

## ABSTRACT

**VIBART, R. E. Continuous Culture Fermentation of Wild-Type Endophyte-Infected, Endophyte-Free, and Non-Toxic Endophyte-Infected (MaxQ™) Tall Fescue Supplemented at Four Energy Levels. (Under the direction of Dr. Steven P. Washburn).**

Eight dual-flow continuous culture vessels (700 ml) were used to compare in vitro effects of wild-type, endophyte-infected (E+), endophyte-free (E-), and non-toxic, endophyte-infected (EN) (MaxQ™) Jessup tall fescue on ruminal fermentation in a grazing simulation at four levels of concentrate supplementation (ground corn). For each of the fescues (E+, E-, and EN), forage to concentrate ratios of 100:0, 85:15, 70:30, and 55:45 were used for a total of 12 experimental diets in a randomized incomplete block design with two replicates. Vegetative grasses were used with compositions as follows: E+ (12.3 % CP, 59.9 % NDF, 29.2 % ADF); E- (13.4 % CP, 60.7 % NDF, 29.4 % ADF); and EN (10.4 % CP, 63.2 % NDF, 31.4 % ADF).

Ruminal cultures were adapted for 48 h before experimental diets were fed and then gradually adjusted to the final diets. Each culture vessel was offered a total of 15 g of DM per day including four equal portions of grass (fed at 0300, 0900, 1500, and 2100 h); and two equal portions of corn (fed at 0900 and 2100 h). Ruminal fluid passage rate was set at 6.25% per h. Headspace gas and liquid samples were analyzed for methane (CH<sub>4</sub>), ruminal culture pH, volatile fatty acids (VFA), and ammonia N (NH<sub>3</sub> N) production to assess the ruminal environment from the pasture-based diets.

Forages had no effect on molar proportion or total VFA production (58.8 mmoles/d). Methane production (24.7 mmoles/d) and ruminal culture pH (6.11) also remained unaffected by forages. Ammonia N output (g/d) varied by grass: EN had lower ( $P < 0.05$ ) values

compared to those of E+ and E-.

Increasing the level of grain linearly decreased ( $P < 0.05$ ) ruminal culture pH, ammonia N, acetate production, and the acetate to propionate ratio, whereas propionate and butyrate production increased ( $P < 0.1$ ) with higher grain supplementation. Overall, ruminal fermentation was minimally altered by the presence or absence of the endophyte. However, forage by grain interactions for methane and ammonia N production were reported. The grain-induced culture pH drop for the highest level of grain (45%) changed the methane production pattern for all three grasses. At that supplementation level, EN was the most energetically efficient forage.

**CONTINUOUS CULTURE FERMENTATION OF WILD-TYPE ENDOPHYTE-  
INFECTED, ENDOPHYTE-FREE, AND NON-TOXIC ENDOPHYTE-INFECTED  
(MaxQ™) TALL FESCUE SUPPLEMENTED AT FOUR ENERGY LEVELS**

by

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## DEDICATION

To Dick, Hazel, Dickie, Andrea and Celina, with love and gratitude

## BIOGRAPHY

Ronaldo Eduardo Vibart was born March 28, 1964 in the city of Buenos Aires, Argentina, the middle son of Richard P. Vibart and Rose M. Millar. He grew up in a farming environment before moving to the beautiful city of Tandil, located in the Southeast of the Province of Buenos Aires.

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A Rotary scholarship allowed him to visit the US for the first time in 1993, touring the State of Wisconsin for five weeks and getting in touch with America's finest dairy tradition. Previously, a four-month working tour to the North Island of New Zealand allowed him to refine his rugby skills and closely interact with the most efficient forage-based dairy agriculture in the world.

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## INTRODUCTION

With over 20 million ha in production, tall fescue (*Festuca arundinacea*) is probably the most common grass component of US pastures (Parish et al., 2003b). The excellent agronomic performance of this cool-season grass has been vastly reported (Bacon and Siegel, 1988; Arachevaleta et al., 1989; Hill et al., 1991; Bacon, 1995). However, impaired animal gain and reproduction has also been reported consistently (Bacon et al., 1986; Crawford et al., 1989; Hoveland, 1993; Thompson and Stuedemann, 1993; Porter, 1995) constituting an ever increasing concern for the livestock industry. The presence of an alkaloid-producing, endophytic fungus (*Neotyphodium coenophyalum*) that resides within the plant in a mutually-beneficial fashion has been associated to both, the desirable agronomic traits and the undesirable animal responses.

Shortly after the grass/endophyte interaction was established, extensive research was placed on fungus removal. As a consequence, animal performance on E- fescues was re-established, but the plant survival characteristics were lost (Ball et al., 2002). The discovery of several endophyte strains that produced only the desirable alkaloids responsible for enhancing hardiness, but not responsible for causing fescue toxicity led to the discovery and release of new, non-toxic, endophyte-infected tall fescue cultivars (Tapper and Latch, 1999; Bouton et al., 2000).

Grazing studies with lactating dairy cattle fed high quality forages have frequently reported that freshly-grazed forage promotes high levels of NH<sub>3</sub> accumulation in the rumen (Reis and Combs, 2000; Soriano et al., 2000), and these levels may exceed microbial needs (Satter and Slyter, 1974). Notorious reductions in N waste can be achieved by adding energy-dense supplements to these forage-based diets. Consequently, lower ruminal NH<sub>3</sub> levels,

higher non-ammonia N (NAN) flow to the duodenum, and improved animal response occur (Kellaway and Porta, 1993; Soder and Rotz, 2001). In addition, fresh forage diets supplemented with grain typically lower CH<sub>4</sub> production, the acetate to propionate ratio, and ruminal culture pH, and improve energy capture and utilization, as compared to unsupplemented diets (Russell, 1998).

It has long been suggested that in order to maximize nutrient utilization, the supplemented carbohydrate source and the N source should have a similar rate and extent of ruminal degradation and utilization (Henning et al., 1993; Huntington, 1997; Dewhurst et al., 2000). However, matching the supply of energy and protein in a synchronized fashion has been extremely difficult to accomplish in grazing situations, and research reports have been somewhat conflicting (Kolver et al., 1998).

Under grazing conditions, forage is usually fed separately from concentrates, altering diurnal pH and acid fermentation patterns (NRC, 2001). The provision of a moderate but readily degradable protein source such as a cool-season, vegetative forage and the most common energy supplement utilized in livestock grazing systems throughout the US, dry corn, were fed in vitro in a systematic fashion in an attempt to partially address this issue. The purpose of our study was to investigate ruminal fermentation characteristics (ruminal culture pH and methane, ammonia, and volatile fatty acid production) in continuous cultures fed diets based on tall fescues with different endophyte infection status that remained unsupplemented or were supplemented at three energy levels.

## REVIEW OF LITERATURE

### Grasslands and Sustainable Agriculture

Sustainable agriculture has been thoroughly discussed in recent years, and renewed interest in livestock intensive grazing systems has been a consequence of such discussion. Forages are often referred to as the backbone of sustainable agriculture, and their contribution to the world's economy, in terms of animal products, has been extensively addressed. Grasslands account for over 30% of the global land area, and in the US they cover approximately half a billion hectares (Barnes et al., 1995), more than half its land surface. In addition to providing the vast majority of nutrients for wild and domestic animals, it also contributes by means of soil structure, fertility and erosion control, water improvement and conservation, recreation and entertainment, among numerous other overall benefits.

Along with the importance of forages in livestock production, the discovery that certain grasses were toxic to livestock and humans has been recognized since early stages of civilization (Bacon, 1995).

### Origin, Introduction, and Adaptation of Tall Fescue

Spread over 20 million ha, Tall fescue (*Festuca arundinacea* Schreb.) is the most widely grown perennial cool-season pasture grass in eastern and northwestern US (Buckner et al, 1979; Hoveland, 1993). It was originally introduced from Europe, although the time of introduction is not clearly known (Terrell, 1979). One Isaac Martindale from Camden, NJ, owned the earliest collection reported by Terrell; although it was not until 1950 that tall fescue was given the name *Festuca arundinacea*. By 1890, numerous collections were reported from a number of locations throughout the US. The Agricultural Experiment Stations of Utah and Kentucky, and the Bureau of Plant Industry in Washington, DC,

conducted the earliest performance tests in late 1800's.

In 1931 Dr. E. N. Fergus, from the University of Kentucky, observed a particular tall fescue ecotype growing on the rolling landscape of W. M. Suiter's farm in Menifee county, KY, and twelve years later the tall fescue cultivar "Kentucky 31" (**KY-31**) was released. Intensively promoted by W. C. Johnstone, agronomist and extension specialist of the University of Kentucky, it became the most popular tall fescue variety, being massively adopted by farmers during the 40's and 50's, supplying for the bulk of the acreage in humid areas of eastern US. Based on winter survival in northwestern US, the Oregon Agricultural Experiment Station, in cooperation with the USDA-ARS, released the cultivar "Alta" in 1940. By this same year, it was estimated that over 160,000 ha of tall fescue was grown in the US (Barnes, 1990).

Its popularity is explained by a number of reasons such as establishment ease; wide adaptation to soil and climate (including periodical drought and flooding conditions); turf grass appearance; nutritive value; tolerance to intensive use; relatively long grazing periods; pest (insect, rust, and nematode) resistance; soil-conserving quality; and seed production (Buckner et al., 1979; Hoveland, 1993; Roberts, 2000).

Throughout the world, the boundaries of tall fescue are imposed by climatic, edaphic, and geographic factors, such as rainfall, temperature, soil texture and moisture, latitude and elevation (Burns and Chamblee, 1979). Widely adapted to Western Europe and scattered humid areas in the northwest and northeast of Africa, it has extended to Eastern Europe, Russia and China. Tall fescue has also been introduced to Japan, Australia, New Zealand, South and East Africa, and North and South America (Burns and Chamblee, 1979). Although best adapted to the transition zone between cool and warm humid areas of the US, tall fescue

is grown under a diverse array of conditions such as northern Florida and southern Canada. Such diversity includes a soil pH gradient of 4.7 to 9.5, and a wide range of soil types, from heavy, poorly drained to droughty, thin-coated soils, where most cool season grasses would not survive. Tall fescue is suitably adapted to North Carolina throughout its three altitudinal subregions (Coast, Piedmont, and Mountains).

### **Plant Description**

Tall fescue is a leafy, wind-pollinated cool-season perennial bunchgrass; although it can produce a thick and even stand when kept grazed or mowed with frequency. Leaf sheaths are smooth, with membranous ligules (up to 2 mm long) and ciliated auricles, which with frequency are reported to be absent. It has numerous heavily nerved, rough to the touch, ribbed green leaves; with leaf blades usually 4 to 6 mm wide and up to 60 cm long (Terrell, 1979). Although early reports describe tall fescue as rhizome-free, short rhizomes are grown in most, but not all plants, inferring that rhizome morphology is dictated by genetic variation (Jernstedt and Bouton, 1985). Longer and thicker rhizomes allow for an improved sward cover, persistence, and plant survival, as suggested by Barnes (1990) and De Battista and Bouton (1990).

Tall fescue reproduces by seed and through tillering. Loosely branched panicles are 10 to 50 cm long, with elliptical spikelets, each with 3 to 10 florets. Reproductive culms possess a large, central cavity with a peculiar disposition of vascular tissue, characteristic of the festucoid family: vascular bundles in three circles and a clearly defined peripheral sclerenchyma wall. The reproductive stage is initiated after vernalization followed by a period of increasingly longer days. Therefore, anthesis is reached in May to early June in most fescue-growing regions of the US. At a same planting location, and in a sequence of

cool-season grasses order, it usually flowers after orchardgrass (*Dactylis glomerata* L.) and before smooth brome grass (*Bromus inermis* L.).

Two of tall fescue progenitors, Meadow fescue (*F. pratensis* Huds.), with a chromosomic conformation of  $2n = 2x = 14$ ; and *F. arundinacea* var. *glaucocescens* Boiss. ( $2n = 4x = 28$ ) are believed to be the ancestor lines in the creation of the hexaploid number of chromosomes ( $2n = 6x = 42$ ) in tall fescue (Terrell, 1979; Jauhar, 1993). Polyploidy adds, at least partially, to the adaptive potential and survivability of tall fescue stands (Berg et al., 1979).

There is a close relationship between the *Festuca* and *Lolium* genera. In previous work, Jauhar (1975) speculated that one of the three related genomes constituents of the allohexaploidy in tall fescue originated from a progenitor of one of the contemporary *Lolium* species. Also, it has been hypothesized that a spike-shaped inflorescence characteristic of the *Loliums* was once derived from a panicle-shaped inflorescence of the *Festucas* by a single mutational event (Jauhar, 1993). Furthermore, the continuous gene exchange among both taxa and the minimal structural chromosomal differentiation support the idea of the close relatedness between both genera (Jauhar, 1993; Barnes et al, 1995).

### **Early Events in the Discovery of the Endophyte**

Tall fescue has had an extensive acceptance due to its grazing persistence, competitiveness, seed production, yield, and summer survival. These characteristics have been linked to the presence of a fungal, ergot alkaloid-producing endophyte (*Neotyphodium coenophialum* (Morgan-Jones & Gams) Glen, Bacon & Hanlin, that resides within the fescue plant.

Initially identified as *Epichloe thyphina* (Fries), and later renamed *Acremonium*

*coenophialum* (Morgan-Jones & Gams), the terms fescue endophyte or fescue fungal endophyte refer to a mutual-benefit relationship, where a plant (fungus) lives within another plant (grass host). This relationship is often referred to as a mutualistic symbiosis, the host providing for nutrition, protection, and a place for dissemination, and receiving in exchange a number of competitive advantages from the endophyte's presence.

This association was reported 70 years ago by K. Sampson, and later confirmed in perennial ryegrass by Neill (1941), in New Zealand, who successfully cultured endophytes from both, tall fescue and perennial ryegrass (*Lolium perenne*). But it wasn't till 1977 that the major breakthrough in the fungal endophyte-grass interaction was established. Two herds grazing separate fescue pastures in the vicinity of Mansfield, Georgia, was the incidental setup for the discovery, with only one of the herds exhibiting fescue toxicity symptoms. In 1973, researchers from the USDA Research Center in Athens, GA J. D. Robbins, C. W. Bacon, and J. K. Porter initiated the process in search for a better understanding of this matter, and three years later, the association was established: the tall fescue pasture exhibiting toxicity was completely infected with intercellular fungi of aerial host tissue from the tribe Balansiae, as opposed to only a 10% infection in the non-toxic pasture (Bacon et al., 1977).

Following, three fungus species taxonomically related to the Clavicipitaceae family were isolated from the toxic tall fescue pasture: *Balansia epichloe* (Weese) Diehl; *B. henningsiana* Moell; and *Myriogensopora atramentosa* Diehl, all three with systemic mycelium running parallel with the long axis of the host cells, and showing no signs of virulence to their grass hosts. Posterior toxicological studies conducted by J. K. Porter demonstrated that these fungi were capable of synthesizing ergot alkaloids (Bacon et al.,

1977).

The association of the endophyte with decreased livestock performance was established in a 4-year grazing study conducted by Hoveland et al. (1983) at Auburn University, confirming previous findings by Bacon and his group. Average daily gains (**ADG**) of steers grazing endophyte-free (**E-**) paddocks (believed to be originated from old fescue seed where the fungus vanished before planting) was 82% higher as compared with gains from a highly infected fescue (0.83 vs 0.45 kg). Increased rectal temperatures and rougher hair coats in steers grazing the infected fescue were also reported (Hoveland et al., 1983).

Unfortunately, the desirable agronomic traits reported earlier in this paper from the fungus-host association are not without their shortcomings. A long sequence of events followed these initial discoveries, in the aid of understanding a large-scale livestock dysfunction centered on infected (**E+**) tall fescue that has been reported vastly in the past 60 years. It has been estimated that over \$ 600 million per year are lost by US beef farmers due to fescue toxicosis (Hoveland, 1993).

### **Incidence and Distribution of the Endophyte**

In the US, over 90% of the tall fescue stands tested are **E+**. Surveys in several fescue-growing states have reported a high fungus infestation level, in a range from 60 to over 80% (Ball et al., 2002). These **E+** levels may be partially attributed to the widespread popularity of the variety KY-31 and its high endophyte infection in origin. An extensive survey conducted by Shelby and Dalrymple (1987), with samples from 26 states, confirmed the heavily infected nature of the cultivar.

Other causes of infection spread are the use of recently harvested seed, the

introduction of infected European ecotypes used in breeding programs, and the perennial nature of fescue pastures (Bacon et al., 1986). Also, it has been reported that 15 fescue species other than tall fescue are infected in the US, spreading the infection to all continental states (Bacon et al., 1986).

The geographic distribution of *N. coenophialum* in fescue populations suggests that no edaphic, climatic or even genetic restriction limits its growth (Shelby and Dalrymple, 1987). Presumed E- tall fescue pastures averaged a 4% annual growth in their levels of endophyte over a period of 9 to 12 years (Shelby and Dalrymple, 1993). The authors hypothesized that this change in endophyte levels was attributable to enhanced competition skills and survival of E+ plants in mixed pastures. Most herbicide treatments are ineffective in killing old stands of E+ fescue, mainly due to the adaptive mechanism of regrowth from rhizomes providing for a significant source of reinfestation in new E- stands (Defelice and Henning, 1990).

### **Toxic Agents and Quantification**

Much of the recent research on tall fescue has focused on isolation of the toxin(s) responsible for poor animal performance. Classified in seven different groups, endophyte-related alkaloids have been implicated in fescue toxicosis, and this matter has been thoroughly studied. However, an extensive body of research has focused on only three of these seven groups as possible toxic agents.

The alkaloid perloine, member of the diazaphenanthrene group, was initially suspected to be the cause of the syndrome, because its concentrations in the plant followed an inverse relationship to that of animal performance (Gentry et al., 1968), and decreased in vitro VFA production (Bush et al., 1972), and protein (Boling et al. 1975) and cellulose

(Bush et al., 1970; Bush et al., 1972; Boling et al. 1975) digestibility had been reported; but this hypothesis was excluded after conclusive data was presented showing decreased animal performance with a low-perloline fescue, as compared with a high-perloline cultivar (Hemken et al., 1979). Other anti-quality factors had to be involved, and this was later confirmed by Strahan et al. (1987) that reported similar concentrations of perloline in KY-31 stands with high and low endophyte infection levels (256 and 331  $\mu\text{g/g}$  of perloline respectively).

Following the initial discovery of the endophyte by Bacon et al. (1979), a second group of alkaloids, the pyrrolizidines, with N-formyl (**FL**) and N-acetyl (**AL**) lolines as the major alkaloids, received a great deal of attention. Both compounds are saturated pyrrolizidines only found in E+ fescues (Bush and Burrus, 1988). Associated to a number of endophyte-related pathologies, Hayek et al. (1991) reported that FL and AL had inhibitive effects on mitogenic growth of blood lymphocytes. However, an extensive review by Cheeke (1988) revealed that the lack of a 1,2 double bond and a branched side-chain in this group of alkaloids make them relatively weak in terms of liver damage, as compared with pyrrolizidine extractions from *Senecio* and other hepatotoxic species. Also, these compounds have been more strongly linked to insect-deterrence (Siegel et al., 1985) than to causing animal toxicity. Interestingly, the toxic effects of this group of alkaloids seem to be potentiated when ergot alkaloids are present (Porter, 1995).

The third group suspected to be the causal agents of fescue toxicosis is the ergot alkaloid group. Produced primarily by *Claviceps spp.*, the ergot alkaloids are divided into five classes, but only four of these have been isolated from *N. coenophialum*-infected tall fescues: ergopeptines, lysergic acid, lysergic acid amides (**LAA** = ergines), and clavines

(chanoclavine, penniclavine, elymoclavine, and agroclavine) (Porter, 1995; Hill et al., 2001).

Ergovaline (**EV**) is the most abundant ergopeptine alkaloid, accounting for almost 80% of this class and ranging from 0.1 to 6  $\mu\text{g/g}$  in E+ stands (Porter, 1995). It is suspected to be the primary agent in fescue toxicosis. Lysergic acid amides contents of tall fescue have been reported to approach 45% of that of ergovaline (Oliver et al., 1993), although it has also been reported that LAA can coexist in similar concentrations to EV in E+ tall fescues (Shelby, unpublished). The clavine alkaloids are considered precursors in the biosynthesis of the other two classes isolated from E+ fescue (Porter, 1995).

Strickland et al. (1992), after extensive work with rat anterior pituitaries, suggested that the agents causing reduced serum prolactin in cattle were the ergopeptine group of alkaloids, represented by  $\alpha$ -ergocryptine. Aldrich et al. (1993) reported similar findings, but attributed this effect to EV, a member of the same group of alkaloids, in beef cattle fed diets with 20% DM of an E+ KY-31. Treatment of the cranial branch of the bovine lateral saphenous vein by Oliver et al. (1993) with LAA resulted in vasoconstriction.

In previous studies, the vasculature constriction was attributed to the presence of the pyrrolizidine alkaloid AL (Oliver et al., 1990). Later work revealed ergonovine (a simple LAA-type alkaloid) and ergotamine, an ergopeptide alkaloid, were responsible for the same vasoconstrictive mechanism (Oliver et al., 1991). The ergot alkaloids are capable of exhibiting dopamine (**DA**)-like activity within physiological toxic ranges. Ergotamine, a member of the ergopeptine group, was shown to have  $\alpha$ -adrenergic activity (Browning et al., 2000), and both, ergopeptines and LAA have been reported to bind and agonize with the D<sub>2</sub> DA receptor system (Larson et al., 1999).

Among the ergot alkaloids, lysergic acid exhibited the highest transport potential

across the ruminal wall, suggesting a stronger toxicosis causal effect from this compound (Hill et al., 2001). However, endophyte infection frequency of tillers and EV concentration in E+ grasses continue to be the most broadly accepted measure used in assessing fescue toxicity (Rottinghaus et al., 1991; Porter and Thompson, 1992; Agee and Hill, 1994). In cattle stressed by heat, the minimum level of EV reported to be toxic from grazing E+ tall fescue stands has been 50 ng/g of grass (Porter, 1995), and signs of toxicosis have been observed in grazing trials with infestation levels as low as 20% E+ (Marsalis et al., 2000).

### **Endophyte-related Disorders**

Poor animal performance has been consistently reported from herds feeding on tall fescue, and this reputation is in contrast with forage quality data from extensive research trials gathered throughout the US and worldwide. The presence of the endophyte affects grazing livestock in a number of ways. Syndromes in livestock from E+ tall fescue often fall in one of the following three groups: fescue foot; bovine fat necrosis; and summer syndrome.

Relatively infrequent, although clinically visible, fescue foot is usually confined to the upper north-growing region or during winter months in the southern region. The presence of a red line (hyperemia) at the coronary band of the hoof is the first manifestation associated to this disease (Bush et al., 1979). Clinical signs include elevated respiration rate, soreness, swelling, lameness, and altered hoof growth. Vasoconstriction at the extremities, which derives in local death and tenderness of soft tissues due to loss of blood supply, cause a gangrenous condition leading to the partial or complete loss of hooves, ears and tails (Bush et al., 1979; Ball et al., 2002).

The presence of hard, necrotic masses of fat in adipose tissue of the abdominal cavity that lead to gastrointestinal disorder, kidney failure, and difficult births, are all characteristic

of bovine fat necrosis. Stuedemann et al. (1975) established a clear link between the incidence of the disease and cattle grazing pure stand of infected tall fescue, heavily fertilized with N from either broiler litter or mineral sources.

Reduced intake, animal gains, conception rates, and tolerance to heat, and increased body temperature and salivation, are signs of fescue toxicity or summer syndrome, although the most visible sign in cattle is the lack of a slick hair coat due to the failure in shedding their winter coat. Fescue toxicity is the most common and costly syndrome associated to the presence of the endophyte (Hoveland, 1993; Ball et al., 2002).

### **Animal Response**

Comparative analysis from studies with animals fed high-endophyte (**HE**) as opposed to feeding low-endophyte (**LE**) or E-, tall fescues have consistently shown a combination of the following production, behavioral, physiological and appearance responses: decreased intake, weight gains, milk production, time spent grazing, blood serum prolactin levels, and overall reproductive function; increased respiration rates, body temperatures, salivation, time spent near or in the water and shade areas; and a rough, curly aspect of their hair coat, frequently covered with mud and manure.

### *Grazing Patterns and Behavior*

Altered ingestive behavior and grazing patterns of cattle grazing E+ tall fescue have been reported in the past with frequency (Hoveland et al. 1983; Bond et al., 1984; Coffey et al., 1988; Peters et al., 1989; Howard et al. 1992; Peters et al, 1992; Seman et al., 1999). Although most of these studies suggest, or even report decreased voluntary intake, some studies lack a consistent intake response that could be linked to endophyte infection (Howard et al., 1992; Peters et al, 1992; Beconi et al., 1995).

In contrast to data reported by Bond et al. (1984), where steers that grazed E+ fescues experienced a reduction in total grazing time, Stuedemann et al. (1985) noted a shorter grazing time between 1200 and 1800 h only, but total time spent grazing was not affected. Similarly, in an experiment with steers grazing E+ KY-31 (65% infection), or E- KY-31 fescues, Coffey et al. (1988) observed that forage endophyte status did not affect total grazing time, but E+ grazers exhibited more nighttime grazing ( $P < 0.01$ ) than the E- group.

In a 2-yr study conducted by Peters et al. (1992), voluntary OM intakes from an E- (Mozark) and a HE (KY-31) variety for the months of June and August showed the following: Intake did not differ for 3 out of the 4 periods (June 1988 and 1989, and August 1989). This was not the case for August 1988, where OM intake was higher ( $P < 0.05$ ) for the E- variety, and the environmental temperatures consistently exceeded 32° C. Such evidence suggests that high ambient temperatures have a negative impact on forage intake (and gain) by cattle consuming E+ tall fescue, and this behavior has been well documented over time.

Steers grazing a LE cultivar (Johnstone, < 1% E+) spent 65 more minutes grazing during daytime ( $P < 0.01$ ) compared with HE KY-31 (60% E+). However, voluntary intake of OM was not different among both groups across four periods of time (from late May to mid September) studied (Howard et al., 1992). Similar findings were reported by Seman et al. (1997) in a behavioral study with steers fed a HE KY-31 (100% E+) vs an E- KY-31 cultivar, although voluntary DMI was not reported. Steers consuming E+ spent less time grazing ( $P < 0.05$ ) and lying, and more time standing ( $P < 0.05$ ) than steers fed E- tall fescue, indicating that E+ steers were undergoing a higher degree of thermal stress.

Considerably fewer research efforts have gone into measuring animal selectivity

responses to E- and E+ tall fescue pastures (Schmidt and Osborn, 1993). Endophyte-free fescue stands were preferred over clover, but steers seemed to have a preference for clover in E+ mixed stands (Fribourg et al., 1991). It has been observed frequently that cattle have a preference for E- pastures (Paterson et al., 1995). Palatability tests conducted by Emile et al. (2000) confirmed a strong preference of sheep for E- hay, a two-fold increase in consumption as compared with an E+ hay (100% E+).

#### *Intake, Ruminal Function and Forage Quality*

The presence of the endophyte has been linked to reduced feed intake and/or feed utilization, and these inhibitory effects have been reported consistently throughout the literature (Goetsch et al., 1987; Strahan et al., 1987; Peters et al., 1992; Aldrich et al., 1993; Humphry et al., 2002). In addition, elevated temperatures have notoriously exacerbated the effects of E+ tall fescue. Decreased DMI from E+ tall fescue consumption has been reported to range from an 8% decrease in beef steers fed hay diets (Schmidt et al., 1982) to a 38% decrease in dairy cows fed freshly chopped E+ tall fescue stands (Hemken et al., 1979).

Ruminal microorganisms and ruminal tissue play a major role in the potential development of fescue toxicity: ruminal microorganisms liberate alkaloids from the infected plant tissue, which in turn alter metabolism in the rumen by means of microbial detoxification (Westendorf et al., 1993; Moyer et al., 1993). In addition, the ruminal wall constitutes the primary absorptive site for alkaloids linked to fescue toxicosis (Hill et al., 2001).

Although chemical measures of forage quality (digestible DM, CP, fiber contents) are usually reported to be similar in both, E- and E+ fescue stands (Bush and Burrus, 1988; Arachevaleta et al., 1989; Howard et al., 1992; Ball et al., 2002), the effects of toxins altering

ruminal culture fermentation were suggested by early in vitro work.

Bush et al. (1970, 1972) reported decreased cellulose digestion and VFA production when perloline was added to the ruminal culture, consistent with later findings reported by Boling et al. (1975) with lambs fed perloline-added diets. Adding to these inhibitive effects, apparent CP digestibility ( $P < 0.05$ ) was also decreased. However, perloline has been reportedly found in both, E+ and E- fescue stands.

In vitro dry matter disappearance (**IVDMD**) utilized as a measure of digestibility, has shown a lack of consistency in trials where E+ and E- diets were compared. Although a number of studies show no influence from the presence of the endophyte (Bush and Burrus, 1988; Harmon et al., 1991; Stamm et al., 1994), Schmidt et al. (1982) reported increased IVDMD of E+ seed and hay ( $P < 0.01$ ), but the authors doubted the 3-unit increase would have any meaningful effect on animal performance. Similar in vivo findings were presented by Neal and Schmidt (1985) with seed-based diets using a rat model.

In a study with growing dairy steers fed increasing dietary proportions of E+ hay, Goetsch et al. (1987) reported a linear reduction in DMI (as a % of BW) of .06% for each 10% increase in E+ hay in the diet. Total tract DM, NDF and N digestibilities increased ( $P < 0.05$ ) linearly with E+ level. Conversely, particulate passage rates in the rumen decreased ( $P < 0.05$ ) with increased infestation level (3.5 to 2.8%/h for the E- and 100% E+ hay diet, respectively). These results suggest that a higher intake from E- diets increased ruminal outflow of potentially fermentable substrate, and this may have decreased fractional overall digestion.

However, a study with sheep by Hannah et al. (1990) reported decreased ( $P < 0.05$ ) ruminal and total tract digestibilities of OM, NDF, and cellulose in HE fescue seed-based

diets (3 ppm EV vs 0 ppm in E- diets). Also, higher ( $P < 0.05$ ) fluid dilution rates and particulate passage rates in the E+ diet seem to contradict previous findings by Goetsch et al. (1987). However, digestibility and passage rate differences may be attributable to intake levels (ad lib in the previous study by Goetsch et al., 1987; restricted and equalized in the study by Hannah et al., 1990) and ergovaline (and possibly other alkaloids) levels fed. Similar digestibility results to those obtained by Hannah et al. (1990) were reported by Fiorito et al. (1991) with lambs fed equal amounts of LE (< 1% E+) or HE (> 95% E+) fescue hay.

In summary, decreased digestibility could be partly responsible for reduced performance when livestock consume E+ fescue. In light of previous findings, Barth et al. (1991) concluded that the reduction in digestibility of E+ tall fescue diets as compared to that of E- diets, occurs only when the following specific conditions are met: a threshold level of alkaloid presence in fescue seeds, heat stress conditions as in summer grazing trials, and similar intake levels between both diet treatments.

### *Beef Production*

The presence of the endophyte has a profound effect on livestock weight gains. A number of studies conducted throughout the 80s on growing steers postulated that there was an overall reduction in ADG of 45 g/d for each 10% increase in endophyte level on an annual basis, and 68 g/d during spring-summer grazing trials (Stuedemann et al., 1985; Crawford et al., 1989). However, the considerable variability in the data of these studies suggested that the total variance was only partly due to the fungus frequency in the fescue stands.

An animal performance summary presented by Stuedemann and Hoveland (1988) showed that a shift from HE to LE fescue stands led to increased steer ADG in a range of 30

to 100%. Gain per ha was also increased, but to a lesser extent, probably because of a higher carrying capacity in HE pastures due to the associated effect of lower intakes.

Although the results from pooled-data studies show a clear linear relationship between fescue endophyte levels and ADG in growing steers (Crawford et al., 1989; Thompson et al., 1993), a 3-yr study conducted by Fribourg et al. (1991) showed a curvilinear response of ADG and gain per ha to increasing E+ levels. It needs to be noted that the fescue stands in the latter study contained clover, whereas those pastures cited in the two former ones essentially refer to pure fescue stands.

In a multi-state, pooled-data analysis involving most of the eastern, fescue-growing region, Thompson et al. (1993) reported spring ADG of 841, 757, and 633 g/d with steers grazing low endophyte (LE,  $\leq 5\%$  E+), medium endophyte (**ME**,  $\geq 20$  to  $\leq 35\%$  E+), and high endophyte (HE,  $\geq 50$  to  $\leq 97\%$  E+) –infected tall fescue stands respectively. Summer ADG was 542, 525, and 374 g/d for LE, ME, and HE tall fescue stands respectively.

Tall fescue toxicosis can be alleviated, at least partially, by the presence of legumes in mixed pastures, at a minimum range of 10 to 25% clover in the stand (Fribourg et al., 1991; Hoveland et al., 1997). During springtime, steers grazing LE fescue plus clover (25% in the stand) gained an additional 130 g/d ( $P < 0.05$ ) as compared with LE fescue alone. On the other hand, ADG obtained from HE, in pure stands or in combination with clover were similar, suggesting that the dilution effect from the presence of clover in the mixed stand was not sufficient to reduce toxicosis from HE fescues (Thompson et al., 1993). Contrasting the spring results, ADG from LE in mixed stands obtained by steers during the summer did not differ from the gains from FE alone. In HE fescues, the response from adding clover to the stand was meaningful: clover-added pastures obtained 138 g/d more ( $P < 0.05$ ) than that by

steers on HE in pure stands (Thompson et al., 1993).

### *Milk Production and Reproduction*

Decreased milk production, growth rates, embryo survival, pregnancy and calving rates; delayed onset of puberty; and calves with reduced weights of both, dairy and beef cows grazing E+ fescues have been reported in a number of studies. Cows feeding on E+ tall fescue have shown a broad-ranged reduction in milk production, and reported to be as high as 45% in beef cows (Schmidt et al., 1983), and 60% in dairy cows (Schmidt and Osborn, 1993). Beef cows grazing E+ produced 25% less milk than cows on E- or orchardgrass pastures (Peters et al., 1992). Similar findings were reported by Schmidt et al. (1983), where crossbred beef cows grazing an E+ fescue pasture produced almost 45% less milk than those on E- stands.

Because elevated environmental temperatures exacerbate symptoms, the fescue toxicosis syndrome is more clearly expressed during the summer months. In a 2-yr summer grazing trial conducted by Strahan et al. (1987), milk production by mid-lactation dairy cows consuming E- fescues was similar to that of cows grazing orchardgrass or alfalfa. The consumption of E+ (KY-31, 63% E+) resulted in decreased milk production ( $P < 0.05$ ) and body weight loss ( $P < 0.05$ ), both attributable to reduced forage intake ( $P < 0.05$ ), when compared with LE (Kenhy and Johnstone, 8.1% E+) and with E- (KY-31) fescues.

Previously, Hemken et al. (1979) had reported similar results, although fungus infection levels, as we know them today, were not reported and the results were somehow unexpected: the consumption of a low-perloline (believed to be at the time the major alkaloid responsible for fescue toxicity) experimental strain resulted in decreased DMI ( $P < 0.01$ ) and milk production ( $P < 0.01$ ) as compared with suspected LE cultivars.

Mizinga et al. (1992) reported similar milk production for spring-calving primiparous beef cows fed E+ and E- fescue seed diets, although ADG was reported to be lower ( $P < 0.05$ ) in cows fed E+ seed (10 to 20% of the diet as-fed).

Cattle consuming E+ fescues have consistently shown reduced conception rates compared to cattle fed E- pastures (Boling, 1985; Schmidt et al., 1986; Gay et al., 1988; Porter and Thompson, 1992; Paterson, 1995). Calving rates from cows grazing a HE fescue were 67%, compared with 86% in those grazing a LE pasture (Boling, 1985). Similarly, a 42% reduction for E+ grazers over the initial 96% pregnancy rate in cows grazing E- was reported by Schmidt et al. (1986). A linear relationship was established between conception rates and infestation level: conception rates decreased approximately 3.5% (Schmidt et al., 1986) to 4.5% (Danilson et al., 1986) for each 10% increase in fungal infection. Results were partially attributed to the greater weight loss and poorer body condition of the E+ groups.

In a 3-yr study conducted by Washburn and Green (1991), reproductive function in beef heifers grazing E+ fescue stands was severely affected: the number of cyclic heifers before the breeding period started (11 vs 37%); pregnancy rates (47 vs 68%); and total heifers with surviving calves (39 vs 65%) in heifers fed a HE (KY-31, 50 to 70 E+) as compared to those fed a LE (KY-31, 50 to 70 E+) were all lower ( $P < 0.05$ ). Caution as to what forage(s) are to be chosen by grazing farmers was strongly suggested.

Hoveland (1993) conducted an extensive survey with data from 21 states bordering and pertaining to the eastern fescue-growing region (from Nebraska to Maryland and south to Texas and Georgia). It has been estimated that the average calving percentage of beef cows grazing tall fescue for the transition zone is 74%, as compared with an achievable 90% on E- pastures, representing almost 900,000 unborn calves annually due to decreased reproductive

function.

#### *Associated Neuroendocrine Function*

The presence of the endophyte has been associated to decreased circulating prolactin (**PRL**) (Hemken et al., 1979; Boling, 1985; Chestnut et al., 1991; Emile et al., 2000), serum cholesterol (Stuedemann and Hoveland, 1988; Oliver et al., 2000), and melatonin (**MEL**) (Porter and Thompson, 1992; Porter et al., 1993). Steers fed E+ fescue stands exhibit altered pituitary and pineal dopaminergic and serotonergic metabolism, both linked to lower PRL and MEL levels, and animal performance (Porter et al., 1990).

Although a number of pituitary PRL suppressive pathways have been hypothesized, it is the anterior pituitary-acting, neurotransmitter Dopamine (**DA**) that acts as the main inhibitory regulator of this lactogenic hormone (Lamberts and Mcleod, 1990). Serotonin (**SER**), elevated environmental temperature, and increased photoperiod stimulate PRL secretion (Porter et al., 1990).

The DA receptor on the pituitary gland is known as a D<sub>2</sub> receptor, and ergot alkaloids bind to these sites and initiate second messenger responses in a similar way to that of DA (Larson et al., 1995). The inhibition of PRL release has been a biological measure by which ergopeptide damage by E+ fescue consumption can be assessed (Thompson and Stuedemann, 1993). The use of compounds that antagonize with DA activity allow for higher circulating PRL in cattle and have been proved to be successful in therapeutic treatment of fescue toxicosis (Porter and Thompson, 1992; Aldrich et al., 1993). However, the supplementation of E+ diets with a DA antagonist in lambs resulted in an increased DMI ( $P < 0.05$ ), but PRL levels were similar to that of the unsupplemented treatment, and skin vaporization as a way of heat dissipation was not alleviated by the use of a DA antagonist (Aldrich et al., 1993).

Chronic ergot alkaloid ingestion resulted in reduced activity of the hypothalamic dopaminergic neurons and homovanillic acid (HVA, a DA metabolite) concentration, and these results were correlated with reduced pituitary PRL (Schillo et al., 1988). In a study by Porter et al. (1990), steers grazing a HE fescue (100% E+) had reduced serum PRL levels (9.2 vs 32.5 ng/ml,  $P < 0.001$ ) as compared to the E- group, but almost a two-fold increase in pituitary dihydroxyphenylacetic acid (DOPAC, another metabolite of DA). No differences among treatments were reported for HVA levels. These results, along with increased levels of 5-hydroxyindolacetic (5HIAA, a major metabolite of serotonin) in E+ stands suggest increased turnover without increased synthesis of DA and SER, which in turn affect thermoregulatory mechanisms (Porter et al., 1990). Differences among previous reports may be attributable to alkaloid concentration, infection level, tissue section analyzed, environmental temperatures, and photoperiod during the timeframe of the studies.

The D<sub>2</sub> DA receptor is also known to be implicated in many physiological roles other than affecting PRL release and thermoregulatory controls. It has been associated to the nervous, adrenal, and even cardiovascular systems (Larson et al., 1999; Oliver et al., 1998). Although reduced plasma PRL has been reported consistently, a few recent studies have also shown alterations in plasma concentrations of luteinizing hormone (**LH**) and growth hormone (**GH**) when cattle were fed E+ tall fescue (Porter and Thompson, 1992; Paterson et al., 1995). Ergot alkaloid-related reductions in LH concentrations from E+ fescue diets may contribute to poor reproductive performance in cows by altering luteal function. In a study by Browning et al. (1997) with beef steers, intravenously injected ergotamine (ergopeptine) or ergonovine (LAA) resulted in reduced plasma PRL concentrations ( $P < 0.01$ ) and transient elevated plasma GH ( $P < 0.01$ ), as compared with a saline vehicle; but only ergotamine

reduced plasma concentrations of LH ( $P < 0.01$ ). In a similar cross-over design, both ergot alkaloids reduced plasma concentrations of PRL ( $P < 0.01$ ) and LH ( $P < 0.01$ ) in cycling dairy cows during their luteal phase (Browning et al., 1998).

Recent findings by Browning et al. (2000) revealed increased plasma concentrations of cortisol, triiodothyronine ( $T_3$ ), and glucagon; and decreased plasma insulin concentration in dairy heifers and cows intravenously injected with ergotamine (19 and 20  $\mu\text{g}/\text{kg}$  BW, respectively), indicating that this endophyte-linked compound is physiologically capable of altering hormone-mediated nutrient metabolism and thermoregulation, resulting in suboptimal cattle performance.

### *Thermoregulation*

Cattle and sheep consuming E+ fescue have frequently been reported to have increased basal body temperatures (Rhodes et al., 1991; Aldrich et al., 1993), rectal temperatures (Hemken et al., 1979; Schmidt et al., 1983; Strahan et al., 1987;), respiration rates (Hemken et al., 1979; Browning et al., 1997; Browning et al., 1998), and time spent in the shade (Aldrich et al., 1993; Oliver et al., 2000), along with excessive salivation (Thompson and Stuedemann, 1993), all adaptive mechanisms that aid in dissipating excessive heat.

The ability to dissipate body heat is frequently associated to alterations in peripheral blood flow. The diminished blood flow to tissues results in tissue death and inability to dissipate body heat (Paterson et al., 1995). In a 3-yr study conducted by Oliver et al. (1998) continuous grazing of E+ fescue stands led to a contractile response in  $\alpha_2$ -adrenergic receptors in blood vessels, which resulted in vasoconstrictive effects such as damaged peripheral vascular perfusion and an impaired heat regulation mechanism. Previously, an in

vitro study by Strickland et al. (1996) showed the effects of AL and several ergot alkaloids (ergonovine,  $\alpha$ -ergocryptine, and EV) on vascular smooth muscle cells of cattle, and partially supports the hypothesis of an alkaloid-mediated contribution to vascular impairment (thickening of blood vessels) in cattle grazing E+ stands.

In a study by Rhodes et al. (1991) wethers fed a HE (1.18 ppm EV) diet had decreased blood flow to adrenal glands and inner hind leg skin ( $P < 0.10$ ) as compared to those fed a LE ( $< 0.05$  ppm EV) diet. A similar response was found in steers fed a HE (0.52 ppm EV) diet in contrast with those fed a LE ( $< 0.01$  ppm) diet, where blood flows to skin, brain, and digestive tract were lower for the HE group ( $P < 0.10$ ).

In a study conducted by Aldrich et al. (1993), dairy steers exhibited increased ( $P < 0.05$ ) rectal temperatures when fed a HE (285 ppb EV) diet as compared to those fed E- diets. At 32°C, the steers fed E- diets had greater skin vaporization than at 22°C, but the E+ group had similar values under both temperature treatments, suggesting impairment in the ability of cattle to remove excess body heat through this mechanism at high environmental temperatures. Similarly, Spiers et al. (1995), using a rat model, reported an EV-mediated response, which exacerbated by environmental temperature, caused severe impairment in heat tolerance mechanisms.

### *Horses*

Horses are particularly sensitive to the toxins produced by the fungus resulting in a severely diminished reproductive performance. Mares grazing E+ fescue have been reported to suffer from agalactia after foaling, prolonged gestations, foaling problems, abortion, and thickened placentas (Monroe et al., 1987; Porter and Thompson, 1992; Ball et al., 2002).

In a 3-yr study by McCann et al. (1992), reduced serum PRL and progesterone were

reported in late-gestation mares grazing E+ stands (KY-31, 100 E+), as compared with those grazing E- (KY-31) tall fescue. Within 2 d of grazing the E+ pasture, circulating PRL was lower than the E- grazing group ( $P < 0.01$ ), and by d 5 upon removal from E+ stands, serum PRL levels were similar to those on E- fescue. Similar findings were reported in an earlier study by Earle et al. (1990) with mares in advanced gestation grazing HE pastures and a prompt recovery after their removal to E- stands.

The lowered circulating progesterone from mares grazing E+ fescues reported in the studies by McCann et al. (1992) and Earle (1990) support the idea of an altered endophyte-related function by vasoconstriction (Rhodes et al., 1991). Placental progesterone, the primary source of progesterone during late gestation, increases 2 wk before parturition, and the suppressive effect from E+ diets may provide for increased gestation lengths (Monroe et al., 1987; McCann et al., 1992). Grazing mares had gestation lengths of 360 and 333 d for E+ and E- stands, respectively. Retained placentas and agalactia were increased ( $P < 0.01$ ) in the E+ grazing group. A trend towards increased ( $P = 0.12$ ) placental weights and decreased ( $P = 0.08$ ) body condition score were also reported (Monroe et al., 1987). Similar findings were reported by Porter and Thompson (1992). A 20-d increase in gestation length resulted in foal and placental over weights, with foals having large bone frames but poor muscle development, and a higher risk of mortality.

Dopamine antagonist treatments have proven to be effective for treating horses suffering from fescue toxicosis (Stuedemann et al., 1998), but neither selenium (Monroe et al., 1987) nor energy (Earle et al., 1990) supplementation appeared to have preventive or alleviating effects. A preventive course of action recommended to producers has been the removal of the mares from E+ fescue stands 2 to 3 months before foaling (Schmidt and

Osborn, 1993).

### **Endophyte-Free Tall Fescue**

The removal of the fungus eliminates endophyte-related fescue toxicity problems, and results in increased animal performance (Schmidt et al., 1982; Hoveland et al., 1983; Siegel et al., 1985; Bacon et al., 1986; Agee and Hill, 1994; Bacon, 1995; Ball et al., 2002). The use of E- seeds has been recommended in order to obtain E- fescue stands, but these are frequently in the range of 95 to 99% E-. In the following years, the level of infection is expected to increase due to seed production, and survivability and competitiveness of E+ plants (Bacon et al., 1986).

The production of E- cultivars has been based in the eradication of the fungus by the use of chemicals in fescue stands or specific seed storage conditions, with or without chemical use (Bacon and Siegel, 1988). Length and storage conditions such as temperature and seed moisture content are essential in fungus survival. Although the use of aged seed has proven to be successful in obtaining E- cultivars, certain storage conditions need to be met. After 300 d at 22°C, fescue seed became E-, but fungus viability remained high at lower temperatures. Further, endophyte survival was high after storing fescue seed for over 2 yr at 6°C (Bacon and Siegel, 1988). Also, at a given temperature, fescue seed with higher moisture content reduced viability faster than seed with less moisture, the later in need of a longer storage period (Siegel, unpublished data).

In spite of enhanced animal performance, producers have not extensively adopted E- cultivars, and this may be due to the uniqueness of the symbiotic relationship between the endophyte and its host grass, and the desirable agronomic characteristics linked to this association. Plant growth and survival due to grazing (Fribourg et al., 1991; Marsalis et al.,

2000) and drought tolerance (Siegel et al., 1985; Arachevaleta et al., 1989); and insect and nematode deterrence (Bacon and Siegel, 1988; Clement et al., 1996) are all enhanced by the presence of the fungus. As a consequence, reduced long-term productivity and/or stand persistence in E- pastures has been reported consistently throughout the literature.

A greenhouse study conducted by Arachevaleta et al. (1989) revealed that E+ plants had 50% more herbage growth, responded better to N fertilization ( $P < 0.05$ ), and showed increased response in terms of regrowth when irrigation followed a severe drought, as compared with E- plants. Also, under severe drought conditions, all E+ plants survived, but only 25% survived in the E- stand.

Fungus-free stands were decreased by the end of the first grazing season, and were more severely affected in the following years in a 3 yr-study by Fribourg et al. (1991). Stands of a LE (KY-31, 3% E+) only accomplished 60 to 80% as compared with those stands with infestation levels higher than 22%. A highly-infected tall fescue cultivar (KY-31, 60% E+) yielded higher forage mass (3,649 vs 3,027 kg DM), and a progressively-increased difference as summer advanced, as compared with a LE cultivar (Johnstone, < 1% E+) from late May to mid September (Howard et al., 1992).

In a 3-yr study by Bouton et al. (1993) designed to compare four E+ germplasms (over 90% E+) and their correspondent E- versions, 2 out of three locations revealed increased productivity and stand survival in E+ versions. The decrease in productivity and persistence was attributed to a reduced tolerance to summer drought in the E- versions.

After a 4-yr grazing study with beef steers, Marsalis et al. (2000) concluded that intensive grazing pressures (4.2 vs 2.1 and 2.9; and 5.4 vs 2.5 and 3.7 steers per ha for high vs low and medium stocking rates) during mid-fall and spring, respectively, produced enough

stress on E- pastures to enable their thinning in stands and allowing for an increased number of better adapted E+ plants in the pasture. During these trials, unequalled stress imposed by grazing, in conjunction with dry weather, lead to greater stand losses in E- cultivars.

### **Jesup Tall Fescue**

Jesup tall fescue is the result of a polycrossed, 15-clone synthetic cultivar, developed by the Georgia Agricultural Experiment Stations. Originated from 32 clones collected in the early 80s near Jesup, GA, and bred for long-term plant survival and yield potential, it was thoroughly tested in Tifton and Athens, GA, and finally released in 1995 (Bouton et al., 1997). It is sold commercially as either E+, infected with the fungus (*Neotyphodium coenophialum* Morgan-Jones & Gams) Glen, Bacon & Hanlin; or fungus-free.

An early to medium maturity tall fescue, E+ Jesup is widely adapted to the southern fescue-growing region of the US, including the Southern Coastal Plain and even some marginal areas of the its western range (Bouton et al., 1997).

Although less persistent than E+ Jesup, the fungus-free version was selected for summer survival under high-pressure grazing conditions in central Georgia. A 3-yr study by Hoveland et al. (1997) revealed that both Jesup E+ (89% E+ infection) and Jesup E- (0% E+) stands survived and remained in similar shape after a severe drought during the first grazing season, even under high grazing pressure. Beef steers grazing E- Jesup had higher ( $P < 0.05$ ) spring ADG as compared with E+, E+ plus alfalfa, and single alfalfa pastures (1.13 vs 0.43, 0.56, and 0.97 kg for low grazing pressure, and 0.93 vs 0.30, 0.55, and 0.66 kg for high grazing pressure, respectively). Although not as drought-tolerant as Jesup E+, ADG and plant survival suggested that Jesup E- tall fescue was highly suited, under good grazing management, for excellent animal performance.

## **Novel Endophyte**

Bacon et al. (1986) and later Bacon and Siegel (1988) raised an interesting question involving endophyte-infected fescues and the possibility that this interaction could be modified in ways to produce only desired characteristics. In order for this to happen, the mechanism(s) leading to stress tolerance had to be identified and the causal agents separated from those causing toxicosis. A number of observations suggested that this could be possible (Hill et al., 1991a; Hill et al., 1991b; Adcock et al., 1997), but it was necessary to understand all interactions involved, including grass hosts, endophyte strains, insects, pests, herbivores, and environmental interactions.

The wide range of biologically active compounds synthesized from tall fescue endophytes in culture or within their grass host suggested a diverse population of endophytes, and a possible symbiotic mutualism between non-toxic alkaloid-producing fungus and the genera *Festuca*. (Bacon, 1995). Several endophyte strains producing no alkaloids or only trace amounts of them responsible for fescue toxicosis had been reported (Agee and Hill, 1994; Adcock et al., 1997; Latch, 1998), suggesting that the rate and type of alkaloid production could vary according to the fungus strain involved. Based on these and other observations, extensive research efforts were led to study the peculiar relationship between the grass and the endophyte.

Although some of the alkaloids produced by the fungus in tall fescue lead to fescue poisoning, such as EV and LAA, other endophyte-related alkaloids, such as peramine and the pyrrolizidine group, are responsible for enhancing hardiness, and have been reportedly involved in increased pest resistance. Researchers from AgResearch, New Zealand, were the first to identify the strain that allowed for this particular spectrum of compounds. Strains of

*N. lolii* endophytes from nature were selected by alkaloid production profile (absence of Lolitrem B and EV, and presence of peramine), cultured, and transferred into improved cultivars to minimize or eliminate toxicity from E+ perennial ryegrasses (Tapper and Latch, 1999).

In cooperation with the University of Georgia, the fungus strain was inserted in two tall fescue cultivars, Jesup and Georgia 5, by Dr. Joe Bouton (University of Georgia) and Dr. Garrick Latch (AgResearch, N. Z.). The “novel endophyte” (the term used by researchers when referring to the new fungus strain) is known commercially as MaxQ™.

Initial studies conducted by Bouton et al. (2000, 2002) with the reinfected (endophyte strain AR542), non-toxic cultivars Jesup and Georgia 5 showed some promising results: a two-spring season grazing trial with lambs resulted in ADG similar to those achieved with the E- stands, and a 50% increase in gains as compared with those fed E+ fescues. Also, PRL levels were dramatically increased, and were similar to E-, as compared to PRL levels on E+ pastures. Similar results were reported for steers grazing during the fall and spring of 1999. Summer endurance was also tested: a 10% occupancy for E- stands as compared with 42% (similar to the E+ version) and 25% occupancy for Jesup AR542 and Georgia 5 AR542, respectively, after 18 months, revealed that improved summer survival was also achieved by the MaxQ™ versions.

A 3-yr grazing study by Parish et al. (2003a) was conducted to evaluate lamb performance on nonergot alkaloid-producing, fungus-infected (EN) tall fescue cultivars Jesup and Georgia 5, and their wild-type E+ and E- versions. Total ergot alkaloid concentration in E-, Jesup EN and Georgia 5 EN averaged 31 and 64 ppb, as opposed to 1,184 and 2,997 ppb in E+, during spring and fall, respectively. As a consequence of this,

signs of heat stress during summer grazing were clearly detected in lambs on E+ stands (increased rectal temperatures, decreased serum PRL). Both, ADG and gain per ha were higher ( $P < 0.05$ ) for the E- and EN cultivar versions than on E+ tall fescue.

In addition, a recently published study by Parish et al. (2003b) reported promising results in beef cattle too. The 3-yr study included fall and spring grazing seasons at two locations in Georgia. Steer and heifer gain, response to fescue toxicity, and grazing behavior on three tall fescue cultivars (Kentucky-31, Jesup and Georgia-5) with different endophyte status (non-toxic, endophyte-infected; endophyte-free; and toxic, endophyte-infected) were examined. Each cultivar reduced ( $P < 0.01$ ) serum prolactin concentrations and dry matter intake, and increased spring rectal temperatures ( $P < 0.05$ ), calf ADG ( $P < 0.05$ ), and ergot alkaloid concentrations ( $P < 0.01$ ) for livestock grazing E+ tall fescues, as compared with cattle grazing either endophyte-free or nonergot, alkaloid-producing stands at both locations. Although bite size was similar for all forage treatments, time spent idling ( $P < 0.01$ ), standing ( $P < 0.01$ ), and water consumption during spring were all increased in the group fed endophyte-infected pastures, as compared with the endophyte-free and non-toxic, infected tall fescue groups.

Numerous animal and plant performance trials are currently under study. So far, the presence of nonergot, alkaloid-producing tall fescues have proven to be effective in attenuating the severity of fescue toxicosis while improving plant survival. The total impact that these improved, non-toxic tall fescues have on forage-based livestock production sustainability still remains to be fully assessed.

### **Energy Supplementation of Freshly Grazed Fescue-Based Diets**

Research data have revealed that feeding other forages or feeds in an increased-

dilution fashion has reduced to some extent the undesirable effects of toxicity in grazing animals. One of the methods this dilution effect can be accomplished is by providing supplemental corn to ruminants grazing E+ fescue stands (Paterson et al., 1995). However, animal performance from supplementing E+ diets with energy concentrates has been somewhat disappointing, and lower gain has been reported as compared with that achieved by supplemented E- diets (Forcherio et al., 1995).

On broader terms, livestock grazing herds throughout the US are typically fed supplemental energy, because it has been consistently established that pasture alone does not meet the requirements of high-producing beef and dairy cattle. Research data clearly show that energy supplementation of moderate to high quality pasture-based diets increases total DMI, even when time spent grazing has usually been reported to be lower (Hannah et al., 1989; Judkins et al., 1997), and provides for a more balanced nutrition, and as a consequence, increased gain and milk production are obtained (Elizalde et al., 1998; Kolver and Muller, 1998, Bargo et al., 2003).

However, feeding high levels of supplemental energy concentrates may interfere with forage digestion efficiency, which is pH-associated, and the depression in fiber digestion is known to be the primary cause of non-additive responses of energy-supplemented diets (Orskov, 1986). In spite of this, it is unlikely that reductions in ruminal pH are entirely responsible for reduced intake and digestibility associated to energy supplementation (Caton and Dhuyvetter, 1997). In addition, other ruminal fermentation characteristics are typically altered when supplemental energy is fed such as methane ( $\text{CH}_4$ ) production, ruminal ammonia ( $\text{NH}_3$ ) N accumulation, and the acetate to propionate ratio.

Compared with unsupplemented steers grazing E- tall fescue stands, Elizalde et al.

(1998) reported higher ( $P < 0.05$ ) ADG (0.74 vs. 0.64 kg/d) when these were supplemented with low levels of cracked corn or processed corn concentrates (1.4 or 2.8 kg/d of each). Similarly, a study by Judkins et al. (1997) with yearling steers grazing E- pasture stands reported higher ( $P < 0.05$ ) ADG in a supplemented group (1.01 kg per d with a 0.4% BW of ground corn) than those grazing tall fescue alone (0.82 kg per d) while spending less ( $P = 0.08$ ) time grazing. In addition, similar forage DMI between both groups was reported, in contrast with a previous study where forage intake was reduced in a group of steers grazing the same pastures plus similar amounts (BW-based) of supplemental corn (Hess et al., 1996). This difference may have been attributable to a higher N content of the fescue stand, and presumably a higher rumen-degradable protein content, in the former study.

In a previous study by Forcherio et al. (1995) with cannulated beef steers consuming low quality E+ and E- hay, the effects of feeding different supplemental energy and protein sources were assessed by contrasting E+ vs. E-, and supplemented E+ vs. unsupplemented E+ treatments. Although greater ( $P < 0.05$ ) for E-, hay intake remained similar across supplemental treatments, but total intake was increased when the energy supplement was added to the E+ fescue diet, similar to that of the group fed E- hay alone. In addition, a follow-up grazing experiment with beef cows fed E+ tall fescue stands (64 ppb ergovaline) alone or in combination with the previous supplements, reported that there was no additional ADG when energy concentrates were added to the E+ grazing diets. Also, supplementing the cows led to similar calf ADG compared to those on an all E+ pasture diet.

## MATERIALS AND METHODS

### *Continuous Culture Operation*

Eight continuous culture vessels, each with an overflow system as described by Teather and Sauer (1988) were used to test the dietary treatments. In order to maintain anaerobic conditions, CO<sub>2</sub> was infused to the system at a rate of 20 ml/min and a circulating water bath system kept the temperature of the vessels at 39°C.

A nonlactating, ruminally cannulated Holstein cow (North Carolina State University Dairy Educational Unit) fed alfalfa (*Medicago sativa* L.) hay was used for collection of ruminal inoculant using a hand pump. A 6-L sample was placed in a preheated insulated container and transported about 12 km to the laboratory on campus. Once in the lab, the inoculum was filtered through double-layered cheesecloth, gently but thoroughly mixed, and added to the 700-ml fermentors within 15 min.

A central paddle device set at a speed of 10 rpm continuously mixed the ruminal cultures. Artificial saliva (Slyter et al., 1966) was prepared and infused continuously at a rate of 0.73 ml/min throughout the study. Therefore, ruminal fluid passage rate was fixed and set at 6.3%/h.

### *Dietary Treatments*

Twelve diets were included in the experiment in a three-by-four factorial arrangement of treatments. Jesup endophyte-infected (E+), endophyte-free (E-), and nonergot alkaloid-producing, endophyte-infected (EN) tall fescue (*Festuca arundinacea* Schreb.) were used to evaluate possible endophyte-status effects on ruminal fermentation. Ground corn was either not added or added at one of three levels to allow for a forage:concentrate ratio of 100:0, 85:15, 70:30, and 55:45 for each fescue cultivar.

A week before sampling, 25 mm of water was applied to each plot in an attempt to recover from intense drought conditions. Fresh tall fescue forage samples were obtained from the Butner Beef Cattle Field Laboratory on May 31<sup>st</sup>, 2002. Samples were estimated to leave a 5-cm height by use of a shear-type blade mower from three 37.2-m<sup>2</sup> plots, one for each fescue grass.

After harvesting the grass, two 0.25-m<sup>2</sup> quadrats were clipped to ground level from each plot in an attempt to assess the upper level (potentially “grazable”) and stubble quantity and quality of each fescue cultivar. The fresh samples were collected, immediately cooled, and transported to the forage laboratory on campus, where they were kept frozen (-20°C).

At the forage lab, a sample of each forage treatment was dried at 65°C for 48 h and ground with a Wiley mill (Thomas Scientific, Swedesboro, NJ) to pass through a 1-mm screen. AOAC (1984, 1999) procedures were used to analyze samples for DM and Kjeldahl N. NDF, ADF, cellulose, lignin and ash were sequentially analyzed using an Ankom<sup>200/220</sup> fiber extractor (Ankom Technologies, Fairport, NY) as modified by Van Soest et al. (1991). A trichloroacetic (TCA, Licitra et al., 1996) procedure was used to determine Non-protein N (NPN) or the A crude protein (CP) fraction. The remaining B<sub>1</sub>, B<sub>2</sub>, B<sub>3</sub>, and C fractions (Van Soest, 1994) were determined with the aid of neutral and acid detergent solutions and the residues analyzed for N content.

Final-diet in vitro true dry matter digestibility (IVTDMD) was determined by placing 0.25-g samples in acetone-soaked Ankom bags (Ankom Technologies, Fairport, NY) and incubated in vitro in the Ankom II Daisy batch for a period of 48 h in a solution containing 1.6 L of McDougal’s buffer (Tilley and Terry, 1963) and 400 mL of strained, freshly extracted ruminal fluid. A bomb calorimeter (model C5000, IKA, Wilmington, NC) was used

to determine the gross energy (**GE**) content of the fescue grasses and the corn.

Tall fescue tiller infestation levels and alkaloid production were assessed by the random collection of 60 tillers in 4 replicates for each grass, and analyzed according to the procedure of Hiatt et al. (1999) and Adcock et al. (1997), respectively. Ergovaline concentration of the upper clipped herbage mass was determined from freeze-dried grass samples following the procedure of Rottinghaus et al. (1991).

Adding increasing amounts of ground corn provided supplemental energy to the diets. Ground corn samples, obtained from the NCSU Dairy Educational Unit, were analyzed following the forage quality procedures previously described for fescue samples. Also, a mycotoxin analyses was performed on the energy source (NCSU mycotoxin lab).

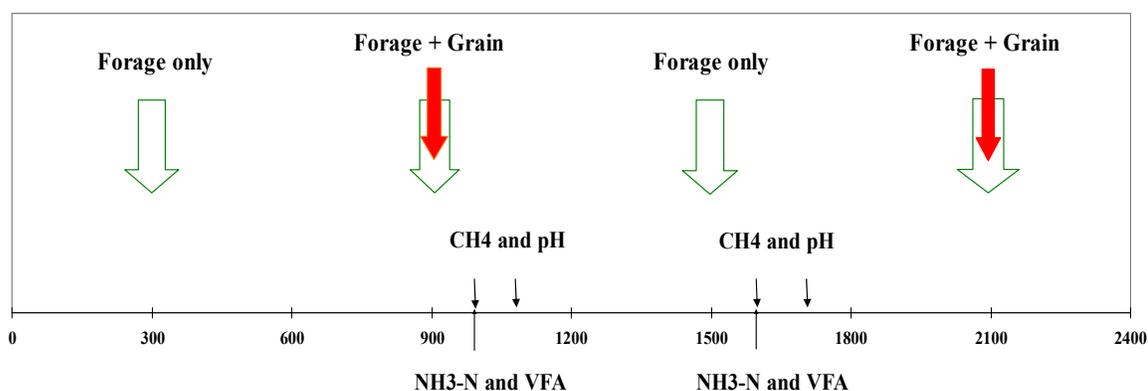
Before feeding, the grass leaves were reduced in size using a food-processor chopper (G. S. Blakeslee & Co., Cicero, IL, localized at the NCSU Metabolism Unit) for 3 min, in an attempt to simulate particle size of ruminal digesta, before entering the fermentors. Estimation of mean and median particle size of the grasses was determined following the procedure of Fisher et al. (1988), after dry sieving (USA Standard Testing Sieve, Fisher Scientific Co.) freeze-dried grass samples (15 g of DM each).

### ***Feeding Schedule***

The experiment consisted of three independent, 6-d runs, such that each treatment combination had two replicates. Microbial populations were allowed to adapt for the first 48 h before any experimental diets were fed. During the adaptation period, the ruminal cultures received alfalfa pellets (15 g DM per d) in two equal aliquots, at 0800 and 1500 h. Fermentation stabilization and dynamics were monitored by measuring gas production and ruminal culture pH.

Once the adaptation period was over, each diet was gradually adjusted to its respective forage:concentrate ratio, and by the initiation of d 4, all diets were final: for each of the fescue cultivars (E+, E-, and EN), the culture vessels received 100:0, 85:15, 70:30 and 55:45 FTGR diets. Each fermentor was offered a total of 15 g of DM per d including: 4 equal portions of grass, fed at 0300, 0900, 1500, and 2100 h; and two equal portions of corn, fed at 0900 and 2100 (Figure 1).

**Figure 1. Daily feeding and sampling schedule: forage (0300, 0900, 1500, and 2100 h); grain (0900 and 2100 h); methane and pH sampling (1000, 1100, 1600, and 1700 h); ammonia N and VFA sampling (1000 and 1600 h)**



### ***Fermentation Assessment***

Headspace gas and ruminal fluid samples were collected at several predetermined intervals to characterize fermentation function. Gas samples (10  $\mu$ l) were withdrawn with the aid of a gas tight syringe (Hamilton Co., Reno, NV) and used to determine CH<sub>4</sub> concentration by gas chromatography (CP-3800 model, Varian Walnut Creek, CA). Ruminal culture pH was measured every time CH<sub>4</sub> samples were extracted (1 and 2 h after the 0900- and 1500-h feeding times) during days 4, 5, and 6 of each run.

Ruminal fluid samples (15 ml) were taken twice daily (1 h after the 0900- and 1500-h feeding times) during the last two days of each run, and analyzed for VFA (5 ml) and for ammonia N (5 ml), using gas chromatography (CP-3380, Varian, Walnut Creek, CA) and a colorimetric assay (Beecher and Whitten, 1970), respectively.

### ***Ruminal Energetics***

The complex interactions within the microbial population and the subsequent plant component conversion to gas and VFA as the main end products have been summarized and simplified in the form of stoichiometric equations (Wolin, 1960; Van Soest, 1994). A number of assumptions were made in order to allow for measurement of CO<sub>2</sub> and microbial biomass, and for calculation of microbial efficiency, based on a theoretical fermentation balance (Wolin, 1960). Carbohydrates are the primary component of ruminant diets. Therefore, it was assumed that all fermentation products were originated from glucose intake (Van Soest, 1994). Total gas production was calculated as the sum of fermentative CH<sub>4</sub> measured by gas chromatography; fermentative CO<sub>2</sub> from stoichiometrical calculations; and buffering CO<sub>2</sub> originated from a CO<sub>2</sub>-saturated medium based on bicarbonate (Blummel et al., 1997; Groot et al., 1998).

Substrate partitioning from total daily DMI in terms of grams per day included the following fractions: VFA; CH<sub>4</sub> plus fermentative CO<sub>2</sub>; and microbial biomass estimated from ATP production (2, 3, and 3 mol/mol of acetic, propionic, and butyric acids, respectively). Also, it was assumed that carbon skeletons from glucose were the primary source of 80% of all bacterial structures (Groot et al., 1998). Based on data presented by Hespell and Bryant (1979) for a dilution rate of 6.0%/h, microbial yield ( $Y_{ATP}$ ) was assumed to be 11.6 mg/mmol of ATP. The contribution of CH<sub>4</sub> (measured) and VFA production (the sum of the energy

contents of acetate, propionate, butyrate, isobutyrate, valerate, and isovalerate) in terms of Kcal per day to total digestible energy (**DE**) were also calculated and examined.

### ***Statistical Analyses***

A split, split-plot model in an incomplete block design was determined to evaluate the effects of fungus presence and grain levels within forage types on fermentation in the rumen. The whole plot, subplot, and sub subplot factors were forage, grain levels, and sampling time relative to feeding (supplemented diets were added grain at 0900 h as opposed to no grain added at 1500 h, respectively).

Ruminal function indicators and end products, along with linear, quadratic and cubic effects from grain supplementation levels were analyzed using the mixed procedure of SAS (1999). For the response variables CH<sub>4</sub>, ruminal culture pH, NH<sub>3</sub> N, and VFA the statistical models included the independent variables forage, grain level, sampling time relative to feeding (with or without corn), and the corresponding two-way and three-way interactions. Error terms included replicate x forage, replicate x grain level (within forage), and the residual term. In the presence of two-way interactions, the PDIFF option was used to separate grain-level or sampling time relative to feeding effects within forages.

In addition, for those response variables that exhibited a sampling time effect, and/or a two-way interaction involving this variable, the data were fitted into new models using the mixed procedure of SAS (1999) and analyzed separately to investigate possible within-forage differences. The correspondent tables from these analyses are shown in the Appendix. Data are presented as the least squares means ( $\pm$  SEM). Significance and trends were established at  $P \leq 0.05$  and  $P \leq 0.10$ , respectively.

## RESULTS AND DISCUSSION

### *Forage Quality*

The endophyte-free tall fescue stand had an average of 4.6% infection, but ergovaline was not detected. As expected, EN was free of the presence of detectable ergovaline, in spite of exhibiting a 91.6% infection of the novel endophyte. Although E+ exhibited a high endophyte infection (85.8%), ergovaline was only present in small quantities (30 ppb, Table 1), almost undetectable (G. E. Rottinghaus, personal communication).

A previous beef-steer study using hay harvested from the same fields reported an ergovaline concentration of 120 ppb in E+ fescues (Killebrew-Matthews, 2002). A number of reasons may have contributed to the relatively low ergovaline concentration associated to the presence of the endophyte in the present study: a low N input, vegetative samples collected with a visually high proportion of leaf blades comparable to that removed during relatively light grazing pressure (Table 1), and differences in environmental conditions during plant growth. High N fertilization has proven to increase ergopeptine-alkaloid content substantially (Porter, 1995). Free from the presence of the endophyte, leaf blades usually have the lowest ergovaline concentrations as compared with other plant locations (Rottinghaus et al., 1991). Dry growing conditions may have impaired plant vigor up to some extent, and such conditions seem to be associated with lower ergovaline concentrations (Agee and Hill, 1994).

It has been reported that some clinical signs of toxicity in heat-stressed steers can appear with E+ fescue diets containing as low as 50 ppb of ergovaline/g of infected grass (Cornell et al., 1990). However, other researchers (Garner, unpublished data; Rottinghaus et al., 1991; Porter, 1995) have reported a minimum value of 200 ppb of ergovaline/g infected grass associated to clinical signs of fescue poisoning in cattle.

The mycotoxin analysis performed on the grain reflected only trace amounts of aflatoxin and low levels of other mycotoxins (results not reported), all far from being of any concern when recommended for livestock nutrition (L. W. Whitlow, personal communication).

Data on quality of individual forages and ground corn are presented in Table 2. Mean DM and OM were 31.4% and 90.2% respectively, and were similar among forages. Fiber components (NDF, ADF, and cellulose, expressed as a % of DM) were higher for EN than those reported for E+ and E- (63.2, 31.4, and 28.5 vs 60.3, 29.4, and 26.6, respectively). These results may be partly due to a higher harvesting pressure (26 vs 20%, expressed as a percentage of total DM in the plots, Table 1) in the EN tall fescue as compared with E+ and E-. Lignin did not differ across grasses and averaged 2.8%.

Although numerically lower in the EN version as compared with the other two forages (10.4 vs 12.9%), CP values of the three forages were within a 3.0-percentage unit range and averaged 12.0%. The relatively low CP content in all three grasses is probably attributable to a low-N input strategy due to a prolonged spring drought before sample collection, and the presumption of a relatively impaired plant-absorption mechanism under this condition.

The N fractions A, B<sub>1</sub>, B<sub>2</sub>, B<sub>3</sub>, and C expressed as a % of CP, averaged 8.8, 8.4, 77.5, 4.2, and 1.0, respectively, and were similar across grasses. However, soluble protein (A and B<sub>1</sub> fractions), expressed as a % of CP, was different among grasses: E- had the highest (20.5%) and EN the lowest soluble protein level (14.5%), with E+ being intermediate (16.9%).

Except for IVTDMD, which was determined for each of the final diets, the chemical

compositions of dietary treatments were calculated based on their forage:concentrate ratios (Table 3). In vitro true dry matter disappearance was not decreased by the presence of the endophytic fungus. Our results are in close agreement with those reported by earlier in vivo studies where E+ and E- diets were compared (Schmidt et al., 1982; Bush and Burrus, 1988; Chestnut and Fribourg, 1991). However, results from digestibility studies should be considered cautiously due to the influence levels of intake have on digestibility (Van Soest, 1994). A higher DMI from endophyte-free tall fescue diets often observed in vivo increases outflow of potentially fermentable substrate from the rumen, allowing for lower digestion.

From the foregoing discussion, it would appear that forage quality was not affected by the presence of the endophyte or alkaloid concentration. These findings are in close agreement with most studies (Harmon et al., 1991; Stamm et al., 1994; Emile et al., 2000) in which the presence or absence of the fungus did not affect the chemical composition of the forages. Further, the previously mentioned studies had higher ergovaline contents in their endophyte-infected diets (134 ppb ergovaline in E+ KY-31, 475 ppb ergovaline in E+ Titan, and 410 ppb ergovaline in E+ Clarine tall fescue in Harmon et al., 1991; Stamm et al., 1994; and Emile et al., 2000, respectively) than the current study.

### ***Microbial Fermentation***

#### *Ruminal Culture pH, Ammonia, and Methane Production*

Conversely, altering the forage:concentrate ratio had an expected impact on most chemical components of the diet, and these changes were reflected in ruminal fermentation. Ruminal culture pH, NH<sub>3</sub> N, and CH<sub>4</sub> production are presented in Table 4. Ruminal culture pH decreased ( $P < 0.001$ ) linearly with increasing grain supplementation levels (Figure 2). Forage-based diets of moderate to high quality usually exhibit decreased ruminal pH values

whenever grain supplementation is increased, reflecting higher ruminal fermentation activity, and this response has been extensively reported, both in vivo (Lana et al., 1998; Olson et al., 1999) and in vitro (Lana et al., 1998; Bach et al., 1999; Bargo et al., 2003).

Most of these studies have incorporated additional treatments by lowering forage:concentrate ratios in some experimental diets to 30:70 and even lower, usually attempting to exacerbate the effect of decreased pH on fiber digestion and/or rate of passage. In the current in vitro study, the mean ruminal pH of all supplementation treatments was 6.1 with a range of 5.9 to 6.3; therefore, pH is not likely to cause a major inhibiting impact on fiber fermentation (Stewart, 1977; Hoover, 1986; de Veth and Kolver, 2001).

Ammonia N output (g/d) varied by grass (Figure 3): EN had lower ( $P < 0.05$ ) values than those obtained for E+ and E-, and this may be attributable to a lower CP content of EN as compared with the other two forages, adding to the dilution effect of increased levels of ground corn (9.0% CP) across grasses. In addition, ruminal  $\text{NH}_3$  N ( $P < 0.05$ ) decreased linearly with increasing supplemental grain levels (Figure 4). The decreased availability response of ruminal  $\text{NH}_3$  N to higher concentrate levels (and correspondingly lower CP levels) are in close agreement with those reported by other in vitro studies (Kolver et al., 1998; Lana et al., 1998; Bach et al., 1999).

Also, a forage by grain supplementation interaction ( $P = 0.08$ ) on  $\text{NH}_3$  N output was observed: EN yielded less ( $P < 0.05$ )  $\text{NH}_3$  N than E+ across all grain levels, and less than E- for all levels except for 15% grain (Figure 5). Conversely, E+ had a higher ( $P < 0.05$ )  $\text{NH}_3$  N output than E- only at the highest grain level (45% grain).

The effect of altering the forage:concentrate ratio on  $\text{NH}_3$  N accumulation can be attributed to either higher microbial synthesis (and lower circulating  $\text{NH}_3$  N) on high

concentrate diets, or a lower deamination rate on those diets. Our results suggest the latter, because calculated microbial yield was unaffected by grain level, and microbial efficiency, in terms of microbial mass per kg of DM ruminally fermented, decreased with increased grain levels in the diet. Microbial populations from forage-fed cows exhibited increased deamination rates as compared with those fed high concentrate diets (Lana et al., 1998).

Despite the assumption that adding concentrate to forage-based diets typically lowers CH<sub>4</sub> production, this response was not clearly seen in the current study, at least for the forage:concentrate ratios tested in our study. But even when CH<sub>4</sub> production was not affected by concentrate feeding, the quadratic response of CH<sub>4</sub> to grain supplementation (Table 5) suggests that gas production may not be necessarily related to substrate fermentation in a linear fashion.

An interaction of forage-by-grain supplementation ( $P = 0.08$ ) was also observed for CH<sub>4</sub> production (Table 4). Methane was similar across forages for all supplementation levels except for the highest grain level, where the difference between E+ and EN forages became significant ( $P < .05$ ), with E- yielding an intermediate value (Figure 6). The reasons causing this peculiar forage pattern in methane production at the highest grain level are unknown. It can be speculated that the shift in microbial population towards a higher proportion of starch-digesting microorganisms in response to the increased grain level in the diet is the impelling force causing the methane responses from all three forages to be different.

Methane production exhibited ( $P < 0.05$ ) an effect of sampling time relative to feeding (results not shown). Methane readings were higher when samples were taken 1 and 2 h after feeding corn (0900 h) than comparable samples taken after feeding forage alone (1500 h). However, when forages were analyzed separately, the previous effect was not consistent

across all three forages: the non-toxic, infected fescue was not affected ( $P = 0.9$ ) by sampling time relative to feeding (Tables 1a to 1c of the Appendix).

It may be speculated that a delayed response in methanogenesis occurred, where the highest  $\text{CH}_4$  values were originating from the previous meal allotted and not the most recent dietary treatment fed. In our study, the time-gas production sequence was not measured, but in vivo studies support the hypothesis of delayed  $\text{CH}_4$  production indirectly by reporting the lowest daily pH values of grass-based diets after 5 to 8 h of feeding corn supplements (Soriano et al., 2000) or concentrates with a high and low starch content (van Vuuren et al., 1986). These results, the delayed-gas production effect and the previously described quadratic response, are in agreement with those reported by Groot et al. (1998) where fermentation kinetics of cell wall and cell contents of Italian ryegrass was assessed.

#### *Volatile Fatty Acid Production*

Concentrations of VFA and respective molar proportions are reported in Table 6. Regardless of the forage, total and individual VFA concentrations remained similar. Also, molar proportions of VFA were not affected by the presence of the endophyte, except for isobutyrate ( $P = 0.07$ ), that tended to have lower values for EN than those reported for E+ and E-. However, the difference in this branched-chain fatty acid among forages may be of doubtful biological significance. One possible explanation for this difference may derive from a lower oxidative deamination activity in EN-associated microbial function than that of the other forages, an effect that could be related to the lower ammonia levels reported earlier for this grass.

Expectedly, concentrate had a profound effect on the individual VFA concentrations and molar proportions. The molar proportions of acetate (**A**), propionate (**P**), and butyrate (**B**)

for the non-supplemented diets shifted from 62.4, 19.7, and 13.4% (a 4.7:1.5:1.0 ratio related to a B-based unit) to 52.3, 22.6, and 20.6% (2.5:1.1:1.0) for A, P, and B, respectively, for the highest grain level.

Total branched-chain VFA concentration (isobutyrate plus isovalerate) remained unaffected across treatments (data not shown). Branched-chain VFA are essential growth factors for fiber-digesting bacteria (Russell and Sniffen, 1984), and the concentrations of C<sub>4</sub> and C<sub>5</sub> isoacids in our study are unlikely ones to impair cellulolytic activity (Gorosito et al., 1985). However, an in vitro study by Bach et al. (1999) reported limiting levels of branched-chain VFA in a forage-only diet, and energy supplementation (a 55:45 forage:concentrate ratio) increased ( $P < 0.05$ ) branched-chain VFA concentrations as compared with an all forage diet.

Based on the CO<sub>2</sub> infusion rate (20 ml per min), concentrations of VFA were converted to production values (Table 7). Total VFA production was similar across treatments, and averaged 58.8 mmol/d. Our results are in agreement with those reported by Eun et al. (2002) and van Vuuren et al. (1986), where total VFA production remained unaffected across three forage:concentrate ratios (70:30, 50:50, and 30:70) in vitro, and different amounts of starch-containing concentrates fed to lactating dairy cows, respectively. Acetate ( $P < 0.05$ ) and valerate ( $P < 0.05$ ) decreased linearly with increased grain, whereas propionate ( $P = 0.06$ ), butyrate ( $P < 0.05$ ), and isovalerate ( $P = 0.08$ ) increased linearly with grain levels (Figures 7 and 8).

The drop ( $P < .001$ ) in the acetate:propionate ratio with higher concentrate feeding was similar across forages and was caused by both, an increase in propionate and a decrease in acetate production (Figure 9). The reasons for a tendency towards a quadratic response ( $P$

= 0.10) of propionate production to grain supplementation (Table 8) are speculative, but most probably related to the CH<sub>4</sub> production pattern in response to concentrate in the diets. Within the ruminal environment, the disposal of reducing equivalents is typically directed through methane or propionate production, and these means of H disposal seem to act as opposing forces (Wolin, 1960; Hungate, 1966). In our study, both CH<sub>4</sub> and propionate production patterns across all concentrate levels exhibited a nonlinear and inverse, pH-associated relationship.

Also, the acetate:propionate ratio exhibited ( $P < 0.05$ ) a sampling relative to feeding effect (results not shown) where the acetate:propionate ratio was higher after feeding corn (2.82) than after feeding forage alone (2.77), an associated effect attributable to the lag response earlier described for CH<sub>4</sub> production and ruminal culture pH.

#### *Substrate and Energy Partition*

Total gas production (including the indirect contribution of CO<sub>2</sub> from the infused bicarbonate-based saliva), and the substrate partition towards VFA production and microbial biomass were unaffected by forage or grain level (Table 9). An interaction of forage by grain level ( $P = 0.08$ ) was observed for substrate used as CH<sub>4</sub> plus fermentative CO<sub>2</sub> (Figure 10), following the CH<sub>4</sub> production pattern previously described.

Total substrate partitioned towards VFA, gas production and microbial biomass remained unaffected by forage or grain supplementation. However, a forage by grain level interaction trend ( $P = 0.13$ ) was observed for total substrate partitioning: the EN tall fescue linearly decreased substrate partitioning towards fermentation end products (including calculated microbial biomass) as concentrates increased in the diets, as opposed to E+ and E- that increased substrate partitioning towards fermentation end products with the higher

concentrate supplementation. Consequently, ruminal fermentability exhibited the same response pattern, and averaged 54.6% across forage and grain treatments.

Microbial efficiency (grams of microbial DM per kg DM fermented) decreased ( $P < 0.05$ ) linearly with increasing grain levels (Figure 11). Although statistically significant, the differences may not be biologically significant, because an 18-g difference between the highest and lowest microbial efficiency values seemed to have been minimally affected (or even unaffected) by the forage:concentrate ratios tested in our study. In a similar fashion, Hoover and Stokes (1991), summarizing the results of seven continuous culture studies, reported that increasing the levels of non-structural carbohydrates (NSC) as a percentage of total carbohydrates fed, such as occurred in the current study, had a minimal, but negative, impact on microbial efficiency ( $r = -0.14$ ).

The effects of forage and grain supplementation on substrate energy partition are presented in Table 10. Both, the energy provided daily from VFA production in terms of kcal/d and as a percentage of digestible energy intake, remained unaffected by forage or grain level, even when DE fed daily increased linearly with grain supplementation. These results are in agreement with previously reported data on total VFA production.

Conversely, the amount of energy produced by  $\text{CH}_4$  expressed as a percentage of daily DE fed decreased linearly ( $P < 0.05$ ) with supplemental grain. Although methane production remained unaffected by grain levels, the higher DE intakes from supplemented diets allowed for a lower percentage in energy partition towards gas production. In addition, an interaction of forage-by-grain supplementation ( $P = 0.08$ ) was observed. Methane energy as a % of DE intake remained relatively unaffected in the endophyte-infected tall fescue across grain levels. However, the endophyte-free forage and, particularly, the novel

endophyte forage exhibited a significant reduction in CH<sub>4</sub> energy as a % of DE intake for the highest grain level (45% grain), becoming the energy supplementation level at which clear differences in fermentation patterns are observed among forages.

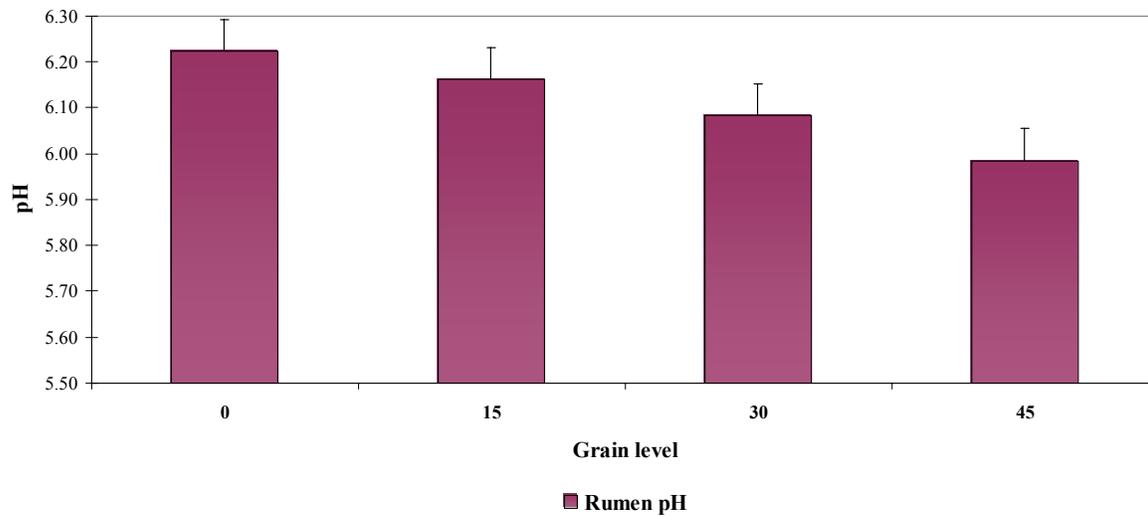
In summary, overall ruminal fermentation characteristics were minimally altered by the forages tested, except at the highest energy supplementation level. The forage results are consistent with those reported by other tall fescue studies where endophyte-infected and endophyte-free cultivars were compared (Harmon et al., 1991; Humphry et al., 2002). In contrast, altering forage:concentrate ratios by increasing the levels of energy in the forage-based diets had a significant impact on fermentation in the rumen. Previous continuous culture studies have reported lower ruminal culture pH, reduced NH<sub>3</sub> N accumulation, and a lower acetate:propionate ratio with increased grain supplementation (Kolver et al., 1998; Bach et al., 1999; Bargo et al., 2003). However, in all three of those studies, bacterial N and NAN flow were similar among treatments, and were only increased as a percentage of N intakes, similar to what can be speculated occurred in our study.

## CONCLUSIONS

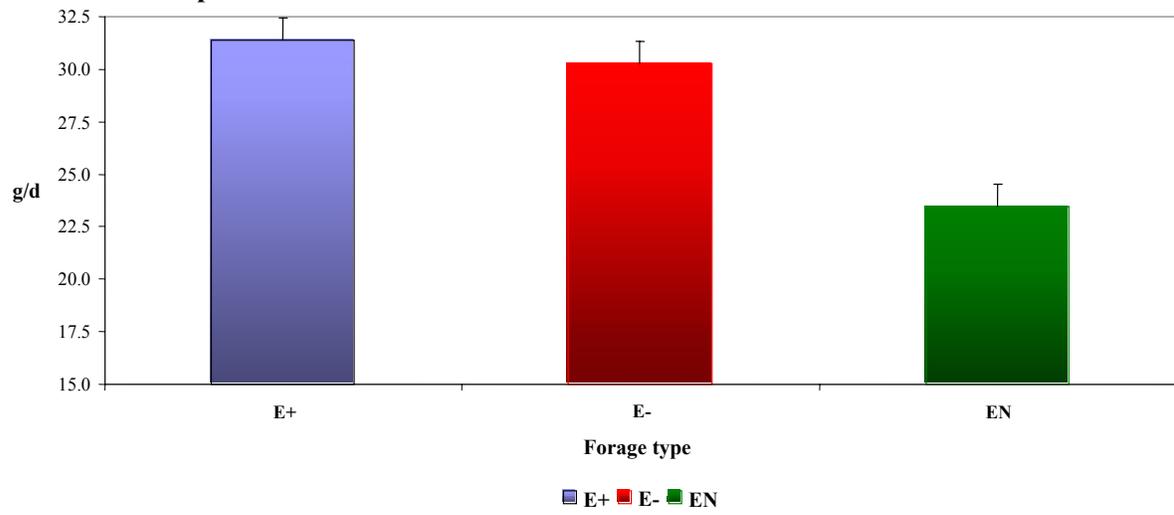
Continuous culture fermentation pattern was followed to investigate possible fungus effects from wild-type or novel-type endophytes present in tall fescue grasses, either alone or in association with diets that altered forage to concentrate ratios. The presence of the endophyte did not seem to alter forage quality, and up to a certain point, microbial fermentation. Increasing levels of grain within forage-based diets decreased ruminal culture pH, ammonia N, acetate, and the acetate to propionate ratio; whereas propionate and butyrate increased with higher concentrate levels.

Under the present study conditions, the grain-induced culture pH drop for the highest level of grain fed (45%) altered the methane production pattern for all three grasses. In addition to having the lowest ruminal ammonia N accumulation, EN produced the least amount of methane, placing it as the most energetically efficient forage tested at this energy supplementation level.

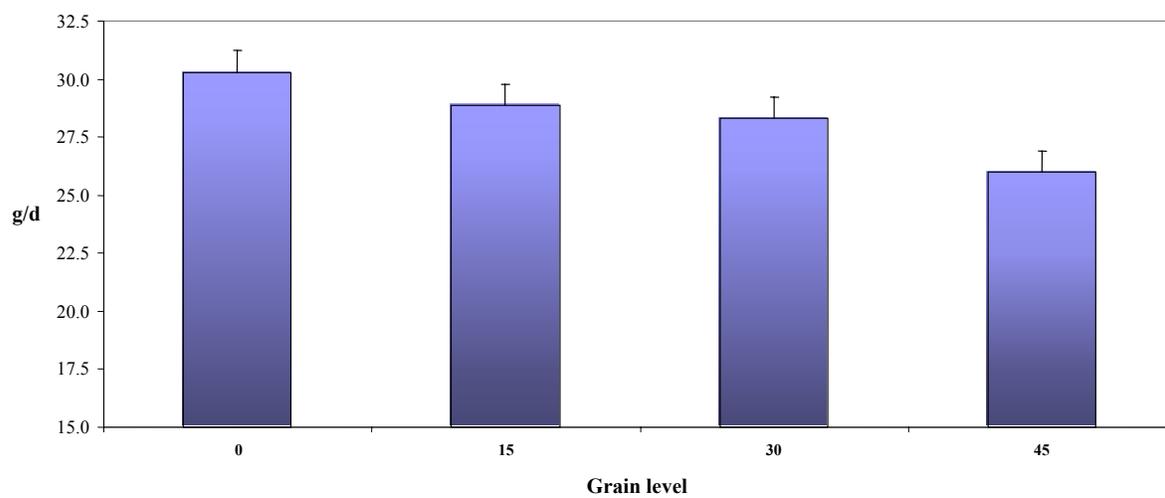
**Figure 2. Grain effect (diets unsupplemented or supplemented with 15, 30, and 45% ground corn, DM basis) on rumen culture pH**



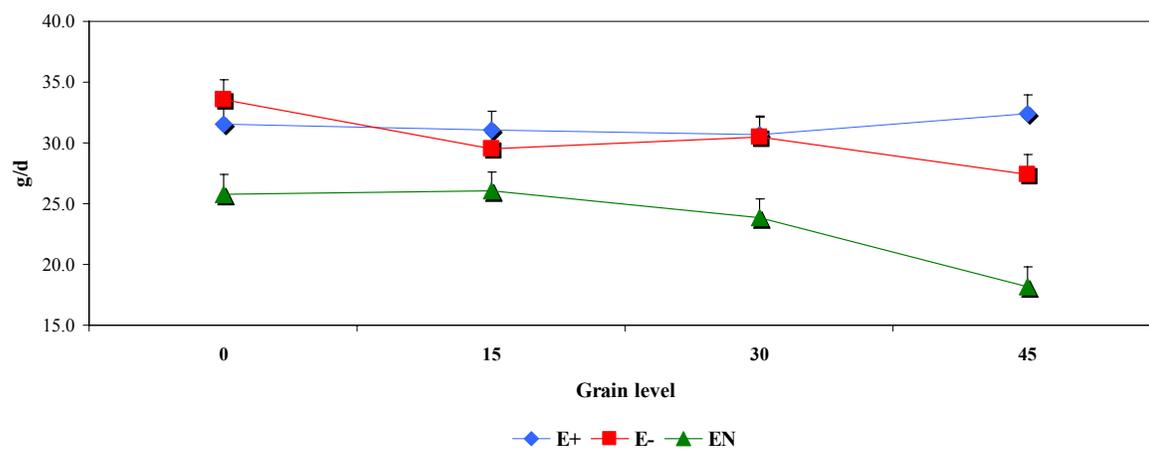
**Figure 3. Effect of forage (endophyte-infected, E+; endophyte-free, E-; and non-toxic, endophyte-infected, EN tall fescue) on ammonia N output**



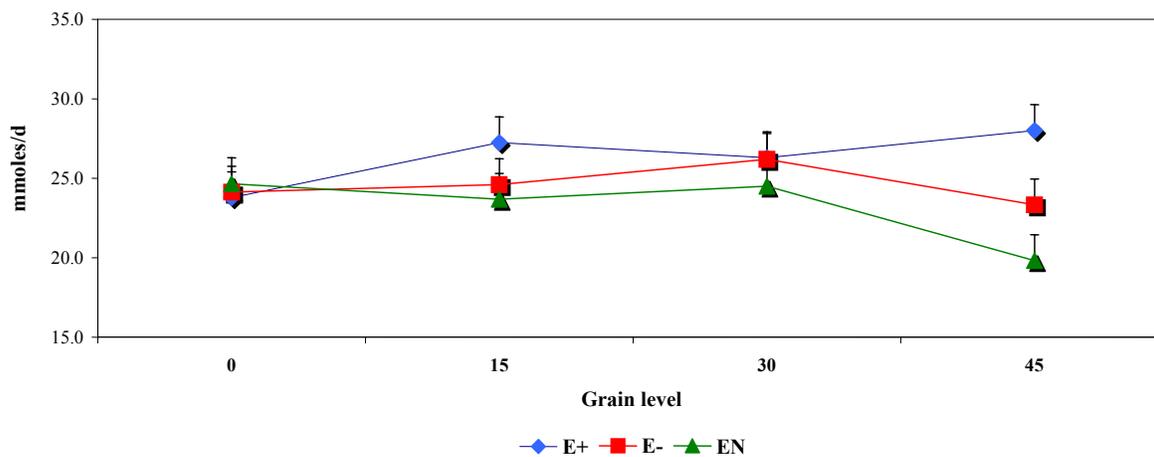
**Figure 4. Grain effect (diets unsupplemented or supplemented with 15, 30, and 45% ground corn, DM basis) on ammonia N output**



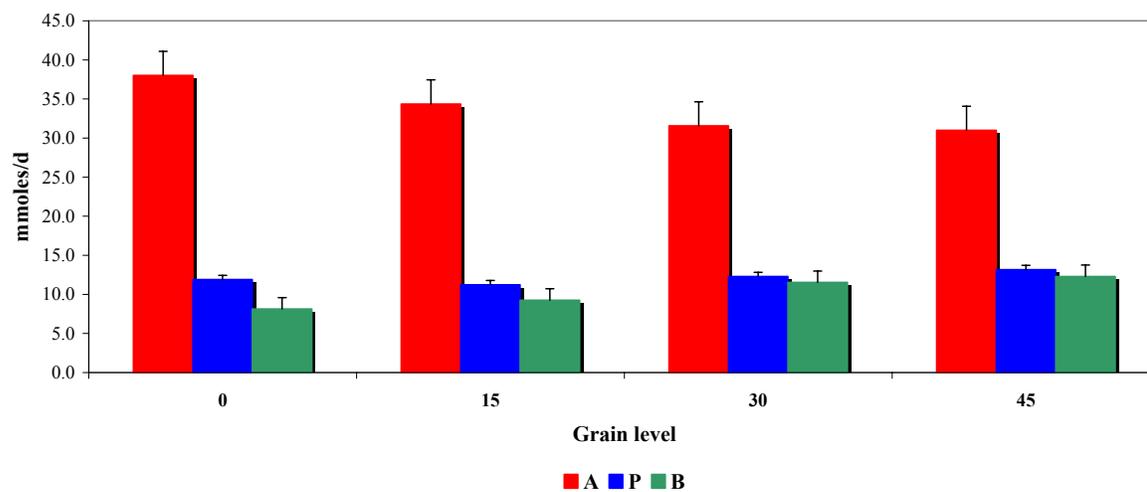
**Figure 5. Ammonia N output: forage (endophyte-infected, E+; endophyte-free, E-; and nontoxic, endophyte-infected, EN tall fescue) by grain (diets unsupplemented or supplemented with 15, 30, and 45% ground corn, DM basis) interaction**



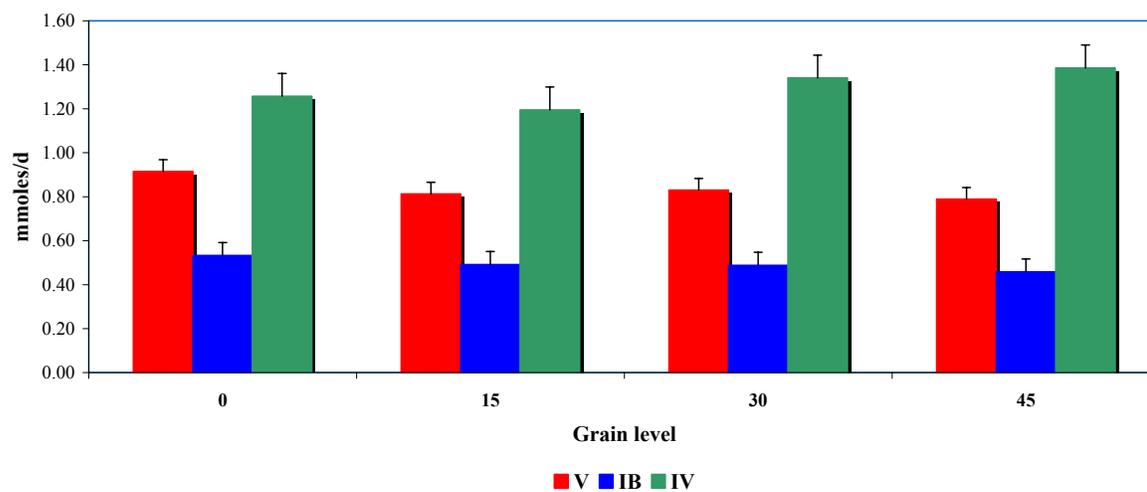
**Figure 6. Methane production: forage (endophyte-infected, E+; endophyte-free, E-; and non-toxic, endophyte-infected, EN tall fescue) by grain (diets unsupplemented or supplemented with 15, 30, and 45% ground corn, DM basis) interaction**



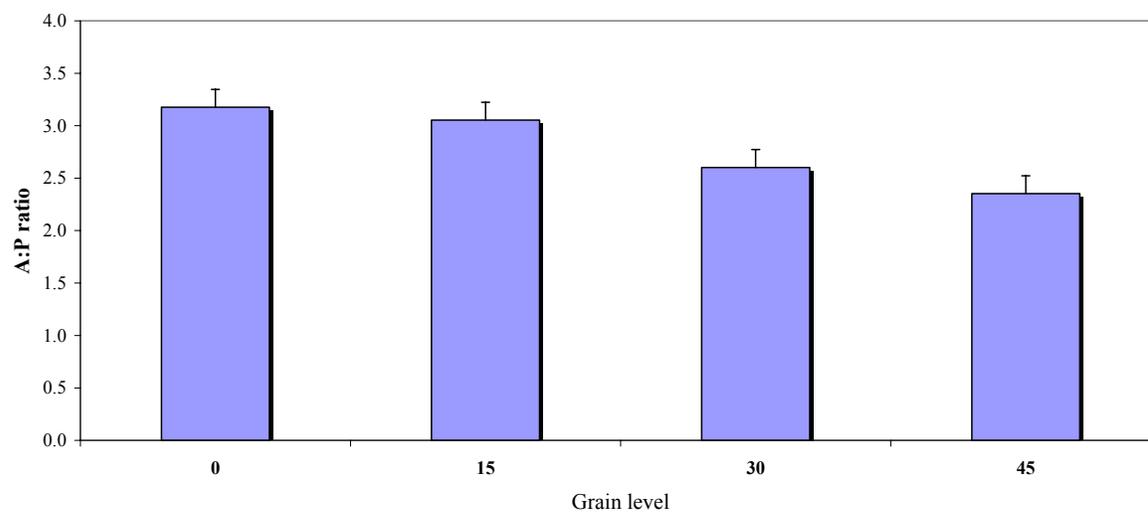
**Figure 7. Grain effect (diets unsupplemented or supplemented with 15, 30, and 45% ground corn, DM basis) on acetate (A), propionate (P), and butyrate (B) production**



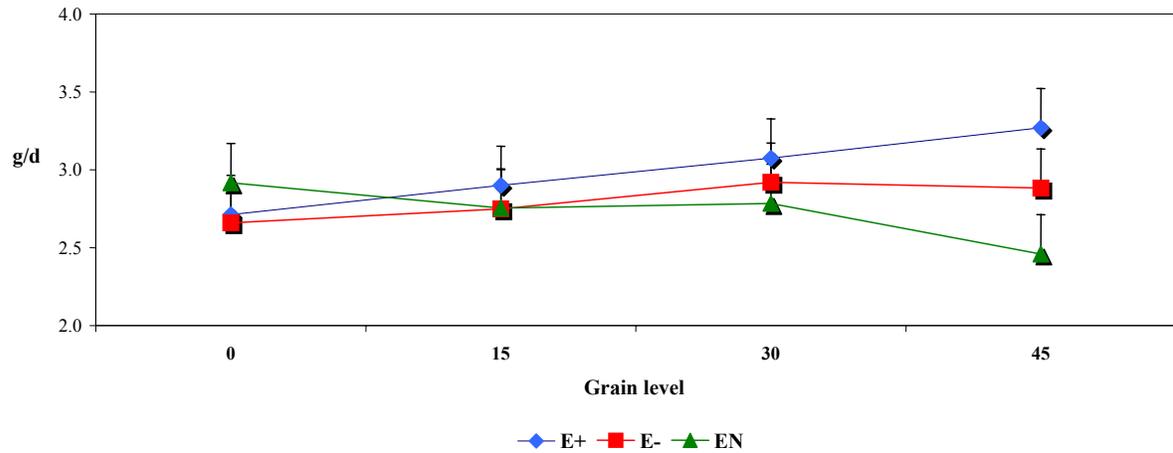
**Figure 8. Grain effect (diets unsupplemented or supplemented with 15, 30, and 45% ground corn, DM basis) on valerate (V), isobutyrate (IB), and isovalerate (IV) production**



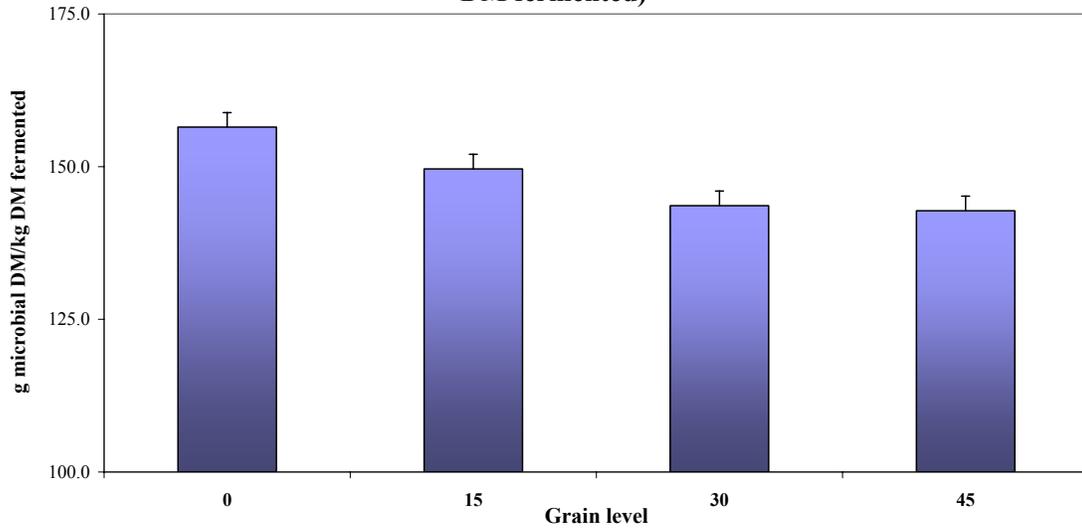
**Figure 9. Grain effect (diets unsupplemented or supplemented with 15, 30, and 45% ground corn, DM basis) on the acetate to propionate ratio**



**Figure 10. Methane plus fermentative CO<sub>2</sub>: forage (endophyte-infected, E+; endophyte-free, E-; and non-toxic, endophyte-infected, EN tall fescue) by grain (diets unsupplemented or supplemented with 15, 30, and 45% ground corn, DM basis) interaction**



**Figure 11. Grain effect (diets unsupplemented or supplemented with 15, 30, and 45% ground corn, DM basis) on microbial efficiency (g microbial DM/kg DM fermented)**



**Table 1. Forage yields (kg DM/ha of harvested and stubble portions), endophyte infection, and ergovaline concentration of endophyte-infected (E+), endophyte-free (E-), and non-toxic, endophyte- infected (EN) tall fescues.**

	Forage type <sup>1</sup>		
	E+	E-	EN
Yield <sup>2</sup> , kg of DM/ha			
Stubble	1536	1752	1701
Harvested	410	419	597
Total	1946	2171	2298
Harvested, % of total	21.1	19.3	26.0
Endophyte infection <sup>3</sup> , %	85.8	4.6	91.6
Ergot alkaloids <sup>3</sup> , % of inf. tillers	100.0	100.0	3.2
Ergovaline, ppb	30	0	0

<sup>1</sup>Forage type: endophyte-infected (E+), endophyte-free (E-), and non-toxic, infected (EN) tall fescue

<sup>2</sup> Plot size: 37.2 m<sup>2</sup>

<sup>3</sup>Tillers sampled Nov. 29, 2001

**Table 2: Chemical composition of the forage and grain ingredients**

Item	Forage type <sup>1</sup>			GC
	E+	E-	EN	
DM, %	29.6	31.6	32.8	87.8
Nutrients, % of DM				
OM	88.9	91.3	90.3	98.6
NDF	59.9	60.7	63.2	9.8
ADF	29.3	29.5	31.4	2.8
Cellulose	26.7	26.5	28.5	2.3
Lignin	2.5	3.0	2.9	0.1
CP	12.3	13.4	10.4	9.0
N fractions <sup>2</sup> , % of CP				
A (NPN)	8.6	10.6	7.4	6.4
B1 (soluble protein)	8.3	9.9	7.1	9.1
B2 (IP - NDIP)	78.1	72.9	81.3	74.0
B3 (NDIP - ADIP)	4.0	5.4	3.2	7.8
C (ADIP)	1.0	1.2	1.0	2.8
Gross Energy <sup>3</sup> , kcal/g	4.1	4.3	4.1	4.0
TDN <sup>4</sup>	69.2	69.0	67.5	86.5

<sup>1</sup>Forage type: endophyte-infected (E+), endophyte-free (E-), and non-toxic, infected (EN) tall fescue

GC: ground corn.

<sup>2</sup>NPN: nonprotein N; IP: insoluble protein; NDIP: neutral-detergent insoluble protein; ADIP: acid-detergent insoluble protein (Van Soest, 1994).

<sup>3</sup>Measured with a bomb calorimeter

<sup>4</sup>For forages,  $TDN = 92.5135 - (0.7965 \times \%ADF)$ ; for GC: NRC (2001) value.

**Table 3. Chemical composition of the final diets**

Item	Forage type <sup>1</sup>											
	E+				E-				EN			
	Grain levels <sup>2</sup>											
	0	15	30	45	0	15	30	45	0	15	30	45
DM, %	29.6	38.3	47.1	55.8	31.6	40.0	48.5	56.9	32.8	41.1	49.3	57.6
Nutrients, % of DM												
OM	88.9	90.4	91.8	93.3	91.3	92.4	93.5	94.6	90.3	91.5	92.8	94.0
NDF	59.9	52.4	44.9	37.4	60.7	53.1	45.4	37.8	63.2	55.2	47.2	39.2
ADF	29.3	25.3	21.4	17.4	29.5	25.5	21.5	17.5	31.4	27.1	22.8	18.5
Hemicellulose	30.6	27.1	23.5	20.0	31.2	27.6	23.9	20.3	31.8	28.1	24.4	20.7
Cellulose	26.7	23.0	19.4	15.7	26.5	22.9	19.2	15.6	28.5	24.6	20.6	16.7
Lignin	2.5	2.2	1.8	1.4	3.0	2.6	2.1	1.7	2.9	2.4	2.0	1.6
CP	12.3	11.8	11.3	10.9	13.4	12.7	12.1	11.4	10.4	10.2	10.0	9.8
N fractions <sup>3</sup> , % of CP												
A (NPN)	8.6	8.3	7.9	7.6	10.6	10.0	9.3	8.7	7.4	7.3	7.1	7.0
B1 (soluble protein)	8.3	8.4	8.5	8.7	9.9	9.8	9.7	9.5	7.1	7.4	7.7	8.0
B2 (IP - NDIP)	78.1	77.5	76.9	76.3	72.9	73.1	73.2	73.4	81.3	80.2	79.1	78.0
B3 (NDIP - ADIP)	4.0	4.6	5.1	5.7	5.4	5.8	6.1	6.5	3.2	3.9	4.6	5.3
C (ADIP)	1.0	1.3	1.5	1.8	1.2	1.4	1.7	1.9	1.0	1.2	1.5	1.8
TDN <sup>4</sup>	69.2	71.8	74.4	77.0	69.0	71.6	74.3	76.9	67.5	70.4	73.2	76.1
IVTDMD <sup>5</sup>	73.1	76.3	80.0	84.3	70.6	74.8	78.7	83.3	69.0	72.8	77.4	80.8

<sup>1</sup>Forage type: endophyte-infected (E+), endophyte-free (E-), and non-toxic, infected (EN) tall fescue

<sup>2</sup>Grain levels in the diet: 0, 15, 30, and 45%

<sup>3</sup>NPN: nonprotein N; IP: insoluble protein; NDIP: neutral-detergent insoluble protein; ADIP: acid-detergent insoluble protein (Van Soest, 1994)

<sup>4</sup>for forages, TDN = 92.5135 - (0.7965 x %ADF); for GC: NRC (2001) value.

<sup>5</sup>IVTDMD: In vitro true dry matter disappearance

**Table 4. Effect of forage and grain supplementation on ruminal culture pH and CH<sub>4</sub> and NH<sub>3</sub>N production (n = 2) using the MIXED procedure of SAS (1999)**

Variable	Forage type <sup>1</sup>												SE <sub>f</sub>	SE <sub>g</sub>	SE <sub>i</sub>	Significance of Effects		
	E+				E-				EN							F	G	F x G
	Grain levels <sup>2</sup>																	
	0	15	30	45	0	15	30	45	0	15	30	45						
<b>pH</b>	6.3	6.2	6.1	6.0	6.1	6.0	6.0	5.9	6.2	6.2	6.1	6.0	0.12	0.07	0.07	0.69	< 0.001	0.60
<b>NH<sub>3</sub>-N, g/d</b>	31.5 <sup>cd</sup>	31.0 <sup>cd</sup>	30.6 <sup>cd</sup>	32.4 <sup>cd</sup>	33.6 <sup>d</sup>	29.5 <sup>c</sup>	30.5 <sup>cd</sup>	27.4 <sup>bc</sup>	25.8 <sup>bc</sup>	26.0 <sup>bc</sup>	23.8 <sup>b</sup>	18.2 <sup>a</sup>	1.05	0.92	1.60	0.06	0.03	0.08
<b>CH<sub>4</sub>, mmoles/d</b>	23.8 <sup>b</sup>	27.2 <sup>c</sup>	26.3 <sup>bc</sup>	28.0 <sup>c</sup>	24.1 <sup>b</sup>	24.6 <sup>bc</sup>	26.2 <sup>bc</sup>	23.3 <sup>ab</sup>	24.7 <sup>bc</sup>	23.7 <sup>b</sup>	24.5 <sup>bc</sup>	19.8 <sup>a</sup>	1.28	1.02	1.63	0.36	0.23	0.08

<sup>1</sup>Forage type: endophyte-infected (E+), endophyte-free (E-), and non-toxic, infected (EN) tall fescue

<sup>2</sup>Grain levels in the diet: 0, 15, 30, and 45%

Least square means in the same row with different superscripts differ ( $P < 0.05$ )

SE<sub>f</sub> = Standard error for forage type

SE<sub>g</sub> = Standard error for grain level

SE<sub>i</sub> = Standard error for the interaction

F = Forage type; G = Grain level; F x G = Interaction between forage type and grain level.

**Table 5. Grain supplementation: probability estimates for linear, quadratic and cubic responses (n = 2) using the MIXED procedure of SAS (1999)**

Variable	Effect <sup>1</sup>		
	Linear	Quadratic	Cubic
<b>pH</b>	<0.001	0.35	0.99
<b>CH<sub>4</sub></b> , mmoles/d	0.77	0.05	0.53
<b>NH<sub>3</sub>-N</b> , g/d	0.003	0.59	0.47

<sup>1</sup>Significance and trends are established at  $P < 0.05$  and  $P < 0.10$ , respectively

Table 6. Effect of forage and grain supplementation on VFA concentration and molar proportions (n = 2) using the MIXED procedure of SAS (1999)

Variable	Forage type <sup>1</sup>												SE <sub>f</sub>	SE <sub>g</sub>	SE <sub>i</sub>	Significance of Effects		
	E+				E-				EN							F	G	F x G
	0	15	30	45	0	15	30	45	0	15	30	45						
VFA, mM																		
<b>Acetate</b>	36.1	32.8	31.4	32.8	32.9	31.7	29.4	29.8	39.5	33.5	29.2	25.8	3.05	2.92	3.57	0.68	0.02	0.29
<b>Propionate</b>	11.3	10.5	12.7	12.6	10.8	11.1	11.9	12.8	11.9	10.6	10.4	12.1	0.65	0.52	0.90	0.85	0.06	0.45
<b>Butyrate</b>	7.4	8.7	12.0	13.1	7.5	8.7	10.7	12.0	8.4	9.0	10.3	10.0	1.35	1.37	1.58	0.54	0.002	0.45
<b>Isobutyrate</b>	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.4	0.4	0.3	0.06	0.06	0.06	0.29	0.12	0.27
<b>Valerate</b>	0.9	0.8	0.8	0.8	0.9	0.8	0.8	0.8	0.9	0.7	0.7	0.6	0.05	0.05	0.06	0.17	0.04	0.61
<b>Isovalerate</b>	1.3	1.2	1.4	1.6	1.2	1.1	1.2	1.3	1.1	1.1	1.2	1.1	0.15	0.10	0.17	0.58	0.09	0.70
Molar proportions, mol/100 mol																		
<b>Acetate</b>	62.8	60.3	53.4	53.5	60.9	58.7	54.0	52.1	63.4	60.5	56.0	51.5	0.44	0.50	0.87	0.25	<0.001	0.28
<b>Propionate</b>	19.7	19.2	21.8	20.7	20.2	20.7	21.8	22.9	19.2	19.2	19.9	24.3	1.12	1.17	1.50	0.53	0.04	0.42
<b>Butyrate</b>	12.8	16.0	20.2	21.2	14.0	16.1	19.6	20.6	13.4	16.3	19.7	20.0	0.99	1.03	1.31	0.95	<0.001	0.95
<b>Isobutyrate</b>	0.9	0.9	0.8	0.8	1.0	0.9	0.9	0.8	0.8	0.8	0.8	0.7	0.03	0.03	0.04	0.07	0.02	0.32
<b>Valerate</b>	1.5	1.4	1.4	1.3	1.7	1.6	1.6	1.5	1.4	1.3	1.4	1.3	0.07	0.05	0.09	0.24	0.04	0.92
<b>Isovalerate</b>	2.2	2.2	2.4	2.5	2.2	2.1	2.2	2.2	1.8	1.9	2.2	2.3	0.16	0.10	0.17	0.59	<0.001	0.15

<sup>1</sup>Forage type: endophyte-infected (E+), endophyte-free (E-), and non-toxic, infected (EN) tall fescue

<sup>2</sup>Grain levels in the diet: 0, 15, 30, and 45%

SE<sub>f</sub> = Standard error for forage type

SE<sub>g</sub> = Standard error for grain level

SE<sub>i</sub> = Standard error for the interaction

F = Forage type; G = Grain level; F x G = Interaction between forage type and grain level.

**Table 7. Effect of forage and grain supplementation on VFA production (n = 2) using the MIXED procedure of SAS (1999)**

Variable	Forage type <sup>1</sup>												SE <sub>f</sub>	SE <sub>g</sub>	SE <sub>i</sub>	Significance of Effects		
	E+				E-				EN							F	G	F x G
	0	15	30	45	0	15	30	45	0	15	30	45						
<b>Acetate</b> (mmoles/d)	37.9	34.5	33.0	34.5	34.6	33.4	30.9	31.4	41.5	35.2	30.7	27.1	3.20	3.08	3.75	0.68	0.02	0.29
<b>Propionate</b> (mmoles/d)	11.9	11.0	13.4	13.3	11.4	11.7	12.5	13.5	12.5	11.1	11.0	12.8	0.68	0.54	0.94	0.85	0.06	0.45
<b>Butyrate</b> (mmoles/d)	7.7	9.1	12.6	13.8	7.8	9.2	11.2	12.6	8.9	9.5	10.8	10.5	1.42	1.45	1.66	0.54	0.002	0.45
<b>Isobutyrate</b> (mmoles/d)	0.5	0.5	0.5	0.5	0.6	0.5	0.5	0.5	0.5	0.5	0.4	0.3	0.061	0.059	0.068	0.30	0.13	0.29
<b>Valerate</b> (mmoles/d)	0.9	0.8	0.9	0.8	0.9	0.9	0.9	0.9	0.9	0.8	0.8	0.7	0.05	0.05	0.07	0.17	0.04	0.57
<b>Isovalerate</b> (mmoles/d)	1.4	1.3	1.5	1.6	1.2	1.2	1.3	1.3	1.2	1.1	1.2	1.2	0.16	0.10	0.18	0.58	0.08	0.69
<b>Total</b> (mmoles/d)	60.4	57.2	61.9	64.6	56.5	56.8	57.3	60.2	65.4	58.1	54.9	52.5	5.40	5.06	6.10	0.72	0.63	0.22
<b>A:P ratio</b>	3.2	3.1	2.5	2.6	3.0	2.8	2.5	2.3	3.3	3.2	2.9	2.1	0.16	0.17	0.21	0.35	<0.001	0.28

<sup>1</sup>Forage type: endophyte-infected (E+), endophyte-free (E-), and non-toxic, infected (EN) tall fescue

<sup>2</sup>Grain levels in the diet: 0, 15, 30, and 45%

SE<sub>f</sub> = Standard error for forage type

SE<sub>g</sub> = Standard error for grain level

SE<sub>i</sub> = Standard error for the interaction

F = Forage type; G = Grain level; F x G = Interaction between forage type and grain level.

**Table 8. Grain supplementation: probability estimates for linear, quadratic and cubic responses (n = 2) using the MIXED procedure of SAS (1999)**

Variable	Effect <sup>1</sup>		
	Linear	Quadratic	Cubic
<b>Acetate</b> (mmoles/d)	0.002	0.26	0.82
<b>Propionate</b> (mmoles/d)	0.03	0.10	0.36
<b>Butyrate</b> (mmoles/d)	<0.001	0.76	0.32
<b>Isobutyrate</b> (mmoles/d)	0.02	0.79	0.48
<b>Valerate</b> (mmoles/d)	0.01	0.26	0.15
<b>Isovalerate</b> (mmoles/d)	0.03	0.29	0.18
<b>Total</b> (mmoles/d)	0.62	0.27	0.67
<b>A:P ratio</b>	<0.001	0.51	0.23

<sup>1</sup>Significance and trends are established at  $P < 0.05$  and  $P < 0.10$ , respectively

**Table 9. Effect of forage and grain supplementation on substrate partition, fermentability and microbial efficiency (n = 2) using the MIXED procedure of SAS (1999)**

Variable	Forage type <sup>1</sup>												SEf	SEg	SEi	Significance of Effects		
	E+				E-				EN							F	G	F x G
	0	15	30	45	0	15	30	45	0	15	30	45						
<b>Total gas, est<sup>3</sup></b> (mmol/d)	117.7	118.2	127.0	133.8	112.5	114.8	119.0	121.5	127.2	116.4	113.7	104.9	10.34	9.61	11.48	0.61	0.86	0.13
Substrate used																		
<b>VFA<sup>4</sup>, g/d</b>	3.8	3.7	4.1	4.3	3.6	3.7	3.8	4.0	4.2	3.8	3.6	3.5	0.36	0.34	0.40	0.74	0.66	0.21
<b>CH<sub>4</sub> + fCO<sub>2</sub><sup>5</sup>, g/d</b>	2.7 <sup>ab</sup>	2.9 <sup>ab</sup>	3.1 <sup>bc</sup>	3.3 <sup>c</sup>	2.7 <sup>ab</sup>	2.8 <sup>ab</sup>	2.9 <sup>b</sup>	2.9 <sup>ab</sup>	2.9 <sup>b</sup>	2.8 <sup>ab</sup>	2.8 <sup>ab</sup>	2.5 <sup>a</sup>	0.23	0.21	0.25	0.49	0.46	0.08
<b>MB<sup>6</sup>, g/d</b>	1.6	1.5	1.6	1.6	1.5	1.5	1.5	1.5	1.7	1.5	1.4	1.3	0.11	0.10	0.13	0.71	0.19	0.20
<b>Total, g/d</b>	8.1	8.1	8.7	9.1	7.8	7.9	8.2	8.4	8.8	8.0	7.8	7.3	0.70	0.65	0.78	0.65	0.85	0.13
<b>Total diet fed</b> (g DM/d)	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0						
<b>Ferm<sup>7</sup>, %</b>	43.7	43.9	47.8	50.3	41.8	42.8	44.6	45.9	47.4	43.5	42.6	39.7	3.89	3.62	4.32	0.63	0.73	0.13
<b>Ferm<sup>8</sup>, %</b>	54.3	54.0	58.1	61.0	51.8	52.7	54.5	55.8	58.8	53.5	51.9	48.5	4.68	4.31	5.21	0.65	0.85	0.13
<b>Microbial eff.<sup>9</sup></b> (g/kg of DM f)	156.0	149.6	143.2	140.8	154.0	150.2	144.4	143.0	154.7	149.1	143.2	144.6	2.30	2.39	3.04	0.94	<0.001	0.91

<sup>1</sup>Forage type: endophyte-infected (E+), endophyte-free (E-), and non-toxic, infected (EN) tall fescue

<sup>2</sup>Grain levels in the diet: 0, 15, 30, and 45%. Least square means in the same row with different superscripts differ ( $P < 0.05$ )

SE<sub>f</sub> = Standard error for forage type; SE<sub>g</sub> = Standard error for grain level; SE<sub>i</sub> = Standard error for the interaction

F = Forage type; G = Grain level; F x G = Interaction between forage type and grain level.

<sup>3</sup>Fermentative CH<sub>4</sub> + fermentative CO<sub>2</sub>(c) + buffering CO<sub>2</sub>(c); (c) = Calculated.

<sup>4</sup>(Acetate, mol/d x 60.05) + (Propionate, mol/d x 74.08) + (Butyrate, mol/d x 88.1)

<sup>5</sup>(CO<sub>2</sub>, mol/d x 44) + (CH<sub>4</sub>, mol/d x 16) + (CH<sub>4</sub>, mol/d x 36)

<sup>6</sup>ATP (mmol) x Y<sub>ATP</sub> (11.6 mg)

<sup>7</sup>Substrate from VFA and gas production expressed as a % of total DM fed per d

<sup>8</sup>Total substrate fermented expressed as a % of total DM fed per d

<sup>9</sup>Microbial efficiency (g microbial DM/kg DM fermented) = [(Microbial biomass, g/DM truly fermented, g) x 1000]

**Table 10. Effect of forage and grain supplementation on energy partition (n = 2) using the MIXED procedure of SAS (1999)**

Variable	Forage type <sup>1</sup>												SE <sub>f</sub>	SE <sub>g</sub>	SE <sub>i</sub>	Significance of Effects		
	E+				E-				EN							F	G	F x G
	Grain levels <sup>2</sup>																	
	0	15	30	45	0	15	30	45	0	15	30	45						
GE fed <sup>3</sup> , kcal/d	61.7	61.3	61.0	60.6	64.0	63.3	62.6	61.9	61.7	61.4	61.0	60.6						
DE fed <sup>4</sup> , kcal/d	45.1	46.8	48.8	51.1	45.2	47.3	49.3	51.5	42.6	44.7	47.2	49.0						
VFA																		
Kcal/d <sup>5</sup>	18.2	17.7	20.3	21.3	17.3	17.8	18.7	19.9	19.6	17.9	17.7	17.3	1.76	1.65	1.96	0.68	0.27	0.20
% of DE	40.3	37.9	41.7	41.7	38.2	37.5	38.0	38.6	46.0	40.2	37.5	35.3	3.67	3.50	4.13	0.72	0.33	0.18
CH <sub>4</sub>																		
Kcal/d <sup>6</sup>	5.0 <sup>b</sup>	5.7 <sup>c</sup>	5.5 <sup>bc</sup>	5.9 <sup>c</sup>	5.1 <sup>b</sup>	5.2 <sup>bc</sup>	5.5 <sup>bc</sup>	4.9 <sup>b</sup>	5.2 <sup>bc</sup>	5.0 <sup>b</sup>	5.2 <sup>bc</sup>	4.2 <sup>a</sup>	0.27	0.21	0.34	0.36	0.23	0.08
% of DE	11.1 <sup>c</sup>	12.3 <sup>d</sup>	11.4 <sup>c</sup>	11.6 <sup>cd</sup>	11.3 <sup>c</sup>	11.0 <sup>bc</sup>	11.2 <sup>c</sup>	9.5 <sup>ab</sup>	12.2 <sup>d</sup>	11.2 <sup>c</sup>	10.9 <sup>bc</sup>	8.5 <sup>a</sup>	0.56	0.45	0.72	0.53	0.01	0.08

<sup>1</sup>Forage type: endophyte-infected (E+), endophyte-free (E-), and non-toxic, infected (EN) tall fescue

<sup>2</sup>Grain levels in the diet: 0, 15, 30, and 45%. Least square means in the same row with different superscripts differ ( $P < 0.05$ )

SE<sub>f</sub> = Standard error for forage type; SE<sub>g</sub> = Standard error for grain level; SE<sub>i</sub> = Standard error for the interaction

F = Forage type; G = Grain level; F x G = Interaction between forage type and grain level.

<sup>3</sup>GE: Gross energy, determined by bomb calorimeter

<sup>4</sup>DE: Digestible energy, from IVTDMD

<sup>5</sup>(Acetate, mol/d x 209.4 kcal/mol) + (Propionate, mol/d x 367.2 kcal/mol) + (Butyrate, mol/d x 524.3 kcal/mol) + (Valerate, mol/d x 681.6 kcal/mol) + (Isobutyrate, mol/d x 524.3 kcal/mol) + (Isovalerate, mol/d x 681.6 kcal/mol)

<sup>6</sup>(CH<sub>4</sub>, mol/d x 210.8 kcal/mol)

## **APPENDIX**

**Table 1a. Endophyte-infected tall fescue: effect of grain supplementation and sampling time on methane and ammonia N production (n = 2)**

Variable	Grain levels <sup>1</sup>								SE <sub>g</sub>	SE <sub>St</sub>	SE <sub>i</sub>	Significance of Effects		
	0		15		30		45					G	St	G x St
	Sampling time <sup>2</sup>													
am	pm	am	pm	am	pm	am	pm	G	St	G x St				
CH <sub>4</sub> , mmol/d	25.1	22.4	27.7	26.8	27.5	25.1	31.0	25.0	2.05	1.91	2.32	0.20	0.05	0.48
CH <sub>4</sub> , % of DE	11.7	10.5	12.5	12.1	11.9	10.8	12.8	10.3	0.91	0.85	1.02	0.46	0.05	0.53
NH <sub>3</sub> -N, g/d	30.6	32.5	30.1	31.9	28.9	32.4	29.7	35.1	1.20	0.94	1.59	0.70	0.04	0.63

**Table 1b. Endophyte-free tall fescue: effect of grain supplementation and sampling time on methane and ammonia N production (n = 2)**

Variable	Grain levels <sup>1</sup>								SE <sub>g</sub>	SE <sub>St</sub>	SE <sub>i</sub>	Significance of Effects		
	0		15		30		45					G	St	G x St
	Sampling time <sup>2</sup>													
am	pm	am	pm	am	pm	am	pm	G	St	G x St				
CH <sub>4</sub> , mmol/d	25.7	22.6	25.6	23.6	27.7	24.7	24.6	22.0	1.39	1.23	1.66	0.32	0.04	0.97
CH <sub>4</sub> , % of DE	12.0	10.5	11.4	10.5	11.9	10.6	10.1	9.0	0.60	0.53	0.71	0.13	0.04	0.95
NH <sub>3</sub> -N, g/d	34.2	33.0	28.9	30.2	32.1	28.9	29.9	24.9	2.27	1.71	2.49	0.29	0.12	0.30

**Table 1c. Non-toxic, endophyte-infected tall fescue: effect of grain supplementation and sampling time on methane and ammonia N production (n = 2)**

Variable	Grain levels <sup>1</sup>								SE <sub>g</sub>	SE <sub>St</sub>	SE <sub>i</sub>	Significance of Effects		
	0		15		30		45					G	St	G x St
	Sampling time <sup>2</sup>													
am	pm	am	pm	am	pm	am	pm	G	St	G x St				
CH <sub>4</sub> , mmol/d	23.6	25.7	23.8	23.5	25.1	23.9	20.1	19.6	1.55	0.82	1.64	0.27	0.94	0.32
CH <sub>4</sub> , % of DE	11.7	12.7	11.2	11.1	11.2	10.7	8.6	8.4	0.69	0.37	0.74	0.11	0.88	0.32
NH <sub>3</sub> -N, g/d	26.8	24.7	25.8	26.3	22.7	24.9	18.3	18.1	1.07	0.67	1.33	0.04	0.89	0.39

<sup>1</sup>Grain levels in the diet: 0, 15, 30, and 45%

<sup>2</sup>Sampling time: 1 and 2 h after the 0900 meal, and 1 and 2 h after the 1500 meal for CH<sub>4</sub>

<sup>2</sup>Sampling time: 1 h after the 0900 meal, and 1 h after the 1500 meal for NH<sub>3</sub>-N

SE<sub>g</sub> = Standard error for grain level

SE<sub>St</sub> = Standard error for sampling time

SE<sub>i</sub> = Standard error for the interaction

G = Grain level; St = Sampling time; F x G = Interaction between grain level and sampling time.

**Table 2a. Endophyte-infected tall fescue: effect of grain supplementation and sampling time on ruminal acetate (A) and propionate (P) molar proportions, and the acetate:propionate (A:P) ratios (n = 2)**

Variable	Grain levels <sup>1</sup>								SE <sub>g</sub>	SE <sub>St</sub>	SE <sub>i</sub>	Significance of Effects		
	0		15		30		45					G	St	G x St
	am	pm	am	pm	am	pm	am	pm						
A, mol/100 mol	62.6	63.0	60.5	60.1	53.6	53.2	53.1	53.9	0.45	0.26	0.51	0.001	0.29	0.43
P, mol/100 mol	19.8	19.6	19.2	19.2	21.7	21.9	20.5	20.8	0.95	0.78	0.96	0.18	0.67	0.43
A:P	3.2	3.2	3.2	3.1	2.5	2.4	2.6	2.6	0.12	0.09	0.12	0.02	0.43	0.36

**Table 2b. Endophyte-free tall fescue: effect of grain supplementation and sampling time on ruminal acetate (A) and propionate (P) molar proportions, and the acetate:propionate (A:P) ratios (n = 2)**

Variable	Grain levels <sup>1</sup>								SE <sub>g</sub>	SE <sub>St</sub>	SE <sub>i</sub>	Significance of Effects		
	0		15		30		45					G	St	G x St
	am	pm	am	pm	am	pm	am	pm						
A, mol/100 mol	61.3	60.5	58.8	58.5	54.2	53.7	52.3	51.6	1.20	0.62	1.23	0.04	0.12	0.94
P, mol/100 mol	20.1	20.4	20.5	20.8	22.0	21.5	22.5	23.4	1.92	1.17	1.92	0.72	0.07	0.03
A:P	3.1	3.0	2.9	2.8	2.5	2.5	2.4	2.3	0.24	0.15	0.24	0.27	0.06	0.28

**Table 2c. Non-toxic, endophyte-infected tall fescue: effect of grain supplementation and sampling time on ruminal acetate (A) and propionate (P) molar proportions, and the acetate:propionate (A:P) ratios (n = 2)**

Variable	Grain levels <sup>1</sup>								SE <sub>g</sub>	SE <sub>St</sub>	SE <sub>i</sub>	Significance of Effects		
	0		15		30		45					G	St	G x St
	am	pm	am	pm	am	pm	am	pm						
A, mol/100 mol	63.1	63.9	61.0	60.1	56.2	55.8	51.9	51.1	0.79	0.41	0.82	0.01	0.26	0.20
P, mol/100 mol	19.2	19.2	18.8	19.4	19.9	20.0	23.9	24.7	1.48	1.15	1.48	0.10	0.06	0.21
A:P	3.3	3.3	3.2	3.1	2.9	2.9	2.2	2.1	0.25	0.19	0.25	0.06	0.14	0.24

<sup>1</sup>Grain levels in the diet: 0, 15, 30, and 45%

<sup>2</sup>Sampling time: 1 h after the 0900 meal, and 1 h after the 1500 meal

SE<sub>g</sub> = Standard error for grain level

SE<sub>St</sub> = Standard error for sampling time

SE<sub>i</sub> = Standard error for the interaction

G = Grain level; St = Sampling time; F x G = Interaction between grain level and sampling time.

**Table 3a. Endophyte-infected tall fescue: effect of grain supplementation and sampling time on substrate partition towards gas production and microbial biomass (n = 2)**

Variable	Grain levels <sup>1</sup>								SE <sub>g</sub>	SE <sub>St</sub>	SE <sub>i</sub>	Significance of Effects		
	0		15		30		45					G	St	G x St
	Sampling time <sup>2</sup>													
	am	pm	am	pm	am	pm	am	pm						
Substrate used as														
<b>CH<sub>4</sub> + fCO<sub>2</sub>, g/d</b>	2.8	2.6	2.9	2.9	3.0	3.2	3.2	3.0	0.29	0.27	0.31	0.14	0.66	0.65
<b>MB, g/d</b>	1.6	1.6	1.5	1.5	1.5	1.6	1.6	1.6	0.13	0.12	0.15	0.80	0.75	0.64

**Table 3b. Endophyte-free tall fescue: effect of grain supplementation and sampling time on substrate partition towards gas production and microbial biomass (n = 2)**

Variable	Grain levels <sup>1</sup>								SE <sub>g</sub>	SE <sub>St</sub>	SE <sub>i</sub>	Significance of Effects		
	0		15		30		45					G	St	G x St
	Sampling time <sup>2</sup>													
	am	pm	am	pm	am	pm	am	pm						
Substrate used as														
<b>CH<sub>4</sub> + fCO<sub>2</sub>, g/d</b>	2.9	2.4	2.9	2.6	3.0	2.8	3.0	2.8	0.31	0.29	0.33	0.60	0.09	0.86
<b>MB, g/d</b>	1.7	1.3	1.6	1.4	1.5	1.4	1.5	1.4	0.17	0.16	0.19	1.00	0.11	0.73

**Table 3c. Non-toxic, endophyte-infected tall fescue: effect of grain supplementation and sampling time on substrate partition towards gas production and microbial biomass (n = 2)**

Variable	Grain levels <sup>1</sup>								SE <sub>g</sub>	SE <sub>St</sub>	SE <sub>i</sub>	Significance of Effects		
	0		15		30		45					G	St	G x St
	Sampling time <sup>2</sup>													
	am	pm	am	pm	am	pm	am	pm						
Substrate used as														
<b>CH<sub>4</sub> + fCO<sub>2</sub>, g/d</b>	2.9	2.9	2.8	2.7	2.8	2.7	2.4	2.5	0.12	0.07	0.14	0.25	0.67	0.89
<b>MB, g/d</b>	1.7	1.7	1.5	1.5	1.4	1.4	1.3	1.3	0.06	0.03	0.07	0.06	0.57	0.65

<sup>1</sup>Grain levels in the diet: 0, 15, 30, and 45%

<sup>2</sup>Sampling time: 1 and 2 h after the 0900 meal, and 1 and 2 h after the 1500 meal for the calculated variables

SE<sub>g</sub> = Standard error for grain level

SE<sub>St</sub> = Standard error for sampling time

SE<sub>i</sub> = Standard error for the interaction

G = Grain level; St = Sampling time; F x G = Interaction between grain level and sampling time.

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