AN ABSTRACT OF THE THESIS OF

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The digestion responses and degradation of ergovaline and production of lysergic acid in the rumen of sheep offered *Neotyphodium coenophialum*-infected tall fescue straw at two ergovaline levels was investigated. Six crossbred wethers (56 +/- 3 kg BW) were randomly assigned to one of two treatment groups in a cross-over design. Each experimental period consisted of 28 d feeding periods with a 14 d wash-out between periods. During the wash-out period all animals received a diet containing <10 ppb ergovaline. Treatments were 1) < 10 ppb ergovaline (E-) and 2) 500 ppb ergovaline (E+). Ergovaline levels were achieved with a combination of tall fescue straw (350ppb ergovaline) plus *Neotyphodium coenophialum*-infected tall fescue seed (~3,300 ppb ergovaline). Diets were isonitrogenous. Rumen fluid was sampled three times (d 0, 3, 28) during the 28-day experimental period for ergovaline and lysergic acid. Samples were collected at time 0 (prior to feeding), 6, and 12 h post feeding. Total fecal and urine collection commenced on d 21 and continued till d 25 of the experimental period. Rumen ammonia, rumen pH, and rectal temperature were not influenced by ergovaline concentration (P > 0.10). Digestion of DM, ADF and CP were not

different between treatments ($P > 0.10$). Feed intake and body weight were different between treatments ($P > 0.10$). Water intake was reduced by ergovaline intake ($P < 0.05$). Serum prolactin was reduced by 27% with ergovaline intake ($P < 0.05$). Ergovaline concentration in rumen fluid expressed as a percent of intake increased over sampling time and sampling day ($P < 0.05$). Lysergic acid concentration in rumen fluid expressed as a percent of intake increased over time from d0 to d3 ($P < 0.05$) but was not different between d3 and d28 at any time point ($P > 0.10$). The feces contained an average of 0.41 μmol/day ergovaline and 0.87 μmol/day lysergic acid. Urine contained no detectable ergovaline; lysergic acid concentration was 1.05 μmol/day. The appearance of lysergic acid in the feces, urine and rumen fluid is likely from the degradation of ergovaline in the rumen due to microbial degradation and further break down in the lower digestive tract. In order to determine if the increased tolerance sheep display to fescue toxicosis is due to ruminal bacteria five major sheep ruminal bacteria were monitored during the adaptation to *Neotyphodium coenophialum* -infected tall fescue straw. This increased tolerance supports the belief that there are microorganisms in the rumen of sheep capable of detoxifying the alkaloids found in infected tall fescue. Rectal temperatures and serum prolactin levels were monitored as an indication of toxicosis. Daily rectal temperatures were not influenced by alkaloid concentration (38.4°C E- vs. 38.4 °C E+, SE 0.0615; $p = 0.40$). Serum prolactin was decreased to 6.4 ng/ml for E+ from 22.9 ng/ml for E- treatment (SE 5.19; $p = 0.023$), indicating subclinical but not clinical fescue

toxicosis. A period effect was detected for most primers used, indicating change in microbial populations due to adaptation to the fescue straw. *Prevotella bryantii* B_14 was detected in low levels through the entire feeding period and levels were approximately the same (95% E- and 89% E+ of day 0 on day 28) from day 0 to 28 but no difference was detected between treatment groups (90% E- vs. 93% E+; $p = 0.45$). The Streptococcus group (*S. bovis, S. caprinus, S. equines)* was detected in low levels through the entire feeding period and E+ treatment tended to lower the concentration of 16S gene but no statistical difference was detected between treatment groups (89% E- vs. 94% E+; $p = 0.39$). *Selenomas ruminantium- Mitsuokella multiacida* JCM6582 was the most abundant organism found in the samples and levels were approximately the same (97% E- and 105% E+ of day 0 on day 28) from day 0 to 28 but no difference was detected between treatment groups (92% E- vs. 106% E+; $p = 0.52$). *Eubacterium ruminatium* (ATCC 17233) was undetectable in most samples over all periods. *Ruminococcus flavefaciens* (ATCC 19208T) sequence was detected in moderate levels through the entire feeding period and levels were approximately the same (97% E- and 99% E+ of day 0 on day 28) from day 0 to 28 but no difference was detected between treatment groups (91% E- vs. 92% E+; $p = 0.28$). *Ruminococcus albus* was detected in low levels through the entire feeding period and levels were approximately the same (95% E- and 83% E+ of day 0 on day 28) from day 0 to 28 but no difference was detected between treatment groups (87% E- vs. 89% E+;

$p = 0.33$). These results imply that none of the five bacteria monitored in this study is responsible for the metabolism of ergot alkaloids.

Metabolism of Ergot Alkaloids by Sheep

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Dr. Shanna Ivey assisted with the design and sample collection for Chapter 2 and 3. Dr. Jennifer Duringer assisted in editing and sample collection. Dr. Linda Blythe assisted with sample collection and was involved with the interpretation of the data. Dr. A. Morrie Craig was involved with the writing and interpretation of data. Zelda Zimmer assisted with sample collection.

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In loving Memory

Of

John Randolph Saput

9/10/1950-11/12/2002

For the one who gave all he could
in order for me to realize my
dreams.

1 Introduction

1.1 Endophyte-infected tall fescue

1.1.1 History and benefits of E+

Tall fescue has a long history in the United States. Believed to have come to the United States as a contaminate in other grains from Western Europe (Stuedemann and Hoveland, 1988), it is now widely used in lawns and golf courses. Tall fescue was originally believed to be good forage for livestock, moderate in neutral detergent fiber (NDF) and having an adequate crude protein (CP) for a grass. Despite this appearance of being good forage, animals did not gain well when grazing fescue pasture and tall fescue acquired a reputation of causing poor animal performance.

Fescue toxicosis, in the form of fescue foot, was documented more than fifty years ago without a known cause of the disease (Cunningham, 1949). An endophtye fungus, originally classified as *Acremonium coenophialum* and later renamed as *Neotyphodium coenophialum,* was discovered in 1941 to have infected some varieties of tall fescue. It was not until ergovaline was isolated in the 1970s (Porter, 1995; Stuedemann and Hoveland, 1988) that the endophyte and its toxins were determined to cause fescue toxicosis. Later research confirmed that this fungus was responsible for fescue toxicosis (Stuedemann and Hoveland, 1988).

Neotyphodium coenephialum lives in the intercellular space within tall fescue in a symbiotic fashion. The grass is the fungus' only means of

reproduction and supplies the fungus with all of the needed nutrients. The endophyte confers several benefits to the plant including drought resistance, increased viability, and protection from insects and herbivores predation (Bacon, 1995). Taken together, these benefits improve the grasses' ability to compete for resources, survive changes in the environment, and produce the next generation.

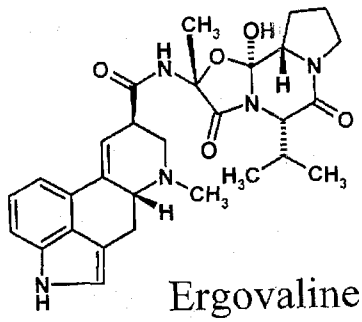
The increase in drought resistance that the endophyte confers to the plant allows the plant to survive harsh conditions. This increased tolerance is in the form of both drought avoidance and drought tolerance behavior. Endophyte infected tall fescue has been shown to develop increased drought avoidance behavior, by increased leaf rolling, leaf senescence and stomatal closure (Arechavaleta et al. 1989; Belesky et al. 1989; Lachno and Baker, 1986). Increased drought tolerance behavior via changes in osmotic potential of the leaf blades, tillers, and basal meristem (Elmi et al. 1989; West et al. 1990) has been observed when comparing infected tall fescue to non-infected varieties. Pinkerton et al. (1990) observed a higher germination rate of endophyte-infected seeds than uninfected seed. Increased viability of infected seedlings were observed by Clay (1987) in the form of higher number of tillers from infected seedlings at 10 weeks after germination and by a decrease in surviving tillers of endophyte-free seedlings at 14 weeks of age. By having a higher germination rate and

increased viability, the infected grass is able to establish itself at a faster rate, therefore securing space and out competing other plants and grasses.

There is some evidence that the endophyte increases the plants ability to resist insect predation after damage. Bultman et al (2004) found endophyte infected tall fescue plants to be more resistant to aphid predation after plants were subjected to artificial grazing damage. The endophyte produces several alkaloids (Table 1-1), which confer protection from herbivores as well as insects. Therefore, from the plant's perspective endophyte is beneficial. From the herbivore's perspective endophyte causes problems with animal health and productivity.

1.1.2 Problems associated with E+

Several ergot alkaloids are produced by the endophytic fungus (Table 1-1). The largest quantity of ergopeptide alkaloid produced, up to 80%, is ergovaline (Lyons et al., 1986). For many years, ergovaline has been believed to be the cause of fescue toxicosis (Porter, 1995) and has been correlated with fescue toxicosis (Tor-Agbidye 2001). There has been some suggestion that the causative agent in



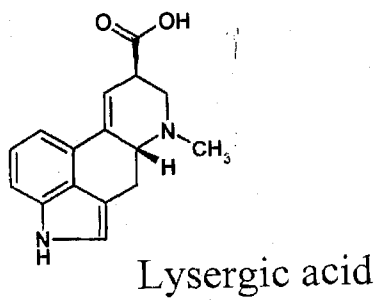


Figure 1-1 Structure of Ergovaline and lysergic acid

fescue toxicosis is the core ring structure (Figure 1) of the ergot alkaloids, lysergic acid (Stuedemann et al., 1998; Hill et al., 2001). Hill et al. (2001) found the transport of the simpler lysergic acid (41.8 mmole) across ruminal tissues with a higher potential mole transport than the bulkier ergopeptine alkaloids (5.8 mmole for ergotamine). The lower molecular weight of lysergic acid and the polar acid group allow lysergic acid to be more soluble then the other ergopeptides that are known to be only slightly soluble (Eckert et al., 1978). Caution should be used when drawing conclusions based on the work by Hill and colleagues because not all ergot alkaloids or even the most abundant alkaloid, ergovaline, were tested for transport across the ruminal tissue.

Also alkaloid concentration was determined by an enzyme-linked immunosorbent assay (ELISA), which as discussed below is sensitive to ergoline alkaloids and not ergopeptide alkaloids.

High-pressure liquid chromatography (HPLC) has been used for many years to quantify various ergopeptides (Rottinghaus et al., 1991; Craig et al., 1994) with highly reproducible results. Until recently there was no analytical method for quantifying lysergic acid. An ELISA has been developed that uses a specific antibody for the lysergic ring moiety (Hill and Agee, 1994). Schnitzius et al. (2001) found this assay to be only semi-quantitative due to high day-to-day variation in the assay; this high amount of variation was also noted by Hill and Agee (1994). While this assay has

the potential for assaying all alkaloids with the lysergic acid ring, Schnitzius et al. (2001) found the ergopeptides have poor affinity to the antibodies due to the large side groups that block the binding of the antibody to the lysergic ring structure. A HPLC assay for quantification of lysergic acid has been developed in our lab and was used for lysergic acid quantification in the present study.

The alkaloids found in endophyte infected tall fescue have two major physical effects on animals that consume it. The first effect is on vasoconstriction, decreasing blood flow. In the northern states during the winter the decreased blood flow can cause ears, tails, and hoofs to become necrotic and slough off. In the summer animals exhibit heat stress, spend less time grassing and more time standing in water. The second effect is on the dopamine (D2) receptor in the brain that causes a drop in serum prolactin levels leading to reproductive and lactation difficulties especially in horses. Horses rely solely on prolactin to initiate lactation unlike cattle and sheep, which use both prolactin and placental lactogen to stimulate prepartum lactogenesis. This explains why complete agalactia is seen in horses and reduced milk production is seen in cattle on endophyte infected feed.

The alkaloids found in tall fescue that have vasoconstriction properties (Dyer, 1993; Strickland et al., 1993, Oliver 1997) are mainly ergopeptides. Dyer (1993) found ergovaline to be 75 times more potent as a

Table 1-1 Alkaloids commonly found in *Neotyphodium coenephialum* infected tall fescue

Ergopeptides	Ergolines	Pyrrolizidine alkaloids	Clavines	Pyrrolopyrazine Alkaloids
Ergovaline	Lysergic acid	Loline	Argoclavine	Peramine
Ergovalinine	Lysergic acid amide	N-acetyl loline	Chanoclavine	
Ergotamine	Lysergol	N-formyl loline	6,7-	
Ergotaminine	Ergonovine	N-formyl norloline	Penniclavine	
α-ergocryptine β-ergocryptine		Perlodine	Elymoclavine	
Ergobine				
Ergobutine				
Ergobutyrine				
Ergocomine				
Ergocristine Ergonine				
Ergoptine				
β-ergoptine				
Ergosine				
β-ergosine				
Ergostine				

vasoconstrictor of the bovine uterine and umbilical arteries than 5-hydroxytryptamine creatinine sulfate (5-HT). The vasoconstriction of the arteries by ergovaline was not reversed even after 3 hours whereas the vasoconstriction caused by 5-HT returned to baseline within 1.5 hours. From this data, he postulated that the reduction in reproductive performance seen in cattle consuming endophyte-infected feed is due to the constriction of blood vessels to the reproductive tract caused by the alkaloids. Animals consuming endophyte-infected feed have less blood flow to the extremities and may show clinical signs of 'fescue foot' where the feet and/or tail becomes gangrenous due to ischemia. Cases of fescue foot have been documented as early as 1949 (Cunningham, 1949). Clinical signs of fescue foot are closely linked to ambient temperature. At colder temperatures, animals consuming an endophyte-infected diet are more likely to show clinical signs of fescue foot (Bacon, 1995). During warmer weather, a different syndrome, summer slump is observed. Summer slump is characterized by poor animal performance, rough hair coat, reduced reproductive performance, intolerance to heat, increased respiration rate, excessive salivation, increased rectal temperature and decreased serum prolactin levels (Paterson et al., 1995; Stamm et al., 1994; Strickland et al., 1993; Stuedemann and Hoverland, 1988). Summer slump is the most common form of fescue toxicosis in the South Eastern United States and

causes the bulk of the economic loss associated with fescue toxicosis, mainly through reproduction dysfunction.

1.1.3 Physiological effects of E+

The effects of endophyte infected tall fescue on the physiological parameters are not clear in the current literature. This is due in part to the lack of knowledge of which compound(s) produced by the fungus are responsible for fescue toxicosis and what levels are needed for the appearance of the disease. Early work done on the effects of infected tall fescue did not correlate them to alkaloid concentrations; instead, treatments were divided into infected (E+) or non-infected (E-).

Hemken et al. (1981) used Holstein calves to investigate the relationship between temperature and disease. In that study no assays for alkaloid concentrations were made. Instead, they used two strains of grass which had been observed to be 'more toxic' (E+) or 'less toxic' (E-) in a grazing situation. They found dry matter intake decrease of 33.2 g/kgBW$^{0.75}$ with E+ feed compared to a control intake of 70.0 g/kgBW$^{0.75}$ at high temperature (34-35 °C). There was no difference in dry matter intake at low (10-13 °C) or moderate (21-23 °C) temperatures. Rectal temperature and respiration rates were also found to be affected by E+ feed only at the higher temperatures. No difference in water intake was detected at any of the temperatures studied.

Neal and Schmidt (1985) used rats as a model to investigate the effect of endophyte on digestion. Their treatments were classified as infected and noninfected using tall fescue seed. The only alkaloid quantitated in the feed was loline (5134 µg/g and 652 µg/g respectively). They found dry matter intake, average daily gain and water intake to be reduced by treatment with infected feed. Surprisingly, they also found rectal temperature to be reduced by the infected seed. Digestibilities for crude fiber, gross energy and dry matter were greater for the E+ diet and crude protein digestibility was not affected by treatment.

Barth et al. (1989) used sheep to examine the effect of endophyte infection status on intake and nutrient digestibility of tall fescue. They classified their treatments as infected (E+) and non-infected (E-) and performed no analysis on the alkaloid levels in the treatments. They found that dry matter intake (1.26 kg/d E- versus 1.25 kg/d E+) and water intake (4.52L E- versus 4.26L E+) was not affected by treatment. However, they found that body weight change (0.207 kg/d E- versus -0.017 kg/d E+) and feed efficiency (weight change/DM intake; 0.163 vs. -0.013) were affected by infection status.

Hannah et al. (1990) used sheep to investigate the effect of endophyte alkaloids on ruminal digestion and digesta kinetics at different environmental temperatures. In a series of experiments, they had four treatment groups with ergovaline at 0, 0.75 ppm, 1.5 ppm and 3.0 ppm

ergovaline measured by HPLC and one treatment group with 20 ppm ergotamine. Water consumption increased in all treatment groups when the animals were placed under heat stress (34 °C with 50% relative humidity) but was not affected by treatment. Rectal temperatures increased when animals were fed treatments with 1.5 ppm ergovaline or 20 ppm ergotamine compared to control feed. Total volatile fatty acid (VFA) concentration in the rumen showed a quadratic reaction to alkaloid concentration with the highest concentration of VFAs occurring at the 3.0 ppm level and the lowest concentration occurring at the 1.5 ppm level. They reported that digestibility was depressed when ergovaline level was above 1.5 ppm or when the animals were under heat stress.

Fiorito et al. (1991) explored the effect of endophyte infected (E+) vs. non-infected (E-) tall fescue on sheep when nutritional intake was kept at a constant level. Treatments were classified as <1% infected and >95% infected, with lolines (Table 1-1) being the only alkaloid assayed (not detected and 909.6 ug/g respectively). During the treatment period, dry matter intake and water intake were kept constant by placing unconsumed feed and water directly into the rumen. Voluntary water intake was lower at the end of the treatment period for E+ treatment. Rumen ammonia, respiration rate, and rectal temperature were not affected by treatment. Heart rate, prolactin levels (24.1 ng/ml for E+ versus 4.7 ng/ml for E-), and total tract digestion of dry matter and fiber were affected by treatment.

Aldrich et al. (1992) used sheep to investigate whether a dopamine antagonist (Metoclopramide (M)) would counteract fescue toxicosis. Diets consisted of <50 ppb and 1,170 ppb (trial 1), <50ppb and 2430 ppb (trial 2) ergovaline measured by HPLC. In both experiments animals were under heat stress (32 °C) at 60% humidity. Water consumption was not effected by E+ feed or by M intake in either experiment. During their first experiment dry matter intake (DMI) and digestibility was reduced in E+ diets however when the sheep's intake was adjusted in experiment 2 to 1.5% of BW these differences were not detected. Core body temperature was increased (38.37°C E- vs. 39.30°C E+) in experiment 1 while rectal (40.05°C E- vs. 40.40°C E+) and skin temperature (38.25°C E- vs. 38.49°C E+) were not different between treatments. In experiment 2 both rectal (39.4°C E- vs. 39.8°C E+) and skin temperatures (37.7°C E- vs. 38.5°C E+) were elevated for animals on an E+ diet. Respiration rates were not different between treatments (143.0 breaths/min E- vs. 149.0 breaths/min E+). Plasma prolactin levels were depressed by E+ feed (136 ng/ml E- vs. 11.4 ng/ml E+). The authors found the dopamine antagonist to eliviate the depression in DMI of animals consuming E+ feed however it did not reduce the increased rectal temperatures or elevate the depressed prolactin levels. Indicating fescue toxicosis is produced by more than one mechanism within the body.

Westendorf et al. (1992) used a rat model to investigate the effect of incubating endophyte infected fescue seed with rumen fluid. In this study alkaloid concentration was determined by HPLC and was 3.66 ppm for E+ non-incubated (E+NON) and 3.78 ppm for E+ incubated with rumen fluid (E+INC). When comparing E- control diet to the E+NON diet, they found the E- diet resulted in better feed intake (22.7 g/d vs. 14.3 g/d), average daily gain (6.04 g versus 2.26 g), and feed conversion (3.8 g of feed/g of gain versus 6.7 g of feed/g of gain). E+INC had better average daily gains (14.6 E+INC g vs. 14.0 g E+NON) and feed conversion (5.9 g of feed/g of gain E+INC vs. 7.5 g of feed/g of gain E+NON) than E+NON. This improvement was not observed in the E- diets that had been incubated with rumen fluid, implying that incubation with rumen fluid altered the toxins found in tall fescue.

Westendorf et al. (1993) used sheep to investigate the effect of endophyte on ruminal digestion. In this study both lolines (N-formyl and N-acetyl loline measured by GLC) and ergovaline (ergovaline and ergovalinine measured by HPLC) was measured. The treatment level of ergovaline was 0, 1.4 ppm, and 1.6 ppm. Intakes were set at a constant amount during the trial (1200 g/d). They found DM digestibility (73.1% E- vs. 69.5% E+) to be reduced by E+ feed. ADF and CP digestibility was also reduced by E+ (ADF: 60.4% E- vs. 52.9% E+; CP: 75.8% E- vs. 73.0% E+). Water intake (3.2 L/d E- vs. 2.8 L/d E+) and respiration rate

(43.7 breaths/min E- vs. 47.3 breaths/min E+) was not affected by alkaloid intake. Rectal temperature was elevated slightly only in the highest treatment (1.6 ppm ergovaline). To determine the site and extent of alkaloid digestion abomasal and fecal samples were tested for ergovaline. Abomasal digesta contained 47.1 - 61.8% of dietary alkaloid ($P < 0.001$) and fecal samples contained 5.9 - 6.9% of dietary ergovaline ($P < 0.001$). This implies that metabolism and absorption occurs both in the foregut and hindgut.

Stamm et al. (1994) investigate the effect of varying concentrations of ergovaline, in the form of E+ straw, on the physiology and performance of beef steers. They used four treatment levels (0, 158, 317, and 475 ppb ergovaline) in a Latin square design. Dry matter intake, rumen ammonia, respiration rate, rectal temperature, weight gain, and gain:feed ratio were not effected by treatment. Serum prolactin levels tended to decrease with increasing levels of ergovaline. The only treatment effect observed was on rumen pH and VFA concentrations, the authors explained this difference as a difference in diet quality and not in response to alkaloid concentration.

Burke et al. (2001) investigated the reproductive response of beef cattle fed an E+ diet. Treatments were infected (E+) and non-infected (E-) with no analysis for any alkaloid. Signs of fescue toxicosis were observed by increased respiration rate (46.6 breaths/min E- vs. 52.0 breaths/min E+, $P < 0.02$), increased rectal temperature (38.8°C E- vs. 39.6°C E+, $P < 0.001$),

and decreased prolactin levels (57.4 ng/ml E- vs. 7.2 ng/ml E+, $P < 0.001$). Reproductive responses were not different between treatments. Estrus detection rate (84.9% E- vs. 80.2% E+), pregnancy rate (89.7% E- vs. 84.8% E+) and calving rate (85.1% E- vs. 85.0% E+) were not influenced by E+ diet. All cows on E+ pastures lost body conditioning.

To evaluate the effect on endophyte infected tall fescue on ovarian function, Burke and Rorie (2002) used mature beef cows that were synchronized for estrus. Treatments were infected (E+) and non-infected (E-) pasture with no alkaloid concentration measured. Signs of fescue toxicosis were observed by a decrease in serum prolactin levels (84.9 pg/ml E- vs. 32.3 pg/ml E+, $P < 0.009$). No difference in mature follicle (> 10mm) numbers or size was detected between treatments. Pregnancy rates were similar between treatments (83.3% E- vs. 88.1% E+) as well as number of days open (84.2 days E- vs. 83.8 days E+), and calving interval (369 days E- vs. 371.6 days E+). Cows on E+ feed had a decrease in body condition.

Burke et al. (2002) investigated the effect of endophyte infected feed on the reproductive performance of sheep. Several experiments were performed to evaluate the seasonal and long term response of sheep to E+ feed. Treatments were endophyte infected tall fescue (E+) and Bermuda grass (E-) and no alkaloid concentration was determined. Serum prolactin levels were decreased for ewes on E+ pasture at all time points. No

difference in pregnancy rate (94.8% E- vs. 92.4% E+), lambing rate (186.4% E- vs. 181.2% E+), birth weight (6.3 kg E- vs. 6.4 kg E+) or lamb weaning weight (34.7 kg E- vs. 33.4 kg E+) of mature ewes was detected. Yearling ewes had a decreased pregnancy rate on fescue pasture (46.0% E- vs. 13.1% E+; $P < 0.05$). Body weight and body condition of ewes on E+ pasture fluctuated between seasons but overall was maintained when compared to sheep on Bermuda pasture. This study shows that sheep are able to be maintained on E+ pasture without loss of body condition where cattle show a marked decrease in body condition when maintained on E+ pasture.

In a attempt to determine the toxin responsible for fescue toxicosis, Gadberry et al. (2003) performed a series of experiments feeding E- seed, E+ seed and pure ergovaline (EV) to sheep under heat stress (33 °C at 50% humidity). Alkaloid concentration was determined by HPLC and ELISA, with treatment level set at 645 ppb ergovaline. They found a decrease in feed intake and skin temperature for E+ but no difference between E- and EV. The calculated thermocirculation index was lower for E+ and EV treatments than E- with no difference observed between E+ and EV. Serum prolactin levels decreased 34% for EV treatment and 94% for E+ treatment when compared to E-. The authors theorize that while ergovaline is a toxin involved with fescue toxicosis other alkaloids are also involved. They also acknowledged the possibility that pure ergovaline

could be degraded faster than ergovaline found in seed, which could explain the differences observed between E+ and EV treatments.

1.1.4 Absorption, excretion, and tracking of EV within the animal

Data is limited on the exact amount and location of absorption of either ergopeptides or lysergic acid. Stuedemann et al. (1998) hypothesized that ergot alkaloids are absorbed from the foregut rather than the hindgut because alkaloids were detected in the urine within 12 hours of ingesting endophyte-infected pasture. In an attempt to address this issue Hill et al. (2001) conducted three experiments evaluating 1) the transport of alkaloids across rumen tissue vs. omasum tissue, 2) transport of alkaloids across rumen tissue vs. reticulum tissue, and 3) transport of alkaloid across living tissue vs. dead tissue (killed with azide). In the first two experiments, individual alkaloids were assayed in order to determine the efficiency of transport of the individual alkaloids. They found lysergic acid crossed all tissues tested more efficiently than lysergol, the lysergic acid amide (ergonovine), or the ergopeptides (ergocryptine and ergotamine) tested when equal molar concentrations of the alkaloid were added to the mucosal side of the tissues in parabiotic chambers. However, their data was not consistent over the experiments that were conducted, for example in their first experiment they found that lysergol was transported at a higher rate than ergonovine but in the second experiment ergonvine was found to have a higher affinity for rumen tissues than lysergol. These

inconsistencies coupled with the lack of data on absorption from the hindgut make it difficult to draw conclusions on the exact amount and site of absorption of ergot alkaloids. Hill and colleagues' work also implies that the transport of alkaloids across the tissues is an active process because tissues treated with azide showed no additional alkaloid transport after 180 minute.

Ergovaline has been shown to be below detection limit in the blood stream 60 minutes after an IV dose was given to sheep (Jaussaud et al., 1998). Durix et al. (1999) found similar clearance rates when a dose of 32ug/kg BW of pure ergovaline toxin (as a tartaric salt) was administered intravenously to lactating goats. No ergovaline was detected in the milk of these goats after 8 hours, implying that milk is not the primary excretion route for ergovaline.

Early work done with radiolabeled alkaloids showed the excretion routes of ergopeptides (ergotamine) to be via the bile and lysergic acid to be excreted via the urinary tract in monkeys, rats and dogs (Eckert et al., 1978). Recent work by Stuedmann et al. (1998) has shown steers consuming endophyte-infected pasture to have the majority of alkaloids excreted in the urine (94%), presumably lysergic acid or ergoline alkaloids, and at a lower level in the bile (4%), presumably an ergopeptide or a mixture of ergopeptides. The method for determining alkaloid

concentrations in this study was with an ELISA, making the determination of the specific alkaloids excreted impossible.

1.2 Rumen detoxification and adaptation

There are three main ways complex microbial systems adapt to changes in its environment: 1) induction of enzymes to process new substrates, 2) growth of a sub-population able to utilize new substrate and 3) genetic selection via mutations in the genome. This last factor is unlikely to play a role in most situations because selection based on mutations is too slow of a process to adapt to the rapidly changing environment bacteria face.

Rumen microbial adaptation to changes in diets is a well-known phenomenon best demonstrated when animals are shifted from a forage diet to a concentrate diet. This change in substrates for rumen fermentation can lead to disease and death if sufficient time for adaptation is not allowed. In this case adaptation is due to growth of sub-populations of bacteria in the rumen. There is considerable evidence of ruminal modification of plant toxins and other compounds. Plant toxins, such as pyrrolizidine alkaloids, oxalate, mimosine and fungal aflatoxins have been shown to be degraded and detoxified in the rumen while other compounds, such as nitrates (Church, 1988), tryptophane (Carlson and Breeze, 1984) and thiamin (Brent and Bartley, 1984), have been shown to be altered into toxic compounds in the rumen.

1.2.1 Tansy ragwort

Tansy ragwort (*Senecio jacobaea*) has historically been the number one toxic plant in Oregon. The principle toxin in Tansy ragwort is pyrrolizidine alkaloids. Pyrrolizidine alkaloids cause liver damage and death in animals who consume a toxic dose. In cattle and horses, a toxic dose is 5% of their body weight of tansy ragwort (dry matter basis) while in sheep the toxic dose is more than 300% of their body weight. At first, the resistance of sheep was believed to be from detoxification of the alkaloids by the liver however; it has been shown that this resistance is due to rumen microbe degradation of the pyrrolizidine alkaloids (Craig et al., 1986, 1992; Wachenheim et al., 1992). Duringer and Craig (2003) tested the in vitro metabolism of pyrrolizidine alkaloids by liver microsomes. While they found differences in the rate of detoxification of pyrrolizidine alkaloids between cattle and sheep this difference was not sufficient to explain the levels of susceptibility displayed by each species. Recent work has the molecular characterization of a consortium of bacteria isolated from sheep fed tansy ragwort capable of degrading pyrrolizidine alkaloids in four hours (Can J paper). This work has shown the consortium to have 6-7 members of previously uncultured ruminal bacteria.

1.2.2 Oxalate

One of the best examples of rumen detoxification by ruminal microbes is the degradation of oxalate. Oxalate is found in tropical grasses such as

buffelgrass, pangolagra and kikuyugrass (Cheek, 1995). Oxalate binds with calcium to form calcium oxalate rendering the calcium unavailable to the animal. Ruminants that are gradually adapted to a diet containing oxalate develop a tolerance and can even thrive on a dose that would be toxic to non-adapted animals (Allison and Reddy, 1984). This tolerance is due to the increase of oxalate degrading bacteria, *Oxalobacter formigenes,* in the rumen (Allison et al., 1985). Specialized bacteria, such as *O. formigenes,* are normally present in the rumen in small quantities unless its specific substrate is present.

1.2.3 Leucaena

Extensive work has also been done with the Leucaena toxin, mimosine. Leucaena species are tropical leguminous shrubs, which have been shown to be toxic to animals. Mimosine is an example of a compound that is made both more toxic and detoxified in the rumen. Mimosine is first converted into 3-hydroxy-4(1H)-pyridone (DHP) in the rumen of all animals. DHP is the toxin that causes goiter, hair loss and reduced performance. Allison et al. (1992) was able to isolate novel ruminal bacteria, *Synergistes jonesii,* from a resistant goat. These bacteria detoxify DHP in the rumen and prevent leucaena toxicity. *Synergistes jonesii* was able to infer protection from leucaena toxicity to cattle when inoculated into the rumen of the cattle (Jones and Megarrity, 1986).

1.2.4 Ergovaline / ergot alkaloids

The evidence of ruminal degradation to ergovaline and other ergot alkaloids is less clear than the previously discussed examples. Westendorf et al. (1992) demonstrated that incubating endophyte infected seed for 24 hours with rumen fluid improved the average daily gains and feed conversion rates in rats not seen with the non-infected seed incubated with rumen fluid. This implies that there is an alteration, presumable the detoxification and degradation of the toxins found in the feed by the rumen fluid. Westendorf et al. (1993) found 47-62 % of dietary ergot alkaloids (ergovaline) in the abomasal digesta and only 6-7% of dietary ergot alkaloids were recovered from the feces of sheep. These results imply both foregut and hindgut degradation and absorption of ergot alkaloids. Sheep have a higher tolerance to the alkaloids than cattle after adaptation (Tor-Agbidye et al., 2001) being able to tolerate ergovaline levels greater than 500 ppb after adaptation, without displaying signs of toxicosis. Sheep are able to maintain their body conditioning on E+ feed better than cattle (Burke et al., 2001; Burke and Rorie 2002; Burke et al., 2002). To date the metabolism of ergovaline in the liver has not been performed, making it hard to determine the liver's role in sheep's tolerance to the alkaloids. It is still unclear if this tolerance is due to a physiological difference, the presence of a detoxifying microbe, or a microbe that increases the toxicity of the alkaloids.

1.2.5 Microbial adaptation to substrates

Microbial adaptation within the rumen is not limited to toxic compounds. Saluzzi et al. (2001) found that adaptation of *Ruminocccus flavefaciens* to ryegrass increased the degradation of ryegrass cell walls by almost 10% after the bacteria had a chance to become adapted to the new substrate, most likely due to the induction of specific enzymes. Shifts in ruminal microbial populations are known to occur when animal diets are changed (Church, 1988). For example, a radical change from a forage-based diet to a concentrate-based diet can cause disease (lactic acidosis); however, animals can have excellent performance after adaptation to the same concentrate diet

1.3 Tracking changes in environmental microbial populations

The biggest problem associated with tracking changes in microbial populations from environmental samples is the contamination of inorganic and organic material. Traditional culture based methods of enumerating bacteria from environmental samples clearly underestimate not only bacteria numbers but also the diversity of bacteria present (Brock, 1987; Wagner et al., 1993; Barns et al., 1994; Amann et al., 1995). The cultivable bacteria from environmental samples often result in bacteria counts 10 to 100 fold less than the direct microscope count (Brock 1987). Numerous studies have investigated the diversity of bacteria from environmental samples and have found that current culturing techniques

only isolate a fraction of the true diversity of bacteria present (Whitford et al. 1998, Tajima et al. 1999, Tajima et al. 2000). Therefore several culture-independent methods of evaluating microbial populations have been developed.

1.3.1 Methods of enumerating bacteria

1.3.1.1 FISH.

Microbial populations are often tracked and enumerated using a method known as fluorescence in situ hybridization (FISH). Fluorescence in situ hybridization involves using a fluorescently labeled oligonucleotide probe specific for ribosomal RNA. Bacteria are first fixed and permeabilized for penetration of the probe, then attached to a slide and allowed to hybridize with the probe for 30 minute to several hours depending on species and sample. After hybridization, the slides are washed and cells are visualized using various techniques such as an epifluorescence microscope, confocal laser scanning microscope or cytometry. FISH has the advantages of being specific for targeted organisms and having a fast detection when compared to culturing methods. A variation of FISH using a stem loop structure for the probe has been reported with rumen bacteria (Schofield et al., 1997). This procedure eliminated the need for washing off the excess probe because the probe would not fluoresce unless it was interacting with its target. This study only used cultures, both pure and mixed, but did not use rumen samples therefore the accuracy of detecting low quantities of a bacteria in a real world sample is still in question.

There are several disadvantages of using FISH to track changes in rumen microbial populations. First, autofluorescence of both the microbial population and the organic material can be problem. Autofluorescence leads to false positive results and an increase in background fluorescence. Archaeal species such as methanogens are known to autofluorescence (Sorensen et al., 1997) as well as some fungal species (Margo and Bombardier, 1985; Graham 1983). Samples containing bacteria that are G + C rich makes optimization of fixation and permeable difficult because fixation is species dependent (Macnaughton et al., 1994; De Los Reyes et al., 1997) leading to false negative results. The biggest problem with using FISH on rumen microbes is the lack of specific probes. To date only a handful of probes specific to rumen bacteria have been designed. Designing probes can be an extremely challenging process. Probes must be specific for the target organism and be complementary to an exposed region of the rRNA. Due to the extensive three-dimensional structure of the rRNA and its associate proteins, not all sequences are accessible to probes. Frischer et al. (1996) hypothesized that this is the reason probes designed using DNA or denatured rRNA fail to perform well with FISH.

1.3.1.2 Cytometry.

Cytomtery has been used in combination with FISH to enumerate anaerobe (Lipoglavšek and Avguštin, 2001) as well as with nonspecific fluorescently labeled cells (Vermis et al., 2002; Sethman et al., 2002).

Vermis et al. (2002) used solid phase cytomtery (SPC) with a fluorescein type ester, which is cleaved, within cells by nonspecific esterases, to enumerate anaerobic bacteria. Their results showed that SPC was equivalent or superior to standard plate count method however, different species of bacteria had different optimal conditions for labeling. The main advantage of using fluorescein esters to enumerate bacteria is they discriminate between live cells and those without an intact cell membrane. The use of fluorescein esters has the disadvantage that it doesn't differentiate between different types of bacteria because the labeling procedure is nonspecific.

Lipoglavšek and Avguštin (2001) used flow cytometry in conjunction with an oligonucleotide probe to monitor changes in the ruminal bacteria, *Prevotella bryantii,* when grown in mixed culture and to detect *P. bryantii* within the rumen. While the mixed culture experiment showed that the probe was accurate for enumerating *P. bryantii,* the rumen sample showed the level of detection was low, estimated to only be 10^5 cells per ml.

1.3.1.3 DGGE.

Denaturing gradiant gel electrophoresis (DGGE) has been used to monitor microbial population changes in several diverse microbial populations such as wastewater treatment reactors (Rowan et al., 2002), soil (Ronn et al., 2002), sausage fermentation (Cocolin et al., 2001), and anaerobic gut populations in humans (Favier et al., 2001; Fromin et al.,

2002). DGGE is a powerful molecular biological assay that takes advantage of the unique melting characteristics of short (150 – 1000bp) sequences of double stranded DNA (dsDNA) as they are run through a denaturing acrylamide gel. When DNA (or RNA) is separated by electrophoresis, the rate at which travel through the gel matrix occurs is limited by the size of the molecule as well as the concentration of the gel matrix. Therefore, larger molecules of DNA will travel a lesser distance than the smaller and thus faster molecules of DNA, during the same amount of time when forced to traverse the gel matrix by an electric current (Fischer and Lerman, 1983). In addition to the use of this property of macro-molecules, DGGE takes advantage of the unique property of DNA where a molecule that has regions of separation as well as other regions that remain helical will travel slower through a gel than a molecule of the same length that is fully helical. Urea and formamide are added to a normal, vertically run, acrylamide gel to create a gradient of denaturant that increases from the top of the gel down. As a double-stranded segment of DNA is forced through the acrylamide matrix by electrophoresis, it also experiences chemical interactions with the denaturants that cause the two strands to begin separating. Since chemical denaturation of DNA is similar to temperature denaturation (Fischer and Lerman, 1983), where as the rate at which a segment of dsDNA will denature is directly related to the nucleotide base content of the segment. Watson-Crick base pairs of

guanine and cytosine have a higher melting temp than thymine-adenine pairs. Therefore, DGGE separates DNA molecules by their composition in addition to their size. The potential for complete denaturation of a segment of DNA has led to the development of a "GC clamp" (Myers, et al., 1987). This term describes a region of multiple guanine/cytosine bases that is added to the end of an oligonucleotide primer used during PCR amplification. This region, when complimented during replication, ensures that the product of interest will not completely denature before the DNA segment of interest has entirely separated. When these properties act in concert, the result is a molecular technique that is robust enough to allow for separation and visualization of mixed groups of organisms, and is sensitive enough to detect differences between members of a strain or species that have a base difference of as little as one pair.

The largest limitation of DGGE is the relatively high detection limit. Cocolin et al. (2001) found the limit of detection of DGGE to be $1.0x10^5$ cells per ml. This level of detection has the potential to fail to detect a change in the population of minor ruminal bacteria which could play a role in the metabolism of ergot alkaloids. The quantification of bacterial populations by DGGE is a relatively new procedure that relies on the quantification of bands by visualizing the bands and analyzing the brightness of them. While DGGE has been published in phylogenetic

analysis it has not been published in a quantitation study without other supporting assays such as real-time PCR.

1.3.2 PCR

1.3.2.1 Conventional PCR.

Conventional PCR is a powerful tool for detecting unique sequences within a mixed DNA sample. PCR involves using two primers, a forward and a reverse, with a thermally stable polymerase to amplify a specific sequence of DNA by several cycles of denaturing to separate the DNA strands, annealing of the primers to the DNA template, and extension of the primer in a sequence specific manner. The specificity of PCR depends on the primer sequences, the reagents used and the reaction conditions. Theoretically, PCR can detect a single copy of a target gene within a mixed sample; however, only qualitative information can be inferred from conventional PCR because it uses end-point evaluation in the form of the presence or absence of a product on a gel. Product accumulation has a direct relationship with the starting amount of template during the exponential phase of amplification; however, after the exponential phase the reaction reaches a plateau and the product accumulation is no longer correlated with the amount of the starting template. After the typical 30-35 cycles of amplification, most products have reached an amplification plateau therefore making it impossible to evaluate the amount of starting material. Therefore, conventional PCR can be used to detect a strain of

bacteria within a sample but it gives no information on the quantity of the bacteria in the sample.

1.3.2.2 Competitive PCR.

In an attempt to overcome the limitations of conventional PCR a method known as competitive PCR (cPCR) was developed and has been used to evaluate a few ruminal bacteria. Competitive PCR involves the coamplification of an internal control sequence that is amplified at the same rate as the target sequence. This allows quantification of the target when a known amount of the control is used. The crucial component of competitive PCR is the design of the internal control. The internal control must amplify at the same rate as the target yet be distinguishable from the target for analysis. This is typically accomplished by cloning the target gene and either adding or removing an internal sequence with a restriction enzyme and reamplifying the resulting product. The validation of the internal control sequence is a lengthy and extensive process involving not only the testing of equal amplification rates but testing of the correct amount of the internal control to use since the most accurate measurements are when the control and unknown have a 1:1 concentration ratio. In order to remove the bias of conventional end-point analysis cPCR is typically amplified for the minimum number of cycles (8-10 cycles) to achieve adequate amplification of template for quantification by electrophoresis.

1.3.2.3 Real time PCR.

Another method, which was developed in order to overcome the limitations of the end-point evaluation of conventional PCR, is Real-time PCR (qPCR). Real time PCR differs from conventional PCR by data being collected after every amplification step instead of at the end. Data is collected in the form of a fluorescence signal which increases as the amount of target increases after each round of amplification. There are two main methods of creating an increase in fluorescence with qPCR, using specific probes or non-specific binding dyes. Specific probes are composed of a sequence specific oliogonucleotide probe attached to a reporter dye and a quencher dye. As the target sequence is amplified the quencher dye is cleaved off the probe, allowing the reporter dye to fluoresce causing an increase in the fluorescent signal. The advantage of using specific probe method is the combination of primers and probes ensure only the amplification of the target gene is being recorded. The disadvantage to this method is the design and validation of the primer and probe combination. Non-specific dyes are typically double stranded DNA binding dyes, which fluoresce when bound to dsDNA. As the amount of double stranded product in the reaction increases so does the fluorescent signal. The main advantage of the non-specific binding method is the ease that it can be adapted to different primers without the need to design and validate a specific probe sequence. The disadvantage of this method is additional analysis must be

performed when using the non-specific method to verify that only the target sequence was amplified. The additional analysis involves performing melting curve analysis and/or visualization with gel electrophoresis.

With either method, the point where the fluorescent signal rises above the background fluorescence is called the threshold cycle (C_T). With qPCR there is a linear relationship between the log of the starting template and the C_T over a large range of magnitudes ranging from 10 to 10^8 copies of a starting template. Quantification of qPCR involves the use of a standard curve of known concentrations made by plotting the log of the starting material verses the C_T. The threshold cycle of the unknowns are compared to the standard curve, resulting in the starting concentration of unknowns without the need for extensive validation of control concentration and post-PCR sample manipulation needed for cPCR.

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2 Metabolism characterization and determination of physiological and digestive effects on lambs fed Neotyphodium coenophialum-infected tall fescue[1]

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2.1 Abstract:

The digestion responses and degradation of ergovaline and production of lysergic acid in the rumen of sheep offered *Neotyphodium coenophialum*-infected tall fescue straw at two ergovaline levels was investigated. Six crossbred wethers (56 +/- 3 kg BW) were randomly assigned to one of two treatment groups in a cross-over design. Each experimental period consisted of 28 d feeding periods with a 14 d wash-out between periods. During the wash-out period all animals received a diet containing <10 ppb ergovaline. Treatments were 1) < 10 ppb ergovaline (E-) and 2) 500 ppb ergovaline (E+). Ergovaline levels were achieved with a combination of tall fescue straw (350ppb ergovaline) plus *Neotyphodium coenophialum*-infected tall fescue seed (~3,300 ppb ergovaline). Diets were isonitrogenous. Rumen fluid was sampled three times (d 0, 3, 28) during the 28-day experimental period for ergovaline and lysergic acid. Samples were collected at time 0 (prior to feeding), 6, and 12 h post feeding. Total fecal and urine collection commenced on d 21 and continued till d 25 of the experimental period. Rumen ammonia, rumen pH, and rectal temperature were not influenced by ergovaline concentration ($P > 0.10$). Digestion of DM, ADF and CP were not different between treatments ($P > 0.10$). Feed intake and body weight were different between treatments ($P > 0.10$). Water intake was reduced by ergovaline

intake ($P < 0.05$). Serum prolactin was reduced by 27% with ergovaline intake ($P < 0.05$). Ergovaline concentration in rumen fluid expressed as a percent of intake increased over sampling time and sampling day ($P < 0.05$). Lysergic acid concentration in rumen fluid expressed as a percent of intake increased over time from d0 to d3 ($P < 0.05$) but was not different between d3 and d28 at any time point ($P > 0.10$). The feces contained an average of 0.41 µmol/day ergovaline and 0.87 µmol/day lysergic acid. Urine contained no detectable ergovaline; lysergic acid concentration was 1.05 µmol/day. The appearance of lysergic acid in the feces, urine and rumen fluid is likely from the degradation of ergovaline in the rumen due to microbial degradation and further break down in the lower digestive tract.

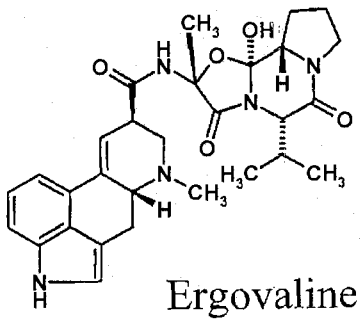
Key words: Sheep, Ruminants, Endophyte, Ergovaline, Tall fescue

2.2 Introduction

In Oregon, grass seed production is the fourth largest agriculture commodity where approximately 160,000 acres are planted in tall fescue mainly used for this purpose (National Ag Statistic Service, 2002). After the seed is harvested, straw is left as a field residue. In the past, straw was eliminated by field burning, but increased environmental restrictions caused producers to investigate other alternatives. The main market for this straw is Japan and other Pacific Rim countries where it is used as forage for livestock. Oregon currently exports approximately 65% of the

straw to Japan, 34% to Taiwan, 1% to Korea; the remaining straw is used domestically in the US.

There is a long history of problems associated with feeding tall fescue to livestock (Strickland et al., 1993; Bacon, 1995; Tor-Agbidye et al., 2001). These problems are associated with the endophyte fungus *Neotyphodium coenophialum,* which infects varieties of tall fescue in a symbiotic fashion. *N. coenophialum* produces ergopeptine alkaloids, with ergovaline being produced in the greatest quantity (Lyons, 1986). Ergovaline is believed to be the cause of fescue toxicosis (Joost, 1995) and has been correlated with fescue toxicosis (Tor-Agbidye et al., 2001). Fescue toxicosis is estimated to cost the beef industry $800 million/year due to reduced reproductive performance and decreased average daily weight gain. However, recent work by Hill et al. (2001) implies the core ring structure of ergopeptine alkaloids, lysergic acid (Figure 1), crosses the rumen wall at a higher rate than any of the other alkaloids, suggesting the toxic agent in tall fescue might be lysergic acid not ergovaline. To date, no one has quantified lysergic acid in the feed or



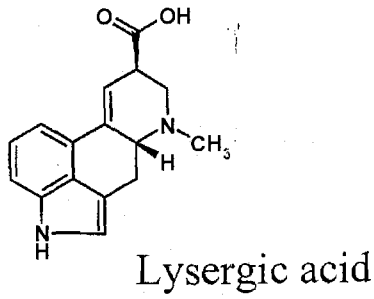


Figure 2-0-1 Structure of ergovaline and lysergic acid

measured the appearance of lysergic acid in rumen fluid, blood, urine or feces in relation to ergovaline levels in the diet. This study assessed the metabolic fate of ergovaline and lysergic acid in sheep consuming *N. coenophilaum*-infected tall fescue containing 500 ppb ergovaline.

2.3 Materials and Methods

2.3.1 Animals.

Six ruminally canulated Poly Pay x Suffolk crossbred yearling wethers (56 +/- 3 kg BW) were randomly assigned to one of two treatment groups in a cross-over design (Kuehl, 2000). Surgical and animal care procedures were approved by the Oregon State University Institution of Animal Care and Use Committee. Wethers were individually housed in metabolism crates for the duration of the study. A two-week adaptation to the metabolism crates and voluntary intake was allowed before the first feeding period. Environmental temperatures were consistent with temperatures in the Pacific Northwest for August through November 2003.

2.3.2 Diets.

The E- treatment consisted of chopped tall fescue straw (90% of diet DM basis) with < 10 ppb ergovaline. The E+ treatment contained chopped tall fescue straw (87.5% of diet DM basis) containing 350 ppb ergovaline; the remainder of the ergovaline was provided by endophyte infected tall fescue seed (~3,300 ppb ergovaline; 6% of diet DM basis) (Table 2-1) to give a final concentration of 500ppb ergovaline. The two treatment straws differed in CP (5.5% E- and 6.5% E+) and the addition of seed (16.2% CP)

increased the CP content of the E+ diet. Therefore soybean meal (SBM; 49% CP) was added to ensure diets were isonitrogenous and CP requirements were fulfilled (NRC, 1985).

Table 2-1 Diet feedstuff and nutrient content (% DM basis)

Item	E- Diet	E+ Diet
E- Straw[a]	90	---
E+ straw[a]	---	87.5
SBM[a]	10.0	6.5
E+ seed[a]	---	6.0
CP, %[b]	9.13	9.14
NDF, %[b]	57	56
Ergovaline[b], ppb	<10 ppb	500 ppb
Lysergic acid[b], ppb	<10 ppb	200 ppb

[a]Percent of diet on DM basis
[b]Value for diet

2.3.3 Experimental Design.

Treatment periods were 28 days with a 14d washout period between treatment periods. Prior to feeding straw (0730), SBM and SBM/seed mix was provided (SBM: 10% and 6.5% of diet; seed 6.0% of diet on a dry matter basis, Table 2-1). Tall fescue straw was provided at 90% of previous 5-d average intake at 0800, with feed refusals from the previous day determined prior to feeding. Intake and orts were monitored through

out the trial; however samples were taken on d 0, 3, 28 and each day during fecal collections (d21-25). Diet grab samples collected on those days and were composited for analysis. Orts were collected during fecal collection and were individually analyzed for ergovaline, lysergic acid, DM, ADF and CP. Straw and ort samples were stored at -20°C until analyzed. Straw, SBM and feces were analyzed for DM, ADF, and CP by Near Infrared Reflectance Spectroscopy (NIR). Seed was analyzed by wet chemistry at Dairy One Forage Laboratory (Dairy One, Ithaca, NY.). NIR is recognized by the Association of Official Analytical Chemists (AOAC) as an official method of analysis. Rectal temperatures were taken daily just after feeding via a handheld digital thermometer with probe placed approximately 3 cm into the rectum. Water intake was measured twice a day, first prior to feeding and then at 1700 and summed for daily water intake. Wethers were weighed at the start and end of each feeding period.

Blood samples for prolactin analysis was collected prior to feeding via jugular venipuncture with 10 ml vacutainers. Tubes were allowed to coagulate at room temperature then centrifuged. Serum was decanted and frozen at -20°C until prolactin analysis was performed. Serum prolactin was analyzed as described by Hockett et al. (2000).

Rumen fluid was sampled for ergovaline and lysergic acid analysis on d 0, 3, and 28 of each period at 0 (prior to feeding), 6, and 12 h after feeding. Additional rumen fluid samples were collected at 0, 3, 6, 9, 12, and 24 h for

pH and ammonia analysis. Approximately 60 ml of rumen fluid was collected with a rumen suction strainer and aliquoted in the following fashion: 9 ml for ammonia was added to 3 ml 25% HCl acid; 13 ml each for ergovaline and lysergic acid analysis; the remaining rumen fluid was used for pH measurement. pH measurements of rumen fluid were taken immediately after collection with a high performance combination probe (Corning, New York, 14831). All other rumen fluid samples were placed on ice immediately after collection then frozen and stored at -20°C.

Collection of total urine and fecal samples commenced on d21 and continued to d25. Urine was collected in plastic pans and emptied twice in a 24 hr period (0800 and 1700). Daily urine was composited, weighed, measured for volume, and a 100 ml subsample was taken at 1700 for ergovaline and lysergic acid analysis. Sheep were fitted with fecal bags at 0800 on d19 of each treatment period for adaptation. Fecal bags were changed twice in a 24 hr period (0800 and 1700) from d 21 to 25. The feces for each day were composited, weighed and hand mixed with a 20% (wet weight) subsample collected each day at 1700. Subsamples were dried in a freeze-drier for 7 days, reweighed for DM, ground, and then composited by lamb and stored at room temperature until analysis. Fecal collections were used to estimate digestibility and calculate ergovaline and lysergic acid absorption.

2.3.4 Chemical Analysis.

2.3.4.1 Ergovaline.

Feed, fecal, urine, and rumen fluid samples were tested for ergovaline concentration by high performance liquid chromatography (HPLC) as described by Craig et al. (1994). Briefly, feed and fecal samples were ground with a Wiley mill to pass through a 1mm screen and stored at -20°C until analysis. Approximately 1.0 g (feed and feces) or 6 ml (rumen fluid) of sample were extracted in chloroform (buffered with NaOH (feed and feces) or KPO_4 (rumen fluid)), with ergotamine added as an internal standard (1µl/ml chloroform). Samples were rotated in the dark for 24 hours (feed and feces) or 5 h (rumen fluid). Five ml of the supernatant was added to an ergosil solid-phase extraction column. The fraction containing ergovaline was then evaporated under N_2 gas, reconstituted in 500 µl methanol and injected onto the HPLC (20 µl). Mobile phase consisted of 30:60 ammonium carbonate (0.2 mg/ml H_2O):acetonitrile mobile phase, with the fluorometer excitation and emission wavelengths set at 250 and 420 nm, respectively.

2.3.4.2 Lysergic acid.

Lysergic acid was determined by HPLC. Briefly, 1.0 g dried, ground straw, orts, seed and feacl samples were extracted by turning overnight with 10 ml acetonitrile:water (50:50 v/v). They were then centrifuged at 2000 rpm for 10 minutes. The supernatant was removed and the pH was

adjusted with 50% acetic acid to pH 5.0-5.5. Rumen fluid was concentrated (6.5 ml) in an ISS-100 centrifugal evaporator (Thermo Forma, Marietta, OH) at high temperature (65 °C). The pellet was resuspended in 3 ml water by vortexing and adjusted for pH (5.0-5.5). Urine was adjusted for pH (5.0-5.5) with no other sample preparation. Three ml of supernatant was passed through a solid phase extraction column (Supelco DSC-SCX SPE column, 500 mg/3 ml; Bellefonte, PA). Lysergic acid was eluted with 5% ammonium hydroxide in methanol, evaporated in a ISS-100 centrifugal evaporator (Thermo Forma, Marietta, OH) at 43 °C, and reconstituted in 200 µl of 0.05 M phosphoric acid:methanol (50:50). Twenty µl of sample was injected onto an HPLC using a mobile phase consisting of 94:6 0.05 M phosphoric acid:acetonitrile and a fluorescence detector set at excitation = 250 nm, emission = 420 nm.

2.3.4.3 Ammonia.

Acidified rumen fluid samples were thawed, centrifuged (1,000 x g) for 15 minutes and analyzed for ammonia by the phenol-hypochlorite method (Broderick and Kang 1980) using a 96-well microtiter plate reader attached to a UV/Vis Spectrophotometer (Elx808; Bio-Tek instruments, Winooski, VT.).

2.3.4.4 Statistical Analysis.

Digestibility, ergovaline and lysergic acid data was analyzed as a crossover by the SAS GLM procedure. Animal, treatment (TRT), period,

sampling day (DAY) and DAY X TRT were included in the model. Rumen pH and ammonia, serum prolactin and physiological variables (rectal temperature, water intake) were analyzed using the REPEATED MEASURED statement with the MIXED procedure of SAS.

2.4 Results and Discussion

Rumen ammonia (4.27 mM E- vs. 4.70 mM E+, SE 0.128; p = 0.90) and pH (6.86 E- vs. 6.97 E+, SE 0.032; p = 0.36) were not different between treatment groups at any time point, consistent with forage diets formulated to be isonitrogenous. Daily rectal temperatures were not influenced by alkaloid concentration (38.4°C E- vs. 38.4 °C E+, SE 0.0615; p = 0.40). This is consistent with findings by Fiorito et al (1991) and Stamm et al. (1994) where no difference in rectal, tail and ear temperature were detected. Other studies (Aldrich et al., 1993; Paterson et al., 1995) have observed differences in daily rectal temperatures. The variation in observed rectal temperatures in response to E+ feed between studies could be due to from the different alkaloid levels used in each study or a difference in ambient temperatures. Higher ambient temperatures and heat stress can cause a greater change in rectal temperature for animals consuming E+ diets when compared to E- diets (Hannah et al. 1990, Gadberry et al. 2003).

Apparent digestibility of DM (53.8% E- vs. 49.8% E+, SE 2.89), ADF (49.4% E- vs. 52.2% E+, SE 4.84), and CP (61.4% E- vs. 63.7% E+, SE 1.62) were not different between treatment groups, which is in agreement with findings by Stamm et al. (1994) and Aldrich et al. (1993). Water intake was decreased by E+ diet (2.95 L/d E- vs. 2.77 L/d E+, SE 0.59; $p = 0.04$) which is consistent with findings by Fiorito et al. (1991) where E+ feed caused a drop in voluntary water intake. However, Aldrich et al. (1993) reported no different in water intake. Serum prolactin was decreased to 6.4 ng/ml for E+ from 22.9 ng/ml for E- treatment (SE 5.19; $p = 0.023$), indicating subclinical fescue toxicosis. This result is consistent with previous research (Porter, 1995; Paterson et al., 1995; Gadberry et al. 2003) which shows depressed blood (serum or plasma) prolactin levels in animals on E+ diets.

Ergovaline released in the rumen, as a percentage of intake, increased from d 0 to d 3 and from d 3 to d 28 at all time points (Table 2-2). Lysergic acid liberated in the rumen, as a percentage of intake, increased from d 0 to d 3 at 0 hr and 6 hr but not 12 hr. No difference in lysergic acid in the rumen was detected between d 3 and d 28 (Table 2-2). Ergovaline appeared in the rumen at a slower rate than lysergic acid, indicating possible degradation of ergovaline to lysergic acid. No carryover effect of ergovaline or lysergic acid in the rumen fluid was detected, i.e. concentration of ergovaline and lysergic acid in rumen fluid at 0 hr on d 0 of

Table 2-2 Ergovaline and lysergic acid concentration rumen fluid of lambs consuming an E+ diet presented as a percentage of intake[a]

		Day			
	Time	0	3	28	SE
Ergovaline	0 hr	0.00[b]	5.12[c]	7.60[d]	0.451
	6 hr	1.42[b]	5.33[c]	7.25[d]	0.511
	12 hr	2.67[b]	6.20[c]	8.42[d]	0.601
Lysergic acid	0 hr	0.00[b]	29.5[c]	27.4[c]	3.01
	6 hr	13.4[b]	37.6[c]	42.3[c]	5.94
	12 hr	21.6[b]	30.1[b]	41.6[b]	8.32

[a] Calculated as amount in rumen fluid/amount in feed
[b,c,d] Within a row, means without a common superscript differ ($P<0.05$)

both treatment periods was undetectable. Hill et al. (2001) theorized that lysergic acid crosses the ruminal wall at a greater rate than other ergopeptine alkaloids because it is smaller and more polar therefore can be transported across tissue easier. That study also showed ergot alkaloids to have a higher affinity and greater passage rate for rumen tissue than reticulum and omasum tissue, implying absorption of ergot alkaloids occurs primarily in the rumen. Studies examining the absorption rate of alkaloids and metabolites from the hindgut have not been conducted; therefore, it is difficult to conclusively determine the site of absorption. Westendorf et al. (1992) found that incubating E+ seed with

rumen fluid then feeding it to rats improved the ADG and feed conversion of the rats when compared to non-incubated E+ seed. The improvement in ADG and FC was not observed in E- seed incubated with rumen fluid, implying the improvement in ADG and FC is due to the detoxification of alkaloids not from an alteration in the digestibility. Taken together this implies that the apparent digestibility of ergovaline results from degradation, not from direct absorption.

Table 2-3 Ergovaline and lysergic acid in the diet, urine and feces of lambs consuming an E+ diet

	Ergovaline[a]	SD	Lysergic acid[a]	SD	Ergovaline: lysergic acid
Diet[b]	0.61	0.07	0.21	0.04	3.00:1.00
Urine[c]	ND[e]		0.33	0.05	
Feces[d]	0.48	0.04	0.51	0.15	0.94:1.00

[a] Actual means, n = 6
[b] mg/kg of Intake
[c] mg/kg of Urine output
[d] mg/kg of Fecal output
[e] Not detectable

Lysergic acid was detectable in the E+ feed at 142 ppb in the straw and 660 ppb in the seed by HPLC analysis. Ergovaline in the straw was 350 ppb and 3,300 ppb in the seed. The E+ diet was formulated to contain 500 ppb ergovaline. Post-trial analysis of feed and ort samples showed an

average ergovaline concentration of 610 ppb (Table 2-3). Ergovaline to lysergic acid ratio in the diet was 3:1 while in the feces the ratio was approximately 1:1 (Table 2-3). As expected ergovaline was undetectable in the urine while lysergic acid was present at approximately 1.4 times the amount that was detected in the feed (Table 2-3). Because of its size, ergovaline is expected to be excreted in the bile and recovered in the feces, as demonstrated by early work done with radiolabled ergot alkaloids (Eckert et al., 1978). These are the first reported values for lysergic acid found in straw and seed as measured by HPLC. Other studies have examined just ergovaline or total ergoline alkaloids measure with an ELISA test.

To estimate the indigestible alkaloids in the diet, the average daily intake of each alkaloid was multiplied by the indigestible DM (1 - 0.498). Ergovaline remaining in the feces was approximately equivalent to the indigestible fraction of the diet (0.25 mg vs. 0.29 mg estimated). Ergovaline is a large, relatively non-polar molecule that would be excreted via the bile and recovered in the feces if absorbed from the digestive tract (Eckert et al., 1978). Therefore, fecal ergovaline levels reflect ergovaline passage through the digestive tract. Lysergic acid remaining in the feces was higher than the amount in the indigestible fraction of the diet (0.23 mg vs. 0.10 mg estimated). Lysergic acid is a smaller more polar molecule than ergovaline and absorbed lysergic acid would be expected to be excreted in the urine

(Eckert et al., 1978). Therefore the increase of lysergic acid seen in the feces is due to degradation of ergovaline as well as other ergopeptides in the digestive tract and incomplete absorption of lysergic acid from the gastrointestinal tract.

The amount of ergovaline in the diet of animals consuming E+ diet was 1.15 µmole/day and lysergic acid was 0.77 µmole/day (Table 2-4). The amount of ergovaline recovered in the feces was 0.41 µmole/day and lysergic acid in the feces was 0.87 µmole/day. Ergovaline was not detected in the urine, while lysergic acid in the urine was 1.05 µmole/day. Total alkaloid concentration (ergovaline and lysergic acid) detected in the diet was 1.92 µmole/day and the alkaloids recovered from the feces and urine was 2.33 µmole/day (Table 2-4), which amounts to 121% recovery of dietary alkaloids from the feces and urine. This is likely due to degradation of other ergopeptides into lysergic acid; while ergovaline can account for up to 80% of the alkaloids produce, many other ergot alkaloids, such as ergocryptine, ergotamine and ergonovine, can be found in endophyte infected tall fescue. All of these ergot alkaloids contain the core ring structure of lysergic acid and vary only in the composition of the side chain. Therefore they can theoretically be degraded to lysergic acid.

The appearance of lysergic acid in the feces and urine in greater amounts than in the feed implies the ergot alkaloids in the feed were degraded to lysergic acid by rumen microbial digestion and degradation in

the lower gastrointestinal tract. Work done by Westendorf et al. (1992) and Hill et al. (2001) suggests that the primary site of degradation and absorption of endophyte alkaloids is the rumen. These data affirms this and allows us to further conclude that most of the ergovaline and other ergopeptides in the diet are most likely converted to lysergic acid in the rumen of sheep.

Table 2-4 Micromoles of ergovaline and lysergic acid consumed and excreted by lambs consuming an E+ diet

	Ergovaline	Lysergic acid	Ergovaline + Lysergic acid
Diet	1.15	0.77	1.92
Urine	ND[a]	1.05	1.05
Feces	0.41	0.87	1.28
Total Excreted	0.41	1.92	2.33

[a] Not detectable

Taken together, these results allow the formation of a model for degradation of ergopeptides alkaloids in sheep consuming endophyte-infected tall fescue (Figure 2-2). Feed containing ergot alkaloids is consumed by sheep and enters the rumen where microbial digestion of the plant material liberates the alkaloids into the fluid. Ergopeptine alkaloids are further degraded by ruminal microbes in to the simpler ergoline or lysergic acid molecules. Ergoline and lysergic acid are absorbed from the rumen in the greatest quantities with ergopeptine alkaloids are absorbed in

minor quantities. Once absorbed, the alkaloids are rapidly removed from the blood stream by the liver. In the liver, alkaloids are possible modified to improve the body's ability to excrete the compounds. From the liver, large ergopeptine alkaloids are sent to the bile for excretion and smaller ergoline alkaloids, lysergic acid and metabolites from liver metabolism are sent to the kidneys for excretion in the urine.

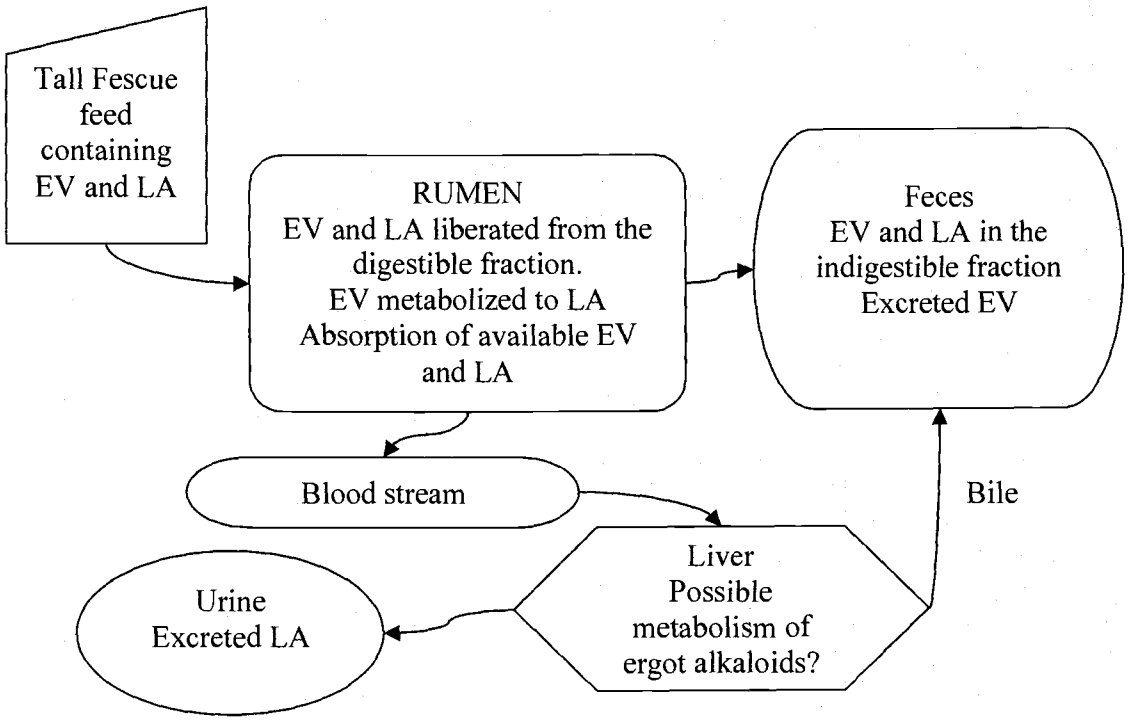


Figure 2-2 Proposed model for the metabolism, absorption, and excretion of ergot alkaloids.

Feed containing ergot alkaloids enter the rumen where microbial digestion of the plant material liberates the alkaloids into the fluid. Ergopeptine alkaloids are further degraded by ruminal microbes in to the simpler ergoline or lysergic acid. Ergoline and lysergic acid are absorbed from the rumen in the greatest quantities with ergopeptine alkaloids are absorbed in minor quantities. Once absorbed, the alkaloids are rapidly removed by the liver. In the liver, alkaloids are possible modified to improve the body's ability to excrete the compounds. From the liver, large ergopeptine alkaloids are sent to the bile for excretion and smaller ergoline alkaloids, lysergic acid and metabolites from liver metabolism are sent to the kidneys for excretion in the urine. EV : ergopeptides (i.e. Ergovaline); LA: ergolines (i.e lysergic acid).

2.5 Implications

This study is the first reported attempt to quantify the metabolism of ergovaline to lysergic acid in the ruminant digestive system using HPLC assays for quantification of these alkaloids. Results of this study may lead to a better understanding of ergovaline metabolism and the causes of fescue toxicosis.

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3 Monitoring major ruminal bacteria in sheep during adaptation to *Neotyphodium coenophialum* -infected tall fescue straw with real-time PCR.[2]

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3.1 Abstract

In order to determine if the increased tolerance sheep display to fescue toxicosis is due to ruminal bacteria five major sheep ruminal bacteria were monitored during the adaptation to *Neotyphodium coenophialum* -infected tall fescue straw. This increased tolerance supports the belief that there are microorganisms in the rumen of sheep capable of detoxifying the alkaloids found in infected tall fescue. Rectal temperatures and serum prolactin levels were monitored as an indication of toxicosis. Daily rectal temperatures were not influenced by alkaloid concentration (38.4°C E- vs. 38.4 °C E+, SE 0.0615; p = 0.40). Serum prolactin was decreased to 6.4 ng/ml for E+ from 22.9 ng/ml for E- treatment (SE 5.19; p = 0.023), indicating subclinical but not clinical fescue toxicosis. A period effect was detected for most primers used, indicating change in microbial populations due to adaptation to the fescue straw. *Prevotella bryantii* $B_1$4 was detected in low levels through the entire feeding period and levels were approximately the same (95% E- and 89% E+ of day 0 on day 28) from day 0 to 28 but no difference was detected between treatment groups (90% E- vs. 93% E+; p = 0.45). The Streptococcus group (*S. bovis, S. caprinus, S. equines)* was detected in low levels through the entire feeding period and E+ treatment tended to lower the concentration of 16S gene but no statistical difference was detected between treatment groups (89% E- vs. 94% E+; p = 0.39). *Selenomas ruminantium- Mitsuokella multiacida*

JCM6582 was the most abundant organism found in the samples and levels were approximately the same (97% E- and 105% E+ of day 0 on day 28) from day 0 to 28 but no difference was detected between treatment groups (92% E- vs. 106% E+; p = 0.52). *Eubacterium ruminatium* (ATCC 17233) was undetectable in most samples over all periods. *Ruminococcus flavefaciens* (ATCC 19208T) sequence was detected in moderate levels through the entire feeding period and levels were approximately the same (97% E- and 99% E+ of day 0 on day 28) from day 0 to 28 but no difference was detected between treatment groups (91% E- vs. 92% E+; p = 0.28). *Ruminococcus albus* was detected in low levels through the entire feeding period and levels were approximately the same (95% E- and 83% E+ of day 0 on day 28) from day 0 to 28 but no difference was detected between treatment groups (87% E- vs. 89% E+; p = 0.33). These results imply that none of the five bacteria monitored in this study is responsible for the metabolism of ergot alkaloids.

3.2 Introduction

3.2.1 Evidence of ruminal degradation

Neotyphodium coenophialum infected tall causes a disease commonly called fescue toxicosis where animals have decreased intake, poor weight gains, elevated rectal temperature, rough hair coat, depressed serum prolactin levels, and reduced blood flow to the extremities. Sheep have a higher tolerance to the alkaloids than cattle after adaptation [1], being able

to tolerate >1200 ppb ergovaline while cattle show signs of fescue toxicosis after consuming feed with 500 ppb ergovaline. It is still unclear if this tolerance is due to a physiological difference, such as different absorption rates or detoxification rates in the liver, the presence of a detoxifying microbe within the rumen of sheep, or a microbe that increases the toxicity of the alkaloids in the rumen of cattle. Rumen alteration of plant toxins and other compounds is a well documented phenomenon. Plant toxins such as pyrrolizidine alkaloids [2][3][4], oxalate [5], and mimosine [6] have been shown to be degraded and detoxified in the rumen while other compounds, such as nitrates [7], tryptophane [8] and thiamin [9], have been shown to be altered into the toxic compounds, nitrites, 3-methyl-indole, and thiamin analogues respectively, in the rumen.

There is indirect evidence that the tolerance to infected fescue observed in sheep is due to rumen alteration of the toxins in the grass. Westendorf et al. [10] demonstrated that incubating endophyte infected seed for 24 hours with rumen fluid improved the average daily gains and feed conversion rates in rats not seen with the non-infected seed incubated with rumen fluid. This implies that there is an alteration, presumable the detoxification and degradation of the toxins found in the feed by the rumen fluid. Westendorf et al. [11] found 47-62 % of dietary ergot alkaloids in the abomasal digesta and only 6-7% of dietary ergot alkaloids were recovered from the feces of sheep. These results imply both foregut and hindgut

degradation and absorption of ergot alkaloids. Stuedemann et al. [12] hypothesized that ergot alkaloids are absorbed primarily from the foregut rather than the hindgut because alkaloids or their metabolites were detected in the urine of cattle within 12 hours of ingesting endophyte-infected pasture. Other work done in this lab [13] has shown the apparent degradation of ergovaline to lysergic acid when endophyte-infected straw was fed to sheep. Lending support to the theory that there is a population of bacteria in the rumen that can degrade ergovaline. To gain insight on the mechanism which gives sheep a higher tolerance, bacteria population shifts in the rumen in response to the toxins must be characterized.

3.2.2 Monitoring ruminal bacteria populations

Traditional culture based methods of determining bacteria populations in environmental samples clearly underestimate the true microbial ecology found *in vivo* [14][15] with direct microscopic count of bacteria greatly exceeding cultivation counts [14][16]. This is especially true when dealing with bacteria that have fastidious growth requirements, such as ruminal bacteria [17]. Traditional methods have lead to the estimation of 22 species of ruminal bacteria [18] yet several studies using the culture-independent molecular technique of sequencing cloned libraries have found the majority of bacteria present in the rumen have not been isolated before [17][16][19]. This method has the limitation of only being able to detect species above 2% of the total populations as well as having biases

introduced by PCR, which will be discussed later. To track ruminal bacteria populations several molecular based methods have been developed. The use of molecular probes has been used to enumerate specific strains of bacteria [20]; however the extensive secondary of the ribosomal RNA makes the design of probes difficult. The detection limit of molecular probes is relatively high, making this technique unsuitable for the enumeration of low density bacterial populations. Several studies have used competitive PCR (cPCR) to enumerate bacteria within the rumen. Competitive PCR relies on the amplification of a known concentration of a control sequence which is identifiable from the target sequence usually by producing a different product size. This technique requires the validation of equal amplification rate of the control and target sequence, the optimization of control sequence concentration, and post-PCR analysis to resolve and quantify the target and control sequences. To date there is only a limited number of cPCR sequences for ruminal bacteria. Recent work has used Real-Time PCR to enumerate and track bacteria populations [21]. Real-Time PCR involves the collection of data, in the form of an increase in a fluorescent signal, after each PCR cycle instead of the end-point analysis that other PCR methods use. This technique has the advantages of being able to use established PCR primers with a theoretical limit of detection of one copy gene per sample.

Rumen bacteria population shifts in response to changes in diets is a well-known phenomenon best demonstrated when animals are shifted from a forage diet to a concentrate diet. Traditional culture methods have shown isolation of different bacteria when animals consume a forage-based diet than when they consume a concentrate-based diet. This observation was supported by 16S rDNA sequencing done by Tajima et al. [19]; however, while their results showed a change in the bacterial population diversity during adaptation, the majority of sequences analyzed were either unidentified bacteria or bacteria only identified with molecular techniques. Tajima et al. [21] used Real-Time PCR to monitor the changes in major cultivable ruminal bacteria populations during the adaptation to a high concentrate diet.

3.2.3 Biases associated with each method of monitoring populations.

Just as traditional methods have biases so do the newer molecular based approaches. The extraction of genomic material from bacterial cells could introduce a bias towards the easier to lyse Gram-negative cells [16]. During PCR several biases can be introduced which confound the results in an experiment. PCR can not distinguish between live and dead cells; it can only show that a targeted template is present in the sample. Whitford et al. [17] found a decrease in the diversity of sequences retrieved after 30 cycles of PCR amplification when compared to 12 cycles. This bias was also noted by Suzuki and Giovannoni [22] during the amplification of three

16S genes from different phylogenetic groups of marine bacteria. Tajima et al. [21] found that different ruminal bacteria DNA amplified at different rates under identical reaction conditions with a universal primer. There are several factors which contribute to the differential amplification such as 1) genome size and rRNA gene copy number, 2) choice of primer and reaction conditions, 3) annealing and extension efficiency, 4) G+C content, 5) DNA concentration and 6) DNA-associated molecules. With identical reaction conditions and template concentration the authors were able to eliminate factors 2 and 5. Previous work had demonstrated that the slowest to amplify template had more 16S copy number on the genome than the quickest template, therefore eliminating factor 1. Annealing and extension efficiency was eliminated as a possibility due to exponential increase in fluorescent signal during amplification of all but one template. The difference in amplification observed by Tajima and coworkers [21] occurred during the lag phase therefore they attributed it to problems with the original DNA template possible caused by DNA-associated molecules.

3.3 Materials and methods

3.3.1 Animals, Diet and Sample collection.

Six ruminally cannulated Poly Pay x Suffolk crossbred yearling wethers (56 +/- 3kg BW) were randomly assigned to one of two treatment groups in a cross over design [23]. Surgical and animal care procedures were approved by the Oregon State University Institution of Animal Care

and Use Committee. Wethers were individually housed in metabolism crates within a barn during the duration of the study. Treatment periods were 28 days with a 14 day washout period between treatment periods. A two-week adaptation to the metabolism crates and voluntary intake was allowed before the first feeding period. Environmental temperatures and photoperiod were consistent with temperatures and day length in the Pacific Northwest during August through November. Treatments were <10 ppb (E-) ergovaline and 500 ppb (E+) ergovaline. For both treatments, chopped tall fescue straw was used. E- diet consisted of straw (95% AF) with <10 ppb ergovaline. E+ diet contained straw (88.5% AF) with 350 ppb ergovaline, the remainder of ergovaline was provided by endophyte infected tall fescue seed (>3,300 ppb ergovaline). The two treatment straws differed in CP and the addition of seed increased the CP of E+ diet, therefore soybean meal (SBM) was added to ensure diets were isonitrogenous and CP requirements were fulfilled (NRC, 1985). Tall fescue straw was provided at 90% of previous 5-d average voluntary intake at 0800, with feed refusals from the previous day determined prior to feeding. Prior to feeding straw (0730), SBM and SBM/seed mix was provided (SBM: 10% and 7% of intake; seed 4.5% of intake on an as fed basis). As an indicator of fescue toxicosis, rectal temperatures and serum prolactin levels were measured. Rectal temperatures were taken daily just after feeding via a handheld digital thermometer with probe placed

approximately 3 cm into the rectum. Blood samples for prolactin analysis was collected prior to feeding on d 0, 2, and 28 of each feeding period, via jugular venipuncture with 10 ml vacutainers. Tubes were allowed to coagulate at room temperature then centrifuged. Serum was decanted and frozen at -20°C until prolactin analysis was performed. Serum prolactin was analyzed as described by Hockett et al. (2000) by the University of Tennessee. Rumen fluid was sampled on d 0, 3, and 28 of each period in a time course at 0 (prior to feeding), 6, and 12 h after feeding. Rumen fluid was collected with a rumen suction strainer, was place on ice immediately after collection, and transported to the lab for DNA extraction.

3.3.2 Pure culture growth and DNA extraction.

Pure cultures listed on Table 3-1, were part of the culture collection maintained in this lab or were obtained from New Mexico State University, Department of Animal and Range Science (Las Cruces, NM). Cultures were grown in 15 ml balch tubes with butyl rubber stoppers and 10 ml of media listed in Table 3-2. DNA was extracted from overnight cultures using Puregene kit (Gentra Systems, Minneapolis, MN.) following manufacture's protocol for Gram positive or Gram negative as appropriate. Bacteria strains and primers used in this study were chosen based on available primers that could be validated.

Table 3-1 Pure cultures and their reaction to primers used.

	Primer						
Bacteria strain	UN	PB	Strep	SR-MM	ER	RF	RA
M. elsdenii T-81	+	-	-	-	-	-	-
Suc. dextrisolvens	+	-	-	-	-	-	-
P. albensis	+	-	-	-	-	-	-
P. bryantii $B_1$4	+	+	-	-	-	-	-
E. rumintium GA 195	+	-	-	-	+	-	-
B. fibrosolvens D1	+	-	-	-	-	-	-
B. fibrosolvens 49	+	-	-	-	-	-	-
B. fibrosolvens nxy	+	-	-	-	-	-	-
Sel. rumintium HD4	+	-	-	+	-	-	-
Strep. bovis IFO 12057*	+	-	+	-	-	-	-
Strep. bovis JB1	+	-	+	-	-	-	-
Strep. caprinus 2.2	+	-	+	-	-	-	-
R. albus 7	+	-	-	-	-	-	+
R. albus 8*	+	-	-	-	-	-	+
R. flavefaciens C94	+	-	-	-	-	+	-
V. parvula	+	-	-	-	-	-	-
C. pasteurainum 5	+	-	-	-	-	-	-
L. vitulinus T185	+	-	-	-	-	-	-

+ indicates PCR product formation
- indicates no PCR product formation
* indicates strain used as standard for real-time PCR

3.3.3 Rumen fluid DNA extraction.

Several methods of DNA extraction were tested on both fresh and frozen rumen fluid (-20 °C) in this experiment prior to the collection of samples. The first method was a bead-beating method described by Whitford et al. [17], briefly 0.7 ml rumen fluid was mixed with equal amount of buffered phenol (buffered with 10mM Tris-HCL, pH 8.0) and 20ul of 20% SDS in a 2.0 ml tube containing 0.5 g of 0.1 mm glass beads. The tubes were shaken three times for 2 minutes on a Mini-Beater and placed on ice for at least 2 minutes between beating steps. Tubes were then centrifuged

and the aqueous phase was removed to a new tube and extracted with buffered phenol and ethanol precipitated. The second method was the Qiagen DNeasy kit (Qiagen, Valencia, CA.) according to manufactures direction. The third method was the Puregene kit (Gentra Systems, Minneapolis, MN.) and the body fluid protocol with the following modifications: 0.25ml rumen fluid as starting material centrifuged for 1 min and supernatant removed, 750ul of Cell Lysis Solution and 4.5ul of Proteinase K Solution was used to resuspend the bacteria pellet then incubated at 55°C for 1 hour. All reagents were scaled to correspond to the 750ul Cell Lysis Solution volume. DNA was hydrated with 200ul DNA Hydration Solution at room temperature overnight. The first method was found to be labor intensive and resulted in samples that had moderate DNA yield, fragmented DNA and protein contamination. The second method was easy to perform yet had the lowest DNA yield. The third method was relatively simple to perform and yielded the highest concentration of DNA without any indication of fragmented DNA or protein contamination. With all methods, fresh rumen fluid had a higher amount of DNA extracted than the frozen rumen fluid. Therefore the third method and fresh rumen fluid was used.

DNA was extracted from whole rumen fluid samples within 30 minutes of collection using Puregene (Gentra Systems, Minneapolis, MN.) kit modified as previously described. All DNA samples were visualized for purity on a

1% argarose gel stained with ethidium bromide under UV light and photographed. Genomic DNA was then stored at –20°C until analysis. DNA concentration was quantified using PicoGreen dsDNA quantitation kit (Molecular Probes, Eugene, OR.) following manufacture's protocol for a 96-well plate. Fluorescence was measured on a plate reader (manufacture). Manufacture supplied lambda dsDNA was used for creating the standard curve use for quantification.

Table 3-2 Complex medium with 40% clarified ruminal fluid (New Mexico State University Rumen Microbiology Laboratory Handbook).

Component	**Quantity per 1 L**
Trypticase	2.0 g
Yeast Extract	1.0 g
Cellobiose	4.0 g
Mineral Solution 1	25.0 ml
Mineral Solution 2	25.0 ml
VFA solution	10.0 ml
Clarified Rumen Fluid	400 ml
Resazurin (0.1%) solution	1.0 ml
Sodium Carbonate, Na_2CO_3	4.0 g
B-vitamins	5.0 ml
1.25% Cysteine sulfide	20.0 ml
Distilled water	519 ml

3.3.4 PCR conditions

Each primer set listed in Table 3-3 was optimized for annealing temperature and Mg concentration using a PTC-200 thermocycler (MJ Research) with 35 cycles of denaturation 95°C for 30 sec. annealing temperature using a gradient of temperatures (from 2 degrees above and below published temperature) for 30 sec. and extension 72°C for 30 sec. with an initial denaturation step at 95°C for 1 min and a final extension step of 72°C for 7 minutes. PCR products were visualized for size and specificity of primer on a 1% argarose gel stained with ethidium bromide under UV light and photographed using Kodak DS Electrophoresis Documentation and Analysis System 120 and Kodak 1D 3.6 software.

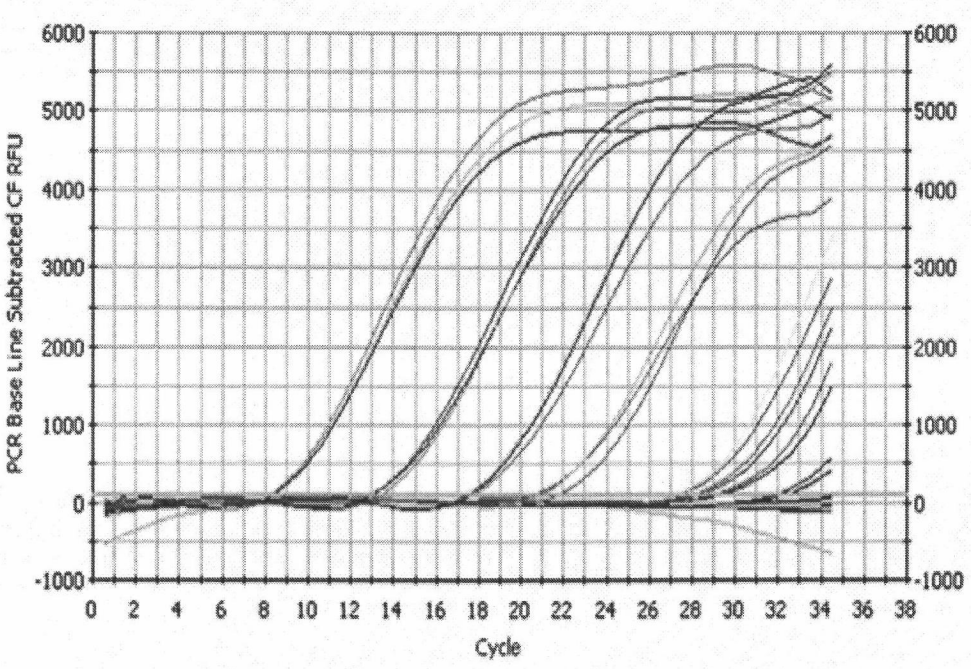


Figure 3-1 Real time PCR amplification graph of standards used. Base line, represented by the bar, was manually set for each reaction and used to calculate the threshold cycle.

Table 3-3 PCR Primers used in real-time PCR reactions

Primer name	Target Organism	Forward	Reverse	Annealing temperature (°C)	Reference
UN	D1(f), P2(r)[a]	AGAGTTTGATCCTGGCTCAG	ACGGCTACCTTGTTACGACTT	62	[16]
PB	*Prevotella. bryantii* B_14	ACTGCAGCGCGAACTGTCAGA	ACCTTACGGTGGCAGTGTCTC	68	[21]
Strep	*S. bovis, S. caprinus, S. equines*	CTTTCCACTCTCACACACG	AGAGTTTGATCCTGGCTCAG	60	[28]
SR-MM	*Selenomas ruminantium-Mitsuokella multiacida*	TGCTAATACCGAATGTTG	TCCTGCACTCAAGAAAGA	55	[21]
ER	*Eubacterium ruminatium*	GCTTCTGAAGAATCATTTGAAG	TCGTGCCTCAGTGTCAGTGT		[21]
RF	*Ruminococcus flavefaciens*	GGACGATAATGACGGTACTT	GCAATGYGAACTGGGACAAT	60	[21]
RA	*Ruminococcus albus*	CCCTAAAAGCAGTCTTAGTTCG	CCTCCTTGCGGTTAGAACA	55	[27]

[a] Universal bacterial 16S primer set

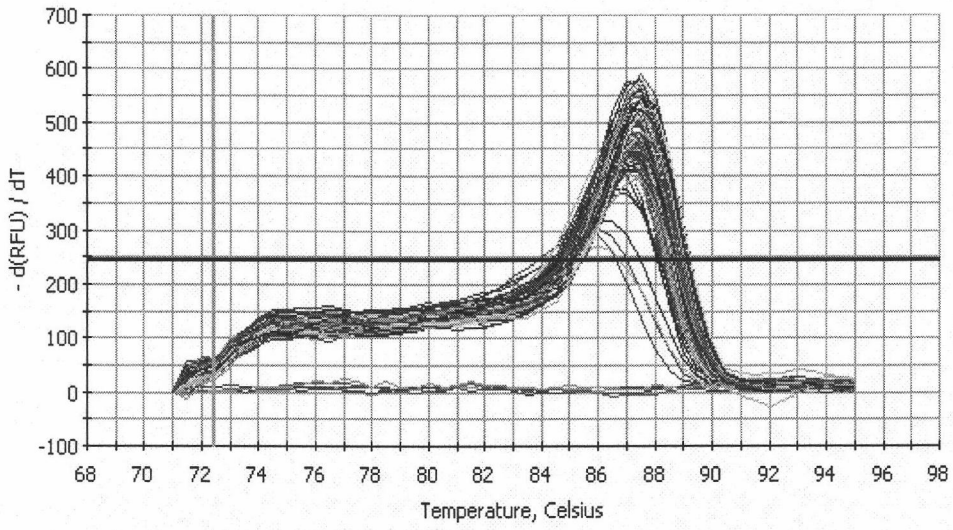


Figure 3-2 Typical melt curve analysis for realt-time PCR reactions showing only one product was formed during amplification.

3.3.5 Real-time PCR

Real time PCR reaction was carried out with SYBR Green using an iCYcler (Bio-rad) with the following conditions; 35 cycles (30 cycles for universal primer) of 95°C for 30 sec, annealing temperature for 30 sec, 72°C for 30 sec with an initial denaturating step of 5 min at 95°C and a final extension step for 7 min at 72°C. Threshold level was manually set for each run. Melt curve analysis was performed after each reaction to assure only one product was amplified during the reaction (Figure 3-2). Each reaction consisted of 30ng genomic DNA template, 200nM forward and reverse primer, 12.5ul SYBR Green Supermix (Bio-rad) and sterile water to a final volume of 25ul. Each sample and standard was ran in triplicate and the average of the three values was used as the sample value. Standards were PCR products generated using pure culture DNA which was amplified

in the PTC-200 thermocycler as described earlier except 30 cycles was used to reduce the possibility of nonspecific amplification and chimeric product formation. A standard curve amplification graph is shown in Figure 3-1. When more than one strain of bacteria that reacted to a primer was available, each bacteria strain was tested for amplification and upon verification that each strain was amplified at an equal rate one strain was arbitrarily chosen to act as the standard (Table 3-1). Products were visualized and quantified using Kodak DS Electrophoresis Documentation and Analysis System 120 and Kodak 1D 3.6 software. A dilution series ranging from 10 to 10^8 copies of the PCR product was used as the standard curve. An example of a standard curve graph is shown in Figure 3-13. All standard curves had an r ≥0.98 and a PCR efficiency of 90-100%.

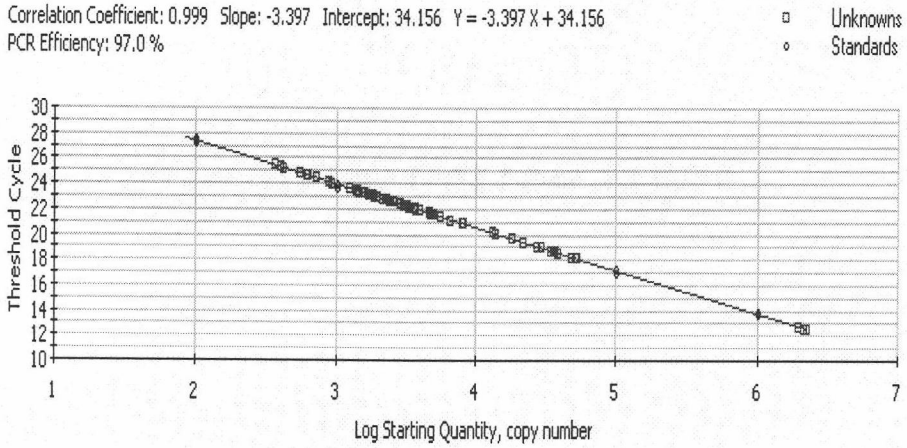


Figure 3-3 Typical standard curve graph for real-time PCR reactions.

3.4 Results and Discussion

Daily rectal temperatures were not influenced by alkaloid concentration (38.4°C E- vs. 38.4 °C E+, SE 0.0615; p = 0.40). Serum prolactin was decreased to 6.4 ng/ml for E+ from 22.9 ng/ml for E- treatment (SE 5.19; p = 0.023), indicating subclinical fescue toxicosis.

Lack of information on the genomic characteristics of ruminal bacteria makes it impossible to infer cell number from the number of 16S rDNA found in a sample. Different bacteria are known to have different numbers of the rRNA gene, occasionally with different sequences within the genome [23]. Also it is unlikely that every organism in the rumen has had its 16S gene sequenced leading to the possibility that a primer set designed for a specific group of bacteria would amplify a sequence of a unrelated organism. Therefore, caution should be used in interpreting the concentration of 16S sequences within a sample and the phrase 'cell number' should be avoided.

Within individual animals there was a significant response of bacteria populations to the diet, but overall there was no difference between diets. A period effect was detected for most primers used, indicating change in microbial populations due to adaptation to the fescue straw. *Prevotella bryantii* B_14 was detected in low levels through the entire feeding period and levels were approximately the same (95% E- and 89% E+ of day 0 on day 28) from day 0 to 28 but no difference was detected between treatment

groups (90% E- vs. 93% E+; p = 0.45). *Prevotella bryantii* is a Gram negative bacteria belonging to the most commonly genus isolated from the rumen of cattle. It is unable to break down plant cell walls in pure culture and is believed to utilize cell wall components liberated by other bacteria for growth. It also has protolytic ability and is believed to play a role in protein degradation in the rumen. In cloning and sequencing experiments the Prevotella group is the most frequently identified bacteria. Whitford et al. [17] showed a preferential amplification of Prevotella sequences by PCR when cycle number was increased from 12 to 30, indicating that this group of bacteria and its impact on rumen diversity might be over estimated in the rumen.

The Streptococcus group (*S. bovis, S. caprinus, S. equines)* was detected in low levels through the entire feeding period and E+ treatment tended to lower the concentration of 16S gene but no statistical difference was detected between treatment groups (89% E- vs. 94% E+; p = 0.39). *Streptococcus bovis* is a Gram positive believe to involved with the development of acidosis in animals on a high concentrate diet. Its preferred substrate for growth is starch; however it can grow on cellodextrins and can persist in the rumen of animals consuming a forage diet. Not much is known about *Streptococcus caprinus.* Only that it is tannin resistant bacteria isolated from the rumen of goats.

Selenomas ruminantium- Mitsuokella multiacida JCM6582 was the most abundant organism found in the samples and levels were approximately the same (97% E- and 105% E+ of day 0 on day 28) from day 0 to 28 but no difference was detected between treatment groups (92% E- vs. 106% E+; $p = 0.52$). *Selenomonas ruminantium* is a Gram negative curved rod detected in the highest number in the rumen of animals on a grain diet. Unable to degrade pectin and xylan but can use the breakdown products of these polymers. These bacteria are considered to be an important acid-tolerant, lactic acid utilizing bacteria in high concentrate diets. On a high forage diet these bacteria would be expected to use the breakdown products of plant cell wall degradation by other bacteria. Not much is known about *Mitsuokella multiacidus* except that it is a Gram negative bacteria, closely related to *Selenomonas ruminantium. M. multiacidus* produces lactate as the major fermentation product. The prevalence of the species of bacteria in the rumen is unknown.

Eubacterium ruminatium (ATCC 17233) was undetectable in most samples over all periods and was not affected by day or treatment. *Eubacterium ruminantium* is a Gram positive bacterium which has been found to represent about 5% of cultivable bacteria isolated from bovine rumens [25]. It is another second line degrader that grows on substrates that are by-products of the breakdown of plants, such as cellobiose, glucose, and fructose but can not grow on cellulose or starch.

Ruminococcus flavefaciens (ATCC 19208T) sequence was detected in moderate levels through the entire feeding period and levels were approximately the same (97% E- and 99% E+ of day 0 on day 28) from day 0 to 28 but no difference was detected between treatment groups (91% E- vs. 92% E+; $p = 0.28$). *Ruminococcus flavefaciens* is a Gram positive bacteria being one of the most active species involved in fiber digestion in the rumen. It is known to be able to degrade recalcitrant forms of cellulose, such as dewaxed cotton fiber [26].

Ruminococcus albus was detected in low levels through the entire feeding period and levels were approximately the same (95% E- and 83% E+ of day 0 on day 28) from day 0 to 28 but no difference was detected between treatment groups (87% E- vs. 89% E+; $p = 0.33$). *Ruminococcus albus* is closely related to *Ruminococcus flavefaciens*, and is also highly active in the breakdown of plant fiber. More numerous than *Ruminococcus flavefaciens* in the rumen, some strains have shown inhibitory effects against *Ruminococcus flavefaciens* by producing bacteriocin. However, Koike et al. [27] found *R. albus* at levels of 10^5 cells/ml while *R. Flavefaciens* to be detected at 10^7 cells/ml of rumen fluid from sheep consuming orchard grass.

All primer sets, except the universal, exhibited an animal effect (Table 3), which helps contribute to the huge standard deviations observed. The animal to animal variation

Table 3-4 Percent change of select organism 16S rDNA copy number per gram of rumen fluid DNA in response to endophyte-infected tall fescue.

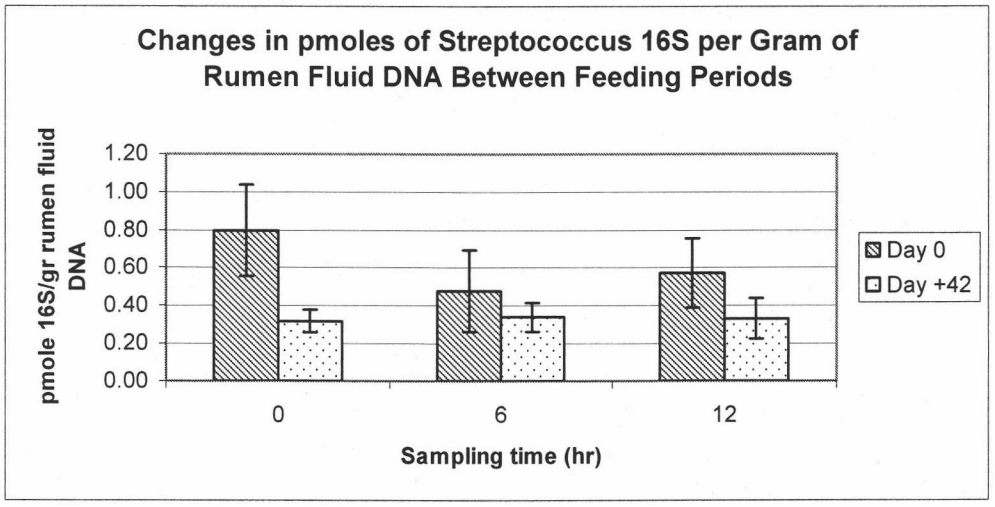
Target Organism	Percent Change	SD	Trt.	Animal	Period	Animal:Trt
D1(f), P2(r)	114	58				
Prevotella. bryantii B_14	92	43	0.45	0.027	<0.0001	0.047
S. bovis, S. caprinus, S. equines	91	45	0.39	0.014	<0.0001	0.040
Selenomas ruminantium-Mitsuokella multiacida	98	45	0.52	0.0026	<0.0001	0.11
Eubacterium ruminatium	ND[a]	NA				
Ruminococcus flavefaciens	92	45	0.28	0.018	<0.0001	0.043
Ruminococcus albus	88	41	0.33	0.0097	<0.0001	0.14

[a] Not detected

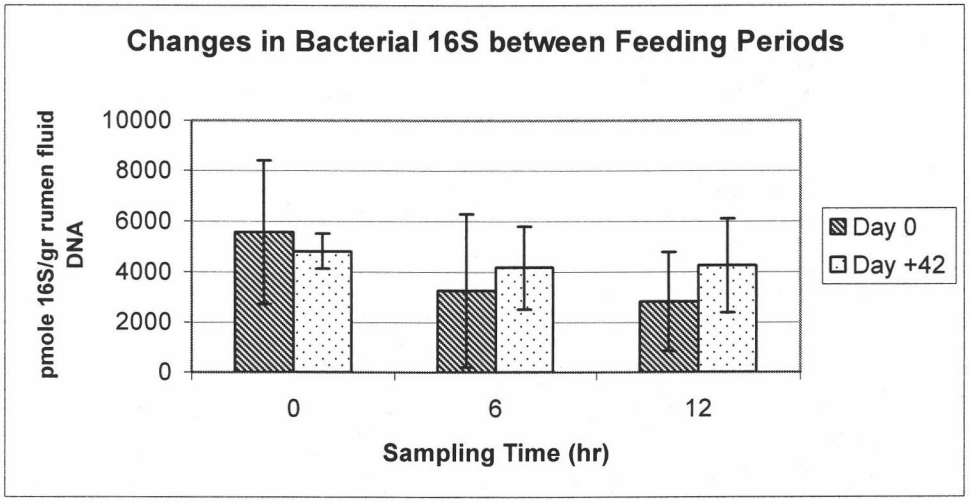
A strong period effect was detected for all primer sets except the universal primer set (Table 3-4). When samples from the first day of each period was examined a decrease in 16S copy number occurred at the time 0 sampling point between periods. At time 0 in the second period the concentration of 16S of each individual primer set was approximately 40% of the concentration found in the time 0 of the first period ($p < 0.0001$). This difference was not detectable at 6 hours or 12 hours, leading to the assumption that the observed change in microbial populations is in response to adaptation to the diet (an example is shown in Figure 3-1a).

The universal primer set did not exhibit this kind of change in concentration (Figure 3-1b), leading to the assumption that another group of bacteria increased in response to the diet.

Figure 3-4 Changes in 16S copy number between feeding periods for (a) *Streptococcus* which is typical of the period response observed in other primer sets and (b) universal primer set.



a



b

Only a small percentage of the bacterial 16S present in the samples were identified using the current primers. For most time points only 2% (ranged from 1-3%) of the 16S gene amplified with the general primer was

represented by the organism used in this study (data not shown). This is consistent with results by Reilly et al. [28] where total bacteria population in the rumen was estimated to be between 1.6 E 10 and 2.0 E 10 and individual strains of bacteria were 10^6-10^7 (*P. bryantii*) and 10^8 (Streptococcus species) in the rumen of dairy cattle. These numbers represent 0.02-0.20% (*P. bryantii)* and 1.0% (Streptococcus species) of total bacteria present. Attwood and Klieve[29] found similar numbers of *Streptococcus bovis* in the rumen of dairy cattle. This demonstrates that the majority of bacteria in the rumen are unidentified and uncharacterized leaving the potential for novel bacteria to be identified that are capable of degrading toxins.

Bacteria that degrade toxins often are present in the rumen in very small quantities. Wachenheim et al. [4] found a consortium of bacteria capable of degrading pyrrolizine alkaloids that exists in the rumen at low concentrations (< 0.10% of total bacteria). It is possible that the theorized ergot alkaloid degrading bacteria is a minor bacteria species that has yet to be identified and cultured.

Another possible explanation of the lack of a treatment response is the level of alkaloid ingested by the animals was not high enough to elicit a population shift. Tor-Agbidye [1] found the level of fescue toxicosis in sheep to be 500-800 ppb ergovaline. This study was at the lower level of

this range and as shown by the rectal temperatures clinical fescue toxicosis was not induced.

This is the first step in process to determine the bacteria or consortium of bacteria responsible for the breakdown and metabolism of ergot alkaloids. Further work in the characterization of the rumen ecosystem and its changes in response to endophyte-infected feed needs to be done in order for the detoxifying bacteria to be identified. This work could include a combination of DGGE (to isolate novel sequences), sequencing, novel primer design, and real-time PCR to track populations.

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