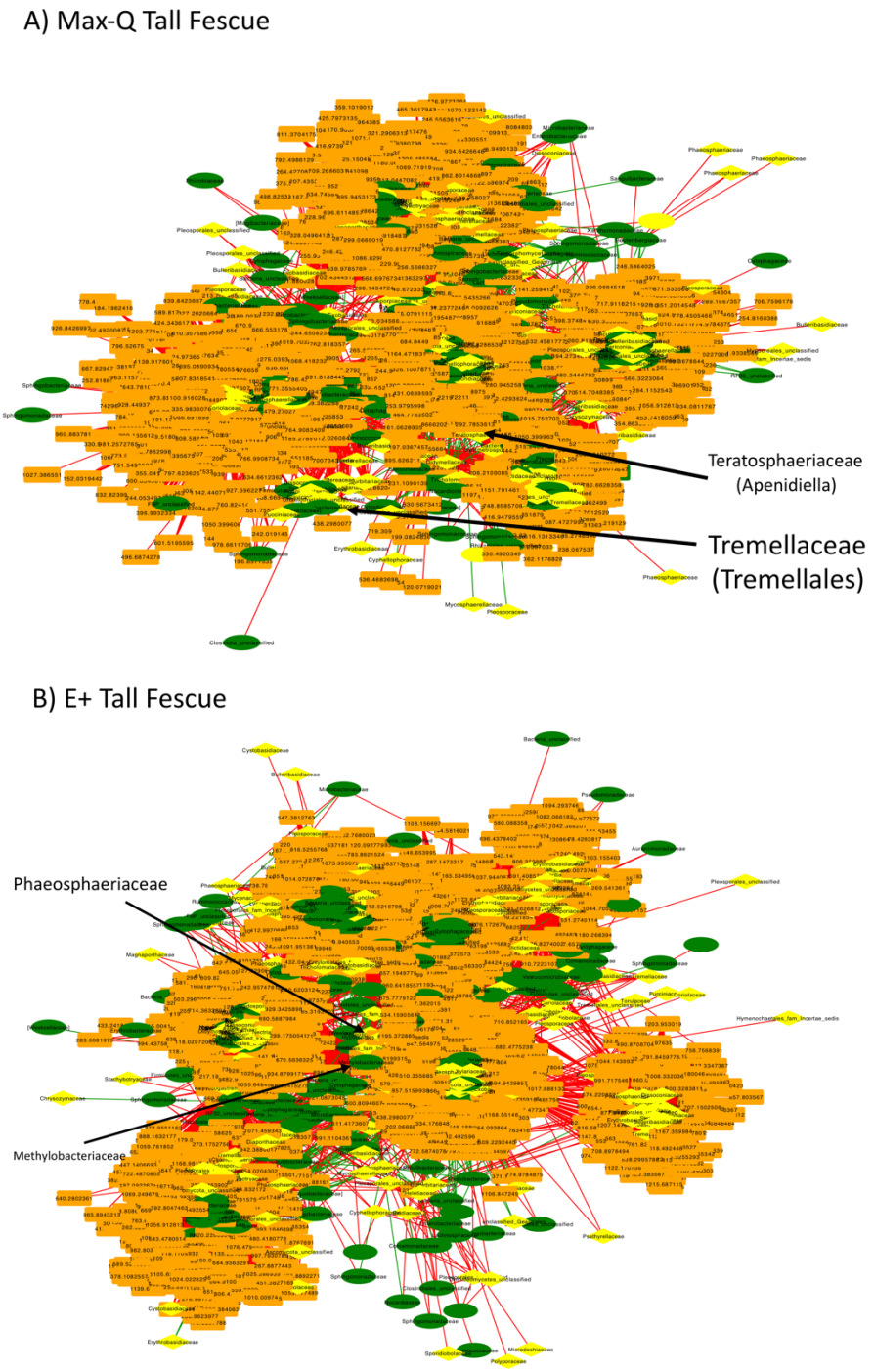


Figure 5.9. Global fescue plant integrative interactomics networks investigating relationships between bacterial (green, circle) and fungal (yellow, diamond) OTUs and metabolites (orange, square) in the tall fescue plant within a non-toxic (**A**; Max-Q; n = 6) or toxic (**B**; E+; n = 6) endophyte-infected plant. Green and red edges indicate positive and negative correlations, respectively. Select nodes of interest have been highlighted by arrows and text.

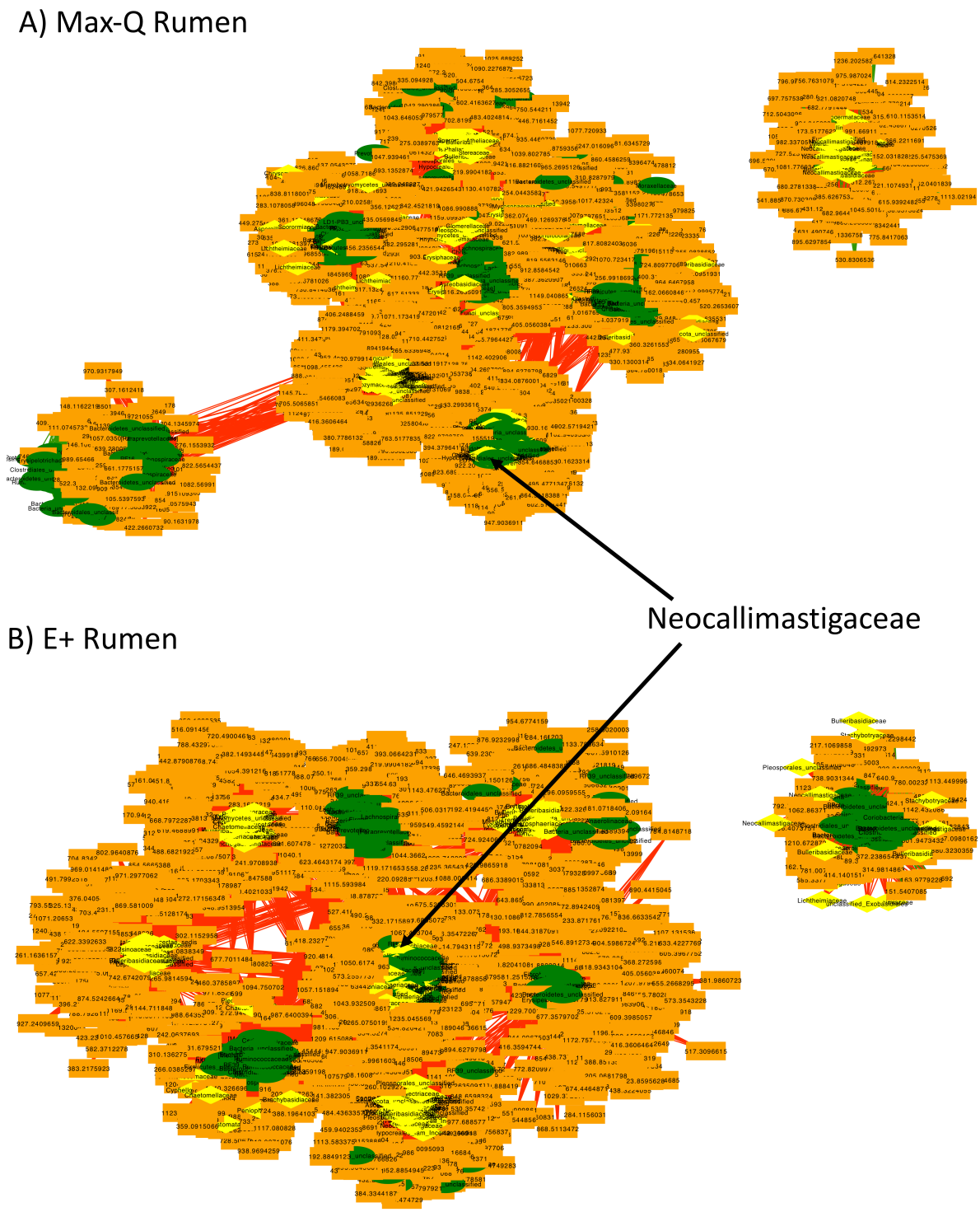


Figure 5.10. Global rumen integrative interactomics networks investigating relationships between bacterial (green, circle) and fungal (yellow, diamond) OTUs and metabolites (orange, square) of non-toxic (A; Max-Q; n = 6) or toxic (B; E+; n = 6) grazing beef steers. Green and red edges indicate positive and negative correlations, respectively. Select nodes of interest have been highlighted by arrows and text.

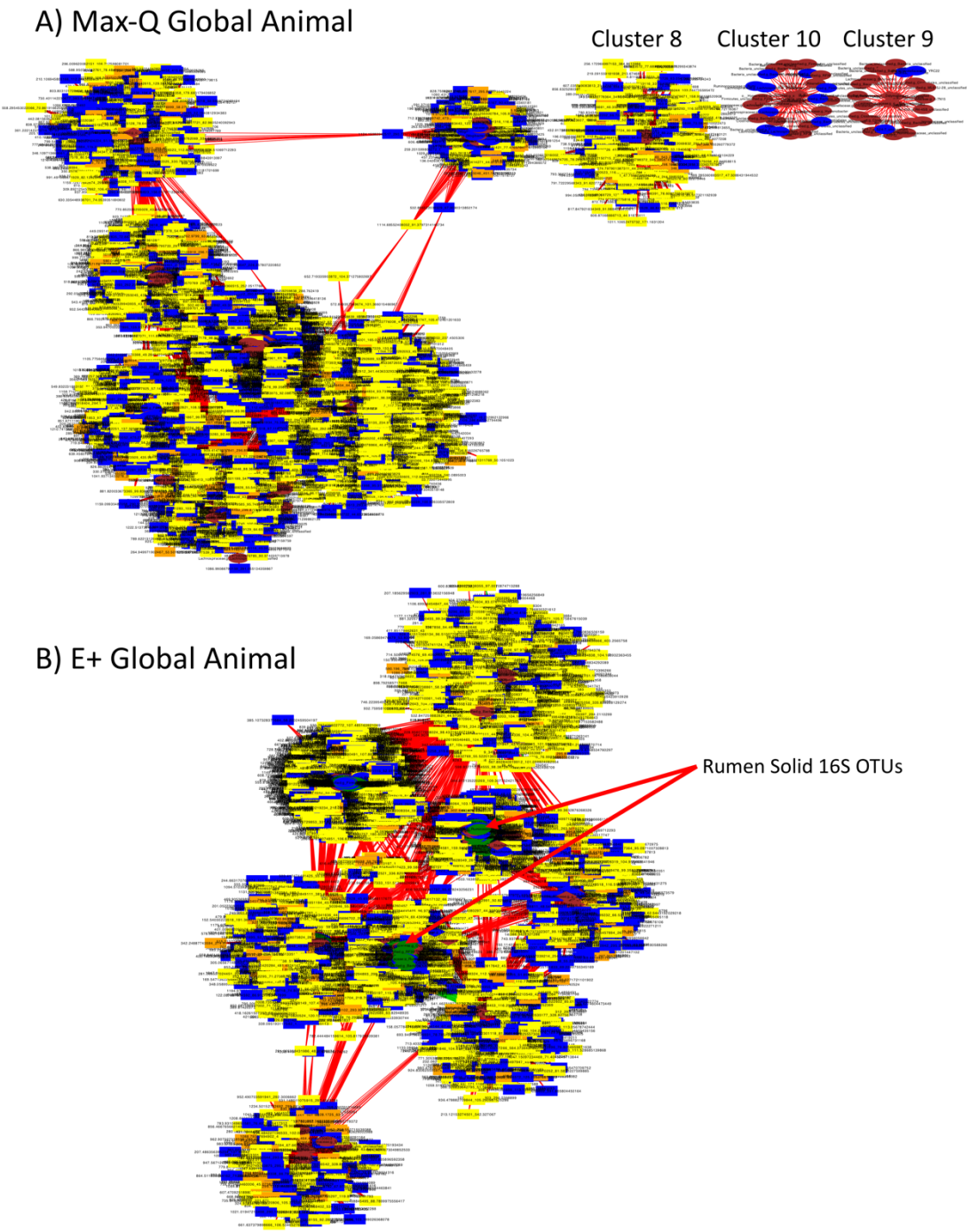


Figure 5.11. Global entire animal integrative interactomics networks investigating relationships between bacterial (circle) and fungal (diamond) OTUs and metabolites in the rumen solid (green), rumen liquid (blue), plasma (orange), urine (yellow), and feces (brown) of either **(A)** non-toxic (Max-Q; n = 6) or **(B)** toxic (E+; n = 6) endophyte-infected tall fescue grazing beef steers. Green and red edges indicate positive and negative correlations, respectively.

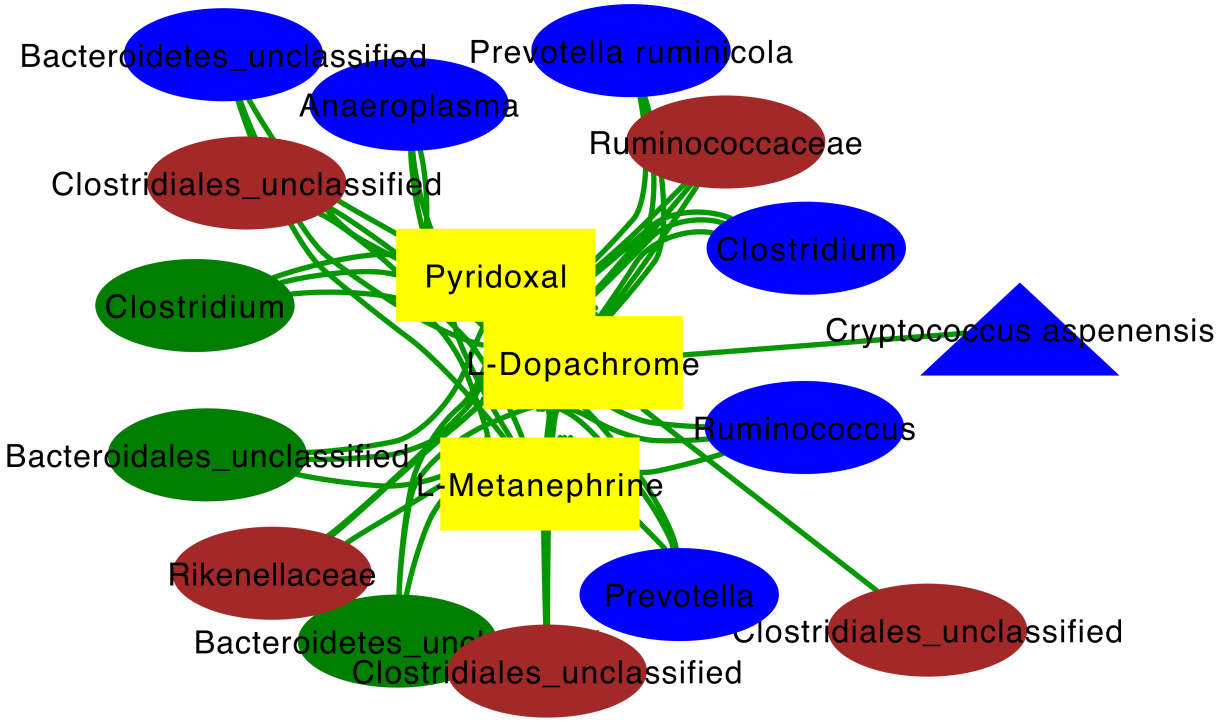


Figure 5.12. Targeted entire animal integrative interactomics networks investigating relationships between bacterial (oval) and fungal (triangle) OTUs and metabolites (rectangle) in the rumen solid (green), rumen liquid (blue), urine (yellow), and feces (brown) of steers on toxic (E+; n = 6) endophyte-infected tall fescue. Metabolic pathways targeted in this analysis include tryptophan, tyrosine, Vitamin B6, steroid hormone, and bile acid metabolism. Green edges indicate positive correlations.

CHAPTER 6

SUMMARY

Fescue toxicosis (FT) is a complex livestock disease that develops when livestock ingest *Epichloë coenophiala* infected tall fescue. While FT negatively impacts multiple animal species worldwide, the beef industry is of major concern in the United States, with an estimated $1 billion negative impact annually. Previous work has suggested ergot alkaloids are the key etiological agents of FT, and, considering the structural similarity between ergot alkaloids and monoamines, many studies have focused on assessing direct effects/interactions of ergot alkaloids within a given biological context (i.e., receptor-alkaloid binding, etc.). Despite the costliness of the disease, the length of time it has been investigated, and the potentially promiscuous nature of ergot alkaloids, no studies to date have systematically investigated global effects of toxic tall fescue or ergot alkaloid exposure in a systemic manner. Further, no studies to date have integrated large 'omics data sets and used the integrate results as a means to interrogate the pathophysiology of FT.

We began in Chapter 2 by using high-resolution metabolomics (HRM), considering the potential for metabolic alterations in FT, to assess effects of toxic tall fescue (E+) exposure on the plasma and urine metabolomes of grazing Angus steers (Figure 6.1.). Using high-resolution mass spectrometry in an untargeted manner and we found E+ grazing significantly alters many HRM metabolic features in the plasma and urine metabolomes. Notably, we found a few metabolites that putatively aligned to monoamines of interest (e.g., dopamine and its metabolites). As effects on monoamines were limited when assessed via a targeted HPLC

analysis, it is most likely that these metabolites are trace amines. This could be of significant FT physiological interest, considering they modulate many monoaminergic activities. Further, we found that amino acid (e.g., tryptophan and tyrosine) metabolism was consistently one of the most significantly perturbed metabolic pathways. This finding was notable, considering that tryptophan is a neurotransmitter and ergot alkaloid precursor. Because tryptophan, among other amino acids, can be microbiota-related and circulating levels are influenced by the enteric microbiota dysbiosis, we next sought to outline effects of E+ on the fecal microbiota in a follow-up study.

So, in Chapter 3, we utilized 16S rRNA gene sequencing to profile the fecal bacterial microbiota of E+ and non-toxic (Max-Q) tall fescue grazing steers under thermoneutral conditions (Figure 6.1.). In this study, we found that E+ grazing significantly alters the abundances of the *Erysipelotrichaceae* family early in the grazing trial (i.e., < 7 days on pasture), while having more significant effects on the *Ruminococcaceae* and *Lachnospiraceae* families later in the grazing trial (i.e., 14 and 28 days). Further, we performed a differential network analysis and found that E+ grazing leads to a unique structure to the interrelationship (i.e., bacteria-bacteria and bacteria-host crosstalk) in the bovine fecal microbiota under thermoneutral conditions. Considering temporal effects of E+ on the dynamic microbiota, the unique structure of this hindgut microbiota, and the potential effects on peristalsis, feeding behaviors, and metabolism, we next sought to see whether these dynamic microbial effects alter the relationship between the microbiota and plasma/urine metabolomes, i.e., focus on the microbiome-metabolome interactions. As cattle exposed to E+ experience thermoregulatory impairments, and under thermoneutral conditions significant perturbations in the microbiota and metabolome exist,

the next study also evaluate if hot and humid environmental conditions modulate metabolome-microbiome interactions in the FT context.

In Chapter 4, grazing beef steers were exposed E+ and a combination of thermoneutral harsh environmental conditions (+temperature humidity index; +THI) and effects on the fecal microbiota, plasma and urine metabolome, and fecal microbiota-plasma/urine metabolome relationship were evaluated (Figure 6.1.). In this study, as in all studies herein, E+ steers had significant reduction of average daily and cumulative weight gains, but the numerical values of both Max-Q and E+ steers were lower than in studies where environmental conditions were not conducive of heat stress throughout those studies durations. This indicates that although +THI may influence muscle accretion regardless of diet, the combination of E+:+THI is more detrimental to animal productivity, as the toxic endophyte still significantly decreased weight gains. Regarding the fecal microbiota, similar results were observed as in Chapter 3 (i.e., increases in *Ruminococcaceae* and *Lachnospiraceae*), but some effects on the microbiota were influenced by environmental conditions, such as less robust increases in *Ruminococceae* under +THI. Further, comparisons between thermoneutral and +THI conditions within cultivar treatment groups showed +THI only had significant effects on the E+ steer microbiota, indicating an increased susceptibility to external environmental stressors. In a similar light, metabolic differences between Max-Q and E+ steers were modulated by +THI, in that some metabolic effects were observed only under thermoneutral or +THI conditions and others were observed regardless of environmental conditions. Finally, complex bioinformatic selection methods (i.e., sparse partial least squares regression) were used to integrate the fecal microbiota and plasma/urine metabolome. Differential network analysis found a unique set of fecal bacterial operational taxonomic units (OTUs) and plasma/urine metabolites were associated and specific

to E+ fescue grazing beef cattle. Three of the OTUs in this E+ specific network were also significantly associated with (patho)physiological endpoints of interest (e.g., weight gains) and HRM features under both thermoneutral and +THI condition. These plasma and/or urinary metabolites may serve as useful biomarkers of effect pointing towards an E+-production reducing hindgut microbiota regardless of environmental conditions that can be detected at the level of the metabolome. Consistent effects of E+ on the hindgut microbiota and plasma and urine metabolomes led to Chapter 5, as the importance of the plant/rumen bacterial and fungal microbiota and metabolomes within FT have not been investigated despite evidence suggesting they may play a significant role in FT etiology.

In Chapter 5, the first interrogation of the multi-compartment microbiota, metabolome, and their integrative relationship was performed in FT (Figure 6.1.). First, it was demonstrated that *E. coenophiala* infection selectively alters both bacterial and fungal taxa in the tall fescue phyllosphere (aboveground) microbiota. Notably, the genus *Epichloë* was significantly increased only in the E+ plant and E+ steers rumen liquids, with minimal detection in other biological matrices. While *Epichloë* was found in the rumen liquids, the detection/abundance of *Epichloë* in the rumen liquids was not a reliable predictor of pasture alkaloids, urinary alkaloids, or weight gains, indicating detection of this particular genus in the rumen liquids may not be a good biomarker of effect. There were mixed effects of E+ grazing on the rumen bacteria, but the ruminal solid and liquid fungi were largely decreased by E+ exposure, pointing to significant negative selection pressure by E+ on ruminal fungi, which are important for plant degradation. Numerous metabolic pathways were significantly affected in the E+ plant and E+ grazing animal. Some pathways we previously found as significantly altered by E+ (e.g., tyrosine and tryptophan metabolism) were again found in multiple, if not all, biological matrices. One

important result identified herein was that E+ may effect specific metabolic pathways in the plant and perturbations in these metabolic pathways (i.e., Vitamin B6) may carry over into and throughout the grazing animal. E+ grazing also significantly altered important growth-related metabolic pathways (e.g., steroid biosynthesis) in the rumen. A *m/z* matching ergovaline was putatively identified using the toxin exposome database (T3DB) and was detected only in the E+ plant and rumen liquids. Interestingly, ergovaline targeted network analysis revealed that metabolic features that were significantly associated with ergovaline were involved in multiple pathways previously identified as significantly perturbed by E+ (e.g., steroid hormone biosynthesis and Vitamin B6 metabolism). Further, targeted microbiota network analysis revealed select fungal OTUs related to the *Phaeosphaeriaceae* family were significantly correlated with *Epichloë* in the plant. In the rumen microbiota targeted network, many OTUs aligned within the *Neocallimastigaceae* and *Lachnospiraceae* families and *Prevotella* genus were correlated with the *Epichloë* OTU. The *Orpinomyces* genus had two OTUs present in the rumen solids and liquids with opposite correlation coefficients, suggesting possible re-partitioning. Integrative analysis revealed the constituents of the beef steer integrome occurs in a tall fescue endophyte-specific manner, supporting the idea of systemic, indirect influences of E+ exposure contributing to FT pathophysiology. Notably, fecal fungal OTUs were the most important in both networks, despite being from classified as different fungal taxa, and only the E+ global xMWAS network had ruminal solid OTUs within the network, indicating this biological matrix may be important. Targeted analysis found three urinary metabolites involved in tyrosine and Vitamin B6 metabolism are associated with the multi-compartment animal microbiota. Urinary L-metanephrine, L-dopachrome, and/or pyridoxal were identified as potential biomarkers of effect or diagnostic biomarkers of dysbiosis for the multi-compartment

microbiota, highlighting potentially readily accessible biomarkers for scientists and producers alike. Not only do these data provide potentially important biomarkers but also foundational evidence that show the previous perturbations we have found using ‘omics methods are snapshots of systemic perturbations that occur in toxic tall fescue grazing steers, and understanding the multi-level multi-compartment integrome will provide more actionable insights moving forward. Overall, this study highlights how the E+ endophyte selectively alters important plant characteristics, with some E+ effects carrying over into the animal, and how E+ exposure in grazing beef cattle alters global physiology in an integrated manner.

CONCLUSIONS AND FUTURE WORK

The data provided herein highlight the complex pathophysiology of FT by using novel molecular biology, analytical, and bioinformatic tools. Throughout, E+ exposure consistently altered amino acid metabolism and numerous bacteria in the *Clostridiales* class. More advanced sampling and computational analysis revealed a highly integrated network of microbial (bacterial and fungal) OTUs and metabolites that outline the pathophysiology of FT and provide a framework for more integrome-based development of FT therapeutics. Future work related to these studies should involve sampling of the fescue plants on the same day as animal samples. Although this increases labor costs, the ability to directly integrate these data with animal data will be insightful. Other notable work evaluating the microbiota and metabolomes in beef steers grazing E+ tall fescue should seek to evaluate how current management strategies, such as rotational grazing, influence the integrated microbiota or metabolic responses. Such data will highlight whether rescue of certain microbial and/or metabolic perturbations is possible and if timing of when management strategy implementation needs to occur to mitigate FT-related production losses will be important. Understanding whether temporal dynamic effects occur is of

special interest when considering microbial and/or metabolic-based therapeutic interventions to help alleviate FT burden as a supplement to additional management practices. Further, substantive, targeted evaluation of the proposed biomarkers are necessary to confirm their utility for development of FT therapeutics and/or management development strategies. Overall, the work provided herein addresses a crucial literature gap in the field of FT and animal productivity in response to toxic tall fescue exposure, provides novel insights into the integrated multi-compartment microbiota-metabolome interaction, and outlines important insights into how to approach the next studies to gain actionable insights for therapeutic development moving forward.

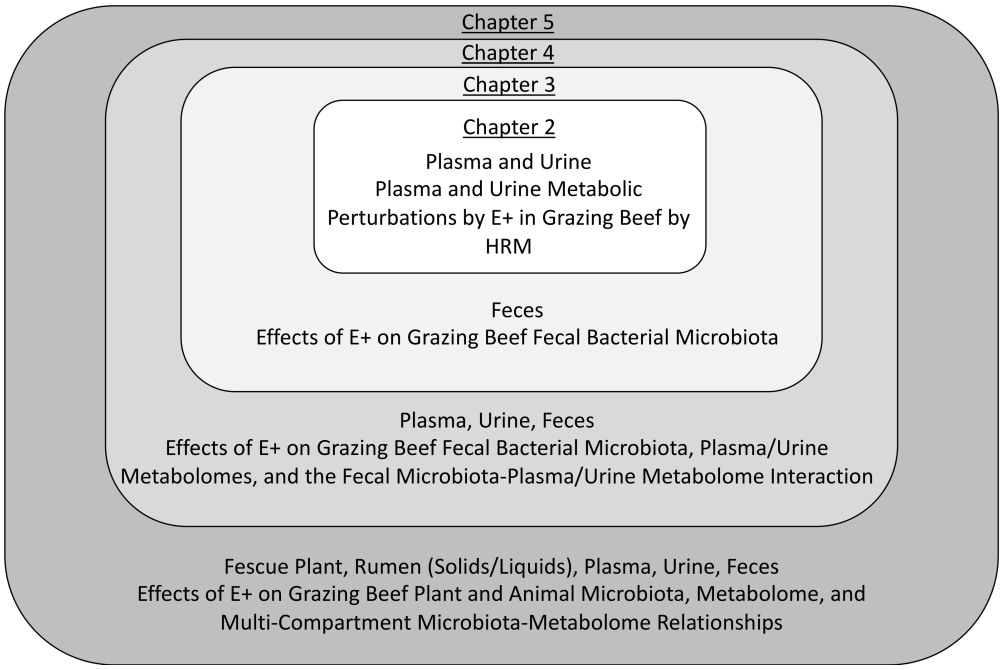


Figure 6.1. Summary figure of Chapters 2, 3, 4, and 5 of dissertation. The first row indicates samples used to generate results. Additional rows describe the major methods and goals of each chapter.

APPENDIX A

CHAPTER 1 SUPPLEMENTARY MATERIAL

Table S1.1. The following tall fescue cultivars performed well in tests conducted between 2013 and 2016. These tests included the current National Turfgrass Evaluation Program (NTEP) study, regional trial, Alliance for Low Input Sustainable Turf (A-LIST) trials, and a sponsored trial. Not all of these cultivars will be available in 2017. Table courtesy of Grady Miller.

List of the Top Performing Tall Fescue Cultivars		
07 Dust	Fayette	Rebounder
07Walk	Fesnova	Regenerate
4th Millennium SRP	Firebird 2	Rhambler 2 SRP
	Firecracker	
Amity	SLS	Rhizing Moon
Aquaduct	Firewall	Rowdy
AST 5112	Foxhound	Rockwell
AST 1736	Golconda	Saltillo
ATF 1612	Grande 3	Screamer LS
ATF 1704	GTO	Supersonic
Avenger II	Hemi	Swagger
Bizem	Hot Rod	Tara
Black Tail	Houndog8	Technique

Bloodhound	Hover	Temple
Bullseye	IS-TF 311	Temptation
Caesar	Kingdom	Terrano
Catalyst	Leonardo	Thor
Crossfire 4	Maestro	Thunderstruck
Dakota	Memphis	Titanium 2LS
		Traverse 2
DTT 43	Michelangelo	SRP
Dynamite SLS QS	Paramount	Turfway
Embrace	Rain Dance	Valkyrie LS
Escalade	Reflection	Xtender
Falcon V	Raptor III	
Faith	Rebel IV	

APPENDIX B

CHAPTER 2 SUPPLEMENTARY MATERIAL

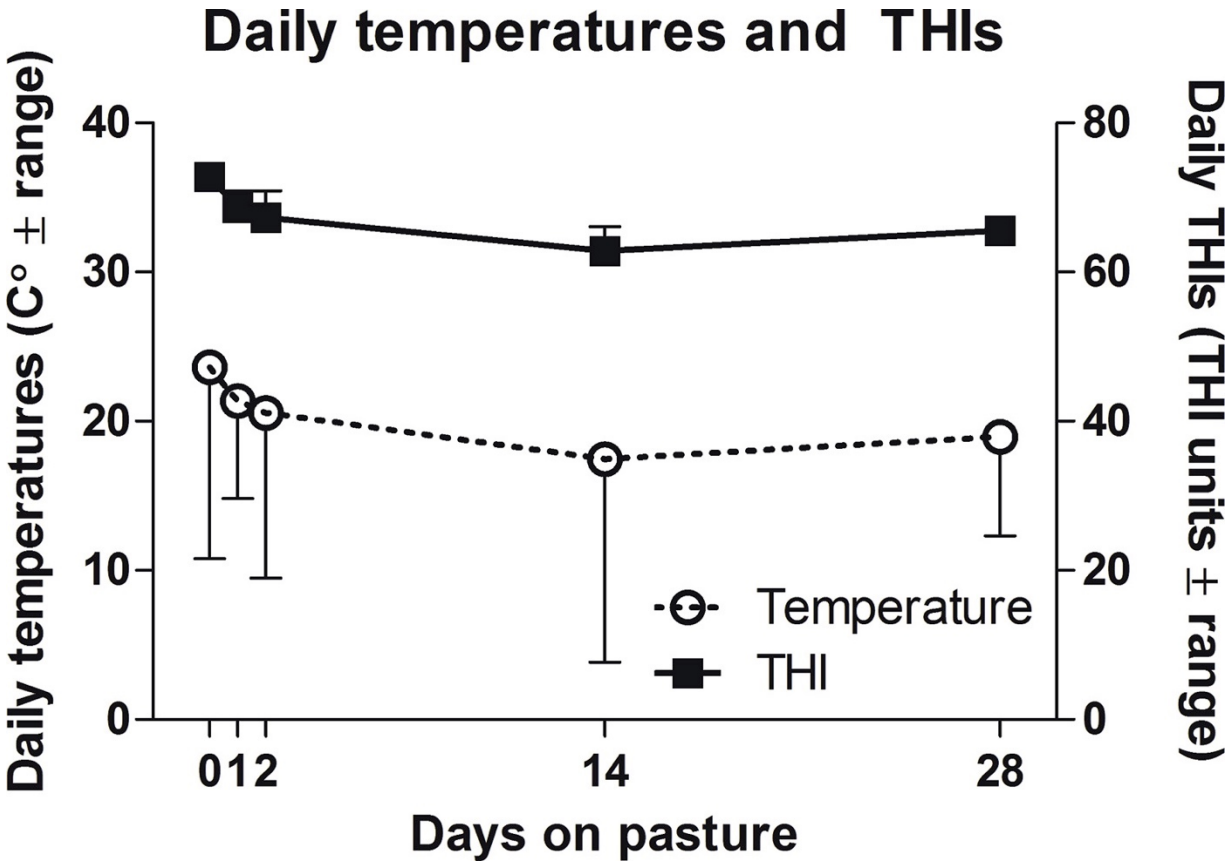


Figure S2.1. Average daily temperature (°C) and temperature-humidity index (THI) units based on data from the weather station located at J. Phil Campbell Natural Resources Conservation Center of the University of Georgia (Watkinsville, GA) for baseline, 1, 2, 14, and 28 days post pasture assignment sampling dates. Data are presented as ± range.

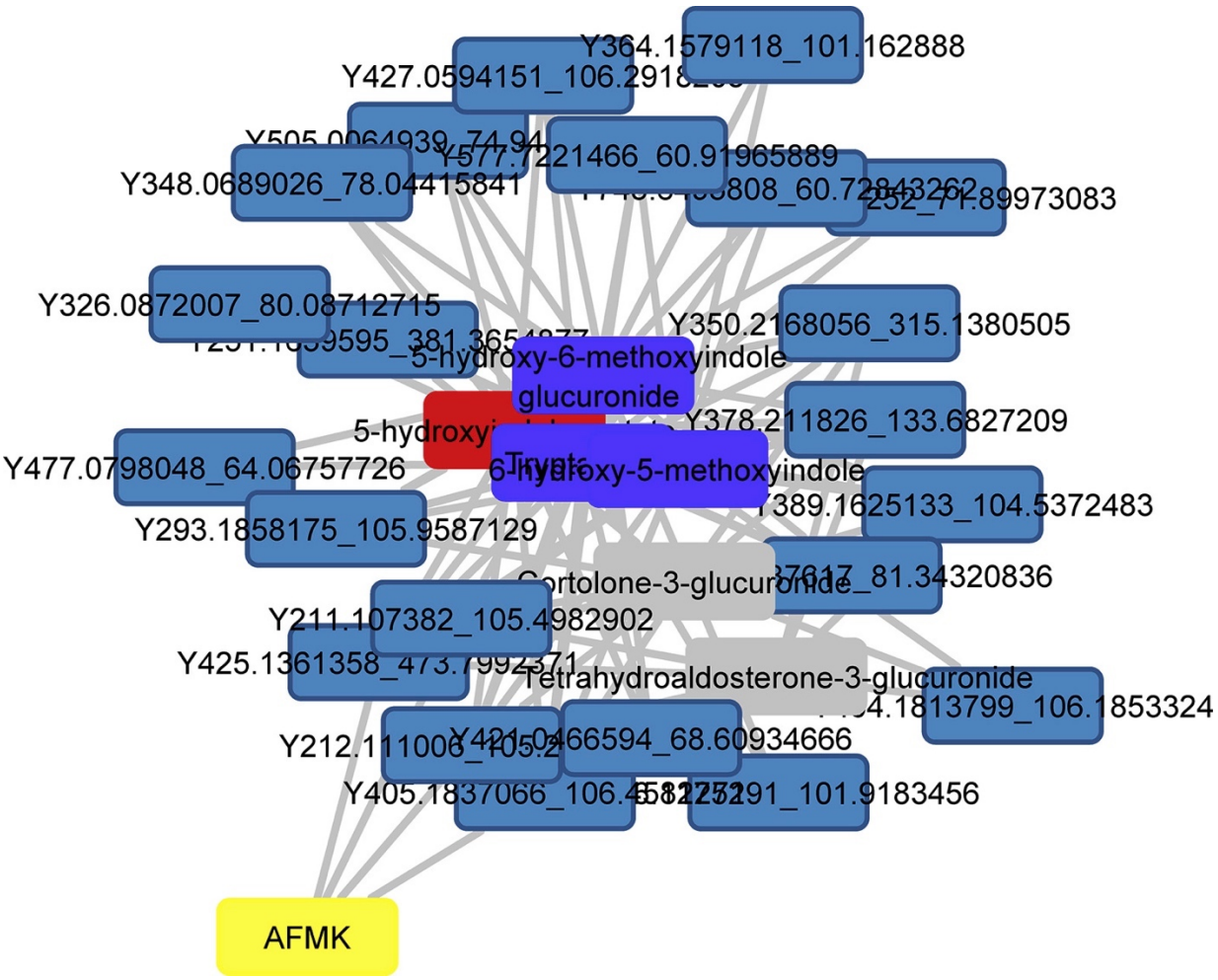


Figure S2.2. Targeted complete Pearson correlation-based network (|r| > 0.5; FDR = 0.05) for bovine urine metabolome of Angus steers grazing wild-type toxic (E+) fescue for 28 days (C18 column data). Numbers indicate the *m/z* and retention time of the putative metabolites. Key anchor metabolite names are included. Red = serotonin metabolite, purple = indole metabolite, yellow = melatonin metabolite, and grey = steroid metabolite.

APPENDIX C

CHAPTER 3 SUPPLEMENTARY MATERIAL

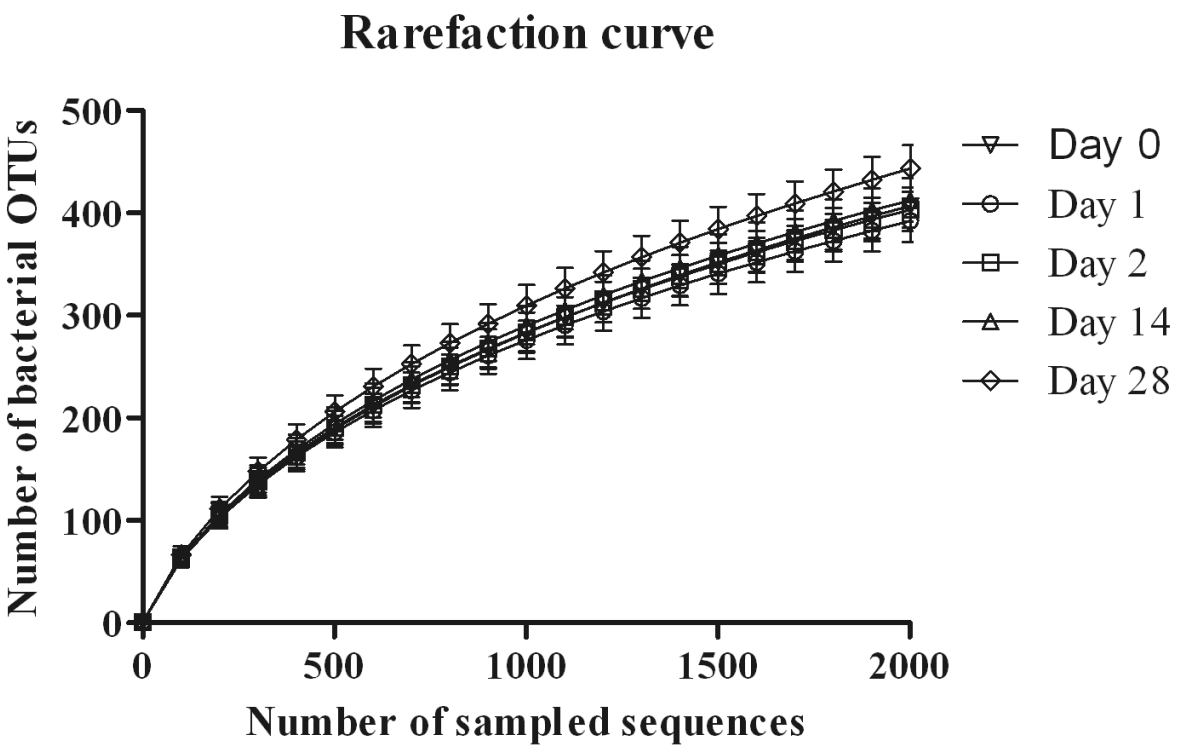


Figure S3.1. Rarefaction curves representing the number of observed sequences versus the sequencing depth derived from operational taxonomic units (OTU) tables generated from the mothur pipeline from fecal samples of Angus steers (n = 12) grazing either a non-toxic endophyte-infected (Max-Q; n= 6) or a toxic endophyte-infected (E+; n = 6) tall fescue before (Day 0) and 1, 2, 14, and 28 days post-pasture assignment. Data are presented as means ± standard deviation.

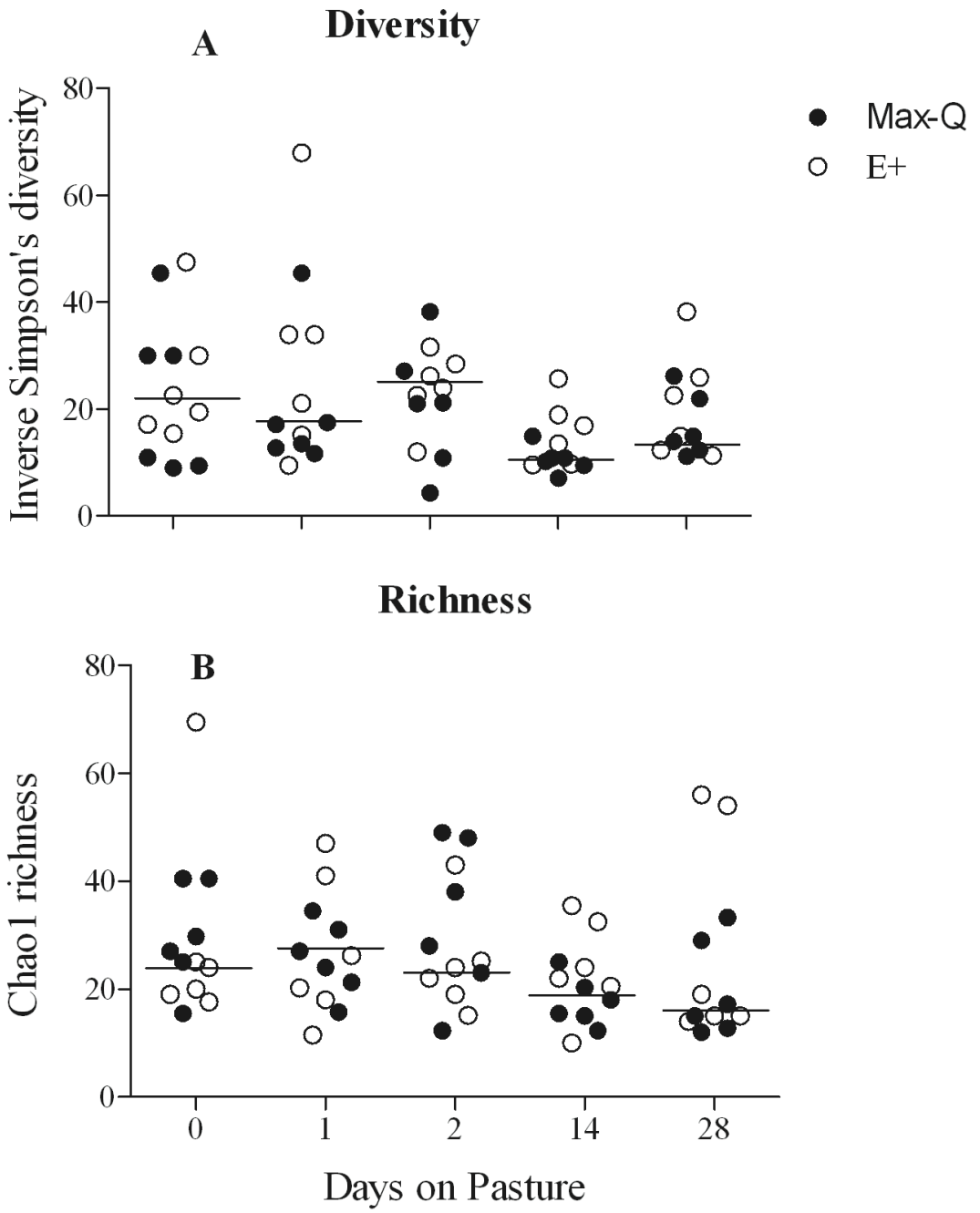


Figure S3.2. Changes in (A) diversity (inverse of Simpson's diversity index) and (B) richness (Chao1 richness) metrics in Angus steers grazing either a non-toxic endophyte-infected (Max-Q; n= 6) or a toxic endophyte-infected (E+; n = 6) tall fescue before placement on pastures (Day 0) and for 1, 2, 14, and 28 days. Diversity and richness means are presented as a line for each sampling date.

File S3.1. Interactive fecal bacterial community composition analyses using Krona Tools 2.7 of the microbiota of Angus steers either prior to pasture placement or after 1, 2, 14, or 28 days of grazing either toxic- (E+; n = 6) or a non-toxic (Max-Q; n = 6) tall fescue. The number of sequences classified within a taxonomic level are expressed as percentages of the total sequence number. To access, follow the link, download the .html file, and open it in a web browser. Then, the interactive Krona plots would appear in the web browser. The link to the .html file is below:

https://drive.google.com/open?id=1AL67gnXwpPvc1SgP6WdOJWEMYTiTj6M7

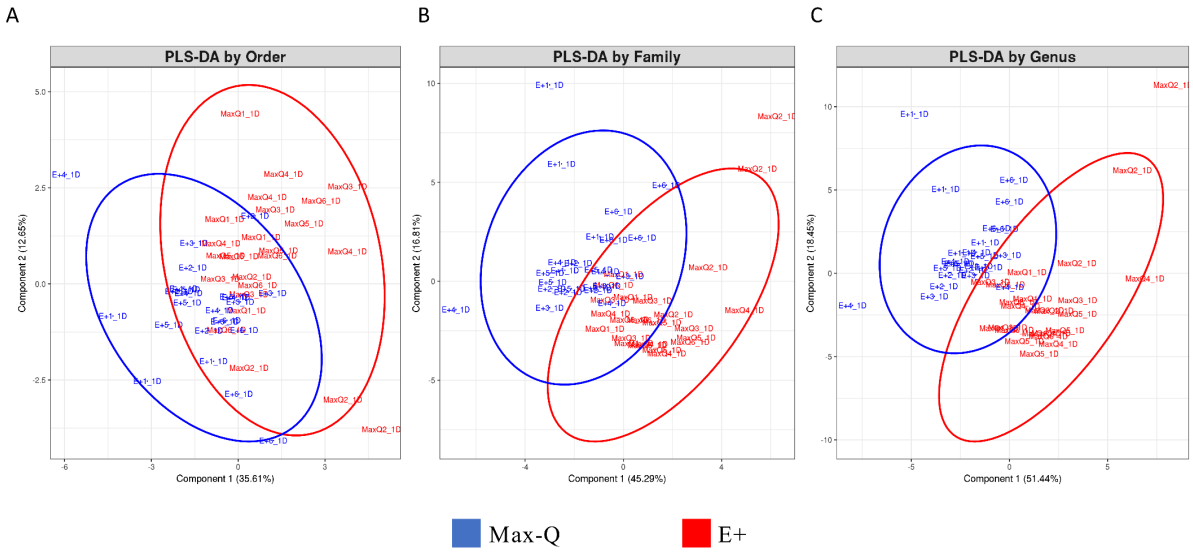


Figure S3.3. Partial least square discriminant analysis (PLS-DA) plots analyzing the fecal microbiota at the **(A)** order **(B)** family and **(C)** genus level of Angus steers grazing either a non-toxic endophyte-infected (Max-Q; n = 6) or a toxic endophyte-infected (E+; n = 6) tall fescue over the course of a 28-day grazing trial.

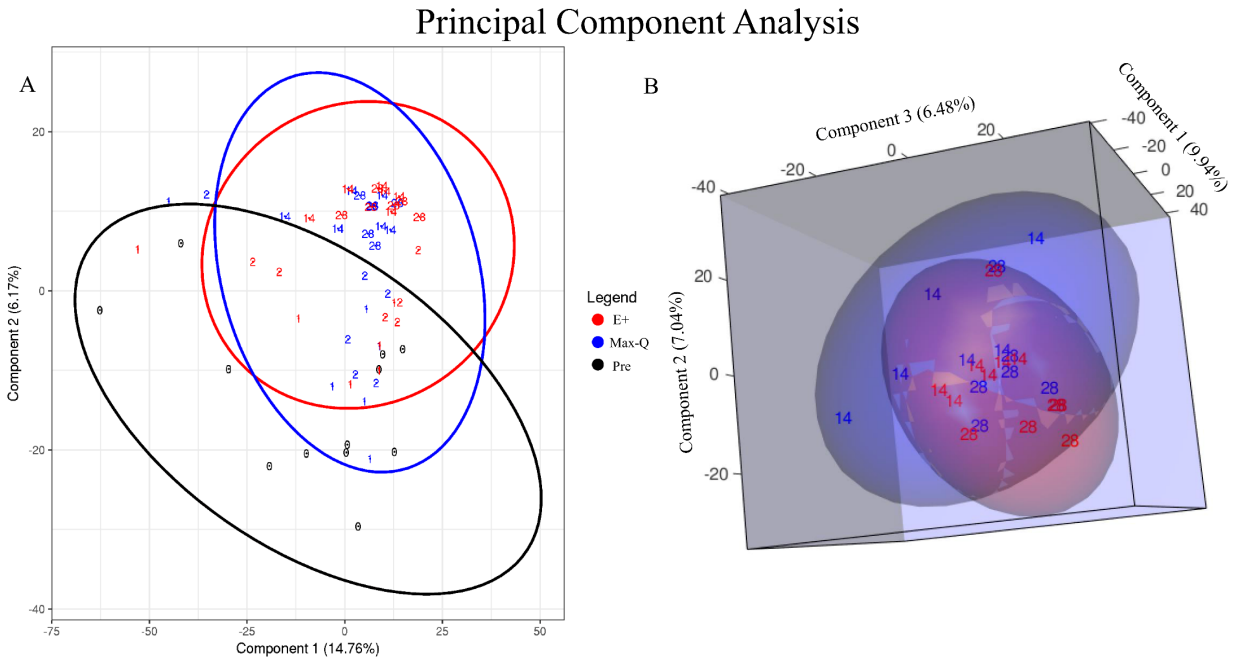


Figure S3.4. Principal component plots analyzing the fecal microbiota of Angus steers grazing either a non-toxic endophyte-infected (Max-Q; n = 6) or a toxic endophyte-infected (E+; n = 6) tall fescue (**A)** throughout the 28-day grazing trial and **(B)** only at 14 and 28 days of the grazing trial.

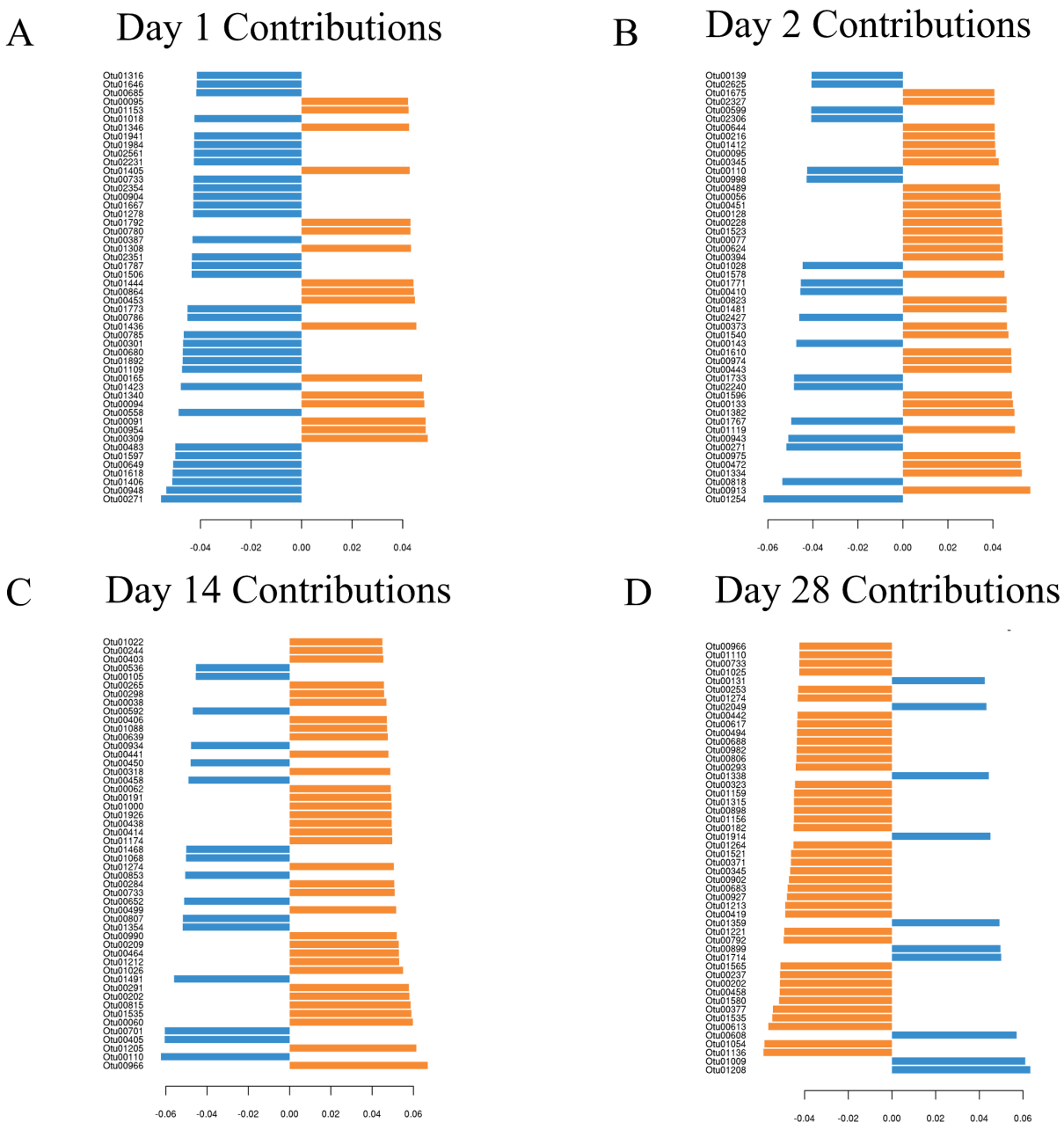


Figure S3.5. Partial least square discriminant analysis (PLS-DA) loadings plots representing the top 50 operational taxonomic units (OTUs) that contributed to separation between Angus steers grazing a non-toxic endophyte-infected (Max-Q; n = 6) and a toxic endophyte-infected (E+; n = 6) tall fescue for the PLS-DA analysis performed on the fecal microbiota at: **(A)** 1, **(B)** 2, **(C)** 14, and **(D)** 28 days. Colors are indicative of: Max-Q = blue, E+ = orange. Bars represent the fescue treatment in which the OTUs had higher abundances and the x-axis is the respective loading weight for each OTU.

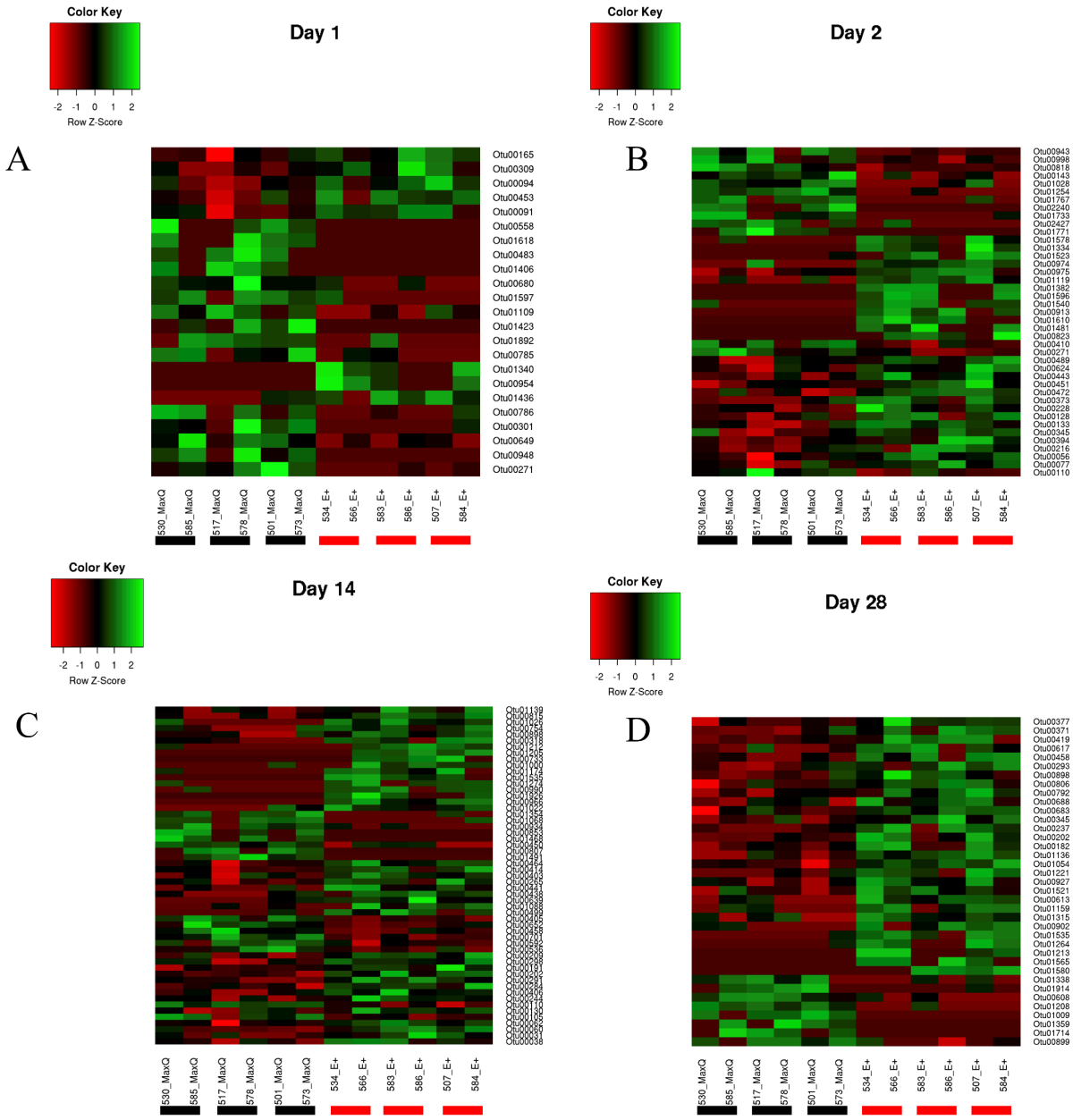


Figure S3.6. Heat maps representing operational taxonomic units (OTUs) that were significantly different ($P < 0.05$) between Angus steers grazing either a non-toxic endophyte-infected (Max-Q; n = 6) or a toxic (E+; n = 6) endophyte-infected tall fescue after **(A)** 1, **(B)** 2, **(C)** 14, and **(D)** 28 days of grazing. Bars underneath the column labels indicate steers on the same pastures: Black = Max-Q steers; Red = E+ steers.

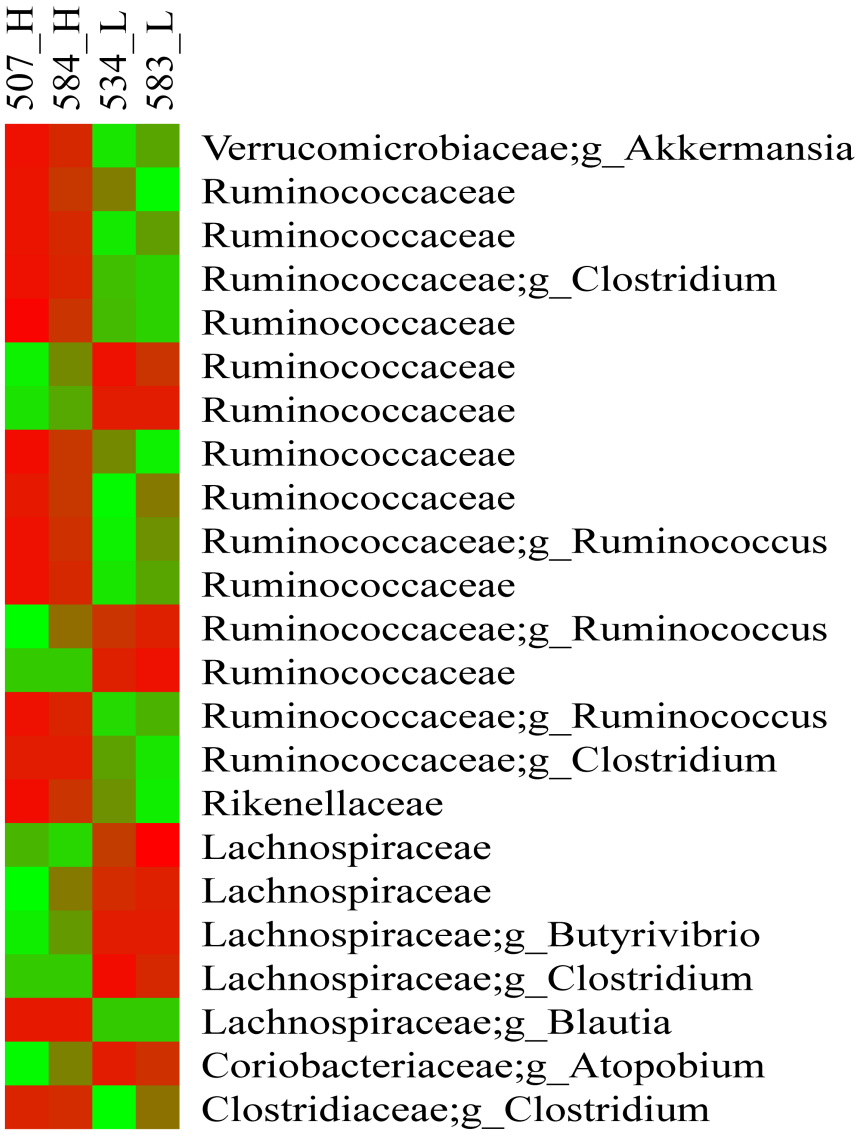


Figure S3.7. Heat maps representing normalized operational taxonomic unit (OTU) sequence counts for Angus steers that grazed a toxic endophyte-infected tall fescue (E+; n = 4) throughout the 28 day grazing trial stratified by weight gains. "_G" indicates the two steers that gained the most weight and "_NG" indicates the two steers that had not gained weight; red represents sequence counts below the respective OTUs means and green represents sequence counts greater than the respective OTU means.

Table S3.1. Bacterial families that had operational taxonomic units (OTUs) that were main contributors to the treatment separations seen in partial least squares discriminant analysis (PLS- DA) and the number of days Angus steers spent grazing either a non-toxic endophyte-infected tall fescue (Max-Q; n = 6) or a toxic endophyte-infected tall fescue (E+; n = 6) where the family OTUs resulted from PLS-DA loadings analysis.

Days on pasture	Bacterial families
1	*Odoribacteraceae* *Veillonellaceae* *Moraxellaceae* *Mycoplasmataceae* *Proteobacteria*_unclassified
2	*Rikenellaceae* *Christensenellaceae* *Mollicutes*_unclassified
14	*ML615J-28*_unclassified *Anaeroplasmataceae* *Eubacteriaceae*
28	*Dehalobacteriaceae* *Paraprevotellaceae*
1 and 2	*S24-7*
1 and 28	*Clostridia*_unclassified
14 and 28	*Lactobacillaceae*
1, 2, and 14	*Mogibacteriaceae* *Bacteroidaceae*
1, 2, and 28	*Erysipelotrichaceae* *Coriobacteriaceae*
1, 14, and 28	*Prevotellaceae* *Clostridiaceae*

Table S3.2. Top four most frequent classified families, broken down by OTUs classified at the genus level, of bacteria that positively correlated with rectal temperatures (RT) in steers that grazed toxic (E+; n = 6) endophyte-infected tall fescue. The number of OTUs within a family/genus, average Spearman correlation coefficient and P-values are presented.

Rectal Temperatures				
Family	Genus	No. of OTUs	Correlation	P-value
Lachnospiraceae		18	0.46	0.026
	Butyrivibrio	2	0.48	0.018
	Roseburia	1	0.54	0.0065
	Coprococcus	1	0.42	0.040
	Dorea	1	0.42	0.040
Ruminococcaceae		4	0.54	0.0093
Coriobacteriaceae		2	0.44	0.031
	Enterococcus	1	0.45	0.028
Paraprevotellaceae		2	0.42	0.042
	CF231	1	0.43	0.037
	YRC22	1	0.41	0.048

APPENDIX D

CHAPTER 4 SUPPLEMENTARY MATERIAL

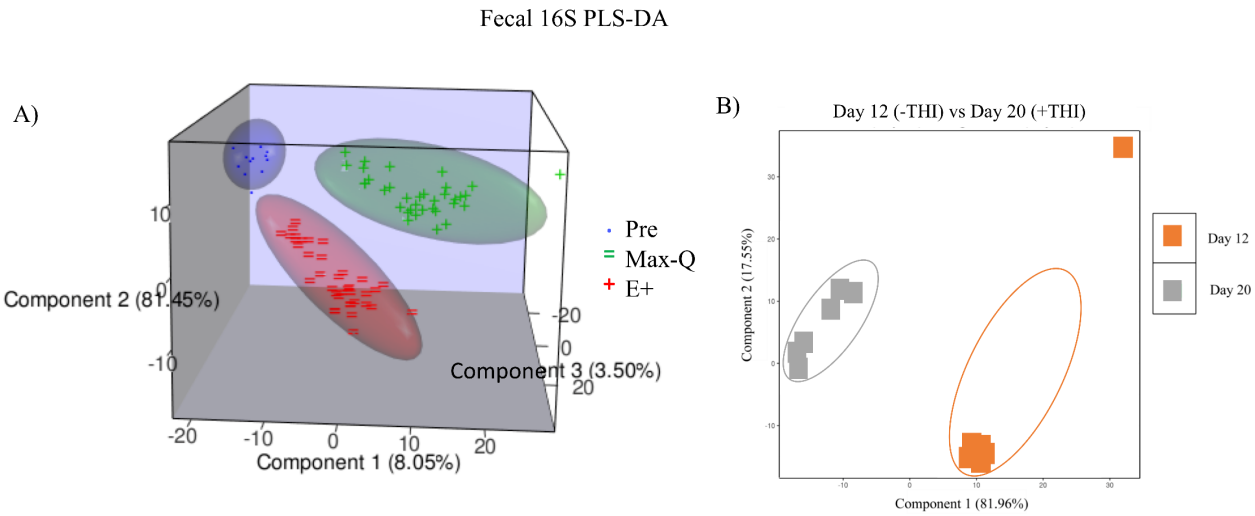


Figure S4.1. Partial least squares discriminant analysis (PLS-DA) plots analyzing the fecal microbiota of Angus steers before pasture placement (blue) or grazing either a novel, non-toxic (Max-Q; n = 6; green) or toxic (E+; n = 6; red) endophyte-infected tall fescue (A) over the course of a 26-day grazing trial or (B) E+ grazing steers on Day 12 (-THI; orange) or Day 20 (+THI; gray) post pasture assignment.

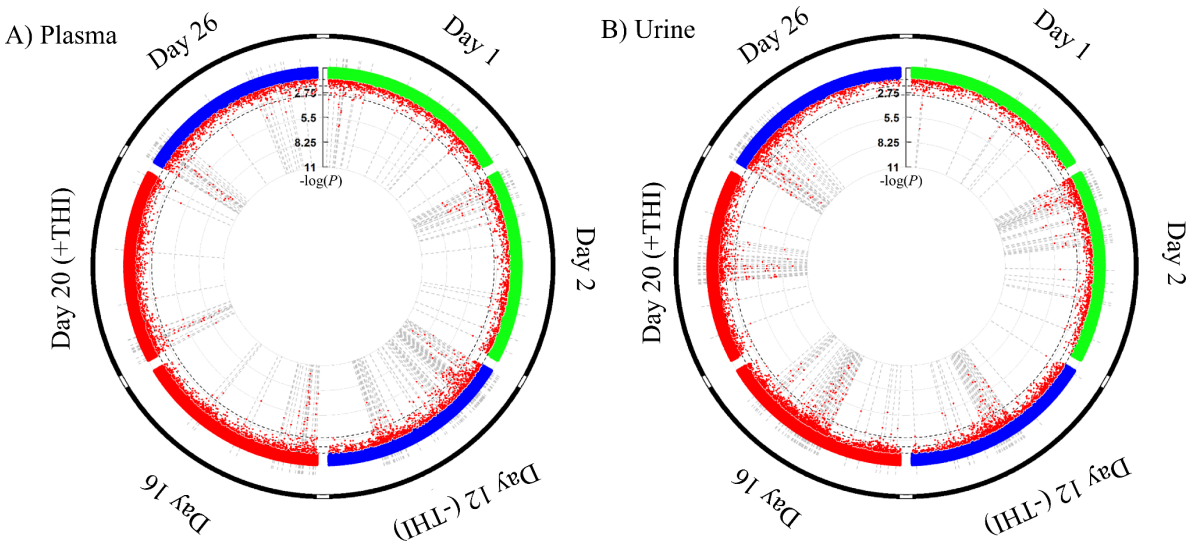


Figure S4.2. Circular Manhattan plots representing high-resolution metabolomics (HRM) features in the (A) plasma and (B) urine of Angus steers grazing either a novel, non-toxic (Max-Q; n = 6) or toxic (E+; n = 6) endophyte-infected tall fescue over the course of a 26 day grazing trial. Red dots indicates HRM features were significantly affected by E+ treatment (FDR corrected $P < 0.05$) and the more central the dots the greater the significance.

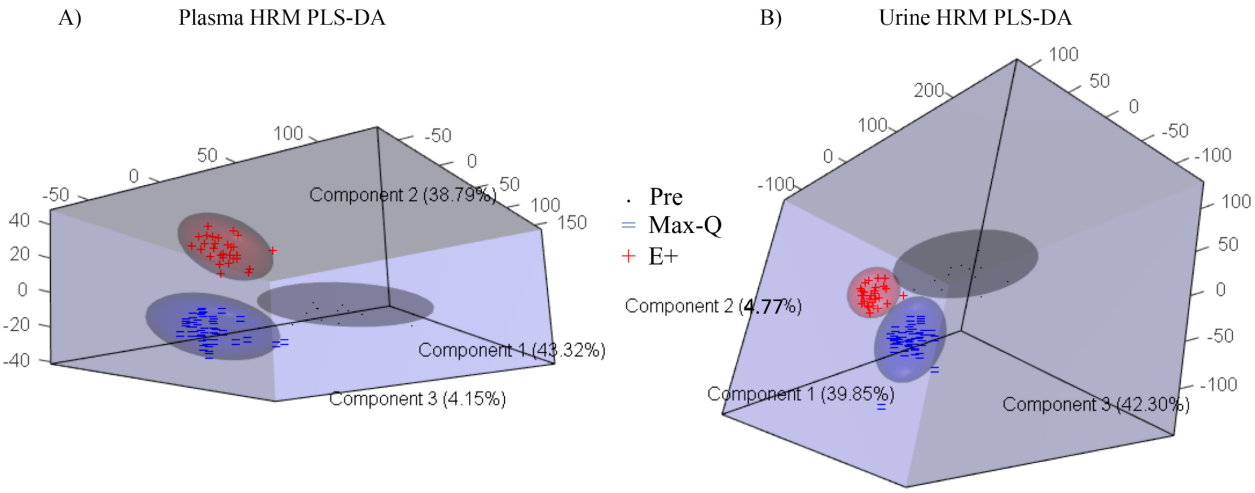
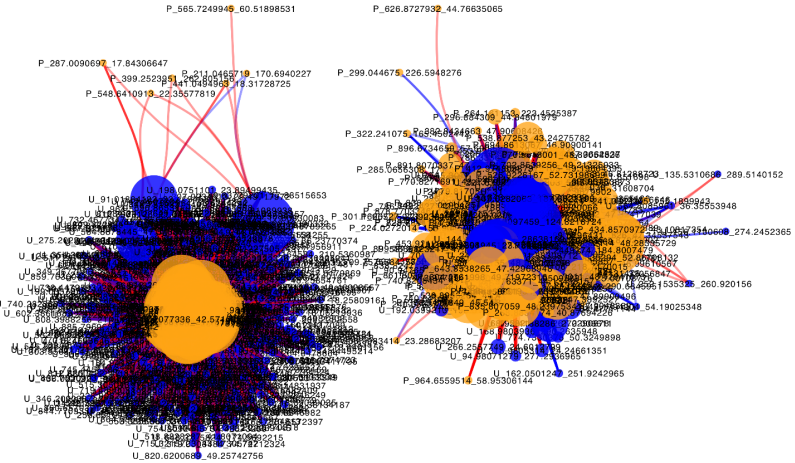
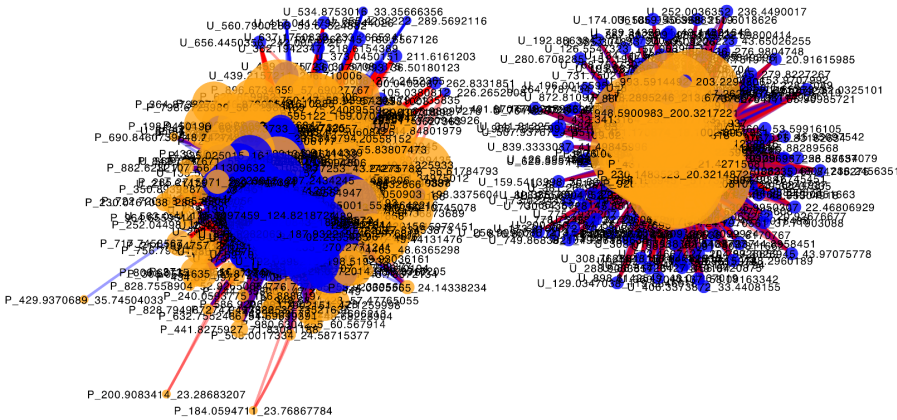


Figure S4.3. Partial least squares discriminant analysis (PLS-DA) plots analyzing the fecal microbiota of Angus steers before pasture placement (blue) or grazing either a novel, non-toxic (Max-Q; n = 6; green) or toxic (E+; n = 6; red) endophyte-infected tall fescue over the course of a 26-day grazing trial in the **(A)** plasma and **(B)** urine.

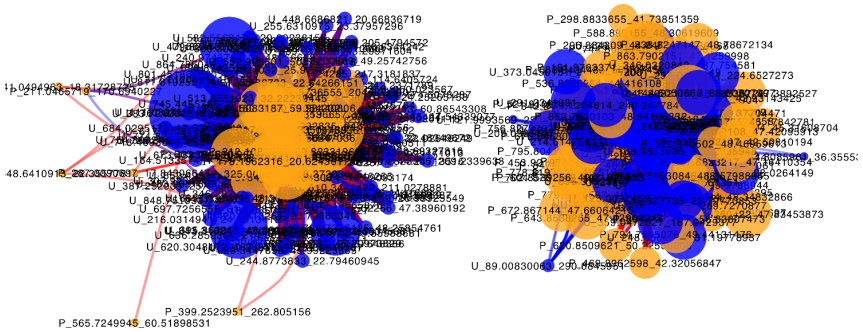
A) Overall; Max-Q



E+



B) Day 12 (-THI); Max-Q



E+

C) Day 20 (+THI); Max-Q E+

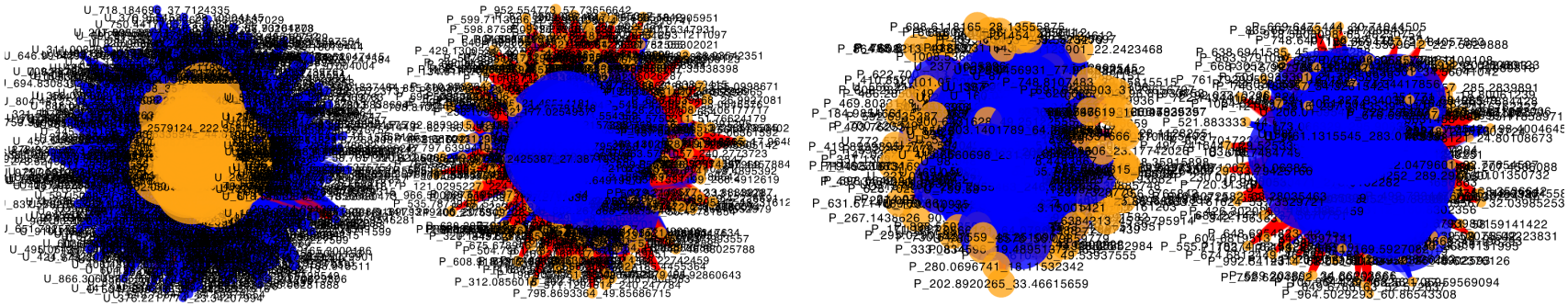


Figure S4.4. Bipartite network of plasma (orange) and urine (blue) high- resolution metabolomics (HRM) features that were selected by sparse partial least squares regression (sPLS; top 100 plasma and urine HRM features [X and Y matrix, respectfully]) using the *mixOmics* R package and were highly correlated (|r| > 0.7) for steers grazing a: **(A)** novel, non-toxic tall fescue (Max-Q) or toxic tall fescue (E+) throughout the grazing trial; **(B)** Max-Q or E+ tall fescue on Day 12 (-THI); **(C)** Max-Q or E+ tall fescue on Day 20 (+THI). Size of the node is respective of the metabolites between centrality within the respective networks.

APPENDIX E

CHAPTER 5 SUPPLEMENTARY MATERIAL

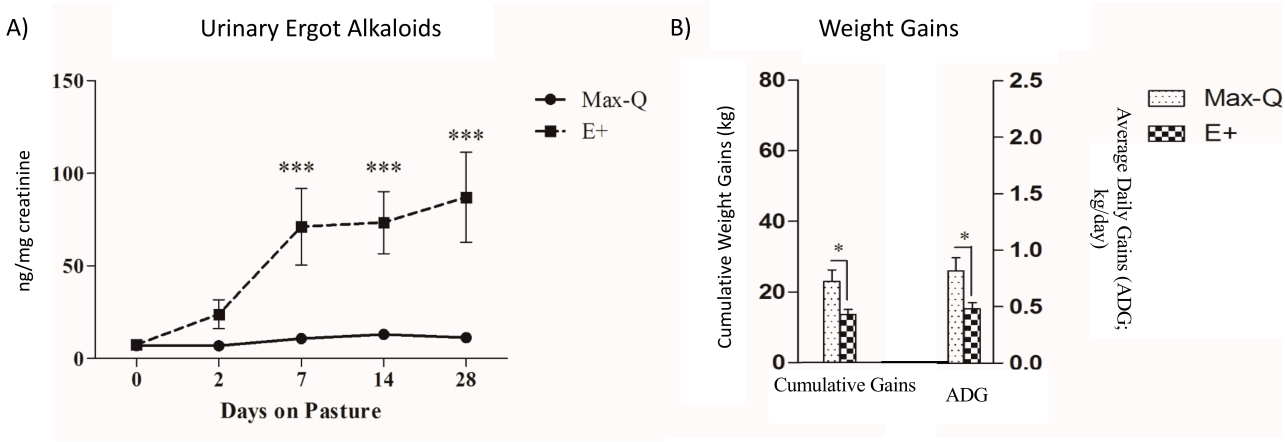


Figure S5.1. (A) Urinary ergot alkaloids (ng/mg creatinine) and **(A)** average daily gain (ADG; kg/day) and cumulative gain (kg) in Angus steers that grazed either non-toxic endophyte-infected (Max-Q; n=6) or toxic endophyte-infected (E+; n=6) tall fescue for a 28-day grazing trial. (*) $P < 0.05$, (***) $P < 0.001$.

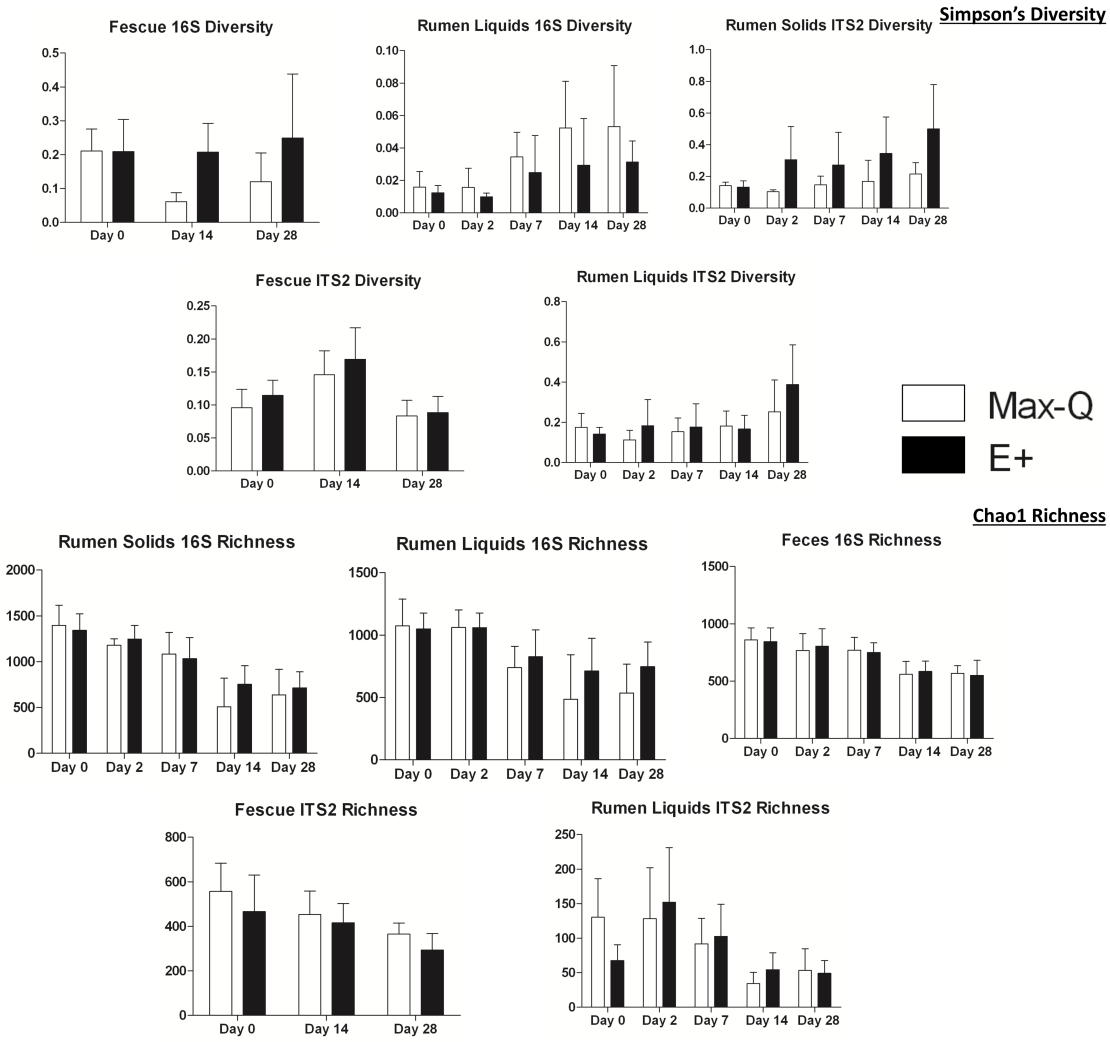


Figure S5.2. Changes in diversity (Simpson's diversity index) and richness (Chao1 richness) metrics in Angus steers and tall fescue grazing or being infected with either a non-toxic endophyte (Max-Q; n= 6) or a toxic endophyte (E+; n = 6) before placement on pastures (Day 0) and for 1, 2, 14, and 28 days (bovine samples) or Day 0, 14, and 28 (fescue samples). Data are presented as mean with standard deviation as error bars. White and black bars indicate Max-Q and E+ tall fescue or grazing steer treatment, respectively.

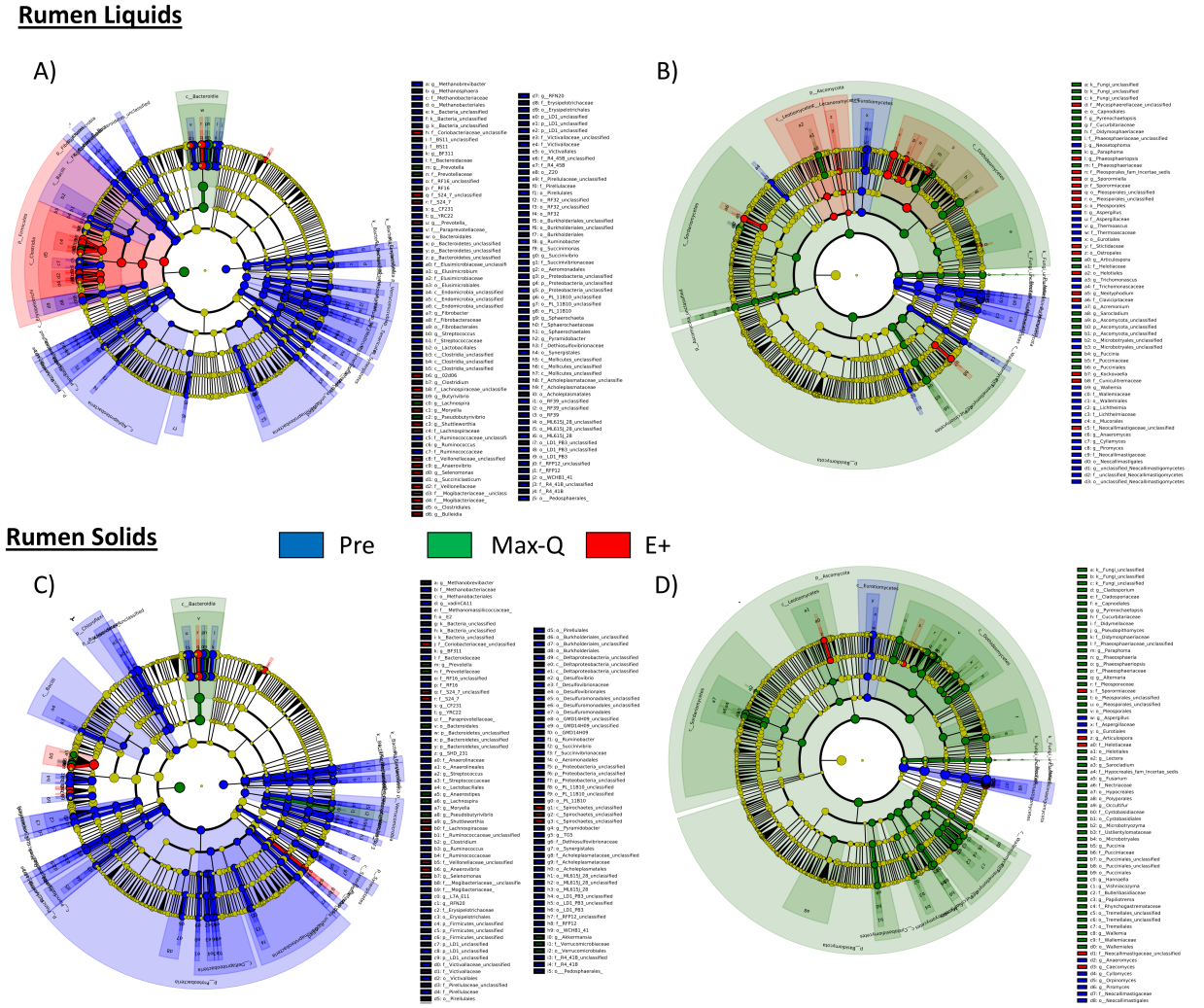


Figure S5.3. Linear discriminant analysis (LDA) effect size (LEfSe; Kruskall-Wallis [$P < 0.05$]; Pairwise Wilcoxon [$P < 0.05$]; logarithmic LDA score > 2.0) of the rumen solid **(A)** bacterial and **(B)** fungal and rumen liquid **(C)** bacterial and **(D)** fungal microbiota of Angus steers across a 28-day grazing trial after placement on either a non-toxic (Max-Q; n = 6) or toxic (E+; n = 6) endophyte-infected tall fescue. Blue, green and red shading indicates greater abundance in Pre (before pasture placement), Max-Q, or E+ steers, respectively. Taxonomic rank labels are provided before bacterial names: "p_; c_; o_; f_; g_" indicate phylum, class, order, family, and genus, respectively. Letters and numbers within the cladogram refer to respective bacterial or fungal names located in the keys to the right of the cladogram. Select taxa of interest have been highlighted by arrows and text.

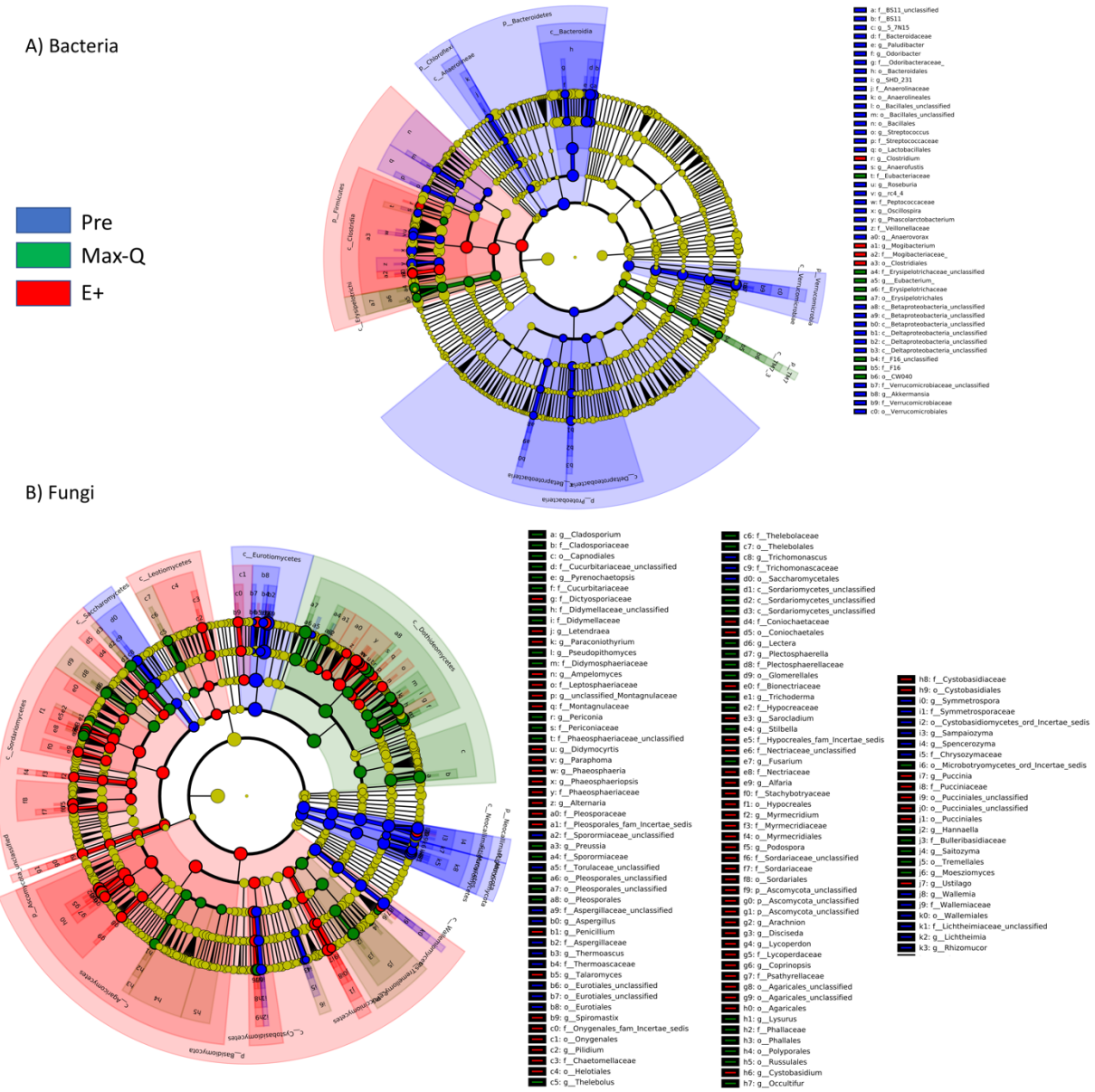


Figure S5.4. Linear discriminant analysis (LDA) effect size (LEfSe; Kruskall-Wallis [$P < 0.05$]; Pairwise Wilcoxon [$P < 0.05$]; logarithmic LDA score > 2.0) of the **(A)** bacterial and **(B)** fungal fecal microbiota of Angus steers across a 28-day grazing trial after placement on either a non-toxic (Max-Q; n = 6) or toxic (E+; n = 6) endophyte-infected tall fescue. Blue, green and red shading indicates greater abundance in Pre (before pasture placement), Max-Q, or E+ steers, respectively. Taxonomic rank labels are provided before bacterial names: "p_; c_; o_; f_; g_" indicate phylum, class, order, family, and genus, respectively. Letters and numbers within the cladogram refer to respective bacterial or fungal names located in the keys to the right of the cladogram. Select taxa of interest have been highlighted by arrows and text.

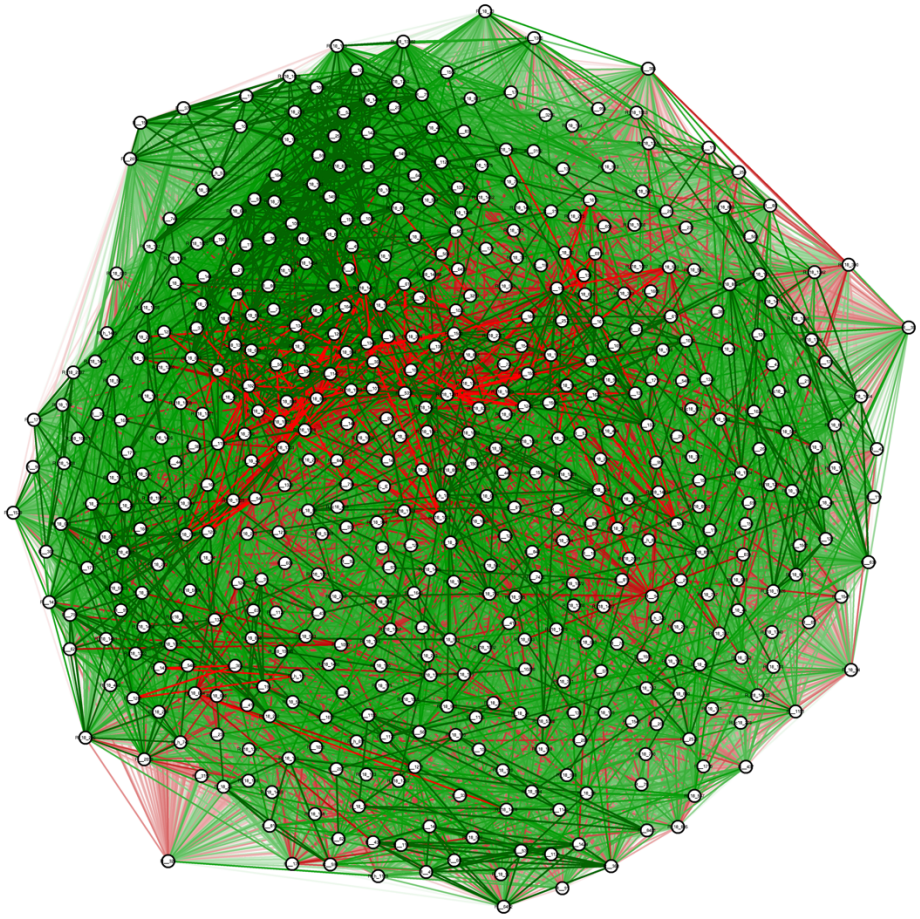


Figure S5.5. Targeted correlation-based network analysis including rumen metabolic features significantly correlated with ergovaline in the overall network of toxic tall fescue grazing steers (E+; $|r| > 0.6$; $P < 0.05$). Green and red indicate positive and negative correlations, respectively.

File S5.1. and S5.2. Containing overlapping OTUs names and biological matrices where present (S5.1.) and xMWAS tables with raw name, cluster, centrality measurement, annotation, shape, and color can be found in the following public folder:

https://drive.google.com/drive/folders/1jYOyZi1Fy4f0UCGlncPs81kDdHZxQyA6?usp=sharing