Yield and Nutritive Quality of Fresh and Ensiled Reduced Lignin Alfalfa in Monoculture and Diculture With Novel Tall Fescue

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YIELD AND NUTRITIVE QUALITY OF FRESH AND ENSILED REDUCED LIGNIN ALFALFA IN MONOCULTURE AND DICULTURE WITH NOVEL TALL FESCUE

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Animal and Veterinary Science

by
Morgan L. Boss
May 2024

Accepted by:
Dr. Matias Aguerre, Committee Co-Chair
Dr. James Strickland, Committee Co-Chair
Dr. Charles Rosenkrans
ABSTRACT

The objectives of this research were to: (1) evaluate the yield, nutritional value, and digestibility of fresh and ensiled reduced lignin alfalfa (RLA; Medicago sativa L.) in monoculture and when mixed with novel endophyte-infected tall fescue (F; Festuca arundinacea) in comparison to a conventional alfalfa (CA) cultivar; and (2) evaluate ensiled samples under varying DM concentrations; with or without inoculant. A trial was prepared as a randomized complete block design with 4 replicates per treatment. Treatments included reduced lignin alfalfa (RLA), conventional alfalfa (CA) and tall fescue (F) plots as well as RLA/F and CA/F plots. Plots were harvested on a 35-day interval, for a total of 5 harvests. Ensiled forage was either treated, or not treated with a lactic acid bacteria (LAB) inoculant, and ensiled at high (50%) or low (35%) DM content. Samples were analyzed for $\alpha$-amylase-treated neutral detergent fiber on organic matter basis (aNDFom), acid detergent fiber (ADFom), acid detergent lignin (ADL), DM yield (DMY), water-soluble carbohydrates (WSC), crude protein (CP), ash, in-vitro DM digestibility (IVDMD), in-vitro NDF digestibility (IVNDFD), undigestible NDF (uNDF), potentially digestible NDF (pdNDF), as well as for fermentation values in ensiled samples. Results from this study indicated that reduced lignin alfalfa can be grown in monoculture or binary mixture with novel tall fescue and produce similar dry matter yield without sacrificing nutritive value. Additionally, while DM percentage, inoculation, and treatment had a significant effect (P<0.05) on silage fermentation, all silages were within established acceptable limits which provides flexibility in silage management to producers.

Keywords: digestibility, reduced lignin alfalfa, tall fescue, mixed forage, yield
DEDICATION

Dedicated in memory of James (Darrell) Brizendine
Lifelong farmer, teacher, and public servant

Thank you for all your love and wisdom throughout the years and for instilling a love of agriculture in me at a young age.

August 18th, 1943 – June 28th, 2023
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CHAPTER ONE

LITERATURE REVIEW

Introduction

Livestock production relies heavily on the availability of high-quality forage as a primary source of nutrition. Forage productivity plays a pivotal role in meeting the nutritional needs of animals and, on a broader scale, in addressing the global challenge of food insecurity. Forage serves as a vital feed resource, supplying essential nutrients, including proteins, fats, carbohydrates, vitamins, and minerals, necessary for the growth, reproduction, and overall health of production animals. Adequate forage productivity tailored to the environment in which it is grown is critical to ensure a reliable and sustainable supply of animal feed, supporting the world's growing population's nutritional needs.

Mixed forage pastures, especially incorporating grass-legume mixtures, play a crucial role in sustainable livestock production systems, providing numerous benefits such as improved forage quality, enhanced animal performance, and environmental sustainability (Sleugh et al., 2000; Albayrak et al., 2011; Sturludóttir et al., 2014; Sheaffer et al., 2020). The inclusion of legumes in mixed forage pastures complements grasses by providing nitrogen fixation, which enhances soil fertility, reduces reliance on external inputs, and increases forage protein content and quality (Ledgard and Steele, 1992; Woodfield and Clark, 2009; Soussana et al., 2010; Tracy et al., 2016; Cherney et al., 2020).

Alfalfa (*Medicago sativa* L.), a cool-season perennial legume, is widely regarded as the "Queen of Forages" as it is known for its high protein content, nitrogen fixation, and excellent digestibility, providing essential amino acids and other nutrients for animal growth and
While alfalfa produces a high-quality, sought-after product, it is recommended to mix with grasses to reduce the incidences of bloat (Sheaffer et al., 2009; Lei et al., 2017). Tall fescue, a cool-season perennial grass, complements alfalfa by providing a reliable source of structural fiber and contributing to overall forage yield (Tracy et al., 2016). Additionally, tall fescue's ability to persist in diverse environmental conditions, including the heat and humidity of the southeastern United States, makes it a valuable component in the region's forage systems (Tracy et al., 2016). Maintaining a balance between legume and grass components is a crucial element in ensuring optimal forage quality, nutrient composition, and animal performance (Tracy et al., 2016; Grev et al., 2017). The combination of alfalfa and tall fescue in forage mixtures contributes to a well-rounded and nutritionally balanced diet for livestock (Tessema and Feleke, 2018).

However, there are still challenges to overcome, such as lignification in plant structures, which can impact digestibility and overall forage quality. Lignin, a complex plant polymer, plays a critical role in plant cell wall structure and affects forage digestibility (Moore and Jung, 2001). Traditional forage varieties often have higher lignin content, which limits their digestibility and nutrient availability for livestock (Li et al., 2008; Arnold et al., 2019). The incorporation of reduced lignin varieties, achieved through genetic engineering, offers the potential to improve forage quality by reducing lignin content and cross-linking interactions (Barros et al., 2019). These reduced lignin varieties have been shown to increase fiber digestibility and overall forage quality, leading to improved animal performance (Grev et al., 2017; Barros et al., 2019). Additionally, reduced lignin cultivars can offer producers flexibility in harvesting schedules without risk of excessive lignification with maturation (Getachew et al., 2018).
Ensiling, the process of preserving forage through anaerobic fermentation, is essential for optimizing forage utilization and reducing losses (Kung et al., 2018). The primary objective of silage production is to minimize available oxygen and rapidly decrease the pH to facilitate the growth of lactic acid bacteria (LAB) and preserve the forage (Ward and de Ondarza, 2008). In order to achieve high-quality silage with preserved nutrients and reduced spoilage, proper ensiling practices should be followed, including maintaining appropriate moisture content, ensuring proper fermentation, and utilizing inoculants (Kung et al., 2018; Muck, 2010). Although alfalfa is highly valued as forage, it can pose challenges during ensiling due to its low water-soluble carbohydrate (WSC) content and high buffering capacity (Nkosi et al., 2016). Despite these challenges, ensiling forages, including alfalfa, aims to preserve high-quality, nutritious feed that remains available throughout the year, thereby improving the sustainability of agricultural production (Dunière et al., 2013).

This literature review aims to explore the importance of mixed forage pastures and grass-legume mixtures, discuss the incorporation of reduced lignin varieties to enhance forage quality, evaluate the impact of lignin on forage digestibility, review forage analysis techniques, including the detergent system, in vitro, and in situ methods, and highlight the significance of ensiling forage. By synthesizing existing research, this review aims to provide valuable insights into optimizing forage resources for sustainable livestock production.

**Mixed Pasture Vs. Monoculture**

Throughout history, mixed pasture forage systems have been a traditional and sustainable approach to agriculture. Farmers would cultivate diverse mixtures of grasses, legumes, and other
plants in their pastures grown in a synergistic manner, providing a varied diet for grazing livestock and promoting soil health. However, with the advent of industrialization and the pursuit of higher yields and efficiency, there was a significant shift towards monoculture farming. (Sylvester and Cunfer, 2009; Franzluebbers and Martin, 2022). Monoculture systems focused on cultivating a single crop over large areas, often leading to soil degradation, increased reliance on synthetic inputs, and environmental concerns (Sylvester and Cunfer, 2009). In recent years, there has been a resurgence of interest in mixed forage systems as a key component of sustainable agricultural practices (Sanderson et al., 2007). This resurgence aligns with the principles of agroecology and sustainable farming, as mixed forage systems contribute to enhanced soil health, improved nutrient cycling, increased forage quality, reduced erosion, and increased biodiversity (Reganold et al., 1990; Heichel and Henjum, 1991; Ledgard and Steele, 1992; Boddey et al., 1995; Veira et al., 2010; Mueller et al., 2013). The reintegration of mixed forage systems acknowledges the importance of diversifying crops, promoting natural processes, and supporting long-term agricultural sustainability (Sanderson et al., 2016).

Legumes in mixed pastures can fix atmospheric nitrogen via their symbiotic relationship with rhizome nodules in their roots. This relationship provides a natural and sustainable source of nitrogen for grasses, thus reducing or eliminating the need for nitrogen fertilizer application (Deak et al., 2010; Tracy et al., 2016; Bélanger et al., 2017; Boddey et al., 2020). Additionally, the symbiotic relationship between legumes and nitrogen-fixing bacteria reduces greenhouse gas emissions and helps mitigate environmental impacts associated with synthetic fertilizer usage (Boddey et al., 2020). Mueller et al. (2013) concluded that interactions between legumes and grasses resulted in root depths reaching twice the expected depth from their individual species, thus improving soil health and water uptake of the stand. Further, grass-legume mixtures have
been known to compensate for the "summer slump" in growth that monoculture stands exhibit during the summer (Sleugh et al., 2000; Ottman and Mostafa, 2014; Tracy et al., 2016). In several studies, dry matter yield (DMY), neutral detergent fiber (NDF), Neutral detergent fiber digestibility (NDFD), crude protein (CP), and in-vitro dry matter digestibility was improved for grass-legume mixtures as compared to grass monocultures (Sleugh et al., 2000; Albayrak et al., 2011; Foster et al., 2014; Brink et al., 2015; Aponte et al., 2019; McDonald et al., 2021; Damiran et al., 2022; Sim, 2022).

Use of mixed forage systems is not without its challenges. Achieving and maintaining the desired balance between grasses and legumes in mixed pastures can be challenging due to differences in growth rates, competitive abilities, and management requirements (Berdahl et al., 2001; Grime, 2006; Tracy et al., 2016). Effective management strategies, including forage species selection, and nutrient management, are necessary to maintain the desired species composition and productivity in mixed pastures (Berdahl et al., 2001; Deak et al, 2007; Bork et al., 2017; Aponte et al., 2019). While mixed forage systems can be more complex to manage and may require additional knowledge and skill, their potential for increased productivity, sustainability, and resilience makes them an attractive option in agricultural systems aiming for long-term productivity and environmental stewardship.

**Tall Fescue**

Tall fescue (*Lolium arundinaceum*) is a cool-season perennial grass that plays a crucial role in agriculture, particularly in the southeastern United States (Sleper and West, 1996). It is highly valued in the United States for its persistence, adaptability, tolerance of high pH soil, and
ability to withstand challenging environmental conditions (Cowan, 1956; Aiken et al., 2012). Additionally, tall fescue possesses a deep root system, allowing it to access moisture and nutrients from deeper soil layers, further enhancing its drought tolerance and overall longevity.

One significant consideration in utilizing tall fescue is the presence of an endophyte \( (Epichloë coenophial) \). This endophyte is naturally present in many tall fescue plants and provides benefits such as improved plant vigor, stress tolerance, and resistance to grazing pests (Hoveland, 1993; Zhuang et al., 2005). However, the endophyte also produces ergot alkaloids, particularly ergovaline, that can cause fescue toxicosis in livestock (Strickland et al., 2012). This condition negatively impacts animal productivity and health, leading to reduced weight gain, poor reproductive performance, vasoconstriction, elevated body temperature, and decreased feed intake, especially in times of heat stress (Paterson et al., 1995; Zhuang et al., 2005). Tall fescue toxicosis continues to pose a major economic loss to producers utilizing tall fescue (Strickland et al., 1993; Strickland et al., 2012).

To mitigate the adverse effects of fescue toxicosis, endophyte-free varieties of tall fescue have been developed. These varieties lack the endophyte, making them a safer alternative for animal consumption. Although utilizing endophyte-free tall fescue reduces the risk of fescue toxicosis and improves animal productivity and well-being, the endophyte-free variety is not as hardy as its endophyte-infected counterpart (Bouton et al., 2002). The need for tall fescue that exhibits plant vigor similar to the endophyte infected cultivars while still preventing the negative animal productivity induced by ergot alkaloids led to the introduction of novel endophyte-infected tall fescue cultivars. Novel tall fescue cultivars mitigates the risk of poor animal productivity associated with tall fescue toxicosis while maintaining healthy, persistent tall fescue stands (Bouton et al., 2002).
Novel endophyte-infected tall fescue can be incorporated into grass-legume mixed pasture as a strategy to optimize productivity of the novel endophyte-infected varieties of tall, without the toxic concerns of the toxic endophyte-infected tall fescue (Bouton et al., 2002; Strickland et al., 2012). Tall fescue complements legumes by providing a reliable source of structural fiber and contributing to overall forage yield. Grass-legume diculture mixes often outperform grass monocultures in terms of DMY (Berdahl et al., 2001; Bélanger et al., 2017; Bork et al., 2017). Additionally, novel endophyte-infected tall fescue can mitigate the risk of bloat, which is a concern of feeding monoculture legumes to ruminants (Aponte et al., 2019). Bloat is a condition characterized by excessive gas accumulation in the rumen. In a study conducted by Veira et al. (2010), when orchardgrass was added as a component of the pasture, bloat in cattle was reduced by 70-90%. This reduction in instances of bloat was likely due to a higher neutral detergent fiber digestibility (NDFD) of the added grasses, which plays a role in regulating the ruminal pH (Aponte et al., 2019). By combining novel endophyte-infected tall fescue with legumes such as clover or alfalfa, the high fiber content of tall fescue is balanced with the protein-rich and easily digestible components of legumes (Khatiwada et al. 2020).

Alfalfa

Alfalfa (*Medicago sativa*), a widely cultivated cool-season perennial legume, stands out as a powerhouse in forage production, and is often referred to as the "Queen of Forages." Introduced to the Southeastern US in the late 1700s to early 1800s, alfalfa is no newcomer to the area (Lacefield et al., 2009). Despite its long-standing past in the Southeast, alfalfa production suffered from alfalfa weevil (*Hypera postica*) infestation, maladaptation to the soil type, and low
nitrogen fertilizer cost resulted in a decline in its production during the mid-1900s (Lacefield et al., 2009). However, the development of more region-adapted cultivars that can resist high pH, drought, intensive grazing, and that incorporate glyphosate-resistance has led to increased use (Bouton, 2012). According to the National Agricultural Statistics Service (NASS) of the United States Department of Agriculture (USDA), the total area of alfalfa or alfalfa-grass hay harvested in the U.S. was approximately 9 million ha, totaling over 47 million tons in 2021 (USDA-FSA, 2022).

Alfalfa exhibits rapid growth and regrowth, allowing for multiple harvests and providing a reliable source of high-quality forage throughout the growing season. Depending on management practices, alfalfa can yield between 2-10 harvests per year and persist for 3-6 years (Sheaffer et al., 2020). It is important to note that nutrient composition changes throughout the vegetative lifecycle of the plant. Younger, more vegetative stands tend to have high CP and higher fiber digestibility but with reduced yields (Moore and Jung, 2001; Lamb et al., 2012). Therefore, producers must aim to harvest stands at intervals that balance nutritive value and yield.

Variation among harvest intervals, typically 28-day, 35-day, and 42-day intervals, can affect stand persistence, CP, yield, and DM percentage (Brink and Marten, 1989; Min, 2016). One study concluded that a 34-day harvest interval yielded significantly more than the 28-day or 42-day harvests (Kallenbach et al., 2002). It is also well documented that CP and fiber digestibility decreases with increased harvest intervals, resulting in reduced yields in subsequent years (Brink and Marten, 1989; Kallenbach et al., 2002; Min, 2016; Sheaffer et al., 2020; Xu et al., 2021). Alternatively, harvesting too frequently can damage the stand, causing a decrease in longevity. This was supported by Brink and Marten (1989), in which they concluded that stand
persistence was reduced with shorter harvest intervals. Probst and Smith (2011) suggest that a 35-day harvest is optimal for most alfalfa cultivars when considering yield and stand persistence.

**Lignin and Reduced Lignin Alfalfa**

Reductions in nutritive quality of alfalfa with stand maturity are linked to the lignification process. Lignin is a complex, indigestible organic polymer that is found in the cell walls of plants. It is an important structural component of plant cell walls, as it provides support and rigidity to the plant, allowing it to stand upright (Guo et al., 2001a). Lignin concentration is negatively correlated with nutrient digestibility in ruminants and is often considered an anti-quality factor (Jung et al., 1997; Getachew et al., 2018). Lignin acts as a physical barrier, making it more challenging for rumen microbes to access the nutrients enclosed within the plant cells (Moore and Jung, 2001). Logically, a reduction in lignin concentration should result in increased forage digestibility and utilization.

In recent years, researchers have made significant progress in downregulating lignin levels in alfalfa through biotechnological approaches (Guo et al., 2001a; Lei et al., 2017; Barros et al., 2019; Cherney et al., 2020). By manipulating the expression of key genes involved in lignin biosynthesis, scientists have been able to develop alfalfa varieties with reduced lignin content. The downregulation of lignin in alfalfa involves targeting enzymes responsible for lignin synthesis, such as caffeic acid 3-O-methyltransferase (COMT) and caffeoyl CoA 3-O-methyltransferase (CCOMT) (Guo et al., 2001b; Barros et al., 2019). By reducing the activity of these enzymes, the lignin biosynthesis pathway is altered, resulting in decreased lignin production, monolignol composition, and lignin accumulation in plant tissues (Marita et al.,
The main constituents of lignin are three monolignols: hydroxyphenyl (H), guaiacyl (G), and syringyl (S). Plants like alfalfa primarily have lignin polymers composed of G and S monomers, with only a minor amount of H monomers (Li et al., 2008). It is unclear whether lignin monomer ratios and composition have a direct impact on forage digestibility as much as overall lignin reduction does (Grabber et al., 1997; Baucher et al., 1999; Guo et al., 2001b; Getachew et al., 2011; Grabber, 2019).

Reduced lignin alfalfa varieties, such as HarvXtra™ (genetically engineered) and Hi-Gest® 360 (selective breeding) are often referred to as "low-lignin" or "reduced-lignin" alfalfa and exhibit improved digestibility, increased forage quality, and enhanced animal utilization (Guo et al., 2001b; Getachew et al., 2018). Various studies conducted over Canada and the United States concluded that reduced lignin alfalfa (RLA) averaged an NDFD increase between 5.8% and 15% when compared to reference alfalfa cultivars (Guo et al., 2001a; Grev et al., 2017; Barros et al., 2019; Cherney et al., 2020; Damiran et al., 2022). Plots grown in a 3-year study in New York, Kentucky, and Montana averaged between 11 and 18.5% less ADL in the RLA cultivar than conventional varieties (Cherney et al., 2020). This reduction in ADL is supported by several other studies reporting between a 6% and 24% decrease in total herbage ADL in RLA cultivars (Grev et al., 2017; Getachew et al., 2018; Barros et al., 2019; Grev et al., 2020; Damiran et al., 2022). It is the general consensus of various experiments that NDF and CP concentrations do not differ significantly between RLA cultivars and conventional alfalfa (CA) cultivars (Guo et al., 2001a; Getachew et al., 2011; Grev et al., 2017; Grev et al., 2020). The yield of RLA harvested on a delayed interval (35-40 days) showed a 20% gain in forage mass as compared to conventional alfalfa harvested on a shorter 30-day harvest schedule (Grev et al., 2017; Getachew et al., 2018). Damiran et al. (2022) similarly found that delaying RLA harvest
increased the forage mass without having to sacrifice the forage quality that typically comes with alfalfa maturation.

While reduced lignin alfalfa shows promise, further research is still needed to fully understand its agronomic performance, persistence, and potential impacts on the environment and overall forage systems. Reduced lignin alfalfa varieties offer producers greater flexibility in the management of nutritive quality, yield, and profitability of their forage plots. The downregulation of lignin in alfalfa represents an exciting advancement in the development of improved forage crops, offering opportunities for sustainable agriculture and enhanced livestock productivity.

**Forage Analysis**

Forage digestibility is a critical aspect of understanding the nutritional quality of animal feed. Several measurements are employed to assess the digestibility of forage, each providing valuable insights into its composition and potential as a feed source. For the purpose of this literature review, emphasis will be placed on digestibility in relation to ruminants. Some key analysis used in this context include Neutral Detergent Fiber (NDF), Acid Detergent Fiber (ADF), Acid Detergent Lignin (ADL), Dry Matter (DM), Neutral Detergent Fiber Digestibility (NDFD), in vitro digestibility, and in situ digestibility.

*Neutral Detergent Fiber*
Neutral detergent fiber (NDF), or $\alpha$-amylase treated neutral detergent fiber (aNDF), is a measure of the total fiber content in a feed or forage sample. It is the sum of the plant cell wall components, including cellulose, hemicellulose, and lignin. The NDF method was originally developed in 1967 by Van Soest and Wine, but a multitude of proposed variations led to dissimilar results and a difficulty measuring NDF (Mertens et al., 2002). The aNDF procedure, though still commonly referred to as NDF, was standardized in 2002 and utilizes sodium sulfite to remove proteinaceous material, and $\alpha$-amylase to degrade starch, in addition to the standard neutral detergent solution (Mertens et al., 2002).

NDF is an important measure in animal nutrition because it is related to digestibility and is directly correlated with dry matter forage intake and animal productivity (Beauchemin, 1996; Arnold et al., 2019). Hansen and Lawrence (2017) found that the best predictor of dry matter digestibility was NDF concentration. As the NDF content of a feed increases, the digestibility and intake generally decrease (Van Soest et al., 1991). This is because the high fiber content makes it more difficult for the ruminal microbes to break down and extract nutrients from the feed. NDF values can vary widely depending on the plant species, maturity stage, and other factors, so it is important to interpret NDF values in context with other nutritional parameters (Van Soest et al., 1991).

Acid Detergent Fiber

Acid detergent fiber (ADF) is a laboratory analytical technique used primarily to determine the amount of cellulose and lignin in a sample of forage or feed (Van Soest et al., 1991). ADF is measured as the residue left after boiling the sample in an acidic detergent
solution that removes the soluble components such as sugars, starches, and proteins, as well as hemicellulose. ADF represents components that are highly resistant to microbial enzymatic breakdown in the rumen. As a result, ADF values give insights into the potential energy available to ruminants from the feed. High ADF values indicate a lower-quality feed that is less digestible, as it contains more structural components such as lignin that cannot be broken down by microbial digestive enzymes and cellulose that is limited in its digestibility due to cross-linking with lignin (Hansen and Lawrence, 2017). As a result, feeds with high ADF levels provide less energy to the animal and may reduce overall performance, particularly in terms of growth rate and milk production in dairy animals (Beauchemin, 1996). Diets with lower ADF are associated with higher voluntary food intake, increased nutrient utilization, and overall improved animal productivity. Although this can be a useful predictor of digestibility, studies have shown that ADF may be a poorer fit for grasses as compared to legumes (Hansen and Lawrence, 2017).

*Acid Detergent Lignin*

Acid detergent lignin (ADL) is a measurement of the component of plant cell walls that is resistant to acid detergent fiber (ADF) analysis. ADL measures the quantity of lignin in forages, which is the structural polyphenolic polymer that provides plants with rigidity and protection. Lignin is indigestible and affects the digestibility of other plant components, such as cellulose and hemicellulose. Thus, ADL is a measure of the amount of lignin present in forages and is used to estimate the digestibility and energy content of the feed (Van Soest et al., 1991). The presence of lignin in forage can negatively impact animal performance. Lignin reduces the availability of nutrients, particularly energy, by encapsulating other cell wall components like
cellulose and hemicellulose. This can limit the access of rumen microbes to digestible portions of the feed, leading to lower fiber digestibility and energy extraction from the forage. A higher ADL is negatively correlated to dry matter intake in ruminants consuming a high forage diet due to increased cell wall components contributing to ruminal fill (Jung and Allen, 1995; Jung et al., 1997). The increased proportion of the indigestible fraction of the cell wall contributes to a reduced ruminal passage rate (Jung and Allen, 1995).

Dry Matter

Dry matter (DM) refers to the portion of feed or forage that remains after all the moisture has been removed. DM is measured as a percentage of the total weight of the feed or forage sample, and it is used to determine the nutritional value of the feed. DM is important in agricultural feed because it allows for accurate calculations of nutrient content and intake (Mertens et al., 2004). Moisture content can vary widely in feed and forage, and the amount of moisture present can significantly affect the concentration of nutrients in the feed (Mertens et al., 2004). By removing the moisture, DM allows for more accurate and consistent comparisons of nutrient content across different feed samples. DM also plays a key role in determining the amount of feed required to meet the nutritional needs of animals. Since animals consume feed on a dry matter basis, DM measurements can be used to calculate the actual amount of nutrients available for the animal to digest and utilize. This information can help farmers and nutritionists create balanced diets that meet the nutrient requirements of different animal species at various stages of growth and production.
Neutral Detergent Fiber Digestibility

Neutral Detergent Fiber Digestibility (NDFD) is a measure of the percentage of NDF that is digested by an animal. NDFD is an important measure in animal nutrition because it helps to predict how much of the forage will be available for digestion by the animal. For example, a forage with a high NDFD value will be more digestible and provide more nutrients than a forage with a low NDFD value. In general, higher NDFD values are desirable because they indicate that more of the fibrous plant material can be utilized by the animal, leading to improved animal performance and potentially reducing feed costs (Oba and Allen, 1999). NDFD is typically performed using an in vitro procedure (IVNDFD), and procedures are described by Ferreira and Mertens (2005). The media and reducing agent typically utilized are defined by Goering and Van Soest (1970).

In situ

In-situ digestibility is a valuable tool in agricultural forage analysis that provides insights into the nutritive value of forages for ruminant animals. It focuses on understanding the digestibility of forage components within the animal's rumen, where microbial fermentation and enzymatic breakdown occur (Vieira et al., 2012). In-situ digestibility involves the placement of small bags containing representative forage samples directly into the rumen of a fistulated ruminant animal. These bags, typically made of nylon, allow the forage to be exposed to the ruminal environment and undergo natural microbial fermentation and degradation. After a predetermined incubation period, typically around 240-270 hours, the bags are retrieved and
analyzed to measure the extent of forage digestion and nutrient breakdown (Ørskov et al., 1980). Huhtanen et al. (2006) explained that data retrieved from in situ and subsequent fiber analysis can divided into meaningful parts: potentially digestibly fiber (pdNDF), neutral detergent soluble (NDS), and insoluble fiber (iNDF).

**Summary**

The development of these procedures has been the result of extensive research and refinement over the years. Scientists and researchers in the field of animal nutrition have continually sought to improve and standardize these methods to ensure accurate and reproducible results. These measurements play a fundamental role in formulating balanced diets for livestock, promoting optimal growth, and maximizing the efficiency of feed utilization. By understanding the digestibility and nutrient content of forage, farmers and nutritionists can make informed decisions to support the health and productivity of their animals.

**Ensiling**

Ensiling is a widely used preservation method in the agricultural industry for storing forage crops to maintain their nutritive value and extend their shelf life. The ensiling process involves harvesting the forage at the optimal stage of maturity, chopping it into small pieces, and tightly packing it into airtight containers, such as silos or pits. Once sealed, the forage undergoes anaerobic fermentation, which is a natural process driven by lactic acid bacteria (LAB) present in the plant material and within the environment, which converts water-soluble carbohydrates to organic acids, primarily lactic acid (McDonald et al., 1991; Stokes, 1992). The main goal of
ensiling forage is to rapidly reduce oxygen, increase acidity, and proper lactic acid bacteria (LAB) growth to create a stable, nutritious feedstuff.

The ensiling process provides several benefits to producers. It allows them to harvest forage at its peak nutritional value, ensuring a high-quality feed source for their livestock throughout the year (Dunière et al., 2013). Ensiling also offers flexibility in managing forage harvest and reduces the risk of weather-related spoilage, as it creates a stable and anaerobic environment that hinders the growth of spoilage microorganisms. Additionally, ensiling is cost-effective and minimizes forage wastage during times of surplus, contributing to overall farm profitability and sustainability (Dunière et al., 2013).

During ensiling, the fermentation process involves the conversion of water-soluble carbohydrates (WSC) in the forage into organic acids by LAB. The primary end product of fermentation is lactic acid and occasionally acetic acid, propionic acid, and butyric acid. Lactic acid production is the most critical aspect of successful ensiling, as it lowers the pH rapidly and inhibits the growth of undesirable microorganisms, preserving the forage effectively (Dunière et al., 2013). Acetic acid formation also contributes to pH reduction and provides additional protection as an antifungal (Kung and Ranjit, 2001). Propionic and butyric acids are produced in smaller quantities and may indicate poor fermentation if present in significant amounts, as they are associated with undesirable microbial activities and decreased forage quality (Kung and Ranjit, 2001). Butyric acid may also be an indicator of protein degradation and is treated as an anti-quality factor (Ward and de Ondarza, 2008). Clostridial growth is associated with the formation of ammonia nitrogen and results in a pungent smell and unpalatable silage (Ward and de Ondarza, 2008; Muck, 2010).
The success of the ensiling process is influenced by several factors, including dry matter (DM) content, WSC levels, and pH of the forage. Forage with a higher DM content (around 30-35%) is preferred for ensiling, as it promotes better packing and reduces the risk of effluent production during fermentation. Silage with high DM content shows characteristics of restricted fermentation and may lead to the growth of undesirable bacteria (Kung et al., 1984; Sheperd et al., 1995; Ward and de Ondarza, 2008). In order to obtain an optimal DM content, forage is wilted before being preserved in order to reduce water activity and to increase WSC concentration (Borreani et al., 2018). Adequate WSC levels are essential, as they provide substrates for LAB to produce lactic acid and promote proper ensilage fermentation (Stokes, 1992; Weinberg and Muck, 1996). Low WSC content may limit the amount of fermentable substrate and negatively affect the ensiling process (Sheperd et al., 1995). Maintaining a low pH (below 4.5) is crucial to inhibit the growth of spoilage microorganisms and ensure stable and well-preserved silage (Weinberg and Muck, 1996; Ward and de Ondarza, 2008). Inoculation of silage with homofermentative LAB has been found to mitigate some fermentation issues of forages with low WSC content or high DM content and to inhibit protein breakdown while also lowering pH, increasing digestibility, and improving aerobic stability (Kung et al., 1984; Kung and Ranjit, 2001; Muck et al., 2018; Huyen et al., 2020). Inclusion of facultative heterofermentative bacteria (previously classified as homofermentative LAB) inoculum such as *Lactobacillus planta rum*, *Lactobacillus casei*, and *Enterococcus faecium* had similar effects as obligate homofermentative inoculants (Muck et al., 2018). Additionally, obligate heterofermentative LAB such as *Lactobacillus buchneri*, *Lactobacillus brevis* and *Lactobacillus fermentum* can result in acetic acid and propionic acid increases along with lactic acid increase (Adesogan et al., 2003; Arriola et al., 2011; Dunière et al., 2013; Santos et al., 2013). This
production of acetic acid has the tendency to inhibit yeasts and fungi that act as anti-quality factors in silage (Reich and Kung, 2010).

Legumes, grasses, and corn are the most widely used products for ensiling, especially in the dairy industry, due to their high nutritional value but can differ in the ensiling process due to variations in their chemical composition and microbial populations (Dunière et al., 2013). Legumes, such as alfalfa, typically have lower WSC content and higher buffering capacity compared to grasses, resulting in slower pH decline during fermentation and risk of clostridial fermentation (Nkosi et al., 2016; Zheng et al., 2017; Borreani et al., 2018). Legumes may also contain higher protein levels, which can lead to increased ammonia production during ensiling and a resulting degradation of the feedstuff. Grasses, on the other hand, may have lower WSC levels, necessitating the addition of a fermentable carbohydrate source to facilitate proper ensilage fermentation (Kung and Ranjit, 2001). Legume-grass mixtures may combine the characteristics of both components, requiring careful management to ensure a successful ensiling process.

In conclusion, ensiling is a valuable method for preserving forage crops, offering benefits to agricultural producers by maintaining forage quality, reducing wastage, and providing a stable feed source for livestock year-round. The fermentation process involves LAB converting WSC into organic acids, primarily lactic acid, leading to a decrease in pH and preventing spoilage. Ensiling success depends on factors such as DM content, WSC levels, and pH, and differences in ensiling between legumes, grasses, and legume-grass mixes should be considered during forage management.
Summary

As pressure increases to provide efficient, nutritious feed to the world's livestock, producers must search for sustainable alternatives to current systems to address global food insecurity. Current literature suggests that mixed pasture systems offer a multifaceted solution for sustainable livestock production in comparison to monoculture stands. By integrating diverse plant species, these systems promote soil health, reduce the risk of diseases and pests, and enhance biodiversity. Moreover, they provide animals with a varied diet, resulting in improved livestock health and productivity. Incorporating alfalfa and grass mixtures holds great promise for enhancing livestock nutrition and forage quality. Alfalfa, with its high protein and mineral content, complements the fiber-rich grasses, further contributing to a nutritious diet for livestock. This approach not only improves animal performance but also promotes nitrogen fixation and reduces the reliance on synthetic fertilizers, thereby contributing to sustainable agriculture.

Reduced lignin alfalfa varieties have been shown to offer producers greater flexibility in harvesting while maintaining forage quality. By reducing the lignin content, forages become more digestible and nutritious for livestock. This innovation allows producers to extend the harvest window without sacrificing nutritional benefits, leading to increased yield and improved feed efficiency. Moreover, research efforts can focus on identifying and developing reduced lignin varieties that are well-adapted to different climates and agricultural systems. More research is needed to assess the environmental impact of reduced lignin alfalfa, particularly in terms of its effect on soil microbial communities, stand persistence, wildlife impact, carbon sequestration, and biodiversity in agricultural ecosystems. Understanding the ecological
implications of introducing reduced lignin alfalfa into the environment is crucial for sustainable agricultural practices.

Lastly, the utilization of proper ensiling techniques enables the preservation of forage with minimal nutrient loss, providing a reliable feed source during periods of scarcity. Ensiling also contributes to reducing the environmental impact of livestock production by minimizing feed wastage. Ongoing research is vital to optimize ensiling practices, develop additives that enhance forage fermentation, and ensure the production of high-quality silage consistently.

Further investigations are necessary to understand the interactions between reduced lignin alfalfa and other forage species commonly used in mixed pasture systems, particularly within the Southeastern United States. Studying the competitive dynamics, nutrient balance, and overall productivity of mixed pastures containing reduced lignin alfalfa will help optimize forage combinations and animal nutrition. In order to address these questions, this study was developed with the primary objectives in mind: (1) the evaluation of yield and nutritional value of a reduced lignin alfalfa cultivar in monoculture and in mix with novel endophyte-infected tall fescue grown in the Southeastern US in comparison to conventional alfalfa and (2) the effect of ensiling alfalfa, tall fescue, and diculture mixtures with and without inoculant at varying DM levels.
References


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CHAPTER TWO

YIELD AND NUTRITIVE QUALITY OF FRESH AND ENSILED REDUCED LIGNIN ALFALFA IN MONOCULTURE AND DICULTURE MIXTURES WITH NOVEL TALL FESCUE

ABSTRACT

The objective of this study was to evaluate the yield, fermentation, and nutritive value of fresh and ensiled reduced lignin alfalfa (RLA) (*Medicago sativa*) grown in monoculture and diculture mixtures with novel endophyte-infected tall fescue (F) (*Lolium arundinaceum*) as compared to a reference alfalfa cultivar (CA) in the Southeastern United States. The study was prepared as a randomized complete block design with four replicates per treatment. Treatments included monoculture RLA, CA and F plots as well as diculture RLA/F and CA/F plots seeded in a 50/50 mix. Plots were harvested on a 35-day interval starting in April 2021 and ending in September 2021 for a total of 5 harvests. Ensiled forage was arranged in a 3x2 factorial of treatments: forage type (RLA, CA, F, RLA/F, and CA/F), non-inoculated or inoculated with a lactic acid bacteria (LAB) inoculant, and ensiled at high (50%) or low (35%) dry matter (DM) content. Throughout the establishment year, RLA (13,034 kg DM/ha), RLA/F (12,338 kg DM/ha) and CA (12,142 kg DM/ha) had the highest dry matter yield, followed by CA/F (9,490 kg DM/ha), and F (5,545 kg DM/ha) (p<0.05).

In terms of chemical composition, RLA and CA exhibited higher average crude protein (CP), acid detergent lignin as a percentage of ash-free α-amylase treated neutral detergent fiber (ADL, %aNDFom), undigestible NDF as a percentage of ash-free α-amylase treated neutral
detergent fiber (uNDF, %aNDFom), in-vitro dry matter digestibility (IVDMD), and in-vitro true
dry matter digestibility (IVTDMD) content as compared to diculutre mixtures and monoculture
tall fescue (p<0.01). Monoculture legumes observed lower (p<0.01) aNDFom and potentially
digestible NDF as a percentage of ash-free α-amylase treated neutral detergent fiber (pdNDF,
%aNDFom), as compared to both their diculture counterparts and tall fescue monoculture. In
general, uNDF (% DM) and uNDF as a percentage of aNDFom increased from the first harvest
to subsequent harvests while in-vitro true dry matter digestibility (IVTDMD) and average
potentially digestible NDF (pdNDF as a percentage of aNDFom) decreased with subsequent
harvests. (p<0.05).

For silage trials, inoculated samples were higher in volatile fatty acid (VFA) content
(p<0.01), lactic acid concentration (p=0.02), and acetic acid concentration (p=0.01) while being
lower in pH and similar (p=0.92) in ammonia nitrogen (NH₃-N) content as compared to non-
inoculated samples . Low DM content (35% DM) was associated with higher total VFA content,
NH₃-N, and lactic acid concentration, while being lower in pH than high DM content (50% DM)
silages (p<0.01). Of the treatment effects observed, alfalfa-tall fescue mixtures had, on average,
12.6% higher total VFA content and 21.2% higher acetic acid concentration when compared to
monoculture alfalfa treatments. Tall fescue silage observed 33.0% lower ammonia nitrogen
(NH₃-N) (DM g/kg) compared to the average of all other treatments. No significant differences
in silage fermentation or quality existed between the reduced lignin alfalfa cultivar and reference
alfalfa cultivar. Inoculation and dry matter percentage had the most impact on the pH, total VFA,
NH₃-N, lactic acid, and acetic acid content in silage (p<0.05). Results from this study indicated
that reduced lignin alfalfa can be grown in monoculture or diculture mixture with novel
endophyte-infected tall fescue and produce similar or slightly more dry matter yield without
sacrificing nutritive value. This study also suggests that novel endophyte-infected tall fescue is a well-suited cool-season grass to pair with either reduced lignin alfalfa or conventional alfalfa in this geographic area. Additionally, while DM percentage, inoculation, and forage type had an effect on silage fermentation, all silages were within established acceptable limits for legume and grass silages, which provides flexibility in silage management to producers.
1. INTRODUCTION

Alfalfa, particularly in diculture mixtures with perennial grasses such as tall fescue, improves animal productivity, stand persistence, nutritive quality, and environmental sustainability when compared to monocultures of alfalfa or grasses alone. The importance of improved forage productivity, animal performance, and agricultural sustainability for producers cannot be understated. Identifying well-suited legume-grass mixtures that are adapted to a particular region should be viewed as a key measure in increasing agricultural sustainability (Tracy et al., 2018).

Previous studies have consistently demonstrated that mixed stands produce higher forage yields, higher nutritive quality, improved animal productivity, and exhibit greater tolerance to environmental stressors (Sleugh et al., 2000; Foster et al., 2014; Sturludóttir et al., 2014; Tracy et al., 2016; Bélanger et al., 2017; Tessema and Feleke, 2018; Aponte et al., 2019; Khatiwada et al., 2020). For instance, Sleugh et al. (2000) concluded that cumulative dry matter yield (DMY), crude protein (CP), and in-vitro dry matter digestibility (IVDMD) of diculture grass-legume mixtures were higher than grass monocultures. Additionally, CP and nutrient digestibility were higher, and NDF concentrations were lower for legume-grass mixes as compared to grass monocultures (Sturludóttir et al., 2014; Brink et al., 2015; Damiran et al., 2022). Research conducted by Sim (2022) concluded that grass-alfalfa yielded 100% greater DMY than grass monocultures when legumes were 12 to 44% of the stand mix. There is some uncertainly if grass-alfalfa mixtures outperform alfalfa monocultures in terms of yield, with Foster et al. (2014), Cherney et al. (2020), and McDonald et al. (2021) concluding that yields were similar among monoculture stands and grass-legumes stands, while Tracy et al. (2016) concluded that
mixtures yielded more than monocultures. Aponte et al. (2019) observed better seasonal forage
distribution of grass-alfalfa mixtures, which then provides producers with a well-distributed
source of nutritious feed throughout the season. This confirmed the claim by Sleugh et al. (2000)
that higher nutritive quality in binary mixes was accompanied by improved seasonal yield
distribution.

In addition to increased productivity, grass-legume mixtures offer other benefits due to
their interactions. Advantages include enhanced soil health, improved nutrient cycling, and
reduced weed invasion. Several studies illustrate that grass-legume mixed plots show increased
resistance to weed invasion as compared to monoculture legume stands (Deak et al., 2007;
Sturludóttir et al., 2014; Brink et al., 2015), even demonstrating a reduction of 5%-55% of weed
presence in a mixed pasture as compared to monoculture (Sanderson et al., 2012). Consequently,
a lower proportion of weed invasion in a pasture increased palatability and intake in ruminants
(Sturludóttir et al., 2014). Another key advantage of mixed pastures is the variation in root depth
between forage types, which enhances resource extraction from the soil. The combination of
legumes and C4 grasses had proportionally deeper root depth distribution when compared to
monocultures (Meuller et al., 2013). Furthermore, incorporating legumes in grass pastures is
particularly beneficial because of their ability to fix nitrogen, increasing both soil and plant
nitrogen content and reducing the need for fertilizer application pastures (Nyfeler et al., 2011;
Schipanski and Drinkwater, 2012; Sturludóttir et al., 2014). Nyfeler et al. 2011 stated that grass-
legume mixtures in 40-60% proportion of legumes result in an efficient transformation of N into
the stand biomass through a symbiotic relationship.

While complex forage mixtures have been suggested to stabilize production outputs
(Deak et al., 2010), the additive effect of using multiple species in increased complexity may not
produce proportionally increased productivity (Sanderson et al., 2016; Foster et al., 2014; Brink et al., 2015). The dominant species in a mixture generally have a greater impact on stand productivity than species richness (Deak et al., 2007), and species mixtures are dynamic in their composition over time (Bork et al., 2017). Foster et al. (2014) observed that complex or very complex alfalfa-grass mixtures did not increase dry matter yield when compared to simple alfalfa-grass mixtures. Therefore, the selection of 2 or 3 forages that are well-suited to the area and complement each other in mixture is often more influential for sustainability than overall complexity (Tracy et al., 2016). This is supported by Cherney et al. (2020) that states perennial grasses to be paired with alfalfa should be selected on a regional basis to produce the best results. Sleugh et al. (2000) and Tracy et al. (2016) stated that tall fescue complements alfalfa in stands and would improve its ability to withstand environmental stressors such as temperature extremes and droughts.

Alfalfa has long been recognized as the "Queen of Forages" and is highly sought after by producers due to its high nutrient content, excellent digestibility, and its ability to fix nitrogen. However, because of its superior digestibility and high protein content, it is also one of the leading bloat-causing legumes grown for livestock consumption (Khatiwada et al., 2020). Bloat, particularly frothy bloat, occurs when rapid fermentation of forage with low fiber and high protein causes gas to be released in the rumen and become trapped in the rumen contents, preventing eructation. This occurs most often when ruminants ingest vegetative legumes after a frost, when moving cattle into a new pasture, or in early spring and late summer (Viera et al., 2010; Sottie et al., 2014). Grass-legume mixtures can mitigate the bloat potential and make it a more attractive grazing option for producers (Tracy et al., 2016).
Additionally, alfalfa's digestibility is inhibited by alfalfa's natural lignin content and its tendency to increase as the plant matures. Lignin is a complex cell wall polymer that plays a critical role in providing strength to the plant, allowing it to stay upright (Guo et al., 2001a; Moore and Jung, 2001). As a plant matures, rapid lignification thickens secondary cell walls and results in a lesser degree of digestibility for potentially digestive components such as cellulose and hemicellulose (Lei et al., 2017). Lignin is considered an anti-quality factor in forages and is directly correlated to a decrease in fiber digestibility in ruminants (Jung and Vogel, 1986; Jung et al., 1997; Moore and Jung, 2001; Getachew et al., 2018). Producers are then faced with the challenge of timing harvests to combat lignification while still maintaining optimum nutritive quality.

Lignin down-regulated alfalfa varieties have been available on the commercial market since 2015 as a result of a scientific collaboration named the Consortium for Alfalfa Improvement (Barros et al., 2019). Downregulation of lignin involves the targeting of enzymes responsible for lignin synthesis, such as caffeic acid 3-O-methyltransferase (COMT) and caffeoyl CoA 3-O-methytransferase (CCOMT) (Guo et al., 2001b; Barros et al., 2019). The CCOMT downregulated variety is what is currently being marketed under the tradename HarvXtra™ (Arnold et al., 2019). Reports of ADL reduction ranged from 6-24% in total herbage ADL when plots were harvested at varying intervals (Grev et al., 2017; Getachew et al., 2018; Barros et al., 2019; Grev et al., 2020; Damiran et al., 2022).

Because of their slower rate of lignification, lignin-downregulated cultivars exhibit higher levels of NDFD at similar maturity when compared to conventional alfalfa cultivars without sacrificing yield (Grev et al., 2017; Getachew et al., 2018; Arnold et al., 2019; Barros et al., 2019; Damiran et al., 2022). Cherney et al. (2020) also pointed out that the impact of RLA on
the NDFD of mixed forage plots is heavily dependent on the proportion of legumes to grass in the stand, with grasses having considerably higher NDFD than legumes. Increasing digestible proportions of the cell wall is vital to animal productivity, as Oba and Allen (1999) found that a 1 unit increase in NDFD was associated with a 0.17kg increase in dry matter intake and a 0.25kg increase in 4% fat-corrected milk. Furthermore, the U.S Dairy and Forage Research Center estimates that a 10% increase in NDFD would result in an annual $350 million increase in domestic milk and beef production due to increased dry matter intake and reduced waste (Oba and Allen, 1999; Reddy et al., 2005; Hatfield et al., 2008). These findings represent a significant advantage to producers as they allow for a flexible, extended optimal harvest window while increasing the forage yield without having to sacrifice nutritive value, ultimately increasing the profitability for producers (Undersander et al., 2009).

While alfalfa and alfalfa-grass mixes are excellent sources of nutrition during the growing season, year-round access to high quality feedstuff is a high priority for farmers. Still, little to no research has been conducted on lignin downregulated alfalfa's performance when ensiled. Ensiling crops is a way that producers can improve the sustainability of their operations by utilizing surplus feed after a harvest to feed during times of low production, such as the winter (Dunière et al., 2013). Ensiling is the anaerobic fermentative process of converting water-soluble carbohydrates into organic acids, particularly lactic acid bacteria (LAB), while rapidly decreasing the pH in order to inhibit the growth of undesirable organisms (Dunière et al., 2013; Wang et al., 2019; Huyen et al., 2020). Although it is one of the most ensiled forages next to corn and grasses, alfalfa is typically more difficult to ensile than others due to its high buffering capacity and its relatively low WSC content (Nkosi et al., 2016; Borreani et al., 2018). To a lesser extent than lactic acid, other products of fermentation can include acetic acid, propionic
acids, and butyric acid. The goal of acetic acid production is typically below 3% in silage (Ward and de Ondarza, 2008), and it is known to contribute to pH reduction and also provides antifungal properties that protect against spoilage from yeasts and fungi. Nonetheless, excessive levels of acetic acid (>5%) have been associated with decreased palatability and, therefore, reduced intake (Ward and de Ondarza, 2008). Levels of propionic acid and butyric acid should ideally be below 0.5% and 0.1%, respectively. The presence of these organic acids may indicate poor fermentation and are often associated with clostridial growth, protein degradation, and poor palatability (Kung and Ranjit, 2001; Ward and de Ondarza, 2008; Muck, 2010).

The success of the ensiling process is impacted by various elements: the dry matter (DM) concentration, levels of water-soluble carbohydrates (WSC), and the pH of the forage. For optimal ensiling, it's preferable to have forage with a DM content of approximately 30-35%, as this encourages adequate fermentation. Silage containing high DM content (>50%) exhibits signs of limited fermentation and could potentially foster the growth of undesirable bacteria (Kung et al., 1984; Sheperd et al., 1995; Ward and de Ondarza, 2008), while low DM content is associated with clostridial growth (Ward and de Ondarza, 2008). To achieve the desired DM content, the forage is first wilted before preservation, aiming to lower water activity and elevate WSC concentration (Borreani et al., 2018). Adequate WSC levels play a pivotal role, serving as substrates for LAB to produce lactic acid and facilitate appropriate ensilage fermentation (Stokes, 1992; Weinberg and Muck, 1996). Inadequate WSC content might curtail the availability of fermentable substances and have adverse effects on the ensiling process (Sheperd et al., 1995). Maintaining a low pH (<4.5) is crucial for inhibiting the growth of spoilage microorganisms, ensuring the stability and proper preservation of silage (Weinberg and Muck, 1996; Ward and de Ondarza, 2008).
The introduction of homofermentative and/or heterofermentative LAB into silage has been shown to mitigate fermentation challenges in forages with low WSC content or high DM content. Findings by Sheperd et al. (1995) demonstrated that inoculation with LAB can cause a quick drop in pH in forages that would normally be restricted by high DM content. Inoculation of forages with heterofermentative and/or homofermentative LAB not only inhibits protein breakdown and clostridial growth but also boosts digestibility and aerobic stability (Kung et al., 1984; Weinberg and Muck, 1996; Kung and Ranjit, 2001; Dunière et al., 2013; Oliveira et al., 2017; Huyen et al., 2020). Trials by Weinberg and Muck (1996) and Muck (1993) illustrated that homofermentative LAB inoculation has positive impacts on milk production, average daily gain, and feed efficiency. Similarly, Oliveira et al. (2017) illustrated that trends exist for increased dry matter intake, leading to increased milk protein and milk fat concentration for inoculated silages.

Although research has been extensively conducted regarding grass-legume mixtures, greater attention is being paid to tailoring mixtures to specific regions and implementing new technologies such as down-regulated lignin alfalfa to improve forage sustainability and productivity. Thus, the objectives of this study were to (1) evaluate the effect of reduced lignin alfalfa in monoculture and diculture mixture with tall fescue on the forage yield, nutritive value, and fiber digestibility, and (2) to investigate the quality of reduced lignin silage in either monoculture or diculture mixtures with tall fescue at high dry matter content, low dry matter content and with or without the addition of an inoculant, particularly in context to forages grown in the Southeastern United States. The hypotheses were that forage performance and nutritive value would be similar among alfalfa monoculture treatments or slightly higher in reduced lignin alfalfa; grasses would exhibit lower forage yield and nutritive value than legume monocultures.
and alfalfa-fescue mixtures; binary mixtures would produce the highest yield; and nutritional values of mixtures would be somewhere between alfalfa and tall fescue results. We also hypothesized that silage inoculated with LAB would exhibit greater fermentation and lower DM silage would perform better than high DM forage but may be mitigated by inoculation. The possible effect of treatment is unknown due to a lack of previous supporting literature.

2. MATERIALS AND METHODS

2.1 Experimental Sites and Climate Data

This study was carried out at the Simpson Research Farm, situated near Pendleton, South Carolina (34°62′10.8″ N 82°73′31.5″W) over a period spanning from March to September 2021. The soil characteristics at the research site were described as Appling sandy loam with 2 to 6 percent slopes (ApB) and Cecil sandy loam (CdB), classified with a land capability rating of 2e, demonstrating moderate restrictions that may limit forage choice and may be susceptible to erosion. (source: web soil survey; www.nrcs.usda.gov). Historical weather data from 1991 to 2021 were collected from a weather station in Clemson, SC, using the National Centers for Environmental Information of the National Oceanic and Atmospheric Administration (NOAA, US Department of Commerce, www.noaa.gov). Table 1 presents the average monthly temperature and precipitation values derived from the collected weather data.

2.2 Experimental Design and Treatments

The study was designed as a randomized complete block design with 5 treatments and plots cut as a repeated measure. During the early spring of 2021, the field was divided into 4 blocks and within each of the blocks, one plot (1.5-m wide and 6.1-m long) was randomly
assigned to one of 5 forage treatments (Figure 1). AmeriStand 455TQ RR alfalfa (CA) (fall dormancy = 4.4, winter hardiness = 2.0), BarOptima® PLUS E34® novel tall fescue (F) and AmeriStand 480 HVXRR (HarvXtra®) reduced lignin alfalfa (RLA) (fall dormancy = 4, winter hardiness = 2.2) were planted in monocultures or in diculture mixtures (CA/F and RLA/F) that were seeded in a 50/50 mix. Plots were planted using a 7-row plot drill equipped with an Almaco cone. Fertilizer was applied to each plot before planting (57.2 kg N/ha, 24.7 kg P2O5/ha, and 48.2 kg K2O/ha) according to recommendations after soil analysis, and after each harvest (23 kg N/ha). Plots were harvested at a 35-day harvest interval for a total of 5 harvests (April 21, May 26, June 30, August 4, September 10). An electric fence was installed around the parameter of the plots as a wildlife deterrent.

2.3 Sample Collection and Analysis

The forage biomass of each plot was harvested 5 times using a Carter plot forage harvester (Carter Manufacturing Co., Brookston, IN, USA). After weighing the harvested biomass in each plot, samples were collected in plastic gallon bags, immediately placed in a cooler with ice, and transferred to the laboratory for processing. Samples were dried at 55 °C in a forced-air oven for 48 h. The resulting dry matter (DM) concentration was used to determine DM yield (kg/ha). Dried samples were ground to pass through a 1-mm screen of a Wiley mill (Arthur H. Thomas, Philadelphia, PA, USA). Ground samples were dried at 105 °C for 16 h to determine analytical DM (Undersander et al., 1993). Ash concentration of each sample was determined after combusting samples in a furnace for 3h at 600 °C (Method 942.05, AOAC) and corresponding samples were ash corrected. For each sample, a subsample was separated and submitted to Cumberland Valley Analytical Services (Waynesboro, PA, USA) to determine the concentrations of crude protein (CP) and water-soluble carbohydrates (WSC) as described by Hall (2009).
Neutral detergent fiber (NDF) and ADF concentrations were determined using an Ankom200 Fiber Analyzer (Ankom Technology, Macedon, NY) and corrected for ash concentration. Blank samples were included in each procedure to correct for loss. Following procedures described by Van Soest et al. (1991), sodium sulfite and heat resistant α-amylase (Sigma no. A3306: Sigma Chemical Co., St. Lou-is, MO, USA) were included for the NDF analysis. NDF and ADF were analyzed using Ankom Technology Method 6 and Method 12, respectively. After determining the ADF, the fiber residue was incubated for 3h in 72% sulfuric acid within 4 L jars that were placed in a Daisy II Incubator (Ankom Technology) to determine ADL concentration.

The care and handling of animals used for collecting rumen contents and in situ incubations was conducted as outlined in the guidelines of the Clemson University Committee on Animal Use (AUP2022-0464). In vitro DM digestibility (IVDMD), in vitro true DM digestibility (IVTDMD), and in vitro NDF digestibility (IVNDFD) were determined using a Daisy II rotating jar in vitro incubator (Ankom Technology). Media and reducing agents were prepared using procedures described by Goering and Van Soest (1970). Samples were incubated for 30 h following the procedures described by Ferreira and Mertens (2005). The composite inoculum was prepared with rumen fluid and solids collected from two rumen-fistulated lactating dairy cows that were fed a diet containing 31.5% corn silage, 3.6% millet baleage, 3.3% bermudagrass hay and 61.6% concentrate mix (DM basis). For in situ forage analyses, 0.25 g of sample was weighed into F57 Ankom bags (Ankom Technologies) and incubated in the rumen of 2 rumen-fistulated and multiparous cows (1 Jersey and 1 Holstein) for 240 h. The cows were fed the same diet described above. After the 240-h incubation, bags were removed, washed, weighed, and subjected to NDF analysis as described previously. The harvested yield of
potentially digestible NDF (pdNDF, kg/ha) was calculated by multiplying the concentration of the pdNDF by the DM yield of the corresponding plot and harvest.

Forage for silage use was pooled by treatment and had been in frozen storage (-18°C) prior to processing. The following treatments were applied to each of the 20 plots: 50% (high) DM with no inoculant added, 35% (low) DM with no inoculant added, 50% DM with inoculant added, and 35% DM with inoculant added for a total of 80 mini silos. Samples were dried to the appropriate DM content in a forced air oven at 50°C. When the target DM% was reached, 125g of sample was weighted and separated into the respective treatment category. Inoculated samples were treated with a multi-strain homofermentative\textsuperscript{a}/facultative heterofermentative\textsuperscript{b} inoculant (SiloSolve\textsuperscript{®} MC) that theoretically contained 68.1 \times 10^{9} \text{CFU/g} of \textit{Lactobacillus plantarum}\textsuperscript{b}, \textit{Lactococcus lactis}\textsuperscript{a}, \textit{Enterococcus faecium}\textsuperscript{a} and was applied at a rate of 15 \times 10^{4} \text{CFU/g} (Chr. Hansen, Inc, Milwaukee, WI, USA). The inoculant was diluted with deionized (DI) water and sprayed onto the inoculated treatments per the manufacturer’s instructions. Forage was ensiled into clear plastic bags, vacuumed-sealed and placed in a dark, room-temperature cabinet for 60 days. The pH of each mini silo was measured immediately after blending 10g of silage with 90mL of DI for 1 minute. Silos were then vacuum-sealed and sent to Cumberland Valley Analytical Services for determination of total volatile fatty acid (VFA) concentration, lactic acid, acetic acid, propionic acid, butyric acid, DM recovery, ammonia nitrogen (NH\textsubscript{3}-N), and pH.

2.4 \textit{Statistical Analysis}

The fit model procedure of JMP Pro v.16.0\textsuperscript{®} (SAS Institute Inc. ©) was used for statistical analysis. The statistical model included the random effect of block, the fixed effect of treatment, the fixed effect of harvest, the interaction of treatment and harvest, and the interaction of block and treatment. Silage statistical data models include the random effect of block, the fixed effect
inoculation, the fixed effect of forage type, the fixed effect of DM level, and the interaction of treatments. All datasets were analyzed in JMP using ANOVA, followed by Tukey's HSD test to consider significance among data. Student's t-test was performed when Tukey's HSD was not feasible, such as a comparison between high and low dry matter content. Figures were obtained using Excel® (Office 365©). Statistical significances and tendencies to differ were declared at p<0.05 and p≤0.10, respectively.

3. RESULTS

3.1 Weather

Rainfall in the 2021 growing season was below the 30-year average for April, May, August, and September, and June/July represented particularly rainy months (Table 1). The average temperatures in 2021 were not significantly higher or lower than the 30-year average.

3.2 Yield and Chemical Composition

Throughout the growing season, RLA (13,034 kg DM per ha) and RLA/F (12,338 kg DM/ha) had statistically higher biomass than CA/F (9,490 kg DM/ha) and F (5,545 kg DM/ha) (Figure 2) (p<0.01). Biomass yield for CA (12,142 kg DM/ha) was significantly higher (p<0.01) than F, but had similar yield to RLA, RLA/F, and CA/F. We observed a significant effect (p<0.01) for average harvest biomass yield, with the most being obtained in harvest 2 (2,514 kg DM/ha), followed by harvest 4 (2,153 kg DM/ha), harvest 1 (2,304 kg DM/ha), harvest 3 (1,796 kg DM/ha), and finally harvest 5 (1,743 kg DM/ha).

The F plots (30.9%) had the highest average DM% (p<0.01) as compared to all other treatments, followed by CA/F (28.4%) and RLA/F (28.1%) (Table 2). The CA and RLA plots
exhibited the lowest DM content (p<0.01) at 26.1% and 26.2%, respectively. There was an observed harvest effect (p<0.01) with harvest 5 exhibiting the highest DM (29.4%), followed by harvest 3 (28.9%), harvest 2 (28.6%), harvest 4 (27.0%), and finally harvest 1 with the lowest DM% at 25.9%. Harvests 5, 3, and 2 were significantly higher (p<0.01) than harvests 1 and 4. There was an observed forage treatment by harvest interaction (p<0.01) reflecting a greater DM difference in F between harvests 1 and 5 (6.11%) than all other treatments. The most pronounced differences in each treatment by harvest are between F harvests 1 and 3 (p<0.01), RLA harvests 1 and 2 (p<0.01), and CA/F harvests 1 and 5 (p=0.03).

Ash did not vary between treatments (p=0.99), but the content did vary between harvests (p<0.01). Harvest 1 (11.8%) ash content was significantly higher than all other harvests and harvest 5 (9.3%) was significantly lower than all others (p<0.01) (Table 2).

There was an observed treatment effect for average crude protein content (p<0.01), with CA (22.9%) and RLA (22.6%) being significantly higher (p<0.05) than all other treatments, RLA/F (20.8%) being slightly higher (p=0.06) than to CA/F (19.0%), while F (15.0%) was significantly lower (p<0.01) than all treatments (Table 2). There was a slight harvest effect regarding CP, where harvest 2 (19.0%) was significantly lower (p<0.05) than all other harvests (Figure 6). There was no difference between CA and RLA CP percentages in any harvest.

Treatment, harvest, and treatment by harvest interaction effects were all significant (p<0.01) for aNDFom. Average aNDFom for CA (33.5%) and RLA (33.2%) were lower (p<0.01) than all other treatments (Table 2). The RLA/F (39.3%) and CA/F (42.3%) plots had similar aNDFom content (p=0.08), while F (52.4%) had the highest (p<0.01) aNDFom. Harvest 4 (42.9%) had higher aNDFom (p<0.01) than harvest 1, 5, and 3 and similar content to harvest 2 (41.4%) (p=0.2). Harvest 1 (37.2%) had lower (p<0.01) aNDFom content than harvests 4, 2, and
3, but similar (p=0.1) content to harvest 5 (38.2%). Differences among harvests by treatment exist between CA/F harvests 4 and 5 (p<0.01), F harvests 1 and 4 (p<0.01), and a tendency to differ exists between RLA harvests 3 and 5 (p=0.1) (Figure 4). There was no difference between CA and RLA within any harvest. CA and RLA both exhibited lower aNDFom than either mixed plot in harvests 3 (p<0.01).

The only significant treatment effect observed for ADFom was that F (30.5%) was found to be higher (p<0.01) than all other treatments (Table 2). Harvest (p<0.01) seemed to play a larger role in differences in ADFom content. In general, ADFom content increased with subsequent harvest, with the lowest value observed in harvest 1 (26.2%) and the highest values observed in harvest 4 and 5 (30.7% and 29.9%, respectively). Interaction among treatment by harvest effects is most pronounced between F harvests 1 and 5 (p<0.01), RLA harvests 1 and 5 (p<0.01), and CA/F harvests 1 and 4 (p<0.01). No significant treatment interactions existed for CA and RLA within any harvest (p=.99). Monoculture F plots were significantly higher in ADFom content than mixed plots in harvests 3, 4, and 5.

Both ADL as a percentage of DM and ADL as a percentage of aNDFom follow the same general trend with CA and RLA containing the most lignin, and RLA/F and CA/F containing moderate levels, and F containing the least (Table 2). Harvests 4 (8.3%) and 5 (8.0%) had higher ADL as a percentage of DM (p<0.01) than harvests 1 (6.6%), 2 (7.0%), and 3 (7.1%) (Figure 5). Treatments CA (8.5%) and RLA (8.2%) had higher (p<0.05) ADL, %DM than RLA/F (6.7%) and F (6.3%), and similar content to CA/F (7.3%) (p=0.9). The ADL as a percentage of aNDFom was higher (p<0.05) in harvest 5 (21.7%) than in harvests 3, 1, and 2 (18.8%, 18.7%, and 17.3%), respectively. Harvest effects were well defined for ADL as a percentage of aNDFom, with CA (25.3%) and RLA (25.0%) exhibiting the highest means, CA/F (17.7%) and RLA/F
(17.2%) displaying similarly moderate ADL content, and F (11.8%) showing a lower mean than all other treatments \((p<0.01)\). There were no differences in ADL content between RLA and CA within any harvest.

Harvest and treatment by harvest interactions were significant \((p<0.01)\) for WSC content (Table 2). Harvest effect, rather than forage type, had the most impact on WSC, which generally decreased with each subsequent harvest. Harvest 1 (11.0%) had the highest WSC content \((p<0.01)\), followed by harvest 5 (8.8%), harvest 2 (8.3%), harvest 3 (8.1%) and finally harvest 4 (7.2%). Treatment by harvest interactions interaction effects exist between F harvests 1 and 4 \((p<0.01)\), CA/F harvest 1 and 4 \((p<0.01)\), and RLA/F harvest 5 and 2 \((p=0.01)\). Monoculture F WSC content was higher than RLA/F in harvests but lower in 5. The same trend can be seen with F and RLA in that WSC content was higher in F in harvests 1 and 2, but lower in harvests 4 and 5. The WSC content in RLA (9.13%) tended to be lower than CA (8.23%) in harvest 5 \((p=0.09)\).

### 3.3 Apparent Digestibility of Nutrients

Treatment, harvest, and treatment by harvest interaction effects were all significant, although treatment was lesser so than other factors for undigestible neutral detergent fiber as a percentage of dry matter \((\text{uNDF}_{240}, \%\text{DM})\) \((p=0.01)\) (Table 3). Tall fescue (13.4%) was higher in uNDF as a percentage of DM than RLA \((p=0.01)\) and RLA/F \((p=0.02)\), and was similar to CA \((p=0.4)\) and CA/F \((p=0.4)\). Harvest 4 demonstrated higher uNDF than harvest 1, 2, and 3 \((p<0.01)\) and tended to be higher than harvest 5 \((p=0.05)\). In general, uNDF as a percentage of DM increased with subsequent harvests with harvest 1 being the lowest (7.4%), followed by harvest 2 (10.1%), harvest 3 (13.2%), harvest 5 (14.5%) and harvest 4 (16.1%) (Figure 6). In the interaction between treatment and harvest, F, CA/F, and RLA/F differed the greatest between harvest 1 and harvest 4 \((p<0.01)\). In harvests 1 and 2, RLA tended to be higher than F, while in
later harvests (4 and 5), F tended to be higher than RLA. Monoculture CA (12.78%) exhibited significantly higher uNDF$_{240}$, % DM than RLA (9.99%) in harvest 2, and it tended to be higher in harvest 4 (p=0.07).

Treatment was significant for undigestible NDF as a percentage of NDF (uNDF$_{240}$, %aNDFom), with CA (37.3%) and RLA (34.4%) being higher than both mixtures (CA/F = 29.5%, RLA/F = 29.5%), and with F (24.9%) being lower than all other treatments (p<0.01) (Table 3). uNDF, %NDF increased with each subsequent harvest, with harvests 4 and 5 having similar (p=1.0) uNDF, %NDF content (37.7% and 37.8% respectively) (Figure 6). The greatest treatment by harvest interactions exists between CA/F harvests 4 and 1 (p<0.01), F harvests 4 and 1 (p<0.01), and RLA/F harvest 5 and 1 (p<0.01). Monoculture CA was significantly higher (p=0.01) than RLA in uNDF$_{240}$, % aNDFom content in harvest 4.

Treatment effects of potentially digestible NDF as a percentage of NDF (pdNDF as a percentage aNDFom) were inverse of uNDF as a percentage NDF in that F (75.1%) was higher (p=0.01) than RLA/F (70.5%) and CA/F (70.5%), and much higher (p<0.01) than RLA (65.6%) and CA (62.7%) (Table 3). The pdNDF as a percentage aNDFom decreased with harvest, with harvest 1 being the lowest (79.01%) and observing a decrease with harvest 2 (74.40%), harvest 3 (66.57%), harvest 4 (62.30%), and harvest 5 (62.18%) (p<0.01) (Figure 6). Harvest 4 and 5 did not differ (p=1.0). RLA/F (3,447 kg DM/ha) produced significantly higher (p<0.01) cumulative pdNDF (kg DM/ha) as compared to F (2,221 kg DM/ha) (Table 3). The greatest treatment by harvest interactions can be observed between CA/F harvests 1 and 5, F harvests 1 and 4, and RLA/F harvests 1 and 5 (p<0.01). The pdNDF content for F plots was significantly higher than CA, RLA, and RLA/F in harvests 1 and 2. The RLF mixed plots were significantly higher than
CA in harvests 1 and 2 as well. There was a significant difference in RLA and CA observed in harvest 4, with RLA (65.50%) exhibiting a higher pdNDF than CA (57.21%).

There is no significant treatment effect (p=0.83) for in-vitro neutral detergent fiber digestibility (IVNDFD) (Table 3), although there is a significant effect for harvest and the treatment by harvest interaction effect (p<0.01) (Figure 7). Harvest 1 was significantly higher than all other harvests (p<0.01). IVNDFD increased as harvests increased, with harvest 1 being the highest (67.7%), followed by harvest 2 (58.8%), harvest 3 (52.7%), harvest 5 (50.3%) and harvest 4 (48.3%). The most significant treatment by harvest differences were seen in F harvest 1 and 4, CA/F harvest 1 and 4, RLA/F harvest 1 and 4, and CA harvest 1 and 3. RLA was the most consistent across harvests, only varying an average .69%. In early harvests (1 and 2), F samples were higher in IVNDFD than RLA, CA, and RLA/F. However, in later harvests, this trend was switched, in that F was significantly lower in harvest 4 than RLA, CA and RLA/F, and it tended to be lower in harvest 5. Monoculture RLA was significantly lower than CA in IVNDFD in harvest 1 (p=0.04) and tended to be higher than CA in harvest 4 (p=0.06).

The highest IVDMD percentages by treatment were RLA (77.1%), CA (77.0%), and RLA/F (75.7%), which were higher than CA/F (73.5%) and F (68.8%) (p<0.01) (Table 3). Harvest 1 exhibited the highest (p<0.01) IVDMD at 82.0% followed by harvest 2 (75.7%), harvest 5 (72.60%), harvest 3 (72.26%) and harvest 4 (69.61%) (Figure 7). Harvest 4 was lower than all other harvests (p<0.01). Treatment by harvest interaction effects were most pronounced between harvests 1 and 4 for F, CA/F, and RLA/F.

IVTDMD was highest (p<0.01) in RLA (84.8%), CA (84.8%), and RLA/F (82.7%), followed by CA/F (80.6%), and finally F (76.4) (Table 3). F was significantly lower than all treatments (p<0.01). IVTDMD generally increased with time and subsequent harvest. Harvest 1
(88.1%) was higher than all other harvests (p<0.01), followed by harvest 2 (83.2%), harvest 3 (80.8%), harvest 5 (79.9%) and harvest 4 (77.4%) (Figure 7). Harvests 3 and 5 were similar in IVTDMD (p=0.6). Treatment by harvest interactions were most pronounced between harvest 1 and 4 for F, CA/F and RLA/F.

3.4 Silage

One sample was excluded from analysis (plot 3, RLA, high DM, inoculated) due to an improper seal on the mini silo causing clear clostridial growth (pH = 5.42, visible mold growth, and sour smell).

Silage pH was most affected by DM content, inoculation, and the inoculation by DM interaction effect (Table 4). The pH was significantly higher in non-inoculated samples (4.60, rather than the inoculated samples (4.37). There were no significant differences in pH by forage type (p=0.16). The higher DM samples observed a significantly higher pH (4.57) than lower DM samples (4.41). While the overall effect was not significant, there was a tendency observed in the inoculation by forage type, with the non-inoculated RLA/F sample observing the highest pH (4.66) and the inoculated F samples observing the lowest (4.25). Non-inoculated samples of both high and low DM observed significantly higher pH than all other samples.

Total VFA content observed a significant inoculation, forage type, DM level, and inoculation by DM level effect with a tendency to differ by inoculation and forage type interaction. Inoculated samples (9.05%) had higher total VFA content as compared to non-inoculated samples (8.05%) (p<0.01). Total VFA content was higher in RLA/F than in CA or RLA (p=0.02), while there was a tendency for CA/F to be higher than CA (p=0.08). Low DM level silage (9.41%) exhibited higher levels of total VFA concentration as compared to high DM silage (7.69%) (p<0.01). While the inoculation by forage type interaction was not significant
(p=0.08), the inoculated RLA/F samples were higher (p<0.01) than all other non-inoculated monoculture samples. Non-inoculated, high DM silage (6.93%) had significantly less total VFA content than all other samples (p<0.01). Inoculated, low DM silage (9.66%) also had higher content than inoculated, high DM silage (8.44%) (p<0.01).

The forage type and DM level effects were significant in ammonia nitrogen concentration. There were no differences observed between inoculated and non-inoculated samples (p=0.92). Fescue silage was significantly lower in ammonia nitrogen than all other treatments (p<0.01). Low DM silage (1.86%) exhibited higher concentrations than high DM silage (1.65%) (p<0.01). While no significant overall effect was observed (p=0.63), non-inoculated F (1.18%) samples were lower (p<0.01) in ammonia nitrogen than all other samples except inoculated F (1.35%) (p=0.98).

Lactic acid showed significant effect by inoculation, and DM level. No significant effect of forage type was observed (p=0.28). Inoculated samples (7.07%) had higher lactic acid concentration as compared to non-inoculated samples (6.44%) (p=0.02). Low DM silage (7.55%) observed higher lactic acids than high DM samples (5.96%) (p<0.01).

Acetic acid concentration was significantly affected by inoculation (p=0.01) and forage type effects (p<0.01), with a tendency to be affected by the interaction effect of forage type by inoculation (p=0.07). Inoculated samples (1.98%) were higher in acetic acid concentration than non-inoculated samples (1.61%) (p=0.01). Acetic acid concentration was significantly higher in RLA/F (2.25%) than in F (1.41%) and CA (1.56%) silage (p<0.05). Similarly, CA/F (2.08%) was higher than F as well (p=0.04).

Propionic acid was not found in all samples, therefore making statistical comparison by mean % impossible. Instead, the rate of instances was analyzed quantitatively for each treatment. Non-
inoculated samples observed 25 (74%) samples that contained propionic acid, compared to 9 (26%) inoculated samples containing propionic acid. High DM silages (68%) contained 23 instances of propionic acid detection as compared to 11 low DM (32%) instances. The CA sample observed the least number of samples with propionic acid (2), followed by RLA (5), RLA/F (8), F (9), and finally 10 CA/F silages containing propionic acid. No low DM level inoculated samples were observed to contain propionic acid, while high DM inoculated, low DM non-inoculated and high DM inoculated silages were observed in 9, 11, and 14 instances, respectively. Propionic acid in CA silage was only observed in the low DM, non-inoculated sample (2).

4. DISCUSSION

4.1 Forage yield and Chemical Composition

Under the conditions of this study, reduced lignin (RLA) monoculture outperformed conventional alfalfa (CA) in both monoculture (6.8% higher) and diculture (27.2% higher) in terms of dry matter yield (DMY). Cumulative forage DM yield was not significantly negatively affected (5.3% decrease) for RLA when grown in a diculture mixture with tall fescue as compared to RLA monoculture. Conventional alfalfa DM yield, however, was 21.8% less grown in mixture with tall fescue as compared to monoculture. Findings correlate with data published by Foster et al. (2014), Cherney et al. (2020), and Sim (2022), which all demonstrated that grass-alfalfa mixtures yielded almost as much as monoculture alfalfa plots, but not more than. The current findings were in contrast with Tessema and Feleke (2018), and their conclusion that grass-legume mixtures exhibited higher DMY than pure stands grasses or legumes as well as
McDonald et al. (2021) that concluded there was no difference in DMY between mixes and alfalfa monocultures. Cumulative DMY for harvest 2 was 8.4%, 14.4%, 28.6% and 30.7% higher (p<0.05) than harvest 1, 4, 3, and 5, respectively. This finding aligns with the findings of Brink et al. 2010, concluding that DM production was greatest in early summer and late summer for somewhat arid regions. Therefore, the results of current and previous studies provide further evidence that RLA can achieve comparable or slightly higher forage biomass yield to its conventional alfalfa counterparts, both in monoculture and in mixtures with tall fescue.

Conventional alfalfa and RLA displayed the highest average crude protein (CP) content, with values of 22.9% and 22.6%, respectively (p<0.01). This aligns with the research findings of Foster et al. (2014), which suggested that alfalfa varieties, including reduced lignin types, can exhibit higher protein content than grass monoculture content. Similarly, the CP content in mixtures and tall fescue followed a decreasing trend: RLA/F (20.8%) and CA/F (19.0%), and tall fescue (F) (15.0%) (p<0.01). This pattern is consistent with multiple studies that indicate grass-legume mixtures may result in lower protein content compared to pure legume stands due to dilution effects but higher than grass monocultures (Sleugh et al., 2000; Sturludóttir et al., 2014; Tessema and Feleke, 2018; Damiran et al., 2022). Reduced lignin alfalfa was not higher (p=0.99) in CP content as compared to conventional alfalfa in any harvest. This is supported by previous studies that found RLA to have similar CP concentrations as compared to reference alfalfa cultivars (Getachew et al., 2011; Grev et al., 2017). In a recent study, higher CP intake was associated with a positive effect on average daily gain and final body weight in beef cattle without resulting in excess nitrogen loss through excretion (Xia et al., 2018).

The presence of weeds in plots should be considered in terms of total yield and nutritional quality. Monoculture stands have been shown to contain higher proportions of weeds
in comparison to mixed stands (Deak et al., 2007; Sanderson et al., 2012; Sturludóttir et al., 2014). Although glyphosate-resistant cultivars were utilized in this study, future research should be done to address this possibility, especially as the stand ages (Sturludóttir et al., 2014).

4.2 Fiber Concentration

In line with the observations of Brink et al. (2015), fiber concentration was higher in the grass monoculture than the legumes or the forage mixes. Conventional alfalfa and RLA had significantly lower average ash-free $\alpha$-amylase treated neutral detergent fiber (aNDFom) concentrations (33.5% and 33.2% respectively) compared to their respective alfalfa-tall fescue mixtures, resulting in a 20.7% reduction in aNDFom between CA and CA/F and a 15.6% reduction between RLA and RLA/F. Grasses usually contain higher fiber concentrations than legumes (Damiran et al., 2022). Therefore, the grass component of the alfalfa-fescue mixtures is the likely cause of increased aNDFom concentration when compared to their monoculture counterparts ($p<0.01$). The similarity in aNDFom concentration ($p=0.99$) between RLA and CA is supported by multiple studies (Guo et al., 2001b; Getachew et al., 2011; Grev et al., 2017; Grev et al., 2020) in which aNDFom concentration was similar or lightly reduced for RLA monoculture as compared to reference cultivars. The harvest effect for aNDFom was significant ($p<0.01$), with harvest 4 displaying the highest mean NDF (42.9%), while harvest 1 had the lowest mean (37.2%). Grev et al. (2017) states that the increasing aNDFom concentration by harvest is likely due to plant maturity. Ultimately, forages containing higher aNDFom are not as desirable in the eyes of producers because increased aNDFom is associated with lower dry matter intake and therefore, reduced average daily gains in beef production and reduced milk production in dairy cows (Arelovich et al., 2008; Undersander et al., 2009).
All harvest, treatment, and treatment by harvest interaction effects were significant for ADL concentration (p<0.01). Conventional alfalfa and RLA exhibited higher acid detergent lignin (ADL) as percentage NDF (p<0.01) than all other treatments, while RLA/F (17.2%) and CA/F (17.7%) had moderate levels, and F (11.8%) had the lowest lignin content as a percentage of NDF (p<0.01). Reduced lignin alfalfa observed a 1% and 3.5% reduction in ADL as a percent of aNDFom and ADL as a perfect of DM, respectfully. Several studies have observed a decrease in ADL concentration in RLA as compared to CA, albeit in higher percentages. Getachew et al. (2018) observed a significantly (23%) lower ADL in RLA than reference cultivars, while Grev et al. (2017), Arnold et al. (2019), Cherney et al. (2020), and Damiran et al. (2022) observed more conservative decreases at 6.8%, 8.4%, 11%, and 8.6%, respectively. On average, ADL as a percent of aNDFom was 30.6% lower in mixtures when compared to monoculture alfalfa treatments (p<0.01). This trend aligns with the observation reported by Damiran et al. (2022) that found alfalfa monocultures had higher ADL than mixtures. If evaluated by harvest, RLA was 3.8%, 4.9% and 18.1 lower in ADL as % of aNDFom in harvests 1, 2 and 4, respectively. On average, harvests 4 and 5 had significantly higher lignin content than harvests 1 and 2. There is a well-established positively correlated link between increased plant maturity and lignin concentration (Albrecht et al., 1987; Jung et al., 1997; Casler and Vogel, 1999), as well as a strong negative correlation between lignin concentration and forage digestibility (Reddy et al., 2005). Finally, drought conditions during the growing season during our trial might have further increased lignin concentration, as water stress is often associated with higher lignin concentration (Moura et al., 2010). Lower than average precipitation was noted in August, which could have likely impacted the lignin concentration in harvest 5 (September 10th).
4.3 Fiber Digestibility

Reduced lignin alfalfa in monoculture observed a 8.8% lower (p=0.2) uNDF$_{240}$ as a percent DM basis, 7.8% lower (p=0.1) uNDF$_{240}$ as a percentage of aNDFom, 4.4% higher (p=0.1) pdNDF as a percentage of aNDFom, a 9.8% increase in average pdNDF per kg DM/ha, and no differences in IVDMD or IVTDMD when compared to conventional alfalfa in monoculture. Similarly, alfalfa-tall fescue mixtures averaged 17.7% lower in uNDF as a percentage of aNDFom, 9% higher in pdNDF a % of aNDFom, 3.2% lower in IVDMD, and 3.7% lower in IVTDMD when compared to monoculture alfalfa treatments. The total pdNDF per kg DM/ha was significantly higher in proportion in the first 2 harvests than the last three harvests.

There was no significance in IVNDFD by forage type (p=0.83), but some differences in IVNDFD can be seen within harvests. Monoculture reduced lignin alfalfa was 6.6% and 9.5% higher in IVNDFD than monoculture CA in harvests 3 and 4, respectively. It should be noted that fiber digestibility is particularly important as Oba and Allen (1999) point out, because even a one unit increase in IVNDFD can result in a significant increase in animal productivity. Casler and Vogel (1999) further support this claim by demonstrating that a 1% increase in IVDMD resulted in a 3.2% in average daily gain in beef cattle. Inconsistencies in IVNDFD could be due to drought experienced during the growth of this harvest, particularly lower than average precipitation in April, May, and August. Further studies would be needed to evaluate the IVNDFD concentration over a larger time period (3-5 years) for RLA grown in this environment. Additionally, perhaps a shortened harvest interval (28 or 30 days) as compared to this study's 35-day interval would result in a more pronounced difference in IVNDFD concentration by treatment.
4.4 Silage

Under the conditions of this study, inoculation effect was significant for all measures of fermentation except ammonia nitrogen (NH3-N) concentration. Inoculated samples were higher in total volatile fatty acid (VFA) content (p<0.01), lactic acid concentration (p=0.02), and acetic acid (p=0.01) concentration while being lower in pH (p<0.01) and similar in ammonia nitrogen content (p=0.92) as compared to non-inoculated samples. Inoculated samples observed 64% fewer instances of propionic acid formation than non-inoculated samples did. In contrast with this study's findings, Sheperd et al. (1995) found that acetate and ammonia nitrate for uninoculated silage were higher than that of inoculated silage. While acetic acid concentration was higher in inoculated samples, in the current study, values were still within the recommended goal (<3%) for acetic acid in silage, as established by Ward and de Ondarza (2008). This increase in acetic acid content in inoculated samples was mostly likely due to the inclusion of Enterococcus faecium and Lactobacillus plantarum. Facultative heterofermentative LAB can ferment pentoses and hexoses, which produce lactic acid as well as acetic acid, while homofermentative LAB can only ferment hexoses. (Muck et al., 2018). Acetic acid in acceptable concentrations has been shown to act as an anti-mycotic and was able to produce a more aerobically stable silage (Ward and de Ondarza, 2008; Santos et al., 2013).

Low DM content was associated with higher total VFA content (p<0.01), NH3-N (p<0.01), and lactic acid concentration (p<0.01), while being lower in pH (p<0.01) than high DM content silages. The decline in pH was slower for treatments with high DM contents, a reflection of restricted fermentation (Kung et al., 1984; Sheperd et al., 1995) and usually results in a roughly 25% decline in total VFAs (% DM) (Ward and de Ondarza, 2008). A study by Kung et al. (2018) corroborates the lower pH observed in low DM silage and explains that it is likely due
to increasingly limited metabolic water available for growth of lactic acid bacteria, which is directly responsible for decrease in silage pH. Furthermore, tall fescue monoculture had 33% lower (p<0.01) ammonia nitrogen as compared to the average of all other treatments. Our findings of higher concentrations of ammonia nitrogen in the low DM forages were supported by a study conducted by Sheperd et al. (1995) that suggested reduction in NH₃-N with increasing DM content was the result of a restriction in protein breakdown, characteristic of higher DM forages.

Alfalfa-tall fescue mixtures had 12.6% higher total VFA content as compared to monoculture legumes. Mixed plots had a 21.2% higher acetic acid concentration on average than monoculture alfalfa silages. Similar acetic acid trends were observed in a study conducted by Niyigena et al. (2022) with varying proportions of tall fescue mixed with alfalfa. Mixed plots also observed a decrease in lignin concentrations relative to monoculture alfalfa plots. Lignin is indigestible and cross-links with other plant structures, therefore inhibiting the ability of cellulose and hemicellulose to be broken down. Hemicellulose is primarily composed of pentoses, while cellulose is composed of glucose (hexose). Hemicellulose (aNDFom – ADFom) content and cellulose (ADF – ADL) were significantly higher in alfalfa-fescue mixtures, lending higher levels of pentoses and hexoses to be utilized by heterofermentative and homofermentative in formation of lactic and acetic acid. Feeding mixed alfalfa and tall fescue has the potential to increase dry matter intake and milk yield in dairy cows as opposed to feeding monoculture tall fescue silage (Richard et al., 2020).

5. CONCLUSIONS
In conclusion, results from the current and previous studies indicate that reduced lignin alfalfa can be grown in monoculture or diculture mixture with novel tall fescue and produce similar or slightly higher dry matter yield than reference alfalfa cultivars without sacrificing nutritive value. Overall, alfalfa-grass mixtures were at least as competitive with monoculture alfalfa stands while consistently outperforming grass monocultures. No differences in reduced lignin monocultures or alfalfa-tall fescue mixtures were observed for silage quality or fermentation, suggesting that forage type (legume or grass), inoculation, and dry matter percentage have a more significant impact on fermentation than lignin downregulation. Additionally, while DM percentage, inoculation, and forage type had a significant effect on silage fermentation, all silages were within established acceptable limits for legume and grass silages, which provides flexibility in silage management to producers and allows them to provide a highly nutritious feed to livestock year-round. Finally, these results offer evidence that reduced lignin alfalfa, and novel endophyte-infected tall fescue complement each other in mixtures when grown in the Southeastern U.S. and contribute to a sustainable livestock production system by offering an extended harvest season, high nutritive value, and excellent silage product.
TABLES AND FIGURES

Table 1. Total monthly precipitation (mm), mean monthly temperature (°C) and 30-yr historical averages for Clemson, SC, during the 2021 growing seasona.

<table>
<thead>
<tr>
<th>Month</th>
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<th>30-year Avg Temperature</th>
<th>30-year Avg</th>
</tr>
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<td>122.2</td>
<td>12.2</td>
</tr>
<tr>
<td>April</td>
<td>83.1</td>
<td>106.4</td>
<td>15.3</td>
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<td>May</td>
<td>88.6</td>
<td>103.4</td>
<td>19.4</td>
</tr>
<tr>
<td>June</td>
<td>143.8</td>
<td>105.9</td>
<td>24.2</td>
</tr>
<tr>
<td>July</td>
<td>153.7</td>
<td>105.4</td>
<td>25.7</td>
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<td>August</td>
<td>98.8</td>
<td>133.4</td>
<td>26.0</td>
</tr>
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<td>September</td>
<td>74.9</td>
<td>95.8</td>
<td>21.9</td>
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Table 2. Nutritional quality of CA, RLA, and F grown in monocultures or in diculture mixtures1.
<table>
<thead>
<tr>
<th>Item</th>
<th>CA</th>
<th>RLA</th>
<th>F</th>
<th>CA/F</th>
<th>RLA/F</th>
<th>SEM²</th>
<th>p-value</th>
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<tr>
<td>DM, %</td>
<td>26.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>26.2&lt;sup&gt; &lt;/sup&gt;&lt;sup&gt;c&lt;/sup&gt;</td>
<td>30.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>28.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.32</td>
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<tr>
<td>Ash, % DM</td>
<td>10.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.15</td>
<td>0.99</td>
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<tr>
<td>CP, % DM</td>
<td>22.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15.0&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>20.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.44</td>
<td>&lt;0.01</td>
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<td>aNDFom, % DM</td>
<td>33.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>33.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>52.4&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>ADFom, % DM</td>
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<td>28.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>30.5&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>27.9&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>&lt;0.01</td>
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<td>8.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.3&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>6.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.27</td>
<td>&lt;0.01</td>
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<td>ADL, % NDF</td>
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<td>25.0&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>WSC, % DM</td>
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<td>8.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.2&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>8.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.28</td>
<td>0.12</td>
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<sup>1</sup>CA = Conventional alfalfa; RLA = reduced lignin alfalfa; F = tall fescue; CA/F = conventional alfalfa + tall fescue; RLA/F = reduced lignin alfalfa + tall fescue. <sup>2</sup>SEM = standard error of means
Table 3. Effect of forage treatments on undigestible NDF concentration and *in-vitro* DM digestibility.

<table>
<thead>
<tr>
<th>Item</th>
<th>CA</th>
<th>RLA</th>
<th>F</th>
<th>CA/F</th>
<th>RLA/F</th>
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<td>uNDF&lt;sub&gt;240&lt;/sub&gt;, % DM</td>
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<td>0.55</td>
<td>0.01</td>
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<td>uNDF&lt;sub&gt;240&lt;/sub&gt;, % aNDFom</td>
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<td>34.4&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>29.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.03</td>
<td>&lt;0.01</td>
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<td>pdNDF, % aNDFom&lt;sup&gt;3&lt;/sup&gt;</td>
<td>62.7&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>IVNDFD, % DM</td>
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<td>55.2&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>58</td>
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<sup>1</sup>CA = Conventional alfalfa; RLA = reduced lignin alfalfa; F = tall fescue; CA/F = conventional alfalfa + tall fescue; RLA/F = reduced lignin alfalfa + tall fescue. 2 uNDF<sub>240</sub> = undegraded neutral detergent fiber (after 240 h of fermentation). 3 pdNDF = potentially digestible neutral detergent fiber. 4 IVDMD = In vitro 30 h dry matter digestibility. 5 IVTDMD = In vitro 30 h true dry matter digestibility.
Table 4. Dynamics of pH, total volatile fatty acid content, ammonia nitrogen, and organic acids of silage.

<table>
<thead>
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<th>Significance</th>
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<td>F</td>
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<td>YL</td>
<td>9.05</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>NH</td>
<td>5.84</td>
<td>***</td>
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<tr>
<td></td>
<td>NL</td>
<td>9.89</td>
<td>***</td>
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<tr>
<td>NH₃N-CPE (DM)</td>
<td>YH</td>
<td>1.65</td>
<td>0.14 ***</td>
</tr>
<tr>
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<td>YL</td>
<td>1.99</td>
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<td>NH</td>
<td>1.71</td>
<td>ns</td>
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<tr>
<td></td>
<td>NL</td>
<td>2.04</td>
<td>ns</td>
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<tr>
<td>Lactic acid (% DM)</td>
<td>YH</td>
<td>5.80</td>
<td>0.60 ns</td>
</tr>
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<td>YL</td>
<td>7.30</td>
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<tr>
<td></td>
<td>NH</td>
<td>4.80</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>NL</td>
<td>7.80</td>
<td>***</td>
</tr>
<tr>
<td>Acetic acid (% DM)</td>
<td>YH</td>
<td>1.34</td>
<td>0.32 **</td>
</tr>
<tr>
<td></td>
<td>YL</td>
<td>1.75</td>
<td>*</td>
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<tr>
<td></td>
<td>NH</td>
<td>1.04</td>
<td>*</td>
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<tr>
<td></td>
<td>NL</td>
<td>2.09</td>
<td>*</td>
</tr>
</tbody>
</table>

ns = not significant; t = tendency to differ (p<0.1); *p<0.05; **p<0.01; ***p<0.001

1CA = Conventional alfalfa; RLA = reduced lignin alfalfa; F = tall fescue; CA/F = conventional alfalfa + tall fescue; RLA/F = reduced lignin alfalfa + tall fescue. 2Y = inoculated silage; N = non-inoculated silage. 3H = High dry matter content (~50%); L = low dry matter content (~35%). 4SEM = standard error of means. 5T = Treatment (forage type); I = inoculation; DM = dry matter content; T x I = the interaction between treatment and inoculations; T x DM = the interaction between treatment and dry matter content; I x DM = the interaction between inoculation and dry matter content; T x I x DM = the interaction between treatment, inoculation, and dry matter content.
Figure 1. Forage plot layout

BLOCK 1
1 2 3 4 5
50:50
Novel Tall Fescue
Conventional Alfalfa

50:50
Novel Tall Fescue
Reduced Lignin Alfalfa

BLOCK 2
6 7 8 9 10
Novel Tall Fescue
Conventional Alfalfa

50:50
Novel Tall Fescue
Reduced Lignin Alfalfa

50:50
Novel Tall Fescue
Conventional Alfalfa

BLOCK 3
11 12 13 14 15
Reduced Lignin Alfalfa

Conventional Alfalfa

50:50
Novel Tall Fescue
Reduced Lignin Alfalfa

50:50
Novel Tall Fescue
Conventional Alfalfa

BLOCK 4
16 17 18 19 20
5' x 20'

50:50
Novel Tall Fescue
Conventional Alfalfa

Novel Tall Fescue

Reduced Lignin Alfalfa

50:50
Novel Tall Fescue
Reduced Lignin Alfalfa

Conventional Alfalfa
Figure 2. Biomass dry matter yield (kg DM per ha) of the different forage treatments.

CA = Conventional alfalfa; RLA = reduced lignin alfalfa; F = tall fescue; CA/F = conventional alfalfa + tall fescue; RLA/F = reduced lignin alfalfa + tall fescue. *Means with different letters differ (P ≤ 0.05). Vertical bars indicate standard errors of the mean.
Figure 3. Interaction of treatment and harvest on CP concentration (% DM).

CA = Conventional alfalfa; RLA = reduced lignin alfalfa; F = tall fescue; CA/F = conventional alfalfa + tall fescue; RLA/F = reduced lignin alfalfa + tall fescue. a-j Means with different letters differ (P ≤ 0.05). Vertical bars indicate standard errors of the mean.
Figure 4. Interaction of treatment and harvest on aNDFom concentration (% DM).

CA = Conventional alfalfa; RLA = reduced lignin alfalfa; F = tall fescue; CA/F = conventional alfalfa + tall fescue; RLA/F = reduced lignin alfalfa + tall fescue. *Means with different letters differ (P ≤ 0.05). Vertical bars indicate standard errors of the mean.
Figure 5. Interaction of treatment and harvest on ADL concentration (% aNDFom).

CA = Conventional alfalfa; RLA = reduced lignin alfalfa; F = tall fescue; CA/F = conventional alfalfa + tall fescue; RLA/F = reduced lignin alfalfa + tall fescue. Means with different letters differ (P ≤ 0.05). Vertical bars indicate standard errors of the mean.
**Figure 6.** Concentration of CP (% DM), uNDF (% DM), and uNDF (% aNDFom) by harvest.

- **CP (% DM)**
  - Harvest 1: a
  - Harvest 2: b
  - Harvest 3: a
  - Harvest 4: a
  - Harvest 5: a

- **uNDF (% DM)**
  - Harvest 1: c
  - Harvest 2: b
  - Harvest 3: ab
  - Harvest 4: a
  - Harvest 5: a

- **uNDF (% aNDFom)**
  - Harvest 1: d
  - Harvest 2: c
  - Harvest 3: c
  - Harvest 4: b
  - Harvest 5: a

a-d Means with different letters differ (P ≤ 0.05). Vertical bars indicate standard errors of the mean.
Figure 7. Concentration of IVNDFD (% DM), IVTDMD (% DM), and pdNDF (% aNDFom) by harvest

Means with different letters differ (P ≤ 0.05). Vertical bars indicate standard errors of the mean.
REFERENCES


Sim, C. (2022). Effect of forage species as monoculture or in binary mixtures on forage characteristics, animal preference, and grazing behaviour (Doctoral dissertation, University of Saskatchewan).


CHAPTER THREE

OVERALL CONCLUSIONS AND FUTURE RESEARCH

This chapter summarizes the results of the experiments reported in this thesis and includes an overview of proposed future studies that will build on the findings from this work. Forage efficiency, meeting the nutritional needs of livestock, and increasing animal productivity are on the forefront of agricultural sustainability and addressing food insecurity. Efficient forage production and utilization is tailored to a particular region’s growing conditions and is paired with complementary species to produce the highest quality livestock feed. Mixing forage species is often a strategy that producers employ to obtain highly nutritious feed, to improve pasture yield, and to mitigate any disadvantageous traits observed in monocultures. Legume-grass mixed pastures offer numerous benefits to producers such as improving animal productivity, stand persistence, nutritive value, and environmental sustainability when compared to monocultures stands of alfalfa or grasses alone. Alfalfa is often included in these mixtures due to exceptionally high protein content and the ability to fix nitrogen for other forages in the mixture. However, it is limited in quality due to high concentrations of undigestible lignin and the tendency to experience lignification at greater rates than other forages. As a result, lignin down-regulated alfalfa varieties have been introduced to the market in recent years in an effort to increase digestibility without sacrificing yield. Following encouraging findings, more studies were warranted to investigate how reduced lignin alfalfa performs diculture mixtures with tall fescue, particularly in the Southeastern United States, and how well it would ensile with variable dry matter content and inoculant.
Conclusions

The primary focus of this thesis was to investigate the effect of lignin reduction of on forage yield and nutritive value on alfalfa grown in monoculture and in mixtures with tall fescue, and to investigate how those treatments, along with inoculant and DM percentage, affect fermentation in silage. Under the conditions of this study, it was found that reduced lignin alfalfa can be grown in monoculture or diculture mixture with novel endophyte-infected tall fescue and produce similar or slightly higher dry matter yield than reference alfalfa cultivars, without sacrificing nutritive value. Overall, alfalfa-grass mixtures were at least as competitive with monoculture alfalfa stands while consistently outperforming grass monocultures. No differences in reduced lignin monocultures or mixtures were observed for silage quality or fermentation, suggesting that forage type (legume or grass), inoculation, and dry matter percentage have a more significant impact on fermentation than lignin downregulation. Additionally, while DM percentage, inoculation, and forage type had a significant effect on silage fermentation, all silages were within established acceptable limits for legume and grass silages, which provides flexibility in silage management to producers and allows them to provide a highly nutritious feed to livestock year-round. Finally, these results offer evidence that reduced lignin alfalfa and novel endophyte-infected tall fescue complement each other in mixtures when grown in the Southeastern U.S. and contribute to a sustainable livestock production system by offering an extended harvest season, high nutritive value, and excellent silage product. To further understand how reduced lignin performs in this geographic area, further studies should evaluate samples beyond the establishment year for yield and nutritive value, the stand persistence, and how the proportion of alfalfa to tall fescue is affected over time.
Possible Future Directions

After evaluating the nutritive value of reduced lignin alfalfa, it is still unclear how animal performance would be affected if fed in monoculture or in combination with novel endophyte-infected tall fescue. Therefore, in a future study, it would be essential to evaluate animal production performance responses of these treatments. Little data exists on the effect of lignin down-regulated alfalfa, particularly in mixture with tall fescue, in high-performing dairy performance. The objective of this subsequent study would be to assess the effects of feeding reduced lignin alfalfa in monoculture and in mixtures with tall fescue on dry matter intake (DMI), fermentation, milk yield, and milk fat composition. Twelve cannulated multiparous Holstein dairy cows would be randomly assigned to each treatment and fed once daily at 1000h. Cows would be randomly assigned treatments diets in a crossover design with four 21-d periods, allowing 16 days for adaptation and 5 days for data and sample collection. Four experimental diets (70:30 forage to concentrate ratio) would be compared: monoculture reduced lignin alfalfa (RLA) haylage; monoculture conventional alfalfa (CA) haylage; reduced lignin alfalfa + tall fescue (RLA/F) haylage; and conventional alfalfa + tall fescue (CA/F) haylage. Diets would be offered as TMR for ad-libitum intake. Dry matter intake would be calculated by recording feed offered and refused. Milk yield would be measured during morning and evening milkings. Milk yield and DMI would both be measured on the last 3 days of each period. Milk samples would be collected on days 17 and 18 and subsequently analyzed for milk fat, protein, lactose, VFAs, and urea nitrogen. Rumen samples would be collected on d 21 of each period at 0, 1, 2, 4, and 6 h relative to morning feeding time. The pH would be immediately measured and then samples would be stored for future VFA and NH₃-N analysis. This potential
future study would better illustrate how laboratory obtained digestibility measures correlate with animal trial results, and how lignin down-regulation in alfalfa affects animal performance.