Total and Nutrient-Specific In Situ Degradation of Kudzu (*Pueraria montana* var. *lobata*) in the Bovine Rumen

by

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DEDICATION

The author would like to dedicate this thesis to his parents, Salvatore and Missie Gulizia, for their endless support and love throughout his life. With their constant support and love, the author will be another step closer to pursuing his career. Thank you, Mom and Dad, for everything that you have done.

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ABSTRACT

Early and late season kudzu (*Pueraria montana* var. *lobata*) leaves were collected to analyze dry matter (DM), crude protein (CP), acid detergent fiber (ADF), and neutral detergent fiber (NDF) *in situ* rumen disappearance to assess kudzu quality. Four studies were conducted during different growing seasons [2 repeated early season (ES); 2 repeated late season (LS)] to determine age variability effects. Kudzu collected from 7 middle Tennessee counties were incubated in the rumen for designated lengths of time (0.25, 1, 12, 24, 36, 48, and 72 h) to determine total rumen degradation (%) and rate of disappearance (% / h). Data were analyzed as a RCBD with repeated measures. Regression analysis was used to determine degradation rate. Overall season effects (across incubation times) for dry matter disappearance (DMD) were significantly different ($P < 0.0001$). Significant season by incubation time interactions were exhibited for total DMD at 12 ($P < 0.0001$), 24 ($P = 0.0004$), 36 ($P = 0.0055$), 48 ($P = 0.0209$), and 72 h ($P = 0.0384$) incubation. *In situ* DMD for ES was 22.65 (0.25 h), 23.72 (1 h), 70.21 (12 h), 81.55 (24 h), 82.57 (36 h), 82.56 (48 h), and 84.17% (72 h). *In situ* DMD for LS was 20.12 (0.25 h), 20.52 (1 h), 51.40 (12 h), 70.57 (24 h), 75.09 (36 h), 76.52 (48 h), and 78.73% (72 h). Slope regression between 1 and 24 h determined a rumen degradation rate of 2.41% / h across both seasons, with no significant seasonal difference ($P = 0.3396$). These results are strong evidence that ES kudzu is more highly rumen degradable than LS. Although there are significant seasonal effects on kudzu DMD during certain incubation times, DMD overall for ES and LS is still indicative of high rumen degradability. Season effects were minimal for NDF, ADF, and CP rumen disappearance.

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CHAPTER I

REVIEW OF LITERATURE

During its active growing season, kudzu rapidly engulfs many woody and herbaceous areas in the Southeastern United States. It is an invasive plant species that has persistently evaded a definitive control method, and, as such, has created land management problems for many land owners. A major problem that land owners face is the destruction occurring from kudzu on their desired forages for agricultural production. Biological control using ruminant animals, specifically browsers, however, can be a beneficial control method for both the owner of the land and the animals. Kudzu is known to have a high nutritive content that can benefit animals. There is, however, limited data on kudzu's efficiency of degradation in ruminant animals. How effective the rumen of the ruminant animal utilizes kudzu is determined using an *in situ* methodology. *In situ* studies provide data on nutrient degradability by analyzing select browse, concentrates, and forages. The *in situ* research included in this thesis will provide data on how kudzu is degraded within the rumen, an indicator of rumen utilization efficiency.

HISTORY AND CHARCATERISTICS OF KUDZU

History of Kudzu

There are 17 species of kudzu in the genus *Pueraria* throughout the world (Table 1), all of which are native to China, Taiwan, Japan, and India (Britton et al., 2002; Everest et al., 1999; Van Der Maesen, 1985). These cultures have implemented various uses of kudzu, including medicinal purposes or making it into cloth and paper (Everest et al., 1999). Kudzu was first introduced into the United States from Japan in 1876 as a display at the first official World's Fair in Philadelphia in the Plant Exhibition section (Everest et al., 1999; Winberry and Jones, 1973). During the late nineteenth century, kudzu's broad leaves and dense growth was initially used as shade for porches and courtyards in the Southern United States. Kudzu quickly became popular and more common among Southern farmers for its advertised multi-purpose uses, including as soil erosion control, a cheap forage for livestock, and various practical uses around their homes (Everest et al., 1999). In the 1930s, the Natural Resource Conservation Service (NRCS) oversaw combating soil erosion from improper agricultural practices (Everest et al., 1999). They dispersed 85 million kudzu seedlings to Southern farmers to establish kudzu plots for soil erosion control and land revitalization (Everest et al., 1999). Kudzu use in soil erosion control was optimal due to its rapid growth of vines that drop roots every few feet which grip the soil and prevent excess movement (Munger, 2002). Land revitalization came through kudzu's property of being a soil nitrogen fixer, which refilled overused, nitrogen deficit soils. In the 1930s, the government offered an $8 per acre incentive to plant kudzu seedlings. By 1946, there were approximately 3 million acres of kudzu in the Southeastern United States (Everest et al., 1999).

Kudzu began gaining negative attention by the early 1950s, as it spread rampantly throughout the South, causing problems for farmland owners (Everest et al., 1999). It was destructive in killing trees, collapsing buildings, and destroying utility poles by aggressively traveling up these structures and forming a dense mass that would strain their integrity. (Missouri Department of Conservation, 2008). The climate of the Southeastern United States was ideal for kudzu to thrive, leading to it being placed on the USDA common weed list in 1970 (Everest et al., 1999). Kudzu became an invasive plant species in the United States because it had no natural competitors in the environment to regulate its growth, as it would in its native Asian countries. In 1997, Congress voted to place kudzu on the Federal Noxious Weed List. There is now an estimated 7 million acres of land in the Southeastern United States that has been engulfed with this invasive plant species (Everest et al., 1999).

Characteristics of Kudzu

Kudzu (*Pueraria montana* var. *lobata*), the species that is predominately found in the Southern U.S., is a large, trifoliate-leaved, semi-woody, perennial vine that belongs to the legume family (Everest et al., 1999; Missouri Department of Conservation, 2008; Munger, 2002). Plant species of the legume family are known for being soil nitrogen fixers. Kudzu vines can grow up to 0.3 m per day in early summer and as much as 18 m total during the growing season (May – October) (Ball et al., 2002; Everest et al., 1999; Munger, 2002). It spreads from the root crown in any direction and will root at the vine nodes every few feet to establish new growths (Everest et al., 1999; Munger, 2002). The spread rate of kudzu can be accelerated by small vines of other plants because kudzu can consistently twine around smaller vines more swiftly than large tree trunks (Munger, 2002; Miller and

Ronald, 1986). Its tuberous roots (descended at the nodes) help maintain a heavy carbon reserve. Roots can reach a depth of 4 m and weigh as much as 91 – 136 kg in older kudzu patches (Everest et al., 1999; Lowenstein et al., 2014; Munger, 2002). The taproot is enlarged and beneficial in that it aids the plant in survival during drought periods (Munger, 2002; Winberry and Jones, 1973).

Asexual regeneration is a frequent and common method by which kudzu multiplies. This occurs every few feet where nodes (the areas on the vine where leaves and roots branch) will send down roots establishing new root crowns (Munger, 2002). There are few fruiting pods that develop viable seeds during the optimal growing season, but its vegetative reproduction continually takes place as the nodes establish roots (Missouri Department of Conservation, 2008). During kudzu's 3rd growing season after germination, seed production will initiate by producing a purple flower in late July to September, if in full sun (Missouri Department of Conservation, 2008; Munger, 2002). When seedpods are produced there are only 1 – 2 viable seeds, and these seed pods are only found on climbing vines (Everest et al., 1999; Munger, 2002). A prolonged exposure to high summer temperatures and increased soil temperatures will accelerate seed germination by affecting seed coat permeability (Munger, 2002; Susko et al., 2001). Attempts to eradicate kudzu by burning may also promote seed germination, where potential new growth would emerge after the burning attempt (Munger, 2002; Susko et al., 2001).

Kudzu is found in many places in the United States and can grow in a wide range of soil types, including sandy soils, acid soils, lime soils, lowlands with high water tables, in over heavy subsoil, and in areas where winter soil temperatures do not drop below -32^{o} C. (Everest et al., 1999; Southeast Exotic Pest Plant Council, 1999). Kudzu can be found in open fields, road sides, and near forest edges, but its spread is at its peak in open fields (Munger, 2002). The widespread distribution of kudzu in the United States and Tennessee is shown in Figures 1 and 2, respectively. These figures show that kudzu has spread from the South and has acquired a level of hardiness to endure colder and dryer climates. Kudzu can endure drought and high temperatures, but will not thrive in wet soils and young vegetative growth will die in low temperatures (Missouri Department of Conservation, 2008). When reaching temperatures between 30^{o} – 35^{o} C, the efficiency of photosynthesis will be affected by increasing heat. (Munger, 2002). Kudzu will grow in many different soils, but the optimal soil type is a deep, loamy soil (Everest et al., 1999; Missouri Department of Conservation, 2008; Munger, 2002; Winberry and Jones, 1973). The most aggressive plots of kudzu are in the Southeastern U.S., with its optimal climates where winters are mild, summer temperatures rise above 27^{o} C, annual precipitation exceeds 102 cm, and sandy loam soils are widespread (Munger, 2002; Winberry and Jones, 1973).

Other factors that can affect growth of kudzu is light availability and the previous existing native plant life. As kudzu starts to encounter shade, growth will dwindle, whereas in direct sunlight the growth rate can increase 3-fold (Munger, 2002). Kudzu contains a high leaf surface area, especially when climbing trees, which enhances the photosynthetic competition for light (Munger, 2002). Kudzu is considered heavily shade intolerant in having the highest light requirement out of 5 native (*Rhus radicans*, *Clematis virginiana*, *Smilax rotundifolia*, *Vitis vulpina*, and *Parthenocissus quinque-folia*) and 3 exotic (*Pueraria lobata*, *Lonicera japonica*, and *Hedera helix*) vine species in the Southeast (Carter and Teramura, 1988; Munger, 2002).

Kudzu differs among species around the world. American kudzu, compared to the Japanese counterpart, is distinctly different in how it overwinters. Kudzu is considered a semiwoody perennial because of its overwintering ability (Munger, 2002). Overwintering is a process where vines develop thick bark, accumulate annual rings of vascular tissue, and attain a desirable stem diameter, usually around 2 cm (Munger, 2002; Tsugawa et al., 1992). American kudzu will produce these overwintering stems only on the vigorous, climbing plants, whereas the Japanese strain will produce the overwintering stems on the portions that lie just above the ground (Munger, 2002; Tsugawa et al., 1992). An additional difference with the North American cultivars of kudzu is that they have limited seed production and are less likely to thrive outside the Southeast U.S. (Munger, 2002).

Uses of kudzu

In China and Japan, kudzu roots are dried and used for medicinal purposes to cure an array of common ailments (Global Invasive Species Database, 2010). Japan, during the 1700s, also attempted to utilize fiber from stems to make grass-like cloth and paper, and also grinding kudzu into flour for use in baking (Everest et al., 1999; Global Invasive Species Database, 2010). Asian grocery and health food stores still import kudzu flour to sell in the U.S. (Lowenstein et al., 2014). Other traditional uses of kudzu are as fiber to stuff cushions and chairs, as a mosquito repellent when burned, and to produce a palatable honey (Global Invasive Species Database, 2010). During the initial years that kudzu was introduced in the U.S., it was used as an ornamental vine (which was appreciated for its grape-like fragrance) to shade many southern homes (Everest et al., 1999).

As previously discussed, kudzu was first introduced to the United States as a means for erosion control but was eventually considered a rampantly unstoppable vegetation that would start to take over the Southeastern United States. Kudzu continues to be an efficient method of soil erosion control on steep embankments, but there are more noninvasive species (e.g., tall fescue and bahiagrass) used now to address this issue (Ball et al., 2002; Global Invasive Species Database, 2010). Being a legume, kudzu has a dual-purpose of hosting nitrogen fixing bacteria that enrich the soil and is also a good source of nutrients when fed to herbivorous livestock (Global Invasive Species Database, 2010).

Use of kudzu as a feedstock

Grazers (e.g., cattle and sheep) and browsers (e.g., goats and deer) will consume kudzu when available, but it is easily overbrowsed or overgrazed (Winberry and Jones, 1973). Kudzu is known to produce a forage of high quality that contains a crude protein (CP) concentration of 15 – 18% and a total digestible nutrient (TDN) value of over 60%, but the use of this plant as a feedstuff has limitations. Kudzu grows rapidly, but it produces a low forage yield of 2 – 4 tons of dry matter per acre per year (Everest et al., 1999). Pairing low forage yield with a vine-like growth habit makes harvesting problematic. During dry periods, producers can harvest kudzu annually or biennially, as it retains moisture for growth deep within the roots (Everest et al., 1999). Grazers and browsers can be enclosed on a plot of kudzu to control its growth, while also receiving a high quality source of nutrients that potentially results in increased animal performance.

In 1945, tropical kudzu (*Pueraria phaseoloides*) was introduced to a herd of Guernsey cows. Initially, few cows ate kudzu, but within days cows consumed it regularly (Emery and Norman, 1947). This experiment was conducted when common pasture crops did not produce a sufficient forage. For one continuously grazing cow during the dry season, an estimated one acre of tropical kudzu was needed. Emery and Norman (1947) determined that tropical kudzu should only be grazed once during the dry season. To use kudzu for grazing, it should not be grazed to the ground to preserve quality and regrowth ability. Tropical kudzu had an estimated 11,000 – 18,150 kg of forage production per year during these experiments. Successful grazing was also established using oxen and goats, and an adapted use for other livestock and poultry. Polk and Gieger (1945) demonstrated that when alfalfa became limited, kudzu meal at 9% of their diet could be substituted in chick rations. Kudzu and various grasses (e.g., pará grass) can form a desirable combination (when grazing) to increase protein content and reduce the need for commercial feeds (Emery and Norman, 1947).

Anti-quality and anti-nutritional factors of Kudzu

Kudzu contains a variety of secondary metabolites varying in concentration (Table 2). These secondary metabolites can act as anti-nutritional or anti-qualitative factors (Allen and Segarra, 2001; Nepomuceno et al., 2013). Legumes, such as kudzu, are beneficial in nitrogen fixation and improvement of animal diets. Secondary metabolites in kudzu, however, can interfere with nutrient intake, absorption, and utilization (Allen and Segarra, 2001; Nepomuceno et al., 2013).

There are both toxic and nontoxic secondary metabolites in plant materials (Reed et al., 2000; Nepomuceno et al., 2013). Alkaloids, cyanogenic glycosides, toxic amino acids, saponins, and isoflavones are toxic compounds present in low concentrations (Reed et al., 2000; Nepomuceno et al., 2013). These compounds have negative effects when absorbed by an animal, including neurological problems, reproductive failure, gangrene, and potential fatalities (Reed et al., 2000; Nepomuceno et al., 2013). Lignin, tannin, cutin, biogenic silica, and volatile terpenoids make up the non-toxic compounds present in high concentrations. Decreased digestibility and palatability can result from these compounds (Reed et al., 2000; Nepomuceno et al., 2013).

Saponins are in high concentrations in tropical kudzu (*Pueraria phaseoloides*), causing tympanism (accumulation of gas), reduced rumen microbial fermentation, and hepatic photosensitivity (Nepomuceno et al., 2013). Saponins also create stable foam in water and impart a bitter flavor to forages, thus decreasing the likelihood of intake by the animal (Nepomuceno et al., 2013). Saponins are major anti-nutritional and anti-qualitative factors, but tannins are the primary negative factor in legumes (Nepomuceno et al., 2013). There are two types of tannins, hydrolysable and the condensed varieties, with the latter being found in legumes, sorghum grains, and tree leaves. Tannins contain a large amount of phenolic hydroxyl groups, allowing them to create links with proteins and other molecules (Fahey and Berger, 1993). A main concern with tannins in feedstuffs are their negative effects on the ruminant digestive system through protein interactions (Fahey and Berger, 1993). Tannins will affect the nutritive value of plant dry matter, reducing the palatability by precipitating salivary proteins and nutrient digestibility by diminishing the permeability of the rumen wall through interactions with the outer cellular layer of the digestive tract. Tannins at a 20 mg/g of dry matter concentration will cause ruminants to reject feedstuffs (Fahey and Berger, 1993). Digestive enzyme activity may also decrease from tannins' ability as a potent inhibitor. Tannins have the potential to cause negative effects to an animal, including impaired ruminal digestion; low milk yield; toxic degenerative changes in the intestine, liver, spleen, and kidney; and constipation (Fahey and Berger, 1993). Both saponins and tannins cause negative effects, but the positive effects these secondary metabolites can have, including diminished ruminal methane production, is still being explored (Sliwinski et al., 2002; Nepomuceno et al., 2013).

Organic acids and reducing sugars are the remaining secondary metabolites that have large concentrations in kudzu. Organic acids can bring about precipitation of calcium ions in the blood, leading to muscle weakness, nephritis, kidney stones, gastrointestinal irritation, and hypocalcemia syndrome in grazing ruminants and horses (Nepomuceno et al., 2013). In large concentrations, reducing sugars can be problematic for equines (Cohen et al., 1999; Nepomuceno et al., 2013). Equines fed a high concentrate diet will produce excess lactic acid, resulting in water retention and decreased pH values in the lumen of the digestive tract (Cohen et al., 1999; Nepomuceno et al., 2013). This risks the possibility of digestive disorders, including osmotic diarrhea and colic (Cohen et al., 1999; Nepomuceno et al., 2013). Additional secondary metabolites, such as coumarin by-products, depsides and depsidones, alkaloids, steroids, triterpenoids, flavonoids, and cardiac glycosides can also cause negative effects when used as a feedstock (Nepomuceno et al., 2013).

BIOLOGICAL CONTROL OF KUDZU USING ANIMALS

Biological control of invasive plant species using animals

Plant populations are controlled naturally by their environment and by natural enemies. Invasive species are unique in disrupting an ecosystem to which it does not belong due to a lack of natural control (Seastedt, 2014). Invasive species are the second largest cause of biodiversity (total variability within and among species of all plant organism and their habitats) loss, behind habitat destruction (Keane and Crawley, 2002). The degree of invasiveness may increase with a lack of natural competitors (Keane and Crawley, 2002; U.S. Fish and Wildlife Service, 2009). Plant species that are established in an environment outside of its natural habitat may be less regulated by the native herbivores in the area, thus resulting in the rapid growth of an invasive plant species (Keane and Crawley, 2002; U.S. Fish and Wildlife Service, 2009). This leads to unwanted imbalances in an ecosystem that have potential to harm native species. These invasive plant species can be controlled by chemical or biological methods. Biological control is a method by which one organism is used to control another and can be used to restore ecosystem balance (U.S. Fish and Wildlife Service, 2009). Biological control of problematic species using animals was recorded as early as 9,500 years ago when cats were domesticated to control rodents (U.S. Fish and Wildlife Service, 2009). Animals used in biological control of invasive plant species can range from insects to ruminant animals.

Tamarix, a small riparian shrub or tree from Eurasia (riparian areas are the interface between land and a river or stream), were brought to North America and planted to combat soil erosion in the 19th century (Seastedt, 2014). It formed a dense, impenetrable culture along streams and rivers located in Southwestern North America. This invasive species was known to be destructive to the environment by spreading excess salts, taking up excessive water, and fueling wildfires (Seastedt, 2014). Several species of *Diorahabda* beetles were released in the late 1990s to infested areas to defoliate the invasive species. When observed in 2002, there was a significant reduction of the invasive species in previous infested areas, but continued biological control with other insects is being used to slowly diminish the species of *Tamarix* (Seastedt, 2014).

Biological control of invasive plant species using insects is widely used. Another invasive plant species is knapweed, a species of *Centaurea*, which drove out native rangeland plant species that cost farmers millions of dollars in the North American grasslands and rangelands. *Larinus minutrus* and *Cyphocleonus achates* were 2 of the 14 species of insects used in reducing the densities of knapweed. Many insect species were used because the invasive plant persisted through previous attempts at biological control (Seastedt, 2014).

Ruminant animals (e.g., goats, cattle, and sheep) have been used for hundreds of years as a form of biological control of invasive plant species and for vegetation management in the United States. There are a number of invasive plants that have been introduced to environments that lack natural control (Hart, 2001). Invasive weeds such as cheatgrass (*Bromus tectorum* L.), medusahead rye (*Taeniatherum caput – medusae* L.), and ventenata (*Ventenata dubia*) are of major management concern to many land owners (Bohnert and Stephenson, 2016). Goats are unique in that they will defoliate invasive plant species infesting an area, allowing more desirable plants to thrive. Goats biologically controlling unwanted foliage have a dual-purpose. The unwanted foliage is biologically controlled and converted to product (e.g., meat and milk products) with a potential monetary profit (Bohnert and Stephenson, 2016; Hart, 2001). Targeted grazing and browsing can aid in the suppression of invasive plant species while maintaining the native plant community (Bohnert and Stephenson, 2016). Containing goats in the infested area of interest could make it economical to use goats for overbrowsing infestations of invasive weedy species (Hart, 2001). Using targeted grazing and browsing on infested areas has proven to be an economical tool for farmers in producing product and preventing invasive species from damaging land in various ways. As an example, fine fuels are created by non-native annual grasses (e.g., cheatgrass) that ease ignition of wildfires and assist in fire spread (Bohnert and Stephenson, 2016; Matchett et al., 2009). Revitalization of native plant species on infested land is a goal when utilizing animals as a method of biological control.

Biological control of Kudzu using animals

Kudzu must undergo constant application of some control method to yield results in lowering its occurrence. Efforts to successfully control this plant is heavily influenced by timed treatments within its life cycle (Missouri Department of Conservation, 2008). Biological control using grazers and browsers can be an effective and cost-efficient method, but it is a slow process (Missouri Department of Conservation, 2008). Elimination of kudzu is possible by frequent defoliation by animals over several years. By over defoliating and reducing photosynthetic carbon, hydrogen, and oxygen (CHO), kudzu will halt its metabolic processes and regrowth will be prevented (Everest et al., 1999; Downs, 2018). Defoliation during the fall will reduce the amount of resources roots receive for survival through the winter, thus accelerating the progression of eradication (Mungers, 2002).

Kudzu can be eliminated using cattle to over graze it at 80% consumption of the vegetative growth for 3 – 4 years (Everest et al., 1999; Missouri Department of Conservation, 2008). Vines which these animals cannot reach may be cut and fed to ensure that defoliation is taking full effect. Remaining plant material after those 3 – 4 years can be spot treated with recommended herbicides (Missouri Department of Conservation, 2008). Furthermore, continuous grazing and browsing of infested areas for approximately 2 months during kudzu's growing season (May – October) can be effective in its eradication. Older infestations become increasingly hard to eradicate. Kudzu over 10 years old will be minimally affected by over grazing and over browsing, so herbicide application may be necessary (Ball et al., 2002; Missouri Department of Conservation, 2008; Miller and Ronald, 1986).

Managed use of invasive species to feed animals

Land owners can manage livestock to use invasive plant species as diet supplements and not only enhance animal production, but also slowly diminish the infestation of the invasive species. Livestock production can be maintained by properly target grazing or browsing invasive species (Bohnert and Stephenson, 2016). When targeting at different times of the year on the invasive species, livestock show an increased efficiency and livestock producers have potential decreased feed cost and an alternate feeding strategy (Bohnert and Stephenson, 2016). As previously described, cheatgrass is an invasive annual grass that has disrupted native plant species on rangelands (i.e., grasslands, shrublands, woodlands, wetlands, and deserts). Cheatgrass contains fine fuels that increase the frequency and spread rate of wildfires (Bohnert and Stephenson, 2016; Matchett et al., 2009). The timing of cattle grazing cheatgrass will yield different results. During the fall, cheatgrass is of poor-quality as a feed, compared to its use in the spring. Dormant cheatgrass in the fall can be grazed to decrease the biomass that will continue into spring (Bohnert and Stephenson, 2016). During the fall, cheatgrass quality is low and must be supplemented with a higher crude protein source to maintain animal body condition (Bohnert and Stephenson, 2016). Heavy targeted grazing on 60 – 80% of the infested areas showed a noticeable reduction of cheatgrass and an increase in native plant species over a 3-year period (Bohnert and Stephenson, 2016). Biological control of cheatgrass using this method is most efficient on large scale rangelands (Bohnert and Stephenson, 2016).

Other data has shown invasive species supplemented into the diet of livestock are equal to high protein feeds used in production. For instance, russian knapweed (*Acroptilon repens*) is an invasive species considered as a feedstuff (Bohnert and Stephenson, 2016). In one study, cattle were supplemented with alfalfa hay or Russian knapweed top dressed on fescue straw. No differences in weight gain or body condition score among the groups were observed (Bohnert and Stephenson, 2016). Using invasive plant species as a supplementation in diets of livestock not only aids in control of aggressive invasive species, but allows for a potential reduction of traditional feeds. This targets two production problems, the management of invasive plant species and providing a feed that reduces costs and increases the efficiency of production (Bohnert and Stephenson, 2016).

EVALUATION OF FEEDSTUFF QUALITY USING THE *IN SITU* PROCEDURE

Rumen physiology

The rumen is located on the left side of the ruminant animal and is the largest compartment of their stomach. The large holding capacity of the rumen allows for the proper time needed for complex components in the diet, such as cellulose and various polysaccharides, to be utilized by microbes (Owens and Goetsch, 1993; Yokoyama and Johnson, 1993). Microbial fermentation of fiber is the main purpose of the rumen. The diverse population of microbes (including bacteria, protozoa, and yeast) are essential in the rumen, and have a symbiotic relationship with the ruminant animal (Bravo and Wall, 2016; Downs, 2018; Owens and Goetsch, 1993; Yokoyama and Johnson, 1993). Microbes are able to survive in the rumen due to the optimal physiological conditions it provides. The rumen has a pH between 6 – 7 and the temperature is typically maintained at 38 – 42^{o} C (Downs, 2018; Owens and Goetsch, 1993). Salivary secretions of bicarbonate and phosphate buffers maintain near neutral rumen pH (Owens and Goetsch, 1993). Microbes receive food (fermentation substrates) and shelter, while the ruminant animal receives microbial protein, vitamins (B complex vitamins and vitamin K), and volatile fatty acids (VFAs) (acetic, butyric, and propionic acid) that meet their requirements (Downs, 2018; Owens and Goetsch, 1993; Yokoyama and Johnson, 1993). Microbes in the rumen break down low-quality feed and form by-products, including VFAs and microbial protein for the ruminant to utilize (Bravo and Wall, 2016; Downs, 2018). Ruminant animals utilize the VFA by-products to produce ATP through the Krebs Cycle and the electron transport chain, whereas a non-ruminant typically use glucose to fuel these cycles (Bravo and Wall, 2016; Downs, 2018). Without microorganisms in the rumen, the ruminant would be unable to efficiently utilize the large amount of fibrous feed it consumes (Owens and Goetsch, 1993; Yokoyama and Johnson, 1993). The rumen contains specialized structures that increases fermentation efficiency. Rumen papillae line the rumen walls to increase surface area for absorption of VFAs and ammonia produced from microbial fermentation. Blindsacs are pocket-like sections of the rumen that helps exclude oxygen to increase the anaerobic environment for microbes to increase efficiency of fermentation (Downs, 2018).

As microbial activities occur, the ingested feed is mixed, via peristaltic movements, between the reticulum and the rumen through the reticulo-rumen orifice. When rumen digesta reaches a threshold particle size, it passes in liquid phase through the reticulo-rumen-omasal orifice, which regulates ingested feed via horn papillae, and into the omasum (Downs, 2018; Owens and Goetsch, 1993). Larger particles that cannot pass through the reticulo-rumen-omasal orifice will be retained until further degradation occurs (Owens and Goetsch, 1993). Microbes that have a slower growth rate will be able to attach to these large particles and avoid potentially being washed out of the rumen, resulting in a high capacity of microbial populations. Aqueous conditions of the rumen serve for optimal microbial activities of bacteria, protozoa, and yeast (Yokoyama and Johnson, 1993).

The bacteria, protozoa, and yeast that survive within the rumen have adapted to the conditions of that ecosystem. The majority of these microorganisms are saccharolytic (causing the hydrolysis of sugars), because carbohydrates, such as cellulose and various polysaccharides, constitute the majority of the ruminant diet (Yokoyama and Johnson, 1993). The rumen lacks oxygen; thus, the majority of rumen microorganisms are obligate anaerobes, which grow efficiently with minimal to no oxygen. There are exceptions for the few facultative anaerobes that survive under aerobic conditions. The estimated composition of gases within the rumen are: 65% carbon dioxide (CO_2), 27% methane (CH_4), 7% nitrogen (N_2), 0.6% oxygen (O_2), 0.2% hydrogen (H_2), and 0.01% hydrogen sulfide (H_2S) (Yokoyama and Johnson, 1993).

Rumen bacteria are mainly obligate anaerobes (e.g., *Ruminococcus flavefaciens* or *Butyrivibrio fibrisolvens*) at $10^{10} – 10^{11}$ cells/g of rumen contents, whereas, the facultative anaerobes (e.g., *Streptococcus bovis*) are present at $10^7 – 10^8$ cells/g of rumen contents (Yokoyama and Johnson, 1993). Bacteria present within the rumen can be classified as cellulolytic, hemicellulolytic, pectinolytic, amylolytic, simple sugar-utilizing, intermediate acid-utilizing, proteolytic, ammonia-producing, lipolytic, or methane-producing (Yokoyama and Johnson, 1993). Competition among rumen bacteria may be influenced by affecting the substrate availability and concentration. Likewise, rumen bacterial populations may fluctuate from variabilities such as, time spent ruminating, the quantity of saliva secreted and its buffering capacity, water consumption, and passage rate of digesta (Yokoyama and Johnson, 1993).

The number of rumen protozoa are approximately 10^5 – 10^6 cells/ml of rumen contents and consist of flagellate and ciliate species, with the bulk being ciliates. Protozoa and bacteria located in the rumen may be equal in mass. Protozoa have been estimated at approximately 2% of the weight of rumen content, 40% of total microbial nitrogen, and 60% of microbial fermentation products (Yokoyama and Johnson, 1993). Rumen protozoa are strictly anaerobic. The predominant genera of ciliates in the rumen are *Isotricha*, *Dasytricha*, *Entodinium*, *Diplodinium*, *Epidinium*, and *Ophryoscolex* (Yokoyama and Johnson, 1993). Ciliates have the ability to degrade and ferment a variety of substrates, including cellulose, hemicellulose, pectin, starch, soluble sugars, and lipids. The genera *Dasytricha* can utilize maltose and other sugars, but not starch, whereas *Isotricha* utilizes starch and sugars, but not maltose (Yokoyama and Johnson, 1993). The concentrations of particular protozoa species vary with the ruminant animal's diet. A diet consisting of large amounts of soluble sugars will show an increase in the population of *Isotricha* (Yokoyama and Johnson, 1993). The effects that protozoa have on fermentation and their benefit to the ruminant animal is still being examined. Studies have shown an overall increase of rumen efficiency, specifically regarding higher rumen ammonia and total VFA concentrations, suggesting there is a higher rate of digestibility when protozoa are present (Yokoyama and Johnson, 1993). Other studies have suggested protozoa do not carry out functions that are essential to the ruminant animal. Rumen efficiency may be increased under certain dietary conditions when protozoa are eliminated. (Yokoyama and Johnson, 1993). If protozoa do not benefit the rumen, they would seem to be stabilizers during fermentation. By controlling the level of substrate available, protozoa can help sustain a more uniform fermentation in between feedings (Yokoyama and Johnson, 1993).

Yeast (anaerobic fungi) concentrations within the rumen are minimal when compared to bacteria and protozoa populations (Downs, 2018; Yokoyama and Johnson, 1993). When the ruminant animal is fed a high-roughage diet, anaerobic fungi has been estimated to make up 8% of the microbial mass. Their significance within the rumen has yet to be determined, but they may play a role in degradation of cellulose and xylans (Yokoyama and Johnson, 1993).

In situ methodology and its comparison to other digestibility measures

When conducting ruminant animal nutrition research, there are three primary methods by which feedstuff quality and digestibility can be determined. *In situ*, *in vitro*, and *in vivo* are common procedures used in ruminant nutrition research, but each yield different results. *In situ* and *in vitro* studies are typically paired to compare rumen degradability data between an artificial and natural rumen environment. Although *in vivo* is an estimate of feedstuff quality (digestibility), it is an entire digestive system comparison that is not typically paired with either *in situ* or *in vitro* (Downs, 2018).

In situ (occurring within its natural environment) techniques are used to study rumen digestion without the need for ruminal simulation. (Downs, 2018; Vanzant et al., 1998). This technique utilizes a ruminally fistulated animal (surgically modified animal with access to desired structure) to isolate the rumen in the natural environment. Analyzing ruminal digestion using the *in situ* technique is efficient when comparing degradation characteristics among feedstuffs and using the data to indirectly determine how well an animal will perform on a feedstuff (Vanzant et al., 1998). *In situ* dry matter disappearance

(DMD) data can be separated into soluble, degradable, and indigestible fractions (Table 3). The soluble fraction is the portion of a feed that degrades quickly (minutes) in the rumen environment and includes starches, sugars, and proteins. Cellulose and hemicellulose principally form the degradable fraction and require a more extended time to digest than the soluble fraction. The indigestible fraction is primarily composed of lignin, an insoluble component of fiber, or undegradable intake protein (UIP) that is utilized by the animal further down the digestive tract. The soluble and degradable fractions are typically summed to determine total *in situ* disappearance. *In situ* studies are modifiable to the type of feed being analyzed. If a concentrate is used in the study, the amount of time needed to complete the study will be less, as compared to a forage, due to concentrates having a higher rate of degradability (Downs, 2018; Elizalde et al., 1999).

In vitro (i.e., in a test tube) organic matter disappearance (IVOMD) techniques for rumen degradation focus on minimizing the variabilities of the animal by creating an artificial rumen environment (Downs, 2018). Rumen fluid, artificial saliva, and the feedstuff of interest are placed in a test tube to mimic rumen fermentation (Downs, 2018; Krizsan et al., 2012). *In vitro* techniques are performed through a two-step process. The first step involves mixing 10 mL of rumen fluid (collected from a ruminally fistulated animal) and 40 mL buffer (to mimic saliva and its buffering capabilities) with 5 g of sample and is incubated for 48 hours at 38^{o}C. Secondly, simulated post-rumen digestion (*in vitro*) is achieved by incubating samples for 48 hours in 50 mL of pepsin-HCl. Insoluble residue is centrifuged and dried at 103^{o}C (Krizsan et al., 2012).

In vivo procedures utilize the entirety of the digestion tract. *In vivo* organic matter digestibility (OMD) analyzes the amount of feed organic matter digested throughout the total digestive tract. Protein evaluation systems use OMD to quantify ruminant energy availability (Gosselink et al., 2004). When determining *in vivo* digestibility, a feedstuff is fed to test animals and fecal samples are collected over a standardized time period. (Downs, 2018). *In vivo* techniques require the entire digestive tract for digestibility determination, whereas *in situ* and *in vitro* procedures isolate naturally or artificially the processes within the rumen environment. *In vivo* techniques to estimate digestibility are used less frequently because of expense and labor requirements (Gosselink et al., 2004).

In situ and *in vitro* procedures are both models for rumen fermentation which measure rate of feedstuff degradation. However, more data can be generated through *in situ* techniques because three fractions of feedstuff degradability (soluble, degradable, and indigestible) can be assessed. The *in vitro* methodology determines overall total disappearance of a feedstuff. Corley et al. (1997) compared *in situ* and *in vitro* rumen degradability of kudzu (Table 3). Total *in situ* dry matter disappearance of leaf and stem was 78% (maximum), whereas *in vitro* degradability was 69.25%. The *in situ* procedure allows components of feedstuff to escape the nylon bag, whereas the *in vitro* methodology is a relatively closed system and less reflective of actual rumen degradability. As shown in Table 3, *in situ* degradation values are typically higher than *in vitro* values. Foster et al. (2007) provided data on *in vitro* digestibility of two legumes, *A. angustissima* (69.79%) and *N. pubescens* (40.93%) to be lower than *in situ* degradability of 71.87 and 48.76%, respectively. Foster et al. (2011) performed *in situ* and *in vitro* rumen degradation procedures on five forages (bahiagrass, annual peanut, perennial peanut, cowpea, and pigeonpea) that resulted in higher values for *in situ* degradability (Table 4). This pattern is not a determination of how efficient the *in situ* method is compared to *in vitro*, yet a difference that stems from one being in a natural rumen environment and the other simulating a more controlled rumen environment.

Limitations of the *in situ* methodology

In situ studies are limited by variations that can occur over the incubation period. Variations can be categorized as animal characteristics, substrate characteristics, bag characteristics, temporal characteristics, other procedural aspects, and mathematical components (Vanzant et al., 1998). The abundance of variables that can affect an *in situ* study creates a problem when standardizing a procedure. Feedstuff characteristics prevent the possibility of a standardized procedure across all feedstuffs, processing techniques, or plant maturities. A defined starting point of a standard *in situ* procedure is presented in Table 5 (Vanzant et al., 1998).

Studies involving bag and sample characteristics have assessed the influence of sample size ratio to bag surface area (SS:SA) on digestibility measurements using the *in situ* method (Vanzant et al., 1998). By increasing the SS:SA, the amount of *in situ* disappearance decreases (Van Keuren and Heinemann, 1962; Vanzant et al., 1998). Suggestion of a negative linear relationship between SS:SA and digestibility of neutral detergent fiber [(NDF; a chemical analysis that separates forage material into cell contents and cell wall components (cellulose, hemicellulose, and lignin)] or dry matter (DM) was concluded through a series of studies (Jurgens et al., 2012; Vanzant et al., 1998). Research also shows that when SS:SA remains constant as bag size changes, there is little effect on digestibility (Vanzant et al., 1998). Bag surface area can be miscalculated when bag dimensions are not doubled to account for both sides (Vanzant et al., 1998). Bag pore size will also affect the degree of ruminal digestion that occurs. An *in situ* bag with large pores will allow for a greater measure of ruminal digestion, but it will also increase the variability when measuring digestion (Vanzant et al., 1998). Large pores will allow for excess particulate matter to escape, which represents the compromise between allowing enough influx of digestive agents, sufficient efflux of digestion end products, and decreasing the influx of digesta residue (Vanzant et al., 1998). Using an *in vitro* method would eliminate these SS:SA and bag characteristic variabilities, but take away the natural principles of the *in situ* procedure.

The diet of the animal used for *in situ* procedures can affect substrate digestion (Nocek, 1988; Vanzant et al., 1998). Both *ad libitum* (as desired) and a maintenance level feeding during *in situ* studies have been recommended (Nocek, 1988; Vanzant et al., 1998). There is a lack of data that concludes a correlation between feed intake and *in situ* degradation. A diet with the potential to influence the microbial population of the rumen also has the potential to affect the rate or degree of ruminal digestion. Animals that consume a more concentrate-based diet will have a lower rate of forage *in situ* degradation, when compared to animals on a roughage-based diet (Vanzant et al., 1998). The type of roughage used as a diet can affect *in situ* degradation (Van Keuren and Heinemann, 1962; Vanzant et al., 1998). For instance, ruminants that consumed brome hay during *in situ* analysis of prairie grass and alfalfa hay demonstrated alterations to degradability of protein in prairie grass (greater effect) and alfalfa hay (smaller effect) (Vanzant et al., 1998). This effect is linked to the amount of dietary soluble proteins and their degradation rates that will affect the *in situ* degradation characteristics of incubated substrates (Vanzant et al., 1998). A low variability diet (i.e., a diet that promotes specific microbial populations) fed to the research animal is recommended when conducting an *in situ* rumen degradation study because of the many dietary factors present (Vanzant et al., 1998). The purpose of ruminant animal's diet is to maximize microbe diversity to make certain there are not individual nutrients that limit rumen digestion (Vanzant et al., 1998).

The feedstuff being evaluated using the *in situ* procedure may have unique characteristics that impede measurements of digestion. Research using grass hay and wheat straw unveiled potential variability, with rate of NDF digestion being influenced by altering the SS:SA. When increasing SS:SA, rate of NDF digestion was decreased in grass hay, but not for wheat straw (Vanzant et al., 1998). Differences between fermentation rates of forages affected the *in situ* degradation rates when the SS:SA was increased. Through buildup of end products, rapidly degrading forages (e.g., grass hay) will be more susceptible to the microenvironment within the sample bag, altering how it degrades (Vanzant et al., 1998). When corn gluten meal is fermented it can form a gelatinous substance that affects the surface area of the bag by blocking bag pores. This is known to greatly reduce *in situ* degradation. Forage particle size can affect rate of *in situ* digestion (Vanzant et al., 1998). Barley, for instance, when ground tends to clump together, thereby affecting rate of ruminal digestion. A 0.4 – 0.6 mm range of ground barley particles digests at a slower rate than a particle range of 0.6 – 0.8 mm (Vanzant et al., 1998).

An important procedural aspect that is often overlooked, but is one of the most impactful, is the *in situ* rinsing procedures. The past 50 years has shown little progression in developments of efficient rinsing procedures. Two types of procedures have been used: hand and machine-rinsing (Vanzant et al., 1998). Variabilities arise when researchers used subjective methods to determine when the rinsing procedure had concluded. For hand washing, researchers have used the clarity of water, a defined time interval, and the number of rinse cycles. Using the clarity of water is a subjective method that can cause variation from researcher to researcher, affecting the amount of calculated DM disappearance. Mechanical washing was created in hopes of eliminating errors and variations of washing intensities between cycles (Vanzant et al., 1998). Using mechanical washing, however, increases the amount of N and DM washed out of the *in situ* bags. This could lead to additional losses of small particles from the bags and overestimation of disappearance (Vanzant et al., 1998).

RUMEN DEGRADATION VALUES OF COMMON FORAGE AND BROWSE SPECIES

Degradation rate data from other *in situ* studies

In situ rumen degradation rates vary among plant species. Researches have conducted *in situ* procedure on various silages, grasses, and legumes (some examples in Table 6). The silages, grasses, and legumes show variations between soluble, degradable, and indigestible fraction.

Corn silage and grass silage have a total *in situ* dry matter degradability (ISDMD) of 86.39 and 84.23%, respectively (Von Keyserlingk et al., 1996). Both silages have similar total ISDMD, yet when broken into the soluble and degradable fractions the numbers vary by 7%, with corn silage having a greater soluble fraction and grass silage having a greater degradable fraction (Table 6). In comparison, corn silage contains more starches, sugars, and proteins, yet a lower cellulose and hemicellulose concentration than grass silage. With little variation, corn and grass silage are highly digestible within the rumen.

Grass hay, bromegrass pasture, and bermudagrass pasture were determined to have between 77 and 87% total ISDMD (Von Keyserlingk et al., 1996; Elizalde et al., 1999;

Galdámez-Cabrera et al., 2003). Grass hay, among the nine forage ISDMD, is the most degradable in the rumen (Table 6). Von Keyseringk et al. (1996) found that it contains a 46.22% soluble fraction and a 40.81% degradable fraction, with approximately 13% indigestible fraction. Bromegrass and bermudagrass have a similar ISDMD, with bromegrass having a smaller soluble (20.90%) and a larger degradable (56.70%) fraction than bermudagrass (26.00 and 53.80%, respectively) (Table 6). Forage age has a significant impact on increasing insoluble fractions due to higher lignin concentration with increased age (Von Keyserlingk et al., 1996).

Legumes typically have a higher rate of rumen degradability when compared to grass species (Foster el at., 2011). This is due to greater crude protein, less water-soluble carbohydrates, and greater buffering capacity than cool-season grasses, which allows proteolysis to occur more in legumes. (Foster el at., 2011). Greater capacity for proteolysis in legumes allows for more crude protein break down during the wilting and ensiling phase. Plant species that contain a high amount of water-soluble carbohydrates will lead to a drop in pH that decreases the occurrences of proteolysis affecting degradability (Foster et al., 2011). Foster et al. (2007), however, found that tropical neptunia contained approximately 40% indigestible fraction, potentially associated with a high lignin or undegradable intake protein (UIP) concentration. It has been demonstrated that velvet bundle flower, prairie acacia, tropical neptunia, and red clover have a total ISDMD of 75.20, 82.89, 61.91, and 85.80%, respectively (Foster et al., 2007; Coblentz et al., 1998). Velvet bundle flower, prairie acacia, and tropical neptunia are all native to the Southern United States. Although, these are native to the same region, the variation among their ISDMD is higher than some silages or grasses (Table 6).

Comparison between alfalfa and kudzu

Kudzu is often compared to alfalfa. Kudzu leaves have a high nutritive value comparable to that of alfalfa (*Medicago sativa*), a common flowering plant used for grazing, hay, and silage for ruminants and other domestic herbivores (Table 7) (Downs, 2018). *In situ* dry matter rumen degradation data provided by Corley et al. (1997) separates kudzu into soluble, degradable, and indigestible fractions between leaf/stem and tuber (roots) (Table 3). Kudzu and alfalfa, being legumes, have a high rate of degradability due to a low concentration of water-soluble carbohydrates, which allows for increased proteolytic activity (Foster el at., 2011). Corley et al. (1997) provided data that kudzu contains 17.5% CP in leaves, similar to alfalfa at 18.7% CP (Table 7). Kudzu leaves contain a high concentration of CP, making it a potential feed for growing ruminants (Corley et al., 1997). The stem and tuber portions of kudzu do not have the same potential in regards to providing optimal nutrients to the ruminant animal. Corley at al. (1997) found a 5 – 7% lower CP level in stems than leaves (Table 8).

In comparison, alfalfa is a high-quality forage characterized by high digestibility and swift ruminal degradation (Kuwahara et al., 2016). Alfalfa and kudzu leaf and stem have similar *in situ* rumen degradation, with alfalfa having an average rate of 73.35% and kudzu having 78% maximum degradability (Coblentz et al., 1998; Corley et al., 1997). Alfalfa leaf and stem have an average soluble fraction of 34.8% and an average degradable fraction of 38.6%, whereas kudzu leaf and stem are 29.1 and 48.6%, respectively (Table 3). This data can potentially predict that alfalfa leaf and stem contain more starch, sugars, and protein than kudzu leaf and stem, but less concentrations of cellulose and hemicellulose. Coblentz et al. (1998) allowed alfalfa to ferment in the rumen for 96 hours, whereas Corley et al. (1997) only allowed 24 hours of fermentation for kudzu. *In situ* dry matter disappearance for whole plant alfalfa was found to be 76.6, 79.6, 79.2, and 81.91% in four studies (Coblentz et al., 1998; Elizalde et al., 1999; Foster et al., 2007; Von Keyserlingk et al., 1996). It is common for alfalfa to have high nitrogen levels that are highly degradable. When compared to red clover and four maturities of eastern gamagrass, Coblentz et al. (1998) provides data showing alfalfa contains higher nitrogen concentration. Despite these high concentrations, high degradability of nitrogen in alfalfa can lead to poor utilization of available nitrogen in lactating dairy cows (Coblentz et al., 1998).

Kudzu has been shown to contain high nutrient concentrations with tremendous promise as a feed for ruminants. Browsing species (e.g., goats) are a potential biological control mechanism for kudzu. There is, however, limited data on rumen degradability of kudzu. The following research will explore the rumen degradability of early and late season kudzu in the middle Tennessee region. Age variability effects will be determined using an *in situ* rumen degradation model.

TABLES AND FIGURES

Table 1. Species of *Pueraria* throughout the world (Van Der Maesen, 1985).

P. tuberosa	*P. lacei*
P. sikkimensis	*P. bella*
P. candollei	*P. pulcherrima*
P. mirifica	*P. phaseoloides*
P. lobata	*P. peduncularis*
P. imbricata	*P. stricta*
P. edulis	*P. wallichii*
P. alopecuroides	*P. rigens*
P. calycina	

Table 2. Summary of qualitative tests from phytochemical screening and analysis of kudzu (Nepomuceno et al., 2013).

Secondary Metabolites	Tropical Kudzu
Organic acids	+++
Reducing Sugars	+++
Saponins	+++
Tannins	+++
Steroids and triterpenoids	++
Saccharides	++
Alkaloids	+
Depsides and depsidones	+
Coumarin in by-products	+
Flavonoids	+
Cardiac glycosides	+
Catechins	-
Lactones	-
Purines	-
Quinones	-

(+++) large presence; (++) medium presence; (+) small presence; (-) absence or inconclusive result of secondary metabolites

Table 3. *In situ* dry matter disappearance (DMD) and *in vitro* dry matter digestion (IVDMD) of kudzu plant parts (Corley et al., 1997).

	In Situ DMD, %			
	Soluble Fraction	Degradable Fraction	Indigestible Fraction	IVDMD, %
Leaf and Stem	29.1	48.6	22.4	64.8 (Leaf) 73.7 (Stem)
Tuber	38.1	31.2	30.7	59.9

Table 4. *In situ* dry matter disappearance (DMD) and *in vitro* true digestibility (IVTD) of ensiled warm-season legumes and bahiagrass (Foster et al., 2011).

	Bahiagrass	Annual Peanut	Perennial Peanut	Cowpea	Pigeonpea
IVTD, %	58.6	74.1	74.5	71.3	45.4
DMD, %	77.8	80.9	79.9	81.3	50.2

Table 5. Standardized rumen *in situ* procedure (Vanzant et al., 1998).

Item	Recommendation
Diet	
Type	60 to 70% forage
Feeding level	Maintenance
Feeding frequency	≥ 2 times/day
Bag	
Material	Polyester
Pore size	40 to 60 μm
Sample size:surface area	10 mg/cm^2
Sample Processing	2-mm screen size (Wiley mill)
Replication	
Number of animals	≥ 2
Number of days	≥ 2
Number of bags	≥ 1
Incubation procedure	
Preincubation	Not necessary
Ruminal position	Ventral rumen
Insertion/removal	Remove simultaneously
Incubation times	To describe curve
Rinsing	Machine (5 times at 1 min/rinse)
Microbial correction	Yes
Mathematical model	Simplest available to adequately describe data
Standard substrate	Yes

Table 6. *In situ* dry matter disappearance (DMD) for forage/silage species.

	DMD, Soluble Fraction (A), %	DMD, Degradable Fraction (B), %	DMD, Indigestible Fraction, %	Total *in situ* DMD%[A]
Silages				
Corn silage[1]	48.22	38.17	13.61	86.39
Grass silage[1]	41.07	43.16	15.77	84.23
Grasses				
Grass hay[1]	46.22	40.81	12.97	87.03
Bromegrass pasture[3]	20.90	56.70	22.40	77.60
Bermudagrass pasture[5]	26.00	53.80	20.30	79.80
Legumes				
Velvet bundle flower[2]	32.95	42.25	24.80	75.20
Prairie acacia[2]	38.40	44.49	17.11	82.89
Tropical neptunia[2]	37.38	24.53	38.09	61.91
Red clover[4]	40.30	45.50	14.20	85.80

[A] (A + B = Total *in situ* DMD%)
[1]Von Keyserlingk et al., 1996
[2]Foster et al., 2007
[3]Elizalde et al., 1999
[4]Coblentz et al., 1998
[5]Galdámez-Cabrera et al., 2003

Table 7. Nutrient composition of alfalfa hay and fresh kudzu leaves (DM basis).

	Alfalfa, hay, sun-cured, midbloom[1]	Kudzu leaves, fresh[2]
Crude protein, %	18.7	17.5
Neutral detergent fiber (NDF), %	46.0	48.1
Acid detergent fiber (ADF), %	36.9	38.2
Ca, %	1.37	0.7
Fe, %	224.60	162.3
K, %	1.56	1.0
Mg, %	0.35	0.3

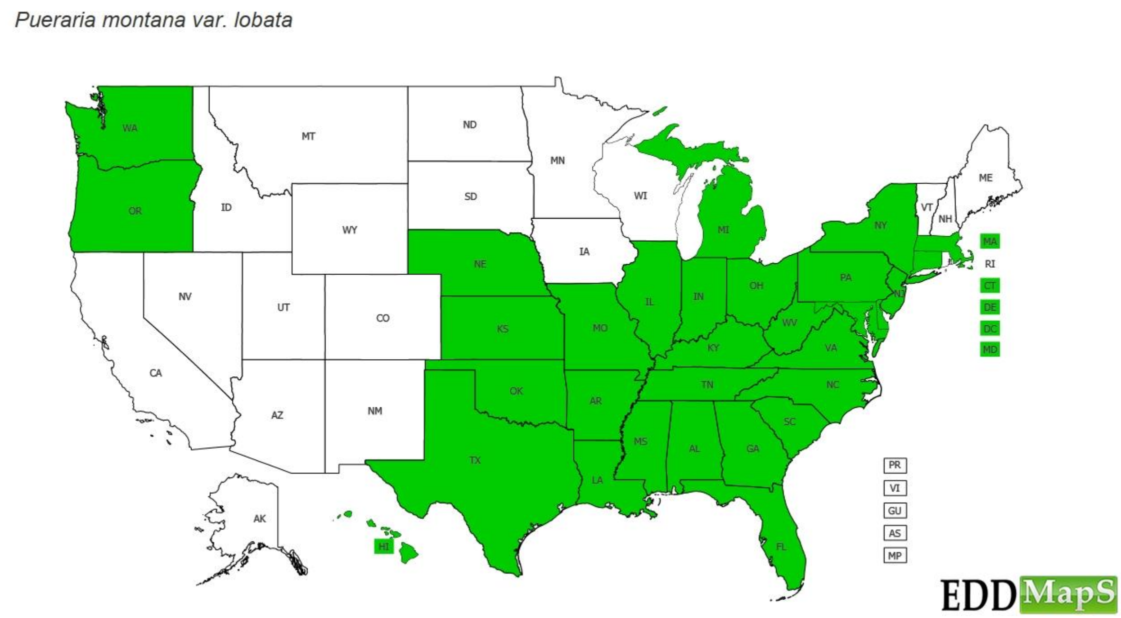
[1]NRC, 2012

[2]Corley et al., 1997

Table 8. Nutritive values of kudzu plant parts (Corley et al., 1997).

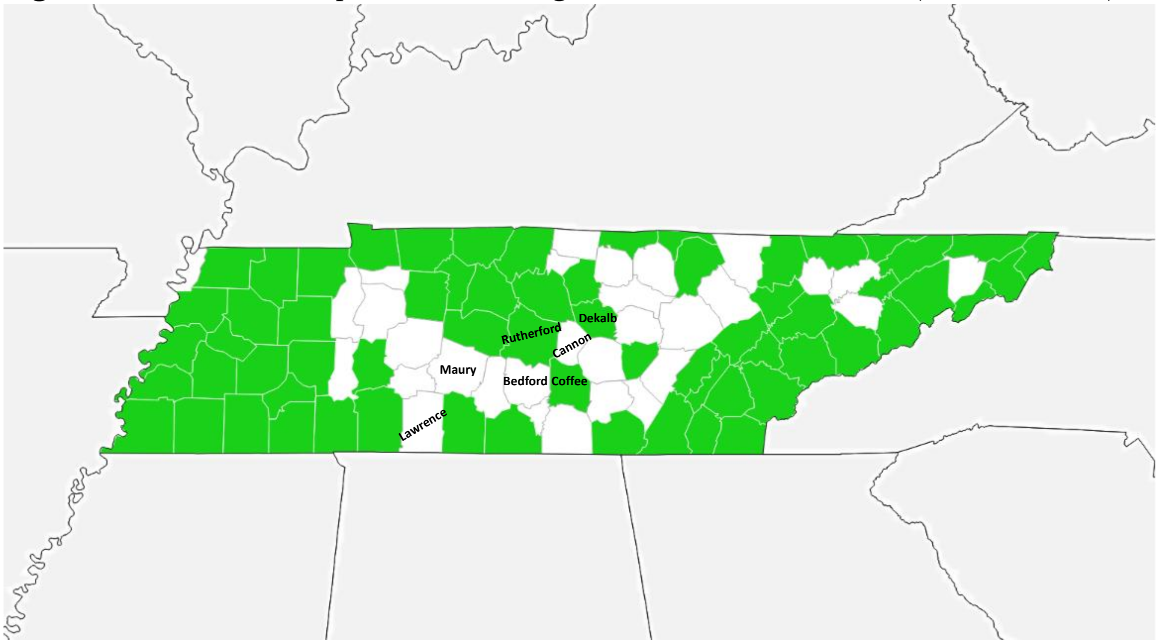
Parameters	Leaf	Stem	Tuber
Crude protein, (CP), %	17.5	10.3	8.6
Neutral detergent fiber, (NDF), %	48.1	73.7	39.8
Acid detergent fiber, (ADF), %	38.2	44.0	53.3
Ca, %	0.7	0.1	0.4
K, %	1.0	1.0	0.3
Mg, %	0.3	< 0.1	0.1
Fe, $mg \cdot kg^{-1}$	162.3	156.6	3600.0

Figure 1. Distribution map of kudzu in the United States (Wallace, 2018).



Green = kudzu found in that state; White = no kudzu found in that state.

Figure 2. Distribution map of kudzu among the counties in Tennessee (Wallace, 2018).



Green = kudzu found in that county; White = no kudzu found in that county.

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Early and late season kudzu (*Pueraria montana* var. *lobata*) age variability effects on total and nutrient-specific *in situ* rumen degradation

CHAPTER II

INTRODUCTION

Kudzu (*Pueraria montana* var. *lobata*), an invasive semi-woody, perennial vine weed species native to eastern Asia was introduced to the United States in the late 1800s as a method of soil erosion control, but by the 1970s the USDA had placed it on the common weed list (Everest et al., 1999). Kudzu crowds out and engulfs native species, preventing them from receiving necessary light to survive. It spreads quickly and grows rapidly, making it a problem for environments in the Southeastern United States. Browsers (e.g., goats and deer) are herbivores that are selective in their consumption of plant materials. Leaves or low growing herbaceous plants are preferred by browsers, and their selective nature makes them a viable option to biologically control kudzu. Potential biological control of kudzu using browsers can yield defoliation of this invasive plant species, allowing more native plants to thrive. Agriculturally important browsers like goats can biologically control unwanted plant species (in this case kudzu), while also yielding products (e.g., meat and milk) with potential monetary profit for producers (Bohnert and Stephenson, 2016; Hart, 2001).

Nutrient composition of kudzu (17.5% CP, 48.1% NDF, and 38.2% ADF) is comparable to alfalfa (18.7% CP, 46.0% NDF, and 36.9% ADF), with both being high quality legumes (Corley et al., 1997; NRC, 2012). There are historic examples of kudzu being used as a supplement in livestock and poultry diets. Polk and Gieger (1945) demonstrated that an inclusion rate of 9% kudzu meal could be supplemented in place of alfalfa meal in chick diets. Emery and Norman (1947) observed that Guernsey cows found tropical kudzu (*Pueraria phaseoloides*) palatable, and was grazed and browsed successfully by oxen, dairy cows, and goats.

If animals are going to be used for biological control of kudzu, it is important to assess its degradability. Data, however, on degradability of kudzu by ruminants is limited. Corley et al. (1997) performed a rumen *in situ* study on kudzu leaf, stem, and tuber over a 24 h incubation time to assess its nutritive value as a feed for ruminants. Further research on kudzu rumen degradability is needed to assess the potential application of using animals as a means of biological control. These four repeated rumen *in situ* studies of early season (ES) and late season (LS) kudzu were conducted to assess the total rumen degradability of dry matter (DM), crude protein (CP), acid detergent fiber (ADF), and neutral detergent fiber (NDF). Potential age variability effects between ES and LS will also be assessed.

CHAPTER III

MATERIALS AND METHODS

Kudzu used in this research was collected from 7 different counties in the middle Tennessee area (Bedford, Cannon, Coffee, Dekalb, Lawrence, Maury, and Rutherford). Sample collection between each county occurred in a 7 d period and samples were grouped by county. Both early [(ES; early June)] and late season [(LS; late July)] kudzu was collected. The method of collection used a 30.5 cm^2 PVC square randomly tossed into clusters of kudzu. Content within the square was collected by clipping an estimated 15 cm depth of kudzu vine. After collection, leaves were removed and dried at 60^{o}C for 48 h. Only kudzu leaves were used in this research. Once dried, kudzu leaves were ground and passed through a 2 mm screen, then compiled into composite samples across all counties for early and late season kudzu. Nutrient composition for samples of early and late season kudzu were determined using near infrared reflectance spectroscopy (NIR) and summarized in Table 1.

Each study used a modified Vanzant et al. (1998) *in situ* procedure. For each study, 5 g of dried, ground kudzu was placed in 24 Ankom® nylon forage bags [(10 x 20 cm; 50 ± 10 micron porosity)]. Each sample bag was prepared in triplicate. Each nylon bag was heat sealed 3 times. A ruminally fistulated animal (*Bos taurus* steer) was used for the *in situ* studies. The research animal was provided *ad libitum* mixed grass hay (Table 2), soybean meal (2.27 kg/d; 46.50% CP, 4.00% CF, 0.50% crude fat), and free-choice vitamin and mineral premix (Table 3) 7 d prior to the initiation of each study and throughout the entirety of the *in situ* incubation periods. All animal handling procedures were approved by the MTSU Institutional Animal Care and Use Committee (IACUC) and conformed to accepted practices (FASS, 1999). This *in situ* procedure was repeated 2 times for ES and 2 times for LS. Twenty-one nylon bags were suspended in the rumen at the time intervals of 0.25, 1, 12, 24, 36, 48, and 72 h. Zero hour bags were processed after each study's completion. Samples were subsequently placed in a large mesh bag (28 x 74 cm) beginning with the 72 h incubation time. After the 0.25 h incubation time was completed, all samples were removed and submerged in water (26^{o}C) to halt fermentation activity. Samples were processed, dried, and analyzed for DMD and disappearance of CP, ADF, and NDF. A modified Vanzant et al. (1998) hand-rinsing procedure was used to process residues. Residues were hand-wash agitated in 39^{o}C water for 1 min and spun dry for 2 min. This protocol was repeated 5 times. Following rinsing procedure, residues were dried at 60^{o} C for 48 h. After drying, residues were allowed to air equilibrate (36 h). Residues were weighed for dry matter disappearance (DMD) determination. After weighing, residues were removed from nylon bags and analyzed for CP, ADF, and NDF (Dairy One, Ithaca, NY) using AOAC and Van Soest procedures. These values were used to determine CP, ADF, and NDF disappearance.

Early (ES) and late (LS) season were considered treatments and data were analyzed as a randomized complete block design with repeated measures using the PROC mixed procedure of SAS (SAS Institute, 2017) to determine treatment (season) and treatment (season) by incubation time interactions. Regression analysis was conducted to determine *in situ* rumen degradation rate (% / h) with linear regression between 1 and 24 h incubation periods. Data were considered significant at the $P < 0.05$ level.

CHAPTER IV

RESULTS AND DISCUSSION

Nutrient composition analysis of ES and LS kudzu leaves demonstrates that it contains a high quality source of nutrients for ruminant animals (Table 1). Early and late season kudzu leaves collected in this research far exceed legume hay forage quality standards. Excellent legume hay contains 18% or more CP and 64% or more total digestible nutrients (TDN) (Rivera and Parish, 2010). Early and late season kudzu in this study contained a CP of 30.5 and 26.7% and TDN of 76 and 59%, respectively (Table 1). However, Corley et al. (1997) analyzed kudzu leaf composition to contain 17.5% CP. The present research analyzed earlier growth season kudzu leaves, whereas Corley et al. (1997) analyzed leaves later in the growing season. Kudzu leaves analyzed by Glass and Al-Hamdani (2016), however, contained 24.46% CP, 44.00% NDF, 28.67% ADF, and 55.99% TDN. Kudzu nutrient composition in this study exceeded the composition of Glass and Al-Hamdani (2016), Corley et al. (1997), and accepted quality standards for legume hay.

In this research, ES and LS kudzu incubated in the rumen for 72 h demonstrated seasonal effects for DM, NDF, ADF, and CP. As indicated in Figure 1, total rumen degradability of ES reaches a maximum at 24 h, and LS kudzu at 36 h of incubation. Slope regression conducted between 1 and 24 h, indicates that ES and LS kudzu leaves have a rumen degradation rate of 2.41 % / h across both time periods. There were no significant seasonal effects on rate of DMD. *In situ* total DMD was significantly different between ES and LS across all incubation times ($P < 0.0001$). Significant season by incubation time interactions occurred for DMD at 12 ($P < 0.0001$), 24 ($P = 0.0004$), 36 ($P = 0.0055$), 48 ($P = 0.0209$), and 72 h ($P = 0.0384$) of incubation (Table 4). *In situ* DMD for ES was 22.65 (0.25 h), 23.72 (1 h), 70.21 (12 h), 81.55 (24 h), 82.57 (36 h), 82.56 (48 h), and 84.17%

(72 h). *In situ* DMD for LS was 20.12 (0.25 h), 20.52 (1 h), 51.40 (12 h), 70.57 (24 h), 75.09 (36 h), 76.52 (48 h), and 78.73% (72 h). Both early and late season kudzu leaves are highly degradable within the rumen, but there are seasonal differences in degradability. With ES kudzu being younger with less fibrous material and insoluble lignin (NDF), it had an overall higher degradability than LS (Table 1). Less NDF in ES kudzu also indicates that it contained a higher concentration of neutral detergent solubles (containing primarily sugars, starches, and proteins) than LS, resulting in a higher degradability. Corley et al. (1997) found that total *in situ* DMD for leaf and stem (1:1, wt:wt) was 78% at 24 h. In the present study, across both ES and LS; DMD for kudzu at 24 h of incubation was analyzed to be 78.8%, which is comparable to the work of Corley et al. (1997).

Figures 2 shows ES and LS kudzu *in situ* rumen disappearance of NDF. Overall, across all incubation times, there was a significant difference between ES (41.27%) and LS (39.81%) kudzu NDF disappearance ($P = 0.0325$), with ES being more degradable than LS kudzu. *In situ* disappearance of NDF for ES and LS kudzu at 12 h was 37.57 and 24.20%, respectively. A significant season by incubation time interaction at 12 h ($P = 0.0004$) was observed (Table 4). This could be indicative of ES kudzu having an increased early rumen degradability. After 12 h of incubation there were no season by incubation time interaction effects. This is likely due to increased lignin content (+ 1.9%) in LS kudzu, thereby affecting NDF disappearance (Table 1).

Early and late season kudzu *in situ* rumen disappearance of ADF is shown in Figure 3. Overall across all incubation times there was not a significant difference between ES (36.09%) and LS (38.39%) kudzu ADF disappearance ($P = 0.0681$). However, there were significant season by incubation time interaction at 12 and 48 h ($P = 0.0420$ and $P = 0.0198$, respectively) (Table 4). *In situ* disappearance of ADF for ES and LS kudzu at 12 h was 36.72 and 28.36% and at 48 h 53.15 and 62.12%, respectively. In this research, kudzu age had minimal effects on ADF degradability.

Minimal seasonal effects were observed between ES and LS kudzu CP disappearance, as indicated in Figure 4. Overall, across all incubation times, there was no significant difference between ES (70.17%) and LS (62.63%) kudzu CP disappearance (P = 0.0984). However, there were significant season by incubation time interactions at 12 and 24 h (P = 0.0005 and P = 0.0349, respectively) (Table 4). Early and late season kudzu *in situ* degradation of CP at 12 h was 78.90 and 55.61% and at 24 h 91.86 and 80.79%, respectively. Although not significant, there is a tendency toward higher CP disappearance in the early season growth of kudzu. This likely due to ES kudzu containing 28.9% available protein, whereas LS contains 24.9% (Table 1). Corley et al. (1997) found kudzu leaves contain 17.5% CP. In the current study, ES and LS kudzu leaves had 30.5 and 26.7% CP, respectively. These differences in composition are likely due to time period of kudzu collection. Corley et al. (1997) collected kudzu between June and October, whereas in this research kudzu was collected between June and July. Later season collections of kudzu would decrease overall CP composition. As indicated by Corley et al. (1997), the leaf part of kudzu contains the highest amount of CP (compared to stem and tuber) and will meet the requirements of most ruminant animals (NRC, 2012).

CHAPTER V

CONCLUSION

Early and late season kudzu nutrient composition and rumen degradability indicates it is a nutrient-rich, highly degradable plant species for ruminant animals. Significant age variability effects influenced kudzu DMD between ES and LS, but rate of kudzu rumen degradability was similar across plant ages. Early season kudzu is more rumen degradable than LS, indicating ES is the most beneficial growing season for animal production during biological control. Using ruminant animals to control kudzu can reap benefits of both invasive plant species control and providing a high quality feedstuff to produce ruminant animals. Browsing early growth kudzu would yield a higher degradability in the rumen, with a potential animal production increase. Likewise, defoliating kudzu during the early growing season will also increase plant damage (due to the plant being young and having limited carbohydrate stores). Further research is needed to assess how anti-quality or anti-nutritional factors inherent to kudzu affects animal performance. Overall, this research indicates kudzu is a highly degradable feedstuff for ruminants, and, when available and accessible, should be considered as a supplement to a ruminant's diet.

Table 1. Near infrared reflectance spectroscopy (NIR) chemical composition of early (ES) and late season (LS) kudzu leaves incubated in experimental fistulated bovine during *in situ* rumen degradation study periods (DM basis).

Analysis	ES	LS
DM, %	88.2	88.5
CP, %	30.5	26.7
Available protein, %	28.9	24.9
ADF, %	18.4	26.2
NDF, %	27.3	45.7
Lignin, %	4.40	6.3
NE_m, Mcal/kg	1.80	1.23
NE_g, Mcal/kg	1.19	0.68
Ca, %	1.54	1.74
P, %	0.43	0.28
Mg, %	0.35	0.39
K, %	2.05	2.05
S, %	0.34	0.39
Cl, %	0.45	0.55
Lysine, %	1.55	1.25
Methionine, %	0.48	0.40

Table 2. Near infrared reflectance spectroscopy (NIR) chemical composition of mixed grass hay fed to experimental fistulated bovine during *in situ* rumen degradation study periods (DM basis).

Analysis	Study 1 and 2	Study 3 and 4
DM, %	93.00	93.60
CP, %	11.40	14.00
ADF, %	40.60	39.50
NDF, %	63.70	63.60
Lignin, %	4.20	4.00
NE_m, Mcal/kg	1.19	1.30
NE_g, Mcal/kg	0.64	0.73
Ca, %	0.42	0.49
P, %	0.29	0.49
Mg, %	0.17	0.33
K, %	2.04	2.48
S, %	0.19	0.25
Cl, %	0.45	1.43
Lysine, %	0.40	0.49
Methionine, %	0.15	0.18

Table 3. Composition of free-choice vitamin and mineral supplement provided to experimental fistulated bovine during *in situ* rumen degradation study periods.

Ca, %	13.5
P, %	8.0
NaCl, %	13.5
Mg, %	4.0
K, %	0.1
Cu, ppm	2,000
Mn, ppm	6,000
Se, ppm	52.8
Zn, ppm	6000
Vitamin A, IU/kg	715,000
Vitamin D3, IU/kg	165,000
Vitamin E, IU/kg	880

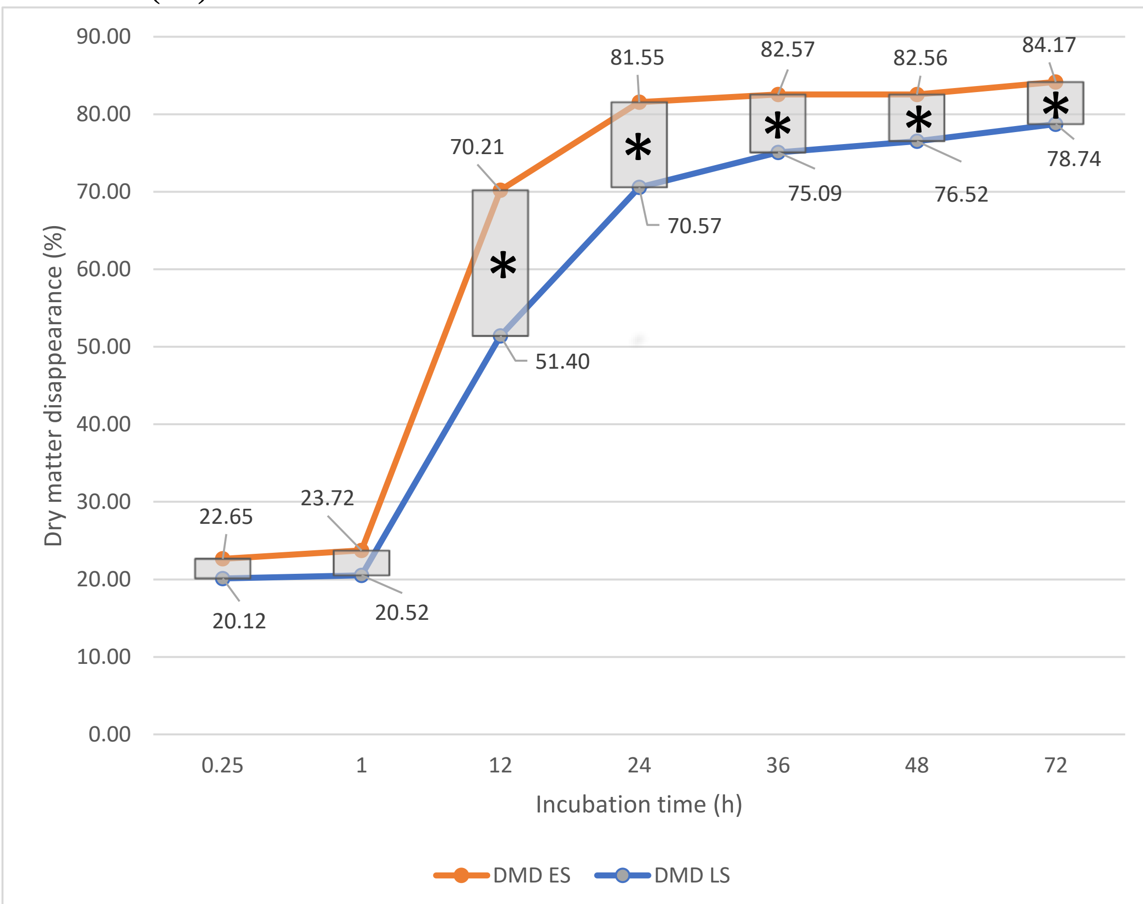
Table 4. Kudzu age by *in situ* incubation time interaction effects for DM, NDF, ADF, and CP disappearance.

	In situ disappearance (%)			
Inc. time (h)	DM	NDF	ADF	CP
0.25	NS	NS	NS	NS
1	NS	NS	NS	NS
12	*	*	*	*
24	*	NS	NS	*
36	*	NS	NS	NS
48	*	NS	*	NS
72	*	NS	NS	NS

NS = no significance

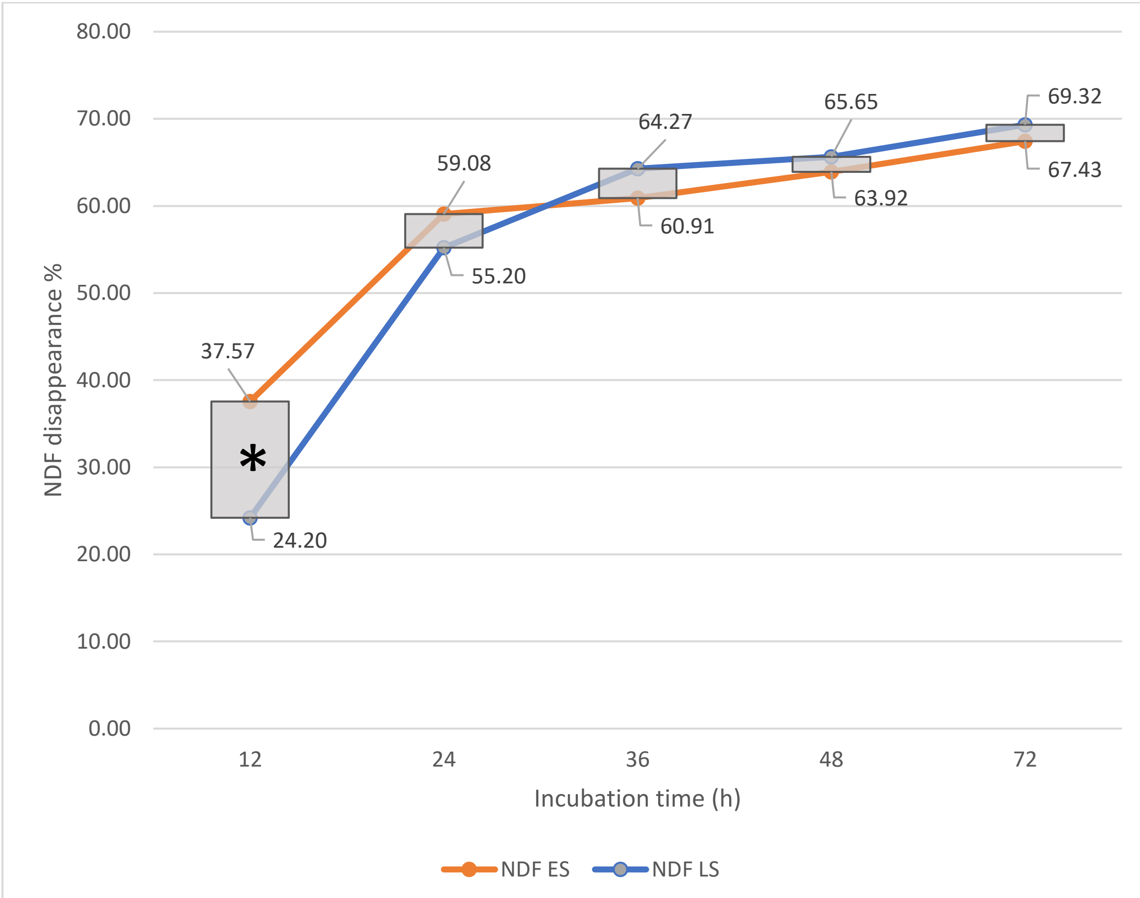
* Significant age by incubation time interaction ($P < 0.05$)

Figure 1. Total *in situ* rumen dry matter disappearance (DMD) of early season (ES) and late season (LS) kudzu.



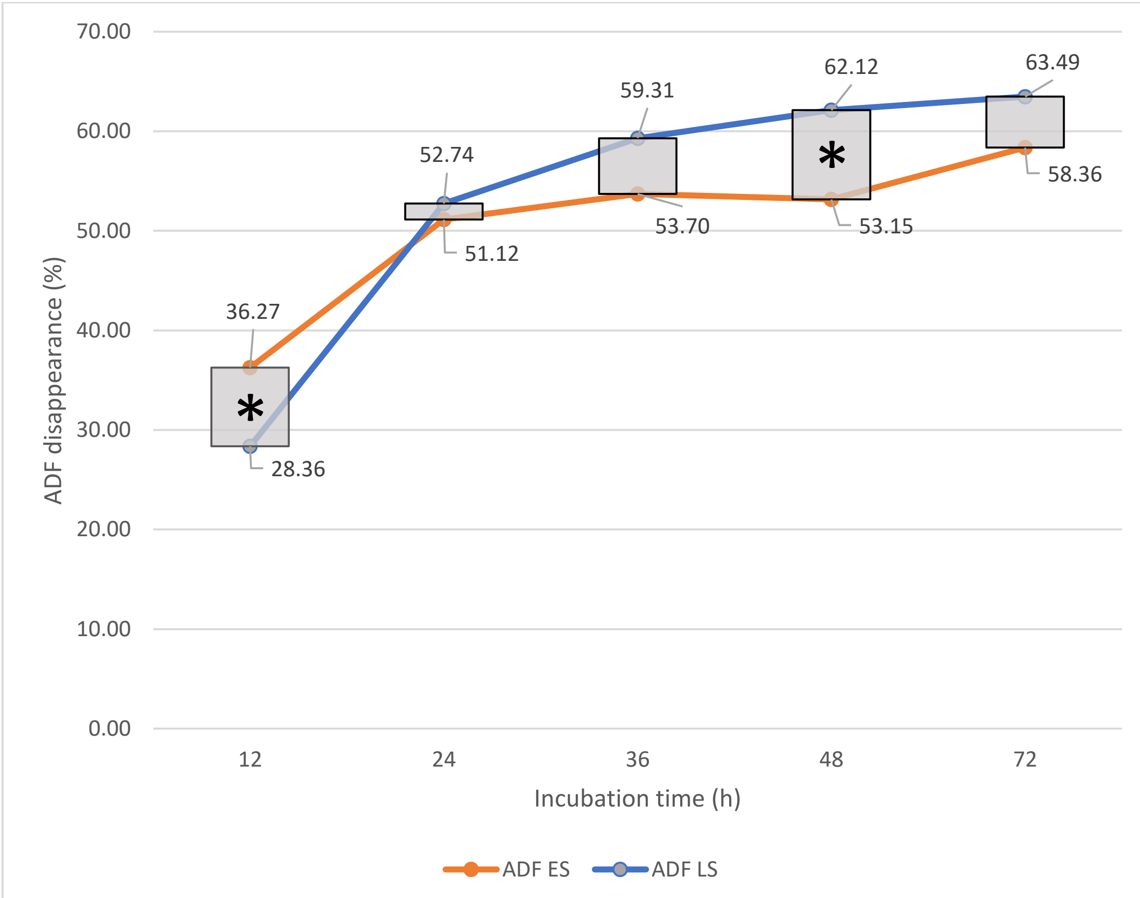
* Significant age by incubation time interaction ($P < 0.05$)

Figure 2. Total *in situ* rumen neutral detergent fiber (NDF) disappearance of early season (ES) and late season (LS) kudzu.



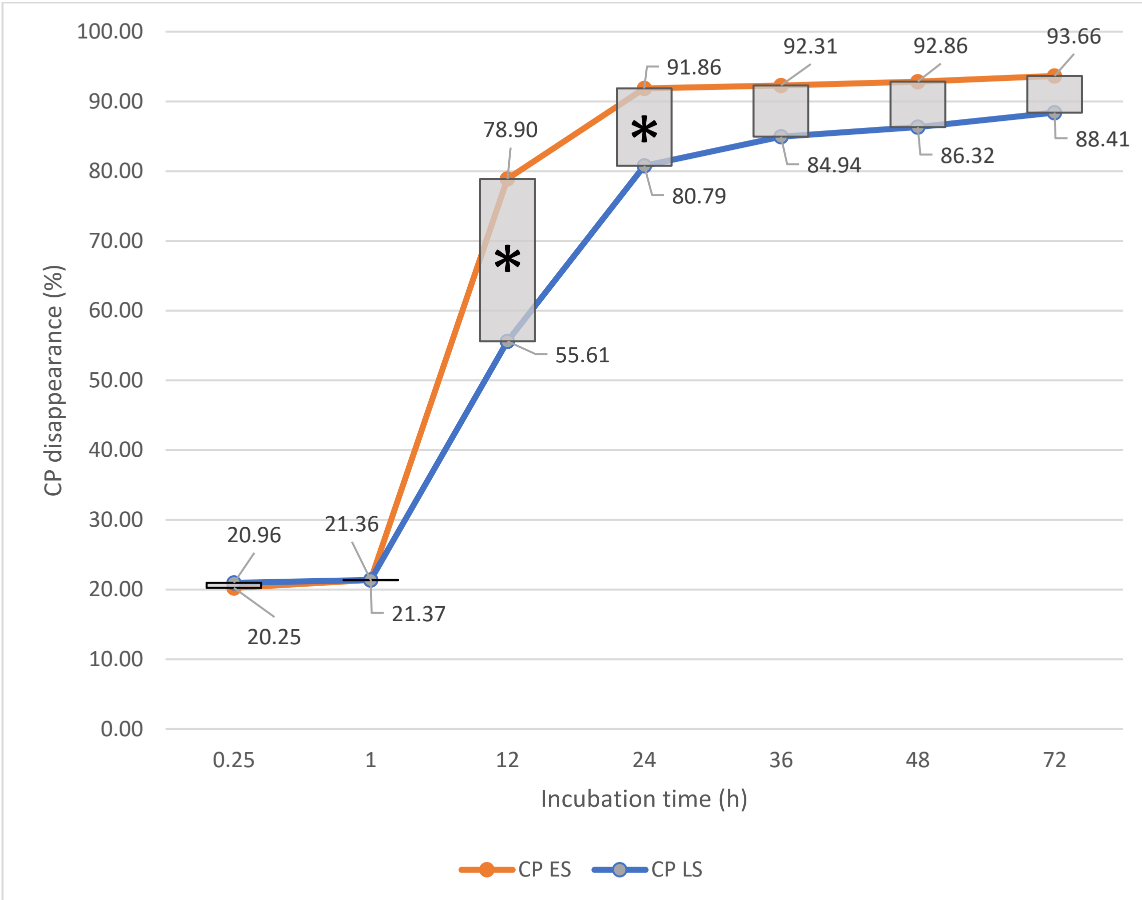
* Significant age by incubation time interaction ($P < 0.05$)

Figure 3. Total *in situ* rumen acid detergent fiber (ADF) disappearance of early season (ES) and late season (LS) kudzu.



* Significant age by incubation time interaction (P < 0.05)

Figure 4. Total *in situ* rumen crude protein (CP) disappearance of early season (ES) and late season (LS) kudzu.



* Significant age by incubation time interaction ($P < 0.05$)

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GLOSSARY

Acid detergent fiber (ADF).	The least digestible plant components, including cellulose and lignin. ADF values are inversely related to digestibility, so forages with low ADF concentrations are usually higher in energy.
Ad libitum.	As much and as often as desired.
Aerobic.	Relating to, involving, or requiring free oxygen.
Anaerobic.	Relating to, involving, or requiring an absence of free oxygen.
Bovine.	Pertaining to cattle.
Browser.	An herbivore that selectively feeds on leaves, soft shoots, or fruits of high-growing, generally woody, plants such as shrubs.
Cellulose.	An insoluble substance that is the main constituent of plant cell walls and of vegetable fibers such as cotton. It is a polysaccharide consisting of chains of glucose monomers.
Crude protein (CP).	Measures the nitrogen content of a feedstuff, including both true protein and non-protein nitrogen.
Defoliate.	Removes leaves from (a tree, plant, or area of land), for agricultural purposes.
Digesta.	Something undergoing digestion (as food in the stomach).
Dry matter (DM).	The part of a feedstuff or other substance that would remain if all its water content was removed.
Facultative anaerobe.	An organism that lives and grows in the absence of molecular oxygen.
Fine fuels.	Fast-drying fuels, generally with a comparatively high surface area-to-volume ratio, which are less than 1/4 -inch in diameter and have a time lag of one hour or less. These fuels ignite readily and are rapidly consumed by fire when dry.
Fistula.	An abnormal or surgically made passage between a hollow or tubular organ and the body surface, or between two hollow or tubular organs.
Forage.	Bulky food such as grass or hay for horses and cattle; fodder.

Grazing. To feed on standing vegetation, as by livestock or wild animals.

Hemicellulose. Any class of substances that occur as constituents of the cell wall of plants and are polysaccharides of simpler structure than cellulose.

Herbivore. An animal that feeds on plants.

In situ. In the natural or original position or place.

In vitro. Performed or taking place in a test tube, culture dish, or elsewhere outside a living organism.

In vivo. Performed or taking place in a living organism.

Legume. Any of a large family (Leguminosae synonym Fabaceae, the legume family) of dicotyledonous herbs, shrubs, and trees having fruits that are legumes (sense 3) or loments, bearing nodules on the roots that contain nitrogen-fixing bacteria, and including important food and forage plants (such as peas, beans, or clovers).

Lignin. A complex organic polymer deposited in the cell walls of many plants, making them rigid and woody. It is practically indigestible.

Microorganism. A microscopic organism, especially, a bacterium, virus, or fungus.

Neutral detergent fiber (NDF). Structural components of the plant, specifically cellulose, hemicellulose, and lignin. NDF is a predictor of voluntary intake because it provides bulk or fill. In general, low NDF values are desired because NDF increases as forages mature.

NIR. Near infrared reflectance spectroscopy is a laboratory analysis of feeds that uses a specific wavelength of near infrared light to estimate nutrient content of feeds based on computerized calibrations of nutrient composition of feedstuffs; a lower-cost analysis compared with traditional wet chemistry. It is dependent on correct calibration to specific feeds for accurate analysis.

Organic matter. Pertaining to substances derived from living organisms or all other compounds containing carbon.

RCBD. Randomized complete block design is a statistical procedure where the experimenter divides subjects into subgroups called blocks, such that the variability within the blocks is less than the variability between blocks. Then, the subjects within each block are randomly assigned to treatment conditions. Compared to a completely randomized design, this design reduces variability within treatment conditions and potential confounding, producing a better estimate of treatment effects.

Rumen. The first and largest compartment of the ruminant stomach, also known as the first stomach. This is the site of fermentation of the consumed feed by microbes.

Ruminant. Even-toed hooved mammals of the suborder Ruminantia. Ruminants usually have a stomach divided into four compartments (called the rumen, reticulum, omasum, and abomasum), and chew a cud consisting of regurgitated, partially digested food. Ruminants include cattle, sheep, goats, deer, giraffes, antelopes, and several other herbivore species.

Secondary metabolite. Small organic molecules produced by an organism that are not essential for their growth, development and reproduction.

Silage. Grass or other green fodder compacted and stored on airtight conditions, typically in a silo, without first being dried, and used as animal feed in the winter.

Steer. A male domestic bovine that has been castrated.

Symbiotic relationship. A close, prolonged association between two or more different organisms of different species that may, but does not necessarily, benefit each member.

Volatile fatty acids (VFA). Any one of several volatile organic acids found especially in rumen or large intestine contents and/or silage. Acetic, propionic, and butyric acids are ordinarily the most prevalent.

APPENDIX

APPENDIX A

INSTITUTIONAL ANIMAL CARE and USE COMMITEE
Office of Research Compliance
010A Sam Ingram Building
2269 Middle Tennessee Blvd
Murfreesboro, TN 37129

IACUCN001: PROTOCOL APPROVAL NOTICE

Wednesday, February 14, 2018

Senior Investigator	**Kevin Downs** (ROLE: Principal Investigator)
Co-Investigators	Joseph Gulizia (Student)
Investigator Email(s)	*kevin.downs@mtsu.edu; jpg3y@mtmail.mtsu.edu*
Department	Agribusiness and Agriscience

Protocol Title	***In situ rumen degradation in kudzu (Pueraria montana) in bovine***
Protocol ID	**18-2007**

Dear Investigator(s),

The MTSU Institutional Animal Care and Use Committee has reviewed the animal use proposal identified above under the ***Designated Member Review (DMR) mechanism*** and has approved your protocol in accordance with PHS policy. A summary of the IACUC action(s) and other particulars of this this protocol is tabulated as below:

IACUC Action	**APPROVED for one year from the date of this notification**	
Date of Expiration	**2/28/2019**	
Number of Animals	1 (ONE)	
Approved Species	**Bos taurus (MTSU herd)**	
Category Subclassifications	☐**Teaching** ☐ Classroom ☐ Laboratory	☒**Research** ☒Laboratory ☐Field Research ☐Field Study ☒Handling/Manipulation ☐Observation
	Comment: NONE	
Approved Site(s)	MTSU Beef Unit, 181 Sam Jared Dr., Murfreesboro, TN	
Restrictions	**Satisfy DMR requirements AND annual continuing review**	
Comments	The official name of this site must be revised in the PHS Assurance	
Amendments		NONE

This approval is effective for three (3) years from the date of this notice. This protocol **expires on 2/28/2021** The investigator(s) MUST file a Progress Report annually regarding the status of this study. Refer to the schedule for Continuing Review shown below; NO REMINDERS WILL BE SENT. A continuation request (progress report) must be approved by the IACUC prior to **2/21/2019** for this protocol to be active for its full term. Once a protocol has expired, it cannot be continued and the investigators must request a fresh protocol.

Continuing Review Schedule: Refer to the following table to request your CR:

Reporting Period	Requisition Deadline	IACUC Comments
First year report	1/31/2019	TO BE COMPLETED
Second year report	1/31/2020	TO BE COMPLETED
Final report	1/31/2021	TO BE COMPLETED

MTSU Policy defines an investigator as someone who has contact with live or dead animals for research or teaching purposes. Anyone meeting this definition must be listed on your protocol and must complete appropriate training through the CITI program. Addition of investigators requires submission of an Addendum request to the Office of Research Compliance.

The IACUC must be notified of any proposed protocol changes prior to their implementation. Unanticipated harms to subjects or adverse events must be reported within 48 hours to the Office of Compliance at (615) 494-8918 and by email – compliance@mtsu.edu.

Post-approval Protocol Amendments:

Date	***Amendment(s)***	***IRB Comments***
NONE	NONE	NONE

All records pertaining to the animal care be retained by the MTSU faculty in charge for at least three (3) years AFTER the study is completed. Be advised that all IACUC approved protocols are subject to audit at any time and all animal facilities are subject to inspections at least biannually. Furthermore, IACUC reserves the right to change, revoke or modify this approval without prior notice.

Sincerely,

Compliance Office (On behalf of IACUC)
Middle Tennessee State University Tel: 615 494 8918
Email: iacuc_information@mtsu.edu (for questions) and
Iacuc_submissions@mtsu.edu (for sending documents)