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Effects of Toxic Fescue Exposure on Vaginal Microbial Communities of Crossbred Beef

Cows

Abby E. Ratton

University of Arkansas

Dale Bumpers Honors College

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Abstract

The consumption of toxic fescue by beef cattle results in several adverse physiological effects such as reduced reproductive success, severe vasoconstriction resulting in hoof sloughing, low body condition scores, hyperthermia, decreased prolactin levels, and reduced hair shedding. The purpose of this study is to characterize bacterial community of the reproductive tract as a potential predictor for toxin exposure. One-hundred fall-calving crossbred cows were allocated to graze Toxic (Toxic: n=50) or Novel (Novel: n= 50) fescue pastures for five months (March-August). Treatments were blocked by sire breed (Charolais or Hereford) and by parity (first, second, third). Animals rotated pastures biweekly and pastures were monitored monthly for ergovaline concentration. Twenty-five animals allocated to Novel treatment were exposed to a toxic pasture two weeks each month in rotation with two weeks exposure to novel pasture. This group was defined as an alternating treatment condition (Alt: n=25). Rectal temperatures, hair score, body condition score, and body weight were collected each month as verification of fescue toxicosis in each animal. In August, the reproductive tract was swabbed using a Morgan swab at the vaginal-fornix junction for 16s rRNA-based microbiota analysis. The V4 region was amplified and sequenced using the Illumina Miseq platform. Data was processed using Mothur v.1.39.5. Significant difference in bovine vaginal bacterial community structure was observed between Toxic and Novel treatments based on Bray-Curtis distance (ANOSIM, R= 0.429223, P< .001). Similarly, the bacterial communities of Toxic pasture also differed significantly from those of the Alt pasture (ANOSIM, R= 0.445697, P< 0.001); however, Novel and Alt treatments did not differ (R = -0.0077, P =.536). Charolais exhibited greater (p= 0.0427) Shannon diversity than Herefords, and diversity increased with parity number with no difference (p=0.7312) observed between parities one and two. Smith-Wilson evenness was greater (p=0.0008) for

Novel animals than Toxic animals. The suppression of *Pseudomonas* was the best indicator of

toxin exposure. Novel animals hosted 16.5 times more (p=0.0057) *Pseudomonas* than toxic

animals. *Ureaplasma* was the second most abundant OTU and 2nd most predictive of pasture

type. Novel animals hosted 3.8 times more (p=0.0004) than novel animals. *Burkholderia* was the

most abundant OTU and the third most predictive of treatment. *Burkholderia* represented

41.74% of the bacterial community in the Toxic treatment reproductive tracts and 14.67% in

Novel animals.

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Introduction

It is estimated that fescue toxicosis impacts 8.5 million head of cattle each year, resulting in nearly 1 billion dollars of annual loss (Strickland et al., 2011). Ergot alkaloids, the major active mycotoxin in toxic tall fescue grass, impact the growth, health, and reproductive success of grazing livestock, particularly in the Southeastern regions of the United States. Fescue grass is routinely used as forage in this region due to its reputation as a high nutrient, hardy grass that is drought, heat, and cold weather tolerant. Toxic fescue grass contains a fungal endophyte that confers heat tolerance, but produces ergot alkaloid mycotoxins that amplify the effects of heat stress and contribute to fescue toxicosis. Cows exposed to moderate to high levels of endophyte infected pasture exhibit lower conception rates and in severe cases, abortion. It is known that the microbial makeup of the reproductive tract has been found to be a determining factor for reproductive health (MacIntyre et al., 2015), and we theorize that it could also have an impact on the reproductive success of cattle exposed to toxic fescue. Therefore, the purpose of this study is to characterize the vaginal microbial community in animals grazing toxic and novel fescue grass and to identify biomarkers in the form of bacterial families that may be indicative of the severity of fescue toxicosis. Identification of bacteria indicative of poor performance on toxic fescue may provide an early warning sign to allow producers ample time to intervene with different management to reduce loss, as well as a means to select for animals more tolerant of toxic fescue grass.

Literature Review

The development of tall fescue as a staple forage in the Southeast U.S.

Tall fescue grass, a cool season perennial of European origin, made its first appearance in the United States in the 19th century; by 1940, upon release of the Kentucky 31 cultivar, tall

fescue was routinely planted across the U.S. as a forage for grazing livestock (Fribourg et al., 2009). Tall fescue was quickly recognized as a hardy, adaptable grass suitable for a wide range of environmental conditions, contributing to is popularity. It proved to be drought, heat, and cold weather tolerant while demonstrating remarkable resistance to mismanagement and overgrazing. These characteristics made tall fescue a staple forage in what is considered to be the "transition zone," referring to regions such as Arkansas, Missouri, Kentucky, Oklahoma, Tennessee, and Virginia whose summers are too warm and humid for cool season grasses and whose winters are too harsh for warm season grasses (Duble, 2018). The longevity and tough nature of tall fescue allowed it to not only serve as a nutritious forage option for grazing livestock, but as a useful tool in erosion control (Kallenbach, 2011). As the demand for tall fescue seed increased, producers began to experience the full effects of tall fescue. Despite its merits, the exceptional forage that captured the attention of the Southeast brought serious consequences with its widespread use. Livestock exposed to tall fescue pastures commonly suffered from an assortment of adverse health effects and performed poorly by most production standards, resulting in great loss (Klotz, 2015). The cause remained unknown until the 1970s when it was discovered that the fungal endophyte contained within tall fescue produced ergot alkaloids detrimental to the health of livestock (Hoveland, 2018). Today, tall fescue is regarded as one of the most prominent grazing forages and covers approximately 35 million acres in the U.S. (Kallenbach, 2011). Several strategies have been developed in an attempt to suppress tall fescue's toxicity while still reaping its numerous benefits. Ergot alkaloids exist at a concentration 5 times higher in the seed head of the fescue plant than the leaves or stems (Dow AgroSciences, 2016); because of this, many producers attempt to control fescue toxicity by minimizing the presence of seed heads in pastures. This can be achieved by the early clipping of seed heads or using chemical agents.

Chemical seed head suppressants such as the herbicide Chaparral [TM] have been found to alleviate some symptoms of fescue toxicosis and boost performance (Williamson & Aiken, 2017). However, these techniques can be time consuming, costly, and do not completely eliminate toxicity. Additionally, negative connotations are often associated with beef grazing herbicide treated forages. Although endophyte-free fescue is available for purchase and has proven to significantly improve animal performance, the absence of the endophyte results in a substantial decrease in hardiness and longevity (Hoveland, 2018), both of which are critical to the appeal of tall fescue. Periods of drought and overgrazing of endophyte-free fescue often result in complete crop loss in a 3 to 4-year period (Beck & Gunter, 2004). It is simply not sustainable. The emergence of novel-endophyte fescue, a fescue variety designed to maintain the hardy characteristics of traditional fescue while eliminating ergot alkaloid production, seemed to mark the end of the problem. Although highly effective, novel-endophyte fescue has proven costly to plant and maintain and therefore is not a feasible option for all cattle producers. Prior to novel-endophyte fescue being successfully planted, toxic fescue must be completely eradicated with several applications of glyphosate. If a single plant avoids contact with the herbicide, it may emerge at a later date and contaminate the novel pasture. Additionally, a single application of glyphosate is useless against tall fescue seeds in the soil, opening up the pasture to contamination long after spraying occurs (Gunter & Beck, 2004). The spray-smother-spray technique is perhaps the most popular approach, which involves applying glyphosate in the spring, then no-till drilling a warm season crop such as sudangrass which is grazed during the summer, and then using a second application at the end of the summer before replanting a cool season grass such as novel endophyte fescue (Ohmes, 2018). However, this process is lengthy and costly, rendering it undesirable for some producers. Fescue toxicosis is considered to be the largest animal health-

related production cost among individuals in the grazing livestock industry (Strickland et al., 2011). Although each of these mitigation strategies have experienced some success, the need for low-cost options persists. No one solution to reduce fescue toxicosis will solve the problem in all regions, but a combination of approaches will likely be more effective. With toxic fescue being difficult and expensive to remove, perhaps one of the best tools to use in concert with these approaches is to produce animals more tolerant of the side effects of ergovaline consumption.

The physiological impact of Fescue Toxicosis on cattle

Toxic fescue can cause an array of adverse physiological effects when ingested, including reduced reproductive success, vasoconstriction, decreased growth rate, poor body condition score, hyperthermia, reduced hair shedding, and in extreme cases, death. Fescue toxicosis is known to interfere specifically with dopamine signaling. Dopamine is a neurotransmitter tasked with the inhibition of prolactin by binding to D2-dopamine receptors. Ergot alkaloids are very similar in structure to dopamine; as a consequence, ergot alkaloids commonly bind to D2-dopamine receptors and further inhibit prolactin production (Strickland et al., 2011). Affected animals often do not perform adequately in terms of milk production, which can be extremely problematic to cow-calf producers. However, both prolactin and milk production are suppressed by heat stress, thus the effects of fescue toxicosis and heat stress are often indistinguishable. The two phenomena may be differentiated by the presence of vasoconstriction (by measuring blood flow or fescue foot) and reduced hair shedding in the summer months when hair score is expected to be low (i.e. animals should be slicked off). "Fescue foot" refers to the sloughing of tissue on extremities due to vasoconstriction and is perhaps the physical abnormality most indicative of fescue toxicosis. Animals may also lose the tips of their ears and tails as a result of vasoconstriction from toxic fescue exposure. Once these symptoms manifest it is often too late

for producers to intervene. In the field, the only indicator of fescue stress observed routinely is

hair shedding score, which is a subjective measure that requires multiple observations overtime.

New measurements that could differentiate early signs of fescue toxicosis from severe cases in a

quantitative way could be very valuable in predicting the severity of fescue toxicosis in advance,

allowing for preventative management.

What is the microbiome?

A microbiome refers to all the microorganisms that inhabit a specific niche in the body

and the collection of DNA sequences that compose these microbes. The abundance of microbes

present in the human body is striking with it being estimated that bacterial cells outnumber

human cells at a ratio of approximately 1.3:1 (Sender et al., 2016). In addition to the large

quantity, a substantial amount of diversity can be observed among microbial communities. In

recent years the relationship between varying microbiomes and health has been evaluated

extensively; studies suggest a vast role for the microbiome, almost as if it were another organ in

its roles impacting health, including infectious, metabolic, and behavioral disease. New roles or

associations for the microbiome are being discovered almost daily.

Utilization of 16s rRNA microbiome sequencing to characterize the microbiome

Much of what is known about microbial compositions has been uncovered via the

utilization of 16s ribosomal RNA (rRNA) microbiome sequencing technology. Bacterial 16s

rRNA regions contain nine hypervariable regions (V1-V9) that demonstrate a substantial amount

of sequence diversity among various bacteria, with the V2-V3 regions being most effective for

differentiation at the genus level (Chakravorty, 2007). Using culture-based approaches in

combination with 16s rRNA sequencing has allowed for a greater understanding of microbial

makeups and helped demonstrate diversity, particularly in the gastrointestinal tract flora of

individuals (Peterson et al., 2009).

Role of the microbiome in disease

Although the mechanisms are not entirely understood, it is clear that the composition of

the microbiome has significant influence on states of health and disease. It has been reported that

at least 10% of variability in human immune response is derived directly from interactions

associated with the microbiome (Gilbert et al., 2015). Recent studies suggest that unfavorable

fluctuations in the microbiome may contribute to the pathogenesis of several diseases. Dysbiosis

refers to any bacterial composition change within a microbiome relative to that of a healthy

individual (Peterson & Round, 2014). Data proposing a linkage between intestinal dysbiosis and

several gastrointestinal diseases including inflammatory bowel disease, irritable bowel

syndrome, nonalcoholic fatty liver disease, as well as obesity, have been observed (Parekh et al.,

2015). One of the most compelling pieces of evidence in favor of this hypothesis has emerged as

the result of gut microbiota transplantation research. A 2016 transplantation study demonstrated

that germ-free mice (gnotobiotic) inoculated with microbiota from obese and lean humans took

on the weight characteristics from their human donors; the mice presented with microbes from

lean donors were also lean, whereas the microbes from obese individuals resulted in overweight

mice (Ridaura et al., 2013).

Role of the microbiome in the reproductive tract

Although the gut microbiota is the subject of the majority of microbiome research, other

microbiomes, such as the reproductive, are theorized to have significant influences on health as

well. As is the case in all microbiomes, deviations from a normal, healthy population may result

in complications. Bacterial vaginosis, one of the most common vaginal conditions affecting

women, is onset by bacterial shifts within the vagina (Bing Ma et al., 2012). Furthermore, reduced reproductive success and reproductive diseases have been associated with the presence of specific microbes and microbial community compositions; in human women, fertility problems and preterm labor are thought to be associated with certain microbial profiles (Clemmons et al., 2017). A "healthy" human vaginal microbial profile is typically characterized by the presence of a substantial amount of *Lactobacillus*. However, numerous individuals lacking large populations of *Lactobacillus* may still exhibit a "healthy" microbiome (Bing Ma et al., 2012), which speaks to the microbiome's diverse nature. Additionally, microbial composition varies among members of the animal kingdom. An abundance of *Lactobacillus* is typically not observed as the hallmark of the bovine vaginal microbiome as seen in most women (Swartz et al., 2014). Despite most microbiome studies being geared towards human health, cattle are equally, if not more susceptible to microbial fluctuations. Although the function of gastrointestinal tract microbiota is of the utmost importance to all species in order to promote health, proper development, and digestion (Yeoman et al., 2018), this phenomenon is even more prevalent in species such as ruminants, who depend heavily on microbial activity for survival.

Previous research into the composition of the bovine vaginal microbiome

In recent years 16s rRNA microbiome sequencing technology has been extended to vaginal microbiome research in cattle. One particular study aiming to characterize the average vaginal microbial makeup of crossbred cows and ewes reported that the most abundant bacterial phyla present included *Bacteroidetes, Fusobacteria,* and *Proteobacteria*; additionally, the most abundant genera included *Aggregatibacter spp.* and *Streptobacillus spp.* (Swartz et al., 2014). A similar study focusing on the vaginal bacterial communities of postpartum lactating Angus cows revealed that the vaginal bacterial populations among sampled individuals were composed

primarily of the phylum *Firmicutes*, followed by *Bacteroidetes, Proteobacteria, Tenericutes,* and *Actinobacteria* (Clemmons et al., 2017). These results were reiterated by a 2015 metagenomic analysis of the vaginal microbial composition of both pregnant and non-pregnant Nellore heifers and cows, where it was determined that the most abundant phyla included *Firmicutes, Bacteroidetes,* and *Proteobacteria* (Laguardia-Nascimento et al., 2015). Furthermore, 45-55% of samples obtained were composed of just 10 Operational Taxonomic Units (OTUs): *Aeribacillus, Bacteroides, Clostridium, Ruminococcus, Rikenella, Alistipes, Bacillus, Eubacterium,* and *Prevotella* (Laguardia-Nascimento et al., 2015). Unfortunately, the resolution of 16s rRNA sequencing is limited, meaning it can be difficult to determine what specific species are present. Therefore, it is interesting to see some overlap in phylum, family, and genus of bacteria but difficult to tell exactly how similar or different the microbial content may be across these studies. Discrepancies between studies could also very likely be the result of breed differences and environmental influence.

Importance of vaginal microbiome in cattle fertility

The possible reproductive issues associated with microbial profiles weigh heavily on producers in the cow-calf industry who depend on annual calving for profits. Perhaps the most notable bacteria-induced illnesses associated with fertility issues in cattle are reproductive diseases such as Brucellosis, Leptospirosis, and Vibriosis, all of which commonly result in abortion (McCormack, 2018). Other culprits of venereal diseases include viral agents and protozoa, which likely disrupt the normal microbiota of the vagina and influence reproduction. The appropriate balance of microbes is critical for preventing the accumulation of pathogenic organisms and maintaining a healthy vagina (Huang et al., 2014), and cattle are no exception.

However, only in recent years have researchers begun to truly explore influence of bovine reproductive microbiomes on fertility.

Potential of microbiome in calf health and growth

Although research regarding the potential of the microbiome in calf health and growth is in its early stages, this concept has been explored extensively in humans. The maternal microbiome is known to have a significant influence on infant microbial composition and is thought to be a determining factor in the health of offspring throughout life (Dunlop et al., 2015). Both elective and acute caesarian section have been associated with an increased likelihood of asthma, laryngitis, and gastroenteritis in offspring (Kristensen & Henriksen, 2016). Furthermore, multiple studies have linked children diagnosed with celiac disease to birth via caesarian section (Decker et al., 2011). With the exchange of microbiota from the mother to the offspring during the birthing process seeming to play a critical role in infant health (Mueller et al., 2015), it is reasonable to think that calf health is affected in a similar fashion. Findings from the limited number of studies exploring the potential long-term effects of the microbiome on calf health suggest that the microbes that colonize the sterile gastrointestinal tract of a newborn calf during and immediately after birth may play a major role in the development of the mucosal immune system, which influences the animal's growth and health throughout its development (Malmuthage et al., 2015). A 2018 study examining the relationship between the maternal vaginal microbiome and the microbial composition of the neonatal bovine gastrointestinal tract revealed striking results. The vaginal microbiome was found to host a variety of specific bacteria essential to digestion of fiber within the rumen and reticulum (fibrolytic); such bacteria are critical components to digestive development, thus indicating that the vaginal microbiome may play a part in colonizing the rumen and reticulum with microbes important to the nutrition of the

adult animal (Yeoman et al., 2018). These findings potentially mean that the role of the vaginal microbiome is much greater than previously thought.

Materials and Methods

Population and Experimental Design

One hundred fall-calving crossbred beef cows were grazed on two pasture types for 5 months (March-August): endophyte-infected tall fescue (P1; n=50) and novel endophyte-infected tall fescue (P1; n=50). Treatments were blocked by sire breed (Charolais or Hereford) and parity (first, second or third). Animals within each pasture type were divided into two groups of 25 and rotated between two pastures of the same forage type every other week. Monthly ergovaline testing was performed to monitor toxicity and pastures testing greater than 300 ppb were classified as toxic. One novel pasture was contaminated with toxic fescue (ergovaline > 300ppb), and 25 animals were exposed to toxic pasture 2 weeks each month, thus it was designated as an alternating treatment (P3; n=25). The resulting allocation of animals to pastures was as follows: toxic (P1; n=50), non-toxic (P2; n=25) and alternating (Alt; n=25). An analysis of the impact of toxic fescue compared to novel fescue indicated significant differences in hair shedding score, body weight and other physiological measurements consistent with fescue toxicosis in the animals on toxic pastures (Chewning et al., 2018). All animals and pastures were located at the University of Arkansas Livestock and Forestry Research Station in Batesville, AR.

Sample Collection

In August 2016, pregnant (n=81; third trimester within 1-2 months of calving) and non-pregnant (n=19) cows were swabbed at the vaginal-fornix junction in the vagina using a double-guarded Morgan swab (Catalogue #:022964, MWI Veterinary Supply Boise, ID) to obtain

vaginal secretions for subsequent 16s rRNA-based microbiota analysis. Visible manure was removed from the exterior of the animal (vulva) prior to sample collection. The swab was sheathed before removal from the vagina, with the outer sheath being discarded before removal of the swab from the inner sheath for storage in a sterile 2.0 mL plastic tube. Samples were transported on ice and stored at -20 $^{\circ}$C until DNA isolation.

DNA Isolation, Quality Control, and Sequencing

DNA was extracted from the swabs using MoBio PowerLyzer® DNA Isolation kit (Catalog #: 12855-100, Carlsbad, CA). NanoDrop$^{\text{TM}}$ OneC (Thermo Fisher Scientific, Madison, WI) was used to check Abs260/280 ratio for the concentration of nucleic acids. PCR was utilized to verify DNA viability for 16s rRNA fragment amplification and sequencing. Forward primer 5'- AGA GTT TGA TCC TGG CTC AG – 3' and reverse primer 5' - GGTTAC CTT GTT ACG ACTT – 3' were the V1 primer sequences used for PCR validation. This region was amplified using the thermocycling protocol: (1) 95 °C for 10 min, (2) 95 °C for15s, (3) 60 °C for 30s, (4) repeat steps 2 and 3 40 times, (5) 60 °C 0.5 °C/cycle for 70 cycles, (6) end, using standard PCR reaction conditions as described for Promega GoTaq® G2 Green Master Mix (Catalog #: M782A; Promega Inc., Madison, WI). Amplification products were observed using 1% agarose gel stained with ethidium bromide and amplification was achieved for all random samples. No amplification was observed from the negative control. PicoGreen$^{\text{TM}}$ dsDNA Reagent (Thermo Fisher Scientific, Madison, WI) was used to check integrity and quality of DNA. The V4 region was sequenced with 250 base pair paired end reads using the Illumina Miseq platform (Illumina Inc., San Diego, CA).

Data Analysis

Sequences were screened for a maximum length of 275 bps. Sequences containing ambiguous bases were removed and sequence noise was reduced using a pre-cluster technique (Huse et al., 2010) where sequences were grouped by similarity and sorted by abundance. Chimeras were identified and removed using the UCHIME v4.2.40 algorithm (Edgar et al., 2011). Sequences were assigned to Operational Taxonomic Units (OTUs) at 97% similarity of the V4 region and classified to the genus level using the Bayesian method against the RDP database. All samples were standardized to the lowest read number found in a single sample by subsampling. Bray-Curtis distances were calculated to evaluate changes in vaginal microbiome community composition and structure between treatments. Data was processed using Mothur v.1.39.5 following the Miseq Mothur SOP (https://www.mothur.org/wiki/MiSeq_SOP) as described by the Schloss lab group (Kozich et al., 2013).

Results

Impact of toxic vs novel fescue on microbiome community structure and diversity

Constant exposure to the toxic pasture drastically changed the bacterial OTUs present in the vagina. A significant difference in vaginal bacterial community structure was observed **(see Table 1)** between toxic and novel treatments based on Bray-Curtis distance (ANOSIM, R = 0.429223, P< 0.001). Toxic communities also differed from those of the alternating treatment (ANOSIM, R= 0. 0.445697, P< 0.001). However, novel and alternating treatments did not differ (ANOSIM, R = -0.0077, P =.536). Charolais exhibited greater Shannon diversity than Herefords (p= 0.0427). Smith-Wilson evenness was greater (p=0.0008) for animals grazed on novel than animals grazing toxic fescue.

Bacterial composition identified in the vaginal microbiome

An OTU can be described as a group of closely related individuals. The top 20 most abundant bacterial OTUs present across all treatments were identified **(see Figures 1A & 1B)**. These figures show how bacterial OTU population representation changed as a function of pasture treatment. The top 5 most abundant OTUs include: *Burkholderia, Ureaplasma, Escherichia/Shigella, Pseudomonas, and Histophilus.*

Across all treatments, *Burkholderia* was present in the highest abundance. However, toxic pastures hosted significantly more *Burkholderia* than both novel and alternating pastures. The second most abundant bacterial OTU, *Ureaplasma,* was represented at a higher abundance in the novel and alternating treatments than the toxic treatment. *Escherichia/Shigella* was identified as the third most abundant OTU. *Pseudomonas*, identified as the fourth most abundant OTU, was found at a much higher abundance in novel and alternating pastures compared to toxic pastures where it was scarce. *Histophilus* was identified as the fifth most abundant bacterial OTU.

Microbes predictive of pasture type

An ANOSIM analysis with Mothur indicated OTU's from *Pseudomonas*, *Ureaplasma*, and *Burkhoderia* were among the best at differentiating pasture treatment. The suppression of *Pseudomonas* was the best indicator of toxin exposure with novel animals hosting 16.5 times more (p=0.0057) *Pseudomonas* than toxic animals. *Ureaplasma* was the second most abundant OTU and second most predictive of pasture type. Novel animals hosted 3.8 times more (p=0.0004) than toxic animals. *Burkholderia* was the most abundant OTU and the third most predictive of treatment. *Burkholderia* represented 41.74% of the bacterial community in the toxic treatment reproductive tracts and 14.67% in novel animals.

Discussion

Bray-Curtis Community structure differentiates animals by pasture type

Bray Curtis is a dissimilarity measure used to quantify population differences between separate entities. An ANOSIM R-value is a statistic quantifying this dissimilarly. Values close to 1 indicate a high degree of dissimilarity whereas a number close to 0 indicates high similarity. Based on Bray Curtis analysis, the microbial community structures among toxic and novel pastures were found to be largely dissimilar to one another (ANOSIM, R= 0.429223, P< 0.001). Interestingly, comparisons between the toxic and alternating treatments indicated an even higher degree of dissimilarity (ANOSIM, R= 0.445697, P< 0.001) while novel and alternating treatments showed virtually no difference (ANOSIM, R= -0.00777315, P=0.536).

These discrepancies are best illustrated by Figures 1A and 1B which show the top 20 vaginal bacterial OTUs most abundant by pasture type represented graphically. Perhaps the most obvious difference is the domination of *Burkholderia* (blue) in the toxic pasture **(Figure 1A)** compared to both the novel and alternating pastures, shown in **Figure 1B.** Another easily perceivable difference is the nearly complete absence of *Pseudomonas* (red) in toxic pastures. Meanwhile, both the novel and alternating treatments hosted a fair amount of *Pseudomonas.* Bray Curtis results can be further illustrated by **Figure 2.** Data points grouped near one another indicate similar community structures. The data points representing toxic treatments (yellow) are largely grouped together and separate from the data points representing toxic and alternating treatments (green). This indicates differences in community structure by pasture type.

Based on these results, it appears that the microbiome may be less susceptible to a shift in population structure when the duration of toxin exposure is limited and animals are alternated frequently and more likely to shift during sustained periods of high toxin exposure. These findings may indicate that animals rotationally grazed between novel and toxic fescue frequently

could avoid some of the impact of toxic fescue in regard to vaginal bacterial composition. If the composition of the vaginal microbiome were found to be an influencing factor in the reproductive success of animals grazing toxic fescue, this information could prove to be useful to producers.

Five vaginal bacterial OTUs appear to classify animals by pasture toxin level

Of the OTUs identified in this study, *Burkholderia, Ureaplasma, Escherichia/Shigella, Pseudomonas,* and *Histophilus* appear to be the best indicators of toxin exposure. Four of the top 5 fall within the same bacterial phylum, *Proteobacteria*. These include *Burkholderia, Escherichia/Shigella, Pseudomonas,* and *Histophilus.*

Although *Burkholderia* is not thought to be associated with reproduction, it has some interesting properties worth mentioning. One particular species, *Burkholderia cepacia*, has been identified as a useful tool in agricultural practices due to its ability to break down toxic compounds in herbicides and pesticides (Holmes et al., 1998). If select species of *Burkholderia* can indeed utilize toxic compounds such as ergovaline, its high abundance within toxic fescue pastures could potentially be explained. Additionally, select species of *Burkholderia* such as *Burkholderia pseudomallei* (the causative agent of Melioidosis disease) thrive in hot, humid climates and are therefore commonly found in tropical and subtropical areas, such as Southeast Asia and Australia (McCombie et al., 2006). Although Arkansas is hardly a tropical region, it does experience a substantial amount of heat and humidity in the summer months, which could have contributed to the high abundance of *Burkholderia* found in the vagina at the August sample collection date. Another potentially influencing factor could be the presence or absence of a pond on pastures. *Burkholderia* is a fairly common environmental bacterium often associated with water. Animals within the toxic and alternating treatments had access to a pond

whereas animals grazing novel pasture did not. It is possible that pond exposure could have influenced the abundance of *Burkholderia* observed, but this is unlikely. Only a small difference in *Burkholderia* abundance was shown between novel and alternating treatments, suggesting that pond exposure was not responsible for the high abundance of *Burkholderia* identified in animals grazing toxic pasture.

Despite *Escherichia* and *Shigella* being classified as separate genera, they are often genetically indistinguishable (Beld & Reubsaet, 2011), and thus were grouped together for this study. Select species of *Escherichia/Shigella* (the third most abundant OTU across all treatments) are commonly associated with postpartum uterine infections in cattle. Previous studies indicate that dairy cows experiencing uterine infections harbor significantly more enteric bacteria in the reproductive tract than healthy cows, particularly *Escherichia coli* (Wang et al., 2013). Chronic uterine infections can result in permanent damage to the endometrium and interfere with ovulation cycles (Sheldon et al., 2008), which could influence fertility.

Like *Escherichia* and *Shigella, Pseudomonas* has been linked to endometritis in cattle (Udhayavel et al., 2013). Furthermore, *Pseudomonas* is known to be one of the bacterial culprits responsible for mastitis in dairy cows, usually spread by contaminated water sources (Swartz & Petersson-Wolfe, 2016). The near absence of *Pseudomonas* among toxic pastures served as the best indicator of toxin exposure and a comparison of the abundance between treatments revealed striking results, with novel animals hosting 16.5 times more (p=0.0057) *Pseudomonas* than toxic animals. One explanation may be that *Pseudomonas* is sensitive to toxic compounds (such as ergovaline) at high levels and as a result fails to thrive in such an environment. Additionally, it is possible that the biological changes brought on by ergovaline consumption could have rendered

affected cows undesirable hosts. This could potentially explain the nearly complete suppression of *Pseudomonas* observed among toxic pastures.

The last member of the phylum *Proteobacteria* identified as a top 5 OTU was *Histophilus*. *Histophilus* is normally located in the nasal cavity of cattle and is one of the most common causes of bovine respiratory disease, an illness that costs the cattle industry millions of dollars in loss each year (Kilma et al., 2014). Both pathogenic and nonpathogenic varieties of *Histophilus* are known to colonize the genitals of male and female cattle. Although the respiratory system is the most common target of pathogenic *Histophilus,* infections can influence other systems as well, including the reproductive and nervous, causing genital infections and blood flow interference, which can lead to localized cellular death (Cooper & Broderson, 2010).

Ureaplasma, a member of the phylum *Firmicutes,* is believed to be closely associated with a multitude of reproductive diseases such as seminal vesiculitis, endometritis, and salpingitis, and is the proven cause of bovine granular vulvitis (Kuhn & Hopkins, 1983), a rapidly spread reproductive infection known to negatively impact fertility. Furthermore, it is thought that *Ureaplasma* may play a role in abortion (Kuhn & Hopkins, 1983). Interestingly, animals grazed on novel fescue hosted 3.8 times more (p=0.0004) *Ureaplasma* than toxic animals. There are several possible explanations for this seemingly contradictory finding. Perhaps the abundance of *Ureaplasma* in the vagina may not be as relevant to the development of reproductive diseases as its mere presence and interaction with other bacterial community members. Additionally, species variation within the genus could serve as a possible explanation, which highlights the sometimes-ambiguous nature of 16s rRNA microbiota analysis. Without species identification, it is difficult to determine if any of these genera could serve as potential biomarkers.

Vaginal Bacterial OTUs identified on pastures compared to those previously reported

Of the top 5 bacterial OTUs, 4 fall within the phylum *Proteobacteria*: *Burkholderia, Escherichia/Shigella, Pseudomonas,* and *Histophilus.* This finding is not surprising, as previous studies into the vaginal microbiome of cows (crossbred, Angus, and Nellore) report *Proteobacteria* being present in high abundance. A 2015 study of the vaginal microbial composition of pregnant and non-pregnant Nellore heifers and cows reported that 45-55% of samples collected were represented by just 10 OTUs: *Aeribacillus, Bacteroides, Clostridium, Ruminococcus, Rikenella, Alistipes, Bacillus, Eubacterium,* and *Prevotella* (Laguardia-Nascimento et al., 2015). Of these 10, only 4 were identified in the top 20 of this study: *Bacteroides, Clostridium, Ruminococcus* and *Bacillus.* The second phylum observed in the top 5 of this study, *Firmicutes,* was previously identified as the most abundant phylum observed in the vagina of postpartum Angus cows (Clemmons et al., 2017).

There are several factors that could account for the similarities and dissimilarities observed between this study and others, making it difficult to accurately compare results. Breed differences and environmental influence are two possibilities. It is likely that Nellore, Angus, and crossbred cattle each have microbial characteristics unique to their specific breed composition (Roehe et al., 2016). Additionally, a group of animals in one region may harbor a different microbial profile than others in separate regions simply as a result of the environment, which is largely due to diet and pasture type. The status of the animal at the time of sampling may also have an impact on what microbes are present, as the microbiome is likely to differ slightly in open, pregnant, and postpartum cows. The manner in which bacterial samples are collected is a potentially influencing factor as well. Some researchers choose to swab the vagina for bacteria whereas others, such as the study regarding the vaginal microbiome of Nellore cattle, use vaginal washes for collection. Furthermore, the hypervariable region (V1-V9) used for analysis can

influence identification, making it difficult to accurately compare any two microbiome studies using 16s rRNA sequencing technology. Although extremely useful for differentiation at the genus level, 16s rRNA analysis can lack the precision needed for specific classification. Species identification would allow for a much better understanding of the potential relationship between the vaginal microbiome and toxic fescue and eliminate some of ambiguity in the results of this study.

Potential reasons for the vast change in the vaginal microbiome of cattle on toxic vs. novel and alternating pastures

Several factors could have potentially contributed to the microbial composition differences observed between animals grazing novel and toxic pastures, including toxin level, environmental influence, and bacterial host preference. Toxin level is a likely possibility, given that nearly all factors other than toxin level were held constant throughout this experiment. Bacterial host preference is another very likely conclusion given the tendency of microbes colonize areas that satisfy their specific environmental preferences, such as temperature. Interestingly, the rectal temperatures of animals grazing toxic fescue were found to be approximately 0.42 ºC higher than those grazing novel (Chewning et al., 2018). This temperature change would be expected to drastically impact conception rate and fertility and would also be expected to influence microbial content. It would be difficult to differentiate the effects of toxic fescue from heat stress since they are confounding factors, but no matter which factor is impacting the microbiome, its likely important in regard to reproductive performance.

Conclusions

The findings from this study implicate that the composition of the microbiome in the vagina differs due to toxin exposure. *Pseudomonas, Ureaplasma,* and *Burkholderia* were identified as the OTUs most predative of toxin exposure and thus the best potential bacterial biomarkers for fescue toxicosis. Four genera identified as top 5 OTUs, including *Pseudomonas, Ureaplasma, Escherichia/Shigella,* and *Histophilus* have been linked to reproductive issues in cattle. This could imply that reproductive success may be altered due to differences in microbial content. This finding could also indicate that it may be possible to improve reproductive success by artificially manipulating the microbes present in any given cow's reproductive tract. Much like a digestive tract can be aided by the introduction of probiotics, perhaps a cow's conception rate can be influenced similarly by introducing microbes found to be positively correlated with reproductive success. If microbes favorable to reproductive health can indeed be identified, it may also be possible to genetically select cattle that provide a conducive environment to a favorable reproductive microbiome. These tools could be used in combination with various fescue mitigation strategies to decrease the overall impact of toxic fescue on reproduction in cattle. However, with the diverse nature of the microbiome and the ambiguity of 16s rRNA microbiota analysis, more precise testing would be necessary to validate results and collect more information at the species or sub-species level of the vaginal microbiome before the possibility of developing probiotic could be explored.

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Figures

Figure 1A: Bacterial OTU population representation as a function of toxic pasture treatment. Different colors represent different bacterial OTUs and the size of the bar in proportion to the full length of the bar depicts the representation of that OTU in comparison to all OTUs identified.
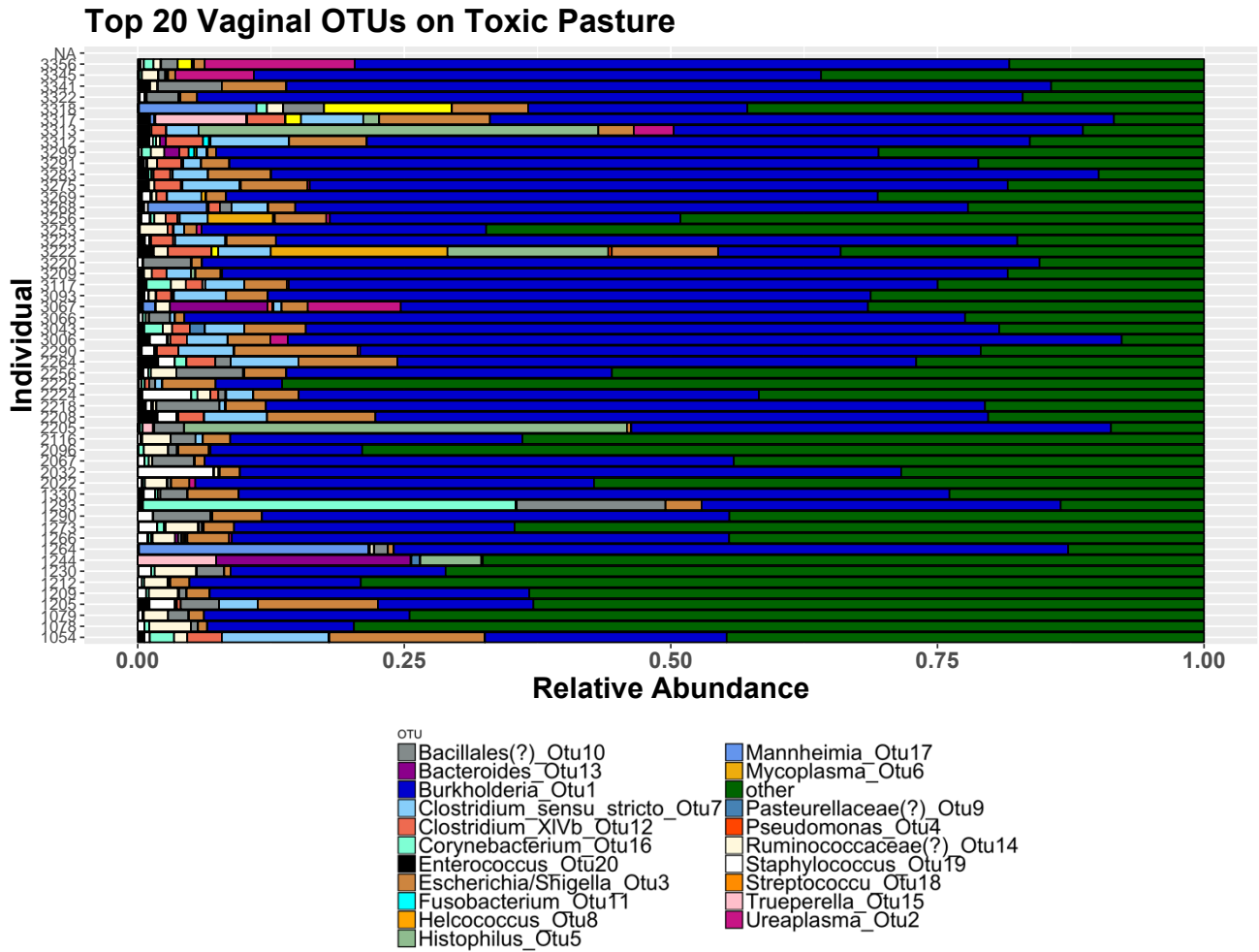

Top 20 Vaginal OTUs on Toxic Pasture

Figure 1B: Bacterial OTU population representation as a function of novel and alternating pasture treatment. Different colors represent different bacterial OTUs and the size of the bar in proportion to the full length of the bar depicts the representation of that OTU in comparison to all OTUs identified.
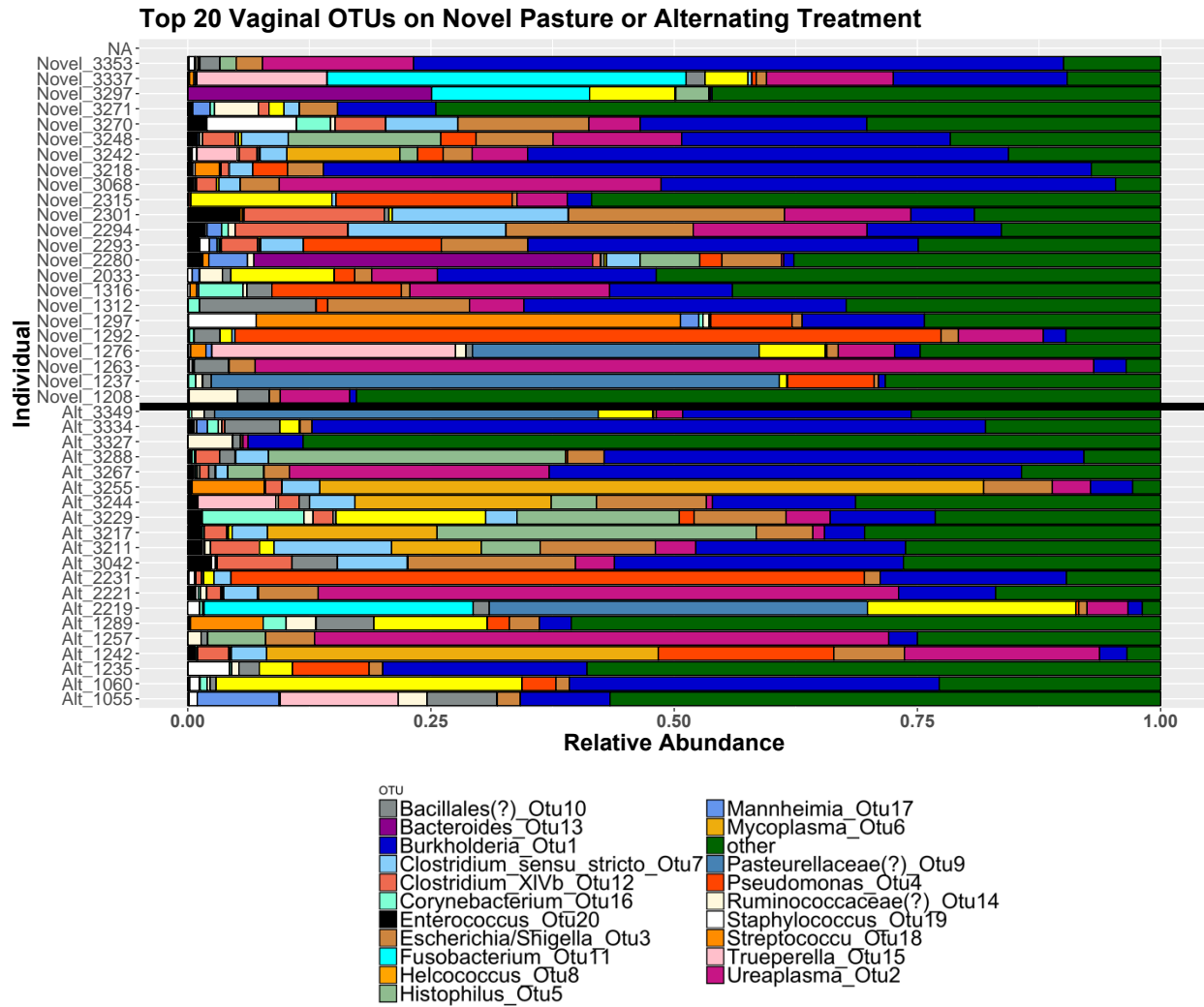

Top 20 Vaginal OTUs on Novel Pasture or Alternating Treatment

Figure 2: Significant difference in bovine vaginal bacterial community structure was observed across pasture type based on Bray Curtis distance (R= 0.429223, P < 0.001). Each point on the plot represents the first and second principal component (PC) which condenses the Brey Curtis structure values across all OTUs into a single value. Plotted points of PC1 vs. PC2 for toxic animals appear to cluster separately from novel and alternating samples, as indicated by the red oval surrounding the majority of the toxic data points.
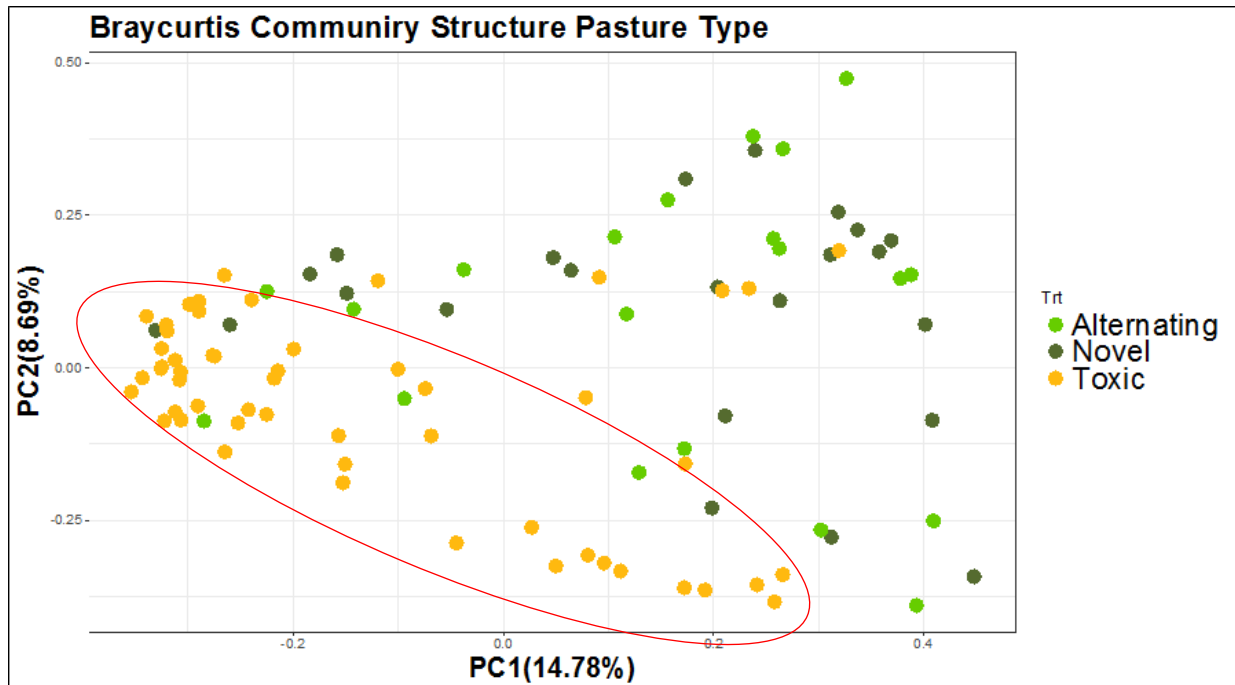
Tables

Table 1: Bacterial Community Structure Comparison across pasture treatments

COMPARISON	ANOSIM, R-VALUE	P-VALUE
N-T-ALT	0.387425	<0.001*
N-T	0.429223	<0.001*
N-ALT	0.00777315	0.536
T-ALT	0.445697	<0.001*

N-Novel, T-Toxic, ALT-Alternating;
* = significant difference (P< 0.001) observed between pasture types
R-value is a measure of dissimilarity between treatments