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Effects of Wilting Extent on the Phytoestrogen Levels, Nutritional Value, Microbial Populations, and In Vitro Ruminal Methane Emissions of Red Clover Hay and Silage Across Stages

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An Abstract of the Thesis Presented
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The main objective of this thesis is to improve the understanding and awareness of methodologies to decrease phytoestrogens in conserved legumes without sacrificing forage nutritive value. In chapter 1, we discussed the main factors influencing each stage of hay production and our current understanding of the hay microbiome dynamics. The primary objective of haymaking is to dry forage enough (80-85% DM) to inhibit the growth of undesirable microbes and halt residual plant enzymatic activity that causes nutrient losses. During the field and storage phases of haymaking, the environment, management practices, and other factors influence the extent of DM losses. This chapter discusses these factors and the strategies that have been developed to mitigate these nutrient losses. A major emphasis was placed on hay microbiota dynamics, as it has been scarcely studied despite its importance on nutrient losses during storage and harvest, especially in high moisture conditions. Since soil particles are a significant source of

undesirable microbes and ash contamination, the effects of cutting height, mower type, and swath manipulation on soil contamination were discussed. Also, the impact of environmental conditions and swath manipulation on wilting time was analyzed for both humid and arid conditions. Special attention was given to design improvements in harvesting equipment to reduce curing time and field losses. Furthermore, we assessed the nutrient losses during storage caused by undesirable microbial and residual plant enzymatic activity resulting from excessive moisture at baling or re-introduced moisture during storage. The extent of spoilage during storage depends not only on bale moisture but also on bale size, density, shape, wrapping, forage type, and storage facilities. A Venn diagram analysis condensed all relevant hay microbiology research and showed that each phase of the haymaking process has a unique microbiome. It also showed that certain fungal and bacterial genera could be shared across more than one hay production phase. For instance, *Aspergillus*, *Cladosporium*, and *Alternaria* are fungal genera that tend to be present throughout the haymaking process. In order to take corrective actions, hay producers need to be aware of the increased susceptibility to nutrient losses associated with particular field and storage practices, environmental conditions, and forage types.

In Chapter 2, we evaluated the effects of insufficient (WET) or ample (CUR) wilting on the phytoestrogen levels, nutritional value, microbial populations, in vitro ruminal methane emissions, and in situ degradability of red clover silage (29.4 and 45.3% DM) and hay (65.1 and 89.1, respectively) across the storage stages. Measurements were taken at the start of storage (STRT), after 14 d (MicA), and once storage processes had stabilized for hay and silage (50 and 78 d, respectively; LATE). Only LATE samples of

hay and silage were tested for the in situ procedure. Data were analyzed as a RCBD (5 blocks) with a 2 (wilting extents) x 2 (conservation methods) x 3 (storage stages) factorial. Results showed that storage DM losses were higher for WET hay than CUR but no differences were observed between WET and CUR silage. Ample wilting of hay and silage preserved better water-soluble carbohydrates during storage relative to insufficient wilting. Due to microbial spoilage, the NH_3-N of WET hay was higher than CUR hay after 14 d of storage, but the opposite was observed after 50 d. For the WET and CUR silage, NH_3-N increased across the ensiling period. The neutral detergent fiber of WET hay increased across storage stages while it remained stable for CUR hay. In contrast, the neutral detergent fiber of WET and CUR silage decreased during the ensiling period. The WET hay favored the growth of molds during storage, while CUR hay reduced their counts after 50 d of storage. For silage, mold counts were lower in WET compared to CUR after 14 d of storage but no differences were observed after 78 d. When the ensiling period is limited to 14 d, CUR silage that was aerobically exposed for 7 d was more susceptible to storage DM losses and subsequent heating relative to WET. However, if the ensiling period is extended to 78 d, no differences were observed between WET and CUR silage in terms of HDD and storage DM losses after being aerobically challenged. Ample wilting preserved the optimal ruminal fermentation kinetics of hay compared to insufficient wilting, while the ruminal fermentation kinetics of silage was not affected by the wilting extent. In vitro ruminal fermentation of WET silage resulted in higher methane yield than CUR, whereas methane yield of WET and CUR hay were not different. For both conservation methods, insufficient wilting reduced methane yield only at the end of storage. The in situ rumen degradability kinetics showed that ensiling increased the

soluble DM fraction relative to haymaking. Ensiling reduced the potentially degradable DM fraction compared to haymaking but increased the rate of degradation of DM. Within insufficient wilting, silage had a higher degradation rate of NDF than hay. Ample wilting was more beneficial for silage than hay in terms of decreasing the levels of phytoestrogens. Across storage stages, hay had lower formononetin and biochanin A than silage. Formononetin and biochanin A of red clover hay decreased after 14 d of storage due to microbial degradation. Overall, ample wilting helped conserve the nutritional quality of hay and silage and decreased the phytoestrogens, especially in silage.

Key words: phytoestrogens, wilting, hay, silage.

DEDICATION

To my friends and the scientific community

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SUPPLEMENTAL MATERIAL

LIST OF ABBREVIATIONS

ADF	Acid detergent fiber
ADIN	Acid detergent insoluble nitrogen
AES	Aerobically exposed silage
CFU	Colony forming units
CH_4	Methane
CP	Crude protein
CUR	Ample wilting
DM	Dry matter
FW	Fresh weight
HDD	Heat degree-days above room temperature
IN	Insoluble nitrogen
IVDMD	*In vitro* dry matter digestibility
IVOMD	In vitro organic matter digestibility
K_f	rate of gas production
LAB	Lactic acid bacteria
M_f	Asymptotic maximal gas production
MTOD	Conservation method
MT	Maximum temperature
NDF	Neutral detergent fiber
NH_3-N	Ammonia nitrogen
NPN	Non-protein nitrogen

NSC	Nonstructural carbohydrates
OM	Organic matter
ppm	Parts per million
RH	Relative humidity
STG	Stage of storage
TDN	Total digestible nutrients
TVFA	Total volatile fatty acids
VPD	Vapor pressure deficit
WET	Insufficient wilting
WILT	Wilting extent
WBG	Wet brewer's grain
WSC	Water-soluble carbohydrates
$\overline{x} =$	Mean

CHAPTER 1

FACTORS AFFECTING NUTRIENT LOSSES IN HAY PRODUCTION

Introduction

Haymaking and ensiling are the primary forage conservation methods in the US (Rotz et al., 2020). Even though silage production has increased considerably in the last 20 years (NASS, 2022), haymaking is still a popular method to preserve forage due to easier marketability and more efficient transportation of the end product (Shinners, 2010; Collins and Moore, 2017). In 2021, the US total hay production was estimated at 120.2 million metric tons, harvested from 20.5 million ha, with an average yield of 5.86 metric tons per ha (NASS, 2022). Total hay production in the U.S. has declined from 152.2 million metric tons to 120.2 between 2000 to 2022, while total silage production increased from 101 million metric tons to 135 during the same period (NASS, 2001, 2022) because producing hay is challenging in humid regions (Han et al., 2014).

The hay production process starts with mowing, followed by a period of wilting to reach 60-70% DM (Rotz et al., 2020), at which point the swath is raked to form a windrow that is further wilted to 80-85% DM before it is baled, stored, and ultimately fed (Pogue et al., 1996; Rotz, 2005). The main objective of haymaking is to preserve the nutrients found in the standing forage by reducing its moisture concentration to halt plant enzymatic activity and inhibit aerobic microbial spoilage (Collins, 1995; Collins and Moore, 2017). However, wilting the forage to >85% DM concentration increases leaf shattering, leading to as much as 25% field DM losses, but reduces storage losses to as little as ~5% (Hoglund, 1964; Orloff and Mueller, 2008). Conversely, as forage DM decreases below

80%, field DM losses will proportionally decrease to less than 5%, while storage losses may increase above 24% (Ball et al., 1998; Coblentz, 2012). In order to reduce total nutrient losses, it is essential to understand the factors causing such losses so optimal management recommendations can be developed. Factors affecting field losses include cutting height, mower type, mechanical conditioning, wilting method, and environmental conditions (Rotz et al., 2020). On the other hand, storage losses depend on bale moisture, shape, weight, density, wrapping type, and storage conditions (Coblentz et al., 2004; Collins and Moore, 2017). The impact of these factors is of great interest because of their effects on the ultimate nutritional value of hay, and the risk of spontaneous combustion, particularly when bale temperature exceeds 70-80°C (Festenstein, 1971; Hancock, 2015)

The microbiome of hay is mainly composed of fungi and bacteria, which are responsible for causing nutrient losses during storage, especially when DM levels are below 80-85% (Roberts, 1995). When microbial spoilage occurs, mycotoxins can be produced by certain molds, which can cause severe animal disease and compromise the animal food safety chain (Korosteleva et al., 2007). The microbiome of hay undergoes considerable changes across harvest and storage stages, particularly as it shifts from field to storage populations (Kaspersson et al., 1984; Alonso et al., 2013). Unfortunately, few studies have evaluated the complex dynamics of the hay microbiome and how they are affected by environmental conditions and management and storage factors. Expanding our understanding of hay microbiology is essential to developing novel approaches to reduce nutrient losses due to spoilage. Understanding how the environment, crop, and management factors affect the haymaking process would help producers reduce field and storage losses, resulting in higher-quality hay. Thus, this

review paper discusses the main factors influencing each stage of hay production and our current understanding of the hay microbiome dynamics.

Hay Production Stages

Mowing Phase

The first step in producing hay involves cutting the stand, ideally once plant maturity is optimal and weather conditions are conducive for curing the swath in less than 5 d (Zhao et al., 2021). Recommendations on optimal maturity at mowing have been provided elsewhere (Sheaffer et al., 1988; Fan et al., 2004; Yolcu et al., 2006) and are usually a compromise between forage quality and yield. However, in many instances, unexpected weather events can force producers to mow their stands outside these recommendations (Collins and Moore, 2017; Coblentz et al., 2020). This first section will discuss cutting height, mower type, and conditioning effects on hay production, emphasizing soil contamination because of its importance as a vector of spoilage microbes (Roberts, 1995).

Cutting height effects

Lowering the cutting height increases DM yield per ha (Yolcu et al., 2006) but also reduces forage quality because of a higher inclusion of fibrous stems in the harvested forage (Sheaffer et al., 1988). Also, it may increase the inclusion of soil particles, leading to higher levels of exogenous ash in the swath (Digman et al., 2013) and counts of undesirable soil microbes (Castagnara et al., 2012). Specific cutting height recommendations to balance yield and quality are available across forage species used

for hay production (Sheaffer et al., 1988). In the case of perennial forages, cutting height is also a critical factor affecting stand persistence because of the differences in crown placement across species, which in turn affects the susceptibility of crowns to mechanical damage during mowing (Undersander, 2006), and also the influence of residual stubble height on the nutrient reserve balance of underground structures (Sheaffer et al., 1988).

Li and Kim (2017) evaluated the effects of cutting height (8 and 15 cm) on the nutritional value of rye (*Secale cereale*) hay (>80% DM) stored in nylon bags. After 2 mo of storage, the CP concentration was higher at 15 vs. 8 cm (10.7 vs. 7.6% of DM, respectively), while NDF ($\bar{x}$= 59.3% of DM), IVDMD ($\bar{x}$= 72.1% of DM), and total fungi count ($\bar{x}$= 7.64 log CFU/g) were not different. DM losses during storage were greater at 15 than 8 cm (10.6 vs. 8%, respectively), which the authors suggested was due to the inclusion of more leaves than stems at higher cutting heights. In a similar study, Digman et al. (2013) assessed the effect of increasing cutting height on exogenous soil ash contamination and the yield of an alfalfa (*Medicago sativa*) stand. This study measured the ash contamination in post-merging windrows. Increasing the cutting height of a sickle cutterbar mower from 6.9 to 10.8 cm reduced the ash concentration in the windrow from 8.13 to 8% of DM. However, increasing the cutting height reduced the yield by 0.16 metric tons ha^{-1} cm^{-1} of stubble lost (R^2= 0.98).

Effects of mower types

The primary mower types used in hay production in North America include the rotary disk and sickle cutterbar mowers (Rotz, 2005). Other designs include flail and rotary drum mowers (Barać et al., 2012). Even though sickle cutterbar mowers are still

used due to their relatively higher reliability and lower cost (Undersander, 2006), rotary disk mowers have largely replaced them in the US (Collins and Moore, 2017) because of their faster ground speed, which enhances productivity, and reduces the tractor time required per ton of hay produced (Rotz et al., 2020). Although studies indicate that mower type does not influence field DM losses, it can affect the DM concentration of the wilted swath (Shinners et al., 1991; Rotz, 2003a). Savoie et al. (1981) reported that after ~36 h of wilting, first-cut alfalfa mowed with a sickle cutterbar mower resulted in a higher DM concentration than the rotary drum mower (62.8 and 54.5%, respectively) because the sickle cutterbar mower produced a wider swath allowing a faster drying rate. In contrast, the rotary drum mower produced a narrower swath, which was harder to dry. Despite differences in DM levels, both mower types produced similar field DM losses ($\bar{x}$= 18.1 kg/ha). Other studies have also reported that under most conditions, the sickle cutterbar and rotary drum mowers produce similar field DM losses (Koegel et al., 1985; Shinners et al., 1991).

Conditioning in hay production

Nowadays, cutting and conditioning mechanisms are typically combined in modern forage mowers (Rotz et al., 2020). Mechanical conditioning of the forage allows additional water loss by physically severing the waxy epidermis, reducing wilting times by 1 to 2 d (Rotz and Shinners, 2007), which reduces the chance of rain damage due to unexpected weather events (Rotz et al., 1987). Conditioning devices can be categorized as either roll or flail types, with the former smashing or breaking plant stems and the latter abrading the waxy surface of the cut forage (Cecava, 1995). Flail conditioners increase the drying rate of forages much more than roll conditioners due to greater physical disruption of the

plant tissues (Rotz and Sprott, 1984) but also increase field DM losses, averaging between 6-11% (Rotz, 1995b), compared to 4-7% for roll conditioners (Shinners et al., 1991). Generally, mechanical conditioning produces higher field DM losses in legumes (4%) than in grasses (1%) (Rotz and Muck, 1994) because of the morphology of legume leaves (slender petioles), which make them more susceptible to shattering losses than sheaths and blades of grasses (Savoie and Beauregard, 1991). Consequently, flail conditioners are recommended for grasses, whereas roll conditioners are suited better for legumes (Greenlees et al., 2000; Rotz, 2001). Koegel et al. (1985) reported higher field DM losses during mowing when comparing flail vs. roll conditioners (18.6 vs. 16.5%) fitted in rotary disk mowers used to cut an alfalfa stand. Similarly, Rotz and Sprott (1984) showed that a flail mower fitted with a flail conditioner resulted in higher field DM losses (6.2%) in alfalfa compared to a rotary disk mower fitted with a roll conditioner (2.9%).

Rotz et al. (1987) observed that the extent of wilting time reduction resulting from mechanical conditioning decreased across alfalfa harvests in a growing season due to stem thickness changes. Using a sickle cutterbar mower fitted with a roller conditioner increased the drying rate of alfalfa by 42.5% in the first harvest, but smaller effects were observed in the second harvest (25%), and no effects were observed in subsequent harvests relative to no conditioning. The authors suggested that the thinner stems found in later harvests could pass through the rolls with minimal damage relative to the thicker stems of the first harvest, which were easily crushed by the rolls. Field losses were similar with or without conditioning in this experiment, but storage losses were indirectly influenced by conditioning through residual moisture levels. The storage DM losses of first-cut alfalfa were lower with conditioning than without (5.5 vs. 8.4%, respectively) due

to a higher DM at baling (78.8 vs. 74.5%). However, conditioning did not affect storage DM losses of the second ($\bar{x}$= 9.85%) and third ($\bar{x}$= 5.65) cuts because there were no differences in DM concentrations at baling due to conditioning ($\bar{x}$= 74.4 and 79.7%, respectively). Seo et al. (2000) evaluated the effects of conditioning on the field drying rate of a mixed stand of grasses (orchardgrass, tall fescue, Kentucky bluegrass, and perennial ryegrass) harvested at three different maturities. Their results showed that after mowing, the wilting time of the stand harvested at the late boot stage was reduced from 5-6 d without conditioning to 3 d with a roller conditioner. A similar trend was observed for the heading (4 vs. 2 d) and blooming stage (3 vs. 1 d, respectively).

Chemical conditioners (i.e., potassium and sodium carbonate) are another alternative to expedite hay curing by disrupting the waxy layer of the cuticle, allowing moisture to pass through (Rotz and Shinners, 2007). This treatment is more effective when used in legumes, such as alfalfa and clover, than in grasses like orchardgrass (*Dactylis glomerata*) and timothy (*Phleum pratense*) (McCartney, 2005), most likely because the cuticle of legumes has a lower moisture loss resistance than grasses (Tsang Mui Chung, 1984). Chemical conditioners can improve the drying rate of all alfalfa cuttings, especially during the summer harvest (Rotz et al., 1987). For instance, Iwan et al. (1993) reported that when alfalfa (first cut) was treated with a chemical conditioner, the drying rate increased by about 20% and 50% for the second and third cuts, respectively, under ideal drying conditions. However, the commercial use of chemical conditioners has been hampered by possible side effects on animal acceptability (Macdonald and Clark, 1987), the need for large volumes of solution, the high cost of

treatment, plant discoloration, and concerns about chemical residues on the sprayed cut forage (Rotz et al., 2020).

Soil microbial contamination

It is estimated that there are up to 10 billion microorganisms per gram of soil and thousands of different species (Delmont et al., 2011). The soil microbiome is highly diverse and consists mostly of heterotrophic microbes, including bacteria, actinobacteria, and fungi (Egan et al., 2018). It is affected by soil properties, environmental conditions, management practices, and soil-plant interactions, among other factors (Jansson and Hofmockel, 2020). Therefore, the impact of soil contamination, which includes undesirable soil microbes, on stored forage nutrient conservation and hygiene varies considerably. Clevström and Ljunggren (1984) observed that fungi most frequently found in fresh forage samples of clover, timothy, and meadow fescue were *Fusarium* (75%) and *Cladosporium* spp. (99% of subsamples), but their respective surrounding soil samples were dominated by *Mucor* (88%), *Fusarium* (72%), and *Penicillium* spp. (65%). Whenever the soil-plant interface is disrupted due to mechanical harvesting or strong wind and rain, soil microbes carried by soil particles will cause a shift in the phyllosphere community (Chaudhry et al., 2020). Drouin et al. (2022) monitored the epiphytic microbial changes of an alfalfa stand during one season. Bacteria from the families *Lactobacillaceae* and *Enterobacteriaceae* were the most often found in spring and summer, and *Xanthomonacaceae* in late-summer. Fungal spores and bacterial endospores are of particular concern as they are very resilient and can cause issues later in the animal production system (Scudamore and Livesey, 1998; Drouin and Lafrenière, 2012).

For this reason, Jouany (2007) argued that increasing cutting height could reduce spoilage losses during hay storage by decreasing soil contamination of forage. Castagnara et al. (2012) measured fungal counts in oats (*Avena sativa*) hay and observed that a lower cutting height (10 vs. 20 cm) resulted in higher counts of *Aspergillus* spp. (5.1 vs. 4.8 log CFU/g) after 30 d of storage, but no effects of cutting height on total fungal counts were observed at cutting and baling. Pedersen and Guttormsen (1975) also studied the effects of cutting height (7 and 15 cm) on the microflora composition of a grassland composed of timothy and red clover (*Trifolium pratense*), with the lower cutting height resulting in higher proteolytic anaerobes in the first cut (2.4 vs. 1.3 log CFU/g). No effects of cutting height were observed in the second cut. Although increasing cutting height reduces the load of undesirable microbes that can cause spoilage downstream, the losses in yield can be impractical (Digman et al., 2013; Undersander, 2013), and each producer should assess the benefits and drawbacks specific to their operations.

Wilting Phase

Field wilting is the most critical step in the process of removing moisture from the swath to obtain suitable DM levels (>80-85%) for storage (Rotz, 1995a). Ideally, this step should not take more than 3-5 d (Collins and Owens, 2003). The rate of drying is affected by environmental conditions such as ambient temperature, relative humidity (RH), wind speed, solar radiation, and soil moisture (Rotz and Shinners, 2007). In addition, crop traits and management decisions play a fundamental role during the wilting phase (Rotz, 1995a). Relevant crop factors include plant species, maturity, and stem-to-leaf ratio (McCartney, 2005). Grasses tend to dry faster than legumes because of their lower stem-to-leaf ratio (Macdonald and Clark, 1987). Among legumes, both alfalfa and birdsfoot

trefoil dry faster than red clover; among grasses, tall fescue dries four times faster than perennial ryegrass (Rotz, 1995a). Mixing legumes (alfalfa, red clover, or birdsfoot trefoil) with grasses (smooth bromegrass) can increase the drying rates of the legume component, likely by modifying the swath structure (Collins, 1985). Appropriate management decisions during wilting can significantly accelerate the swath drying rate, which is critical in areas with poor drying conditions.

Effect of swath width

Swath width is one of the most significant factors affecting the drying rate, with narrow swaths drying much more slowly than wider swaths (Kung et al., 2010). Since increasing the field drying rate is critical to limit nutrient losses due to plant respiration and unexpected rainfall, swaths covering 80% to 100% of a cut area are recommended (Undersander, 2013). Wide swaths expose a greater surface area to solar radiation and can be more porous, promoting air exchange that reduces humidity in the swath micro-environment (Shinners and Friede, 2017). Savoie et al. (1984) measured the drying rate of wide swaths (1.45 m) and narrow windrows (0.89 m) of timothy across four maturity stages (early boot, heading, anthesis, and seed stage). When cut at early boot (18.4% DM), a narrow windrow took more time to reach 60% DM compared to a wide swath (71.1 vs. 54 h, respectively), but swath width had no effect at heading (23.4% DM), anthesis (30.8%), and seed stage (40.9%) in narrow vs. wide swath ($\bar{x}$= 25.4, 51.7, and 4.6 h of wilting, respectively). The authors suggested that differences across maturities were due to a decreasing moisture concentration from the earliest to the latest maturity, and consequently swath width had little effect as maturity increased (Rotz and Muck, 1994; Siles et al., 2015).

Swath manipulation

Swath manipulation is a key step that helps to expedite the forage drying rate and makes this process more uniform across the swath (Rotz and Savoie, 1991). This can be done with various equipment, including tedders, inverters, mergers, and rakes.

Tedding

Tedders use rotating tines to stir and spread wilted forage on the field surface (Collins and Owens, 2003). A rotary and a fluffer tedder are the two main designs, with the latter being preferred to speed up the drying rate, especially after rainfall events. This is because a fluffer has parallel rake bars that engage the windrow without changing its width (Savoie and Beauregard, 1990) as opposed to a rotary tedder that stirs and spreads the swath, altering its width (Rotz et al., 2020). Tedders are estimated to increase the drying rate by 30% (Savoie and Beauregard, 1990). Hartfiel and Digman (2021) found that after 24 h, tedded alfalfa (first cut) resulted in a higher DM concentration than the untedded one (42 vs. 31% DM, respectively). The same trend was observed in the second and third cuts (51 vs. 35 and 53 vs. 48%, respectively). In addition, they reported that the ash concentration in tedded alfalfa (first cut) was similar to the untedded treatment ($\bar{x}$= 10.5% of DM), but it was different in the second and third cut (10.7 vs. 11.7 and 9.4 vs. 10.8%, respectively). The authors explained that for the second and third cuts, soil particles adhered more to the heavier untedded narrower swaths relative to the lighter, tedded swaths. However, no explanation was provided for the first cut results. Tedding can reduce the field drying time, but it also causes field DM losses (Hartfiel and Digman, 2021). For instance, Rotz et al. (2020) reported that when swaths with 70% DM are

tedded, field DM losses can be up to 10%. However, if tedding is done when the swath has <60% DM, the field DM losses are approximately 3% (Rotz and Shinners, 2007). Due to the beating action of tedders, legume crops tend to suffer more leaf losses and, consequently, more CP losses since leaves have higher CP levels (Rotz and Muck, 1994; Du et al., 2021). Savoie (1988) reported that when alfalfa was tedded with a rotary tedder at a DM concentration of 40, 24, and 18%, the field DM losses were 5.9, 3.3, and 3.4%, respectively, while tedding timothy at 50, 36 and 25% DM resulted in losses of 1.2, 2.1, and 0.8%, respectively. Due to the tendency for higher leaf losses with legume hays, Rotz et al. (2020) recommended tedding legume forages within 4 h of mowing to reduce leaf losses (or during the night/sunrise), while grasses can be tedded about 24 h after mowing. Overall, tedding tends to be more compatible with grass hay, where leaf losses are of less concern; its utilization on legumes must be carefully managed, especially when the crop DM concentration is >50% (Rotz, 2003a).

Swath inversion

Inverters provide a gentler alternative to manipulate the swath (Rotz and Muck, 1994) as they swap the moist bottom of the swath with the dry top (Rotz and Shinners, 2007; Rotz et al., 2020). Inverters are configured quite like mergers but have a narrow pick-up that works better with narrow swaths (Savoie and Beauregard, 1990). This equipment results in limited field DM losses, between 0.7-1.5% (Rotz and Savoie, 1991), but only in a 15% drying rate increase on the same day of treatment (Savoie and Beauregard, 1990). Regarding machinery and operating costs, it is important to consider that, compared to tedders, inverters are more expensive and cost about 20% more in labor and fuel (Rotz et al., 2020).

Raking and merging

Rakes and mergers are typically used to form the windrows needed for optimal baling, with the former being more common (Rotz, 1995a). Parallel bar, wheel, and rotary rakes are the most common rake designs (Neu et al., 2017). The first two designs are similar in that both roll the swath into narrower windrows with limited ventilation, while rotary rakes, with their horizontal rotating tines, form a fluffy and aerated windrow that allows for better hay drying (Schuler, 2003). Although inconsistent differences in drying rates have been observed among rake designs (Savoie et al., 1981), raking typically increases the drying rate by 10-20% on the day of baling (Rotz, 1995a; Rotz, 2003b), and the resulting field DM losses range between 1-20% (Rotz and Muck, 1994). Hay should be raked when the DM concentration of the swath is between 60-70% to avoid high field DM losses and a slow drying rate (Rotz et al., 2020). It is also important to keep in mind that rakes work better when swaths are not spread out wide because gathering then becomes increasingly difficult (Rotz et al., 2020). Mergers are better suited to wide swaths because their wide pick-up allows them to combine swaths of different widths into a single windrow (Schuler, 2003). With a wide pick-up and a conveying system, mergers result in lower leaf loss and less inclusion of rocks and soil particles in the windrow compared to rakes, which must have contact with the soil to conjoin the swaths (Neu et al., 2017). Neu et al. (2017) reported a lower concentration of exogenous ash in first cut alfalfa using a merger (11.1% of DM) compared to raking with a wheel rake (15.3% of DM); similar results were also observed in the second cut (10.5 vs. 13.8%, respectively). The DM concentration of the swath may also influence the inclusion of ash into the windrow. Digman et al. (2013) evaluated the effect of swath DM (60.2 and 38.8%) on the

concentration of ash in windrows generated after merging. Their results showed that a higher swath DM (60.2 vs. 38.8%) resulted in a slightly higher ash concentration (8.13 vs. 7.99% of DM, respectively). The authors speculated that when swaths have a high DM concentration, the soil under and around the swath will be drier and more crumbled, making it more likely to contaminate the windrow generated during merging. However, such small differences are unlikely to be of practical concern in the field.

Environmental factors affecting swath drying

Field drying is affected by ambient weather and soil conditions, with the former being more influential than the latter (Rotz, 1995a). Rotz and Chen (1985) ranked the different environmental and crop variables known to affect the rate of field drying of alfalfa according to their average correlation coefficient with drying rate. They found that solar radiation (r= 0.61) was the single most correlated factor, followed by swath surface temperature (0.45), ambient temperature (0.35), vapor pressure deficit (0.34; VPD), crop moisture (0.22), RH (-0.21), swath density (-0.18), vapor pressure (0.15), soil moisture (-0.15), and wind velocity (0.11). However, the authors cautioned that high multicollinearity among variables in their dataset may have obscured relationships. Khanchi and Birrell (2017) reported that when switchgrass was harvested at seed development and seed shattered stage, solar radiation was the most important variable influencing the drying rate, with correlation coefficients of 0.5 and 0.49, respectively. They also reported that the VPD was positively correlated with the drying rate, but their correlation coefficients differed across day and nighttime conditions. During daytime, the correlation coefficients between VPD and drying rate for seed development and seed shattered stage were 0.24 and 0.38, respectively. However, these coefficients increased during nighttime to 0.83

and 0.85, respectively. Overall, solar radiation was reported to be the most important factor affecting the drying rate of switchgrass during daytime but for nighttime conditions, VPD was the most important variable.

On sunny days, a wide swath dries faster than a narrow one because it intercepts more solar radiation, accelerating the drying rate (Rotz, 1995a). When all stomata of the cut forage are open, the drying rate is rapid on the top layers but slow in the bottom part of the swath where RH is high and solar radiation is low. Subsequently, an increase in temperature at the top part of the swath induces the stomata to close (Thompson, 1981). This, in turn, increases the VPD in the swath and raises the evaporation rate in the bottom layers where stomata remain open. The drying process continues until the stomata of the bottom layers close (Thompson, 1981). Thus, the two main factors affecting the VPD are the swath temperature and the RH of the surrounding air (Rotz and Chen, 1985). However, RH has a negligible effect on the drying rate under good drying conditions, such as on sunny days with RH <60%. When energy from the sun strikes the forage, the plant temperature increases up to 20°C above air temperature, causing an increment in the vapor pressure (Dernedde, 1980). Hence, the drying rate of swaths increases. For instance, Rotz and Chen (1985) reported that increasing the VPD by 4.5 kPa increased the drying rate by 28%. On the other hand, plant type and swath structure can reduce the drying rate (Rotz, 1995a). Khanchi and Birrell (2017) found in switchgrass that windrow density was negatively correlated with drying rate during the day (-0.38) and night (-0.1).

In poor drying conditions, such as humid and rainy areas, a high RH and dew presence are undesirable during wilting as they reduce the rate of swath drying (Collins and Moore, 2017). Furthermore, in humid areas, if there is limited solar radiation, the

temperature of the forage may become similar to the surrounding air, causing a low VPD (Rotz, 1995a). Gupta et al. (1989) reported that a low VPD during nighttime causes a decrease in the drying rate of swaths. This is because the temperature difference between the forage and ambient air is relatively small, resulting in a low VPD that slows the drying rate of swaths (Rotz, 1995a). Dew presence in humid areas also affects the drying rate, especially in thick swaths, because they absorb more moisture on the top layers compared to the bottom. In contrast, thin swaths tend to have a more uniform moisture concentration across layers (Gupta et al., 1989). Dyer and Brown (1977) speculated that the rewetting caused by dew during swath wilting is also a function of the swath DM concentration. Although no studies could be found assessing the effect of dew across forage families, the amount of moisture from dew formation absorbed by the forage depends on crop DM, cuticle thickness, leaf-to-stem ratio, and stem thickness (Rotz, 1995a). Soil moisture and temperature also affect swath drying, with the former being the most influential during wilting (Rotz, 1995a). According to Rotz and Chen (1985), an increase of 15% in soil moisture reduces the drying rate of alfalfa swath by 20% because high soil moisture creates a wet surface on the underside of the cut forage. Thus, wet conditions under the swath cause moisture migration from the soil to the swath and reduce its drying rate.

Baling Phase

Once forage has been dried to a suitable moisture concentration, it must be gathered, compressed, and packaged for handling and storage (Rotz et al., 2020). Hay is typically packed into round, rectangular, or small square bales of varying weights to facilitate handling. The differences in bale weight, shape, and density result in distinct

moisture thresholds at baling to avoid nutrient losses during storage (Rotz and Shinners, 2007). Figure 1 shows compiled information from baler manufacturers (collected in December 2021), and Collins and Coblentz (2007) about the range of bale dimensions and weights and moisture thresholds suggested for bale types. Field losses during baling typically vary between 2-5% (DM basis) for small rectangular bales (20-30 kg) (Rotz and Muck, 1994), but those for round bales (227-908 kg) can surpass 10%, especially if bale moisture falls <15% (McCartney, 2005; Collins and Moore, 2017). Because heavier and denser bales need to be dried more extensively, they will suffer more field losses than lighter bales, especially in legumes (McCartney, 2005; Collins and Moore, 2017). Hay producers typically prefer round bales because they reduce the costs of labor, infrastructure, and equipment (Huhnke, 2003). Field DM losses can be reduced by increasing ground speed during baling and forming larger windrows (Grisso and Fike, 2020). In this respect, Anderson et al. (1981) evaluated various field harvesting practices using large round bales of alfalfa hay and found that DM losses at baling were 14, 12, and 5% for single, double, and triple-sized windrows, respectively. However, due to the field losses incurred at each raking needed for double- and triple-sized windrows (5% each event), the overall field losses (including wilting, raking, and baling) were similar across windrow types ($\overline{x}$= 20% of swath yield, DM basis). If mergers had been evaluated in that study instead of rakes, the advantages of producing larger windrows might have been maintained throughout, when considering the overall field losses. Anderson et al. (1981) also reported that windrow size (single, double, and triple-sized windrows) affected the baler feed rate (93, 193, 275 kg/min, respectively), but increasing the field speed from 5.6 to 8.1 km/h did not. Larger windrow sizes increased baler capacity, lowered bale

density, and reduced baling DM losses. The baler capacity, measured as the time required to roll and tie bales of a given weight, increased from 4.1 to 10.7 metric ton/h when the feed rate tripled due to less time spent tying bales at high baling densities. Management decisions and equipment availability during baling will ultimately determine bale weight, shape, density, and size, which are crucial factors affecting the susceptibility to spoilage during storage, depending on moisture concentration at baling (Rotz and Muck, 1994). Vurarak et al. (2017) evaluated the impact of small cylindrical and prismatic balers on the quality of mixed clover hay. Despite the target bale weight being the same for both balers (25-30 kg), the round bales had a lower bale weight (19.2 kg) than the square bales (22.6), but they had a higher density (126.6 vs. 108.7 kg/m^3, dry basis respectively). Furthermore, more leaf losses occurred from the outer surface of cylindrical bales during baling, and this was reflected in a lower CP concentration in cylindrical vs. prismatic bales (13.3 vs. 15.6% of DM).

Currently, manufacturers of both round and square balers offer a variety of features designed to improve hay quality and preservation during storage. Most companies offer features such as additive applicators (Rotz et al., 2020), internal cutting systems that help to increase bale density by 1 to 5% (Shinners and Friede, 2018), and density, moisture, and weighing sensors that provide accurate readings throughout the entire baling process. From data collected in December 2021, Table 1 summarizes the number of models offering these special features for major baler manufacturers.

Baling in arid environments

In arid regions, baling losses due to excessive dryness of plant tissues are the primary concern. In order to reduce these losses, hay is often baled during the night and up to shortly after sunrise. At those times of the day, the RH is typically at its highest, and there is a high chance of dew accumulation in the swath (Muck and Shinners, 2001; Brown, 2015). In arid regions, low RH (<60%) facilitates the loss of moisture from the swath, sometimes to an excess (≥85% DM), which can result in significant field DM losses (>8%) due to leaf shattering when the swath is raked and baled (Rees, 1982). Consequently, in these regions, raking and baling are typically performed when proper ambient conditions are conducive to the rewetting of swaths with dew (Brown, 2015). However, sometimes this is not enough to rehydrate hay to the extent that it can reduce leaf brittleness significantly, which forces producers to actively reintroduce moisture by using equipment like sprayers or irrigation systems (Muck and Shinners, 2001). Because of the limitations of relying on dew and the inadequacy of irrigation devices for rehydrating swaths, Staheli (1998) proposed a system of hay re-hydration that applies steam to windrows at the baler pick-up mechanism. Shinners (2014) reported lower field DM losses when large square bales of alfalfa were prepared using a steam re-hydration mechanism compared to natural dew re-hydration (0.5 and 1.2% DM loss, respectively), even though the steam-rehydrated bales had a higher DM concentration at baling (91.4 vs. 88.8%, respectively). Although not quantified, the authors observed that leaf retention on stems was superior on stem-rehydrated bales than the dew re-hydration treatment. Moreover, greater leaf retention in bales prepared using steam re-hydration technology helped achieve higher bale densities than dew re-hydration (272 vs. 226 kg DM/m^3, respectively).

The steam softens leaves and stems, making them easier to flatten with the plunger. The nutritional composition did not differ between the two treatments. Notably, the levels of acid detergent insoluble crude protein were no different ($\overline{x}$= 3.73% of CP), indicating that the temperature increase due to steam application was insufficient to cause heat damage to plant proteins. In a second experiment, Shinners (2014) assessed the effects of stem re-hydration on alfalfa large square bales and observed a lower bale DM compared to the no re-hydration (87.3 vs. 90.8%, respectively) and lower DM losses at baling (0.9 vs. 2.3%). Bale nutrient composition showed an increase in CP (21.2 vs. 20.5% of DM) and decrease in NDF (39.6 vs. 40.8% of DM), ADF (25.5 vs. 26.1% of DM), and acid detergent insoluble crude protein (4.06 vs. 4.54% of CP) in stem-rehydrated bales compared to no-rehydrated ones, respectively.

Storage Phase

Dry matter concentration is the most important factor influencing hay nutrient losses during storage. Between 70-85% DM, aerobically stored hay nutrient losses will occur mainly due to the activity of aerobic microbes such as molds (Duchaine et al., 1995b; Coblentz et al., 1996). Plant respiration is only a significant contributor to nutrient degradation if the hay has <70% DM (implausible) and is stored at ambient temperatures >20°C (Lowell, 1995). During storage, bales can have DM levels below the recommended thresholds if 1) windrows at baling are wet or 2) adequately dried bales are exposed to rainfall or soil moisture before or during storage (Collins and Moore, 2017). The amount of soil moisture absorbed by bales can be mitigated if suitable surfaces, such as wooden racks, are used to prevent direct contact with the soil, especially after rainfall events (Collins, 1995). Figure 2 summarizes the typical range of DM losses during storage

across various storage conditions. The highest storage losses occur when bales are stored outside on poorly drained soils without any protection and exposed to rainy conditions, while the lowest losses are observed in bales protected by a pole barn or hoop structure.

Key indicator variables that are practical and useful to monitor spoilage during storage include bale temperature (Coblentz et al., 2000) and visual moldiness (Roberts, 1995). Typically, spoilage during storage will result in a decrease in non-structural carbohydrates (Coblentz et al., 1997) and true protein (Rotz and Muck, 1994), and an increase in fiber and non-protein N (Collins and Coblentz, 2007a). It is widely reported that bale temperature changes during storage are highly correlated with the moisture concentration of hay at baling (Coblentz et al., 2004), and both variables are crucial to determining the extent of quality loss during storage (Coblentz and Hoffman, 2009). When hay is stored either inside a shelter or outside, the moisture concentration dynamics of bales will be influenced by forage family, bale type and density, the type of wrapping used, and the arrangement of bales in the storage place (Ivanovs et al., 2013; Collins and Moore, 2017). Equally important are the environmental conditions during storage and how all these factors interact with the storage method (Collins and Moore, 2017).

Bale wrapping type

Collins and Moore (2017) reported that when twine- and net-wrapped bales were stored outside on well-drained soils and exposed to rainy conditions, the storage DM losses reached values of ~30% and ~27%, respectively (Collins and Moore, 2017). These values increased further when twine- and net-wrapped bales were stored outside on

poorly drained soils besides rainy conditions (~45% and ~37% DM, respectively) because water accumulated on the ground surface was absorbed by the bales, increasing microbial activity (Collins and Moore, 2017). However, no differences were observed in storage DM losses (~3.5%) between twine- and net-wrapped bales stored in pole barns or hoop structures (Collins and Moore, 2017). Thus, despite net-wrapping reducing DM losses in bales stored outdoors, relative to twine, the difference is not large enough to suggest that net-wrapped bales could be stored outdoors without any cover.

Alternative wrapping strategies have been explored to allow producers to store hay outdoors without a cover. For example, Reiter (2019) evaluated alfalfa hay (bud stage) baled with different wraps: net-, twine-, and B-wrap. The latter is a breathable bale wrap that sheds precipitation, keeps moisture from going into the bale, and allows water vapor to escape from the bale through microscopic pores. After storage outdoors on wood pallets for 365 d, the DM losses of twine-, net-, and B-wrapped bales were 8.8, 6.6, and 1%, respectively. The NDF and ADF of twine- and net-wrapped bales were not different ($\bar{x}$= 60.2% and 42.2% of DM, respectively) but were much higher than B-wrapped bales (48.5% and 33.8% of DM, respectively). Also, B-wrapped bales had higher NSC levels than twine (11.7 vs. 8% of DM, respectively), while net wrap (9.3) was no different from B- and twine-wrap. Authors speculated that the higher NSC levels observed in B- vs. twine-wrapped bales were due to lower rain penetration into the bale, resulting in less NSC leaching during storage. Crude protein concentration was not different across all wraps ($\bar{x}$= 14.4% of DM), most likely because this nutritional component is less soluble, but further research into the effects of bale wrapping on protein fractions should give more accurate insight into bale protein dynamics. In a continuation of the study, Reiter et al.

(2020) showed that after 16 mo of outdoor storage, net-wrapped bales had a lower DM concentration compared with the B-wrap (84 vs. 87%, respectively) because the net wrap allows more moisture to escape from the bales. The DM of twine-wrapped bales (85%) was not different from either the net or B wrap. The NDF concentration was greater in twine- vs. B-wrapped bales (49 vs. 46% of DM, respectively), but both were not different relative to net-wrapped bales (48). According to the authors, the difference in NDF between B- vs. twine-wrapped bales is explained by a reduced penetration of environmental moisture into the B-wrapped bales. This in turn, resulted in less microbial activity and loss of soluble nutrients, which limited the increase of NDF. Mold counts of net- and twine-wrapped bales were not different ($\overline{x}$= 5.9 x 10^6 log CFU/g), but they were higher than B-wrap (4.8 x 10^4). Finally, CP ($\overline{x}$= 14.7% of DM) and ADF ($\overline{x}$= 32% of DM) were not different across wrap types. Thus, the B-wrap is a promising technology that better sheds precipitation and conserves DM and quality in bales relative to twine and net wraps, especially when producers need to store bales uncovered outdoors for a significant period.

Recently, Coblentz et al. (2021) evaluated the effects of storing alfalfa-orchardgrass bales with a DM of 74.2% at baling, under anaerobic conditions (wrapped with 7 layers of plastic film). This approach is the same as used in baleage (40-70% DM) (Muck et al., 2020). However, it is important to keep in mind that the fermentation of sugars to lactic acid, and consequently acidification, will decrease significantly above 55% DM and completely stop above 70% DM (Coblentz and Akins, 2018; Muck et al., 2020). Coblentz et al. (2021) found that wrap-sealing bales reduced the maximum internal bale temperature and heating degree days (HDD) compared to unsealed bales during storage

(41.5 vs. 61.6°C and 111 vs. 732°C-d above 30°C, respectively) because wrapping with plastic film maintains anaerobic conditions within the bale. After 84 d of outdoor storage, the final DM was lower in sealed vs. unsealed bales (74.7 vs. 79.9%, respectively). The WSC (7.61 vs. 5.04% of DM) and TDN (61.5 vs. 56.9, respectively) were higher in sealed vs. unsealed bales, while NDF (47.4 vs. 52.6), ADF (27.2 vs. 29.6), and ADIN (7.94 vs. 13.9) were lower in sealed vs. unsealed bales, respectively. This is because wrapping in the plastic film helps to limit aerobic spoilage, resulting in sealed bales having superior nutritive value compared to unsealed bales.

Bale moisture

Recently, Coblentz et al. (2020) suggested that the DM threshold for small rectangular bales (~45 kg, fresh basis) should be 80%, while for heavier bales (~500 kg, fresh basis), the threshold should be 82% for large round bales and 84% for large rectangular bales. Figure 3, prepared using a dataset collected for our recent meta-analysis on hay preservatives (Killerby et al., 2022b), shows that round bales are more susceptible to spoilage at the same DM concentrations. Moreover, Collins and Moore (2017) suggested that high-density bales should have a higher DM concentration to "safely" reduce DM losses during storage. For instance, they recommend that high-density (224-256 kg/m^3) large rectangular bales should be stored at 84-88% DM, while round bales at 160-208 kg/m^3 density should be stored at 82% DM, and small low density (128-176 kg/m^3) rectangular bales at 80% DM or above. Denser bales are especially favored for hay exports, and considering the increasing relevance of this market (Banta, 2010), it is important to keep in mind the increased susceptibility of high-density bales to spoilage caused by excess moisture (Collins and Moore, 2017). The water vapor coming

from bales that were stored with excess moisture can condense on the container walls during maritime shipping and then fall back onto the outer bale layers causing mold damage. This, in turn, will cause the rejection of the shipped hay upon arrival (Sokhansanj, 1996). Water condensation can also occur in high-moisture bales wrapped with plastic because the plastic will limit residual moisture losses from the bale during storage, and that moisture can condensate right under the surface resulting in microbial growth (Mirzaee and Bishop, 2010).

Heat damage can be significant in hay bales <80% DM (Coblentz et al., 2004), and its extent is heavily influenced by bale weight and density (Hancock, 2020). Low-DM bales are prone to heat accumulation, DM losses, Maillard's reaction (Nursten, 2005), loss of nutritive value (Collins, 1995), and the presence of mycotoxins (Roberts, 1995). Typical storage heating patterns of hay baled between 70-75% DM show a peak of 54-60°C after 4-6 d of storage that eventually decreases to 27°C after 2-3 wk, whereas the temperature of hay baled at 85% DM was 27°C at the start of storage and decreased to 24°C after 6 wk (Collins and Moore, 2017). The authors did not disclose the environmental temperature in the latter study. Bales with lower DM and higher density can reach higher temperatures and have a prolonged heating curve. The heat generated in the initial stages of storage is due to residual plant respiration (Lowell, 1995) and microbial activity (Supplemental Fig. S1). Internal hay bale temperatures above 40°C are of concern in stored forage (Coblentz et al., 2004), and frequent monitoring of bale temperature is advised when >50°C (Collins and Moore, 2017). Maillard reaction will occur >55-60°C (Van Soest, 1982), and spontaneous combustion is likely >70°C (Coblentz et al., 2004).

Forage type and bale size

It has been reported that legume hay is more susceptible to spoilage than grass hay during storage, in part because of higher levels of water-soluble constituents (Collins, 1995; Collins et al., 1997). Verma and Nelson (1983) indicated that legume-grass mixed hay bales are more susceptible than pure grass bales when stored in conditions conducive to spoilage (outdoors). Coblentz et al. (2004) also observed that at the same bale HDD accumulation (i.e., 600°C-d), small conventional alfalfa hay bales had lower DM recovery (92%) than bermudagrass bales (98%; *Cynodon dactylon*).

Coblentz et al. (2020) compared the heating characteristics of 1.2 m and 1.5 m diameter bales of the same forage type (alfalfa-orchardgrass mix), DM level ($\overline{x}$= 79.4%), and density ($\overline{x}$= 166 kg DM/m^3). The 1.5 m diameter bales heated to a greater extent (46.1°C maximum temperature) and accumulated more HDD (334°C-d above 30°C) than the 1.2 m bales (41.6°C and 106°C-d, respectively). The application of a propionic acid-based preservative ($\overline{x}$= 0.34% of bale weight) successfully preserved more TDN and prevented the increase of NDF in 1.5 m bales compared to untreated bales but had no effects on 1.2 m bales, indicating that the effectiveness of the preservatives on heating variables may be affected by the size of the bale (Coblentz et al., 2020). In addition, our recent meta-analysis on hay preservatives showed that legumes were less responsive to organic acid-based preservatives than grasses, most likely due to a higher buffering capacity (Killerby et al., 2022b). Therefore, it is recommended that baling and storage recommendations should be more closely enforced for legume hay, as they are likely to be more susceptible to spoilage losses during storage relative to grasses. Further studies should be conducted to assess the extent of these differences.

Storage methods

The approach that is taken to store bales significantly impacts the overall nutrient losses in the hay production chain. For instance, the storage DM losses of net-wrapped round bales stored outdoors with no protection were 30-45%, while only 5-10% losses were reported when the same type of bales was covered under a plastic tarp (Coblentz, 2009; Hancock, 2020). Similarly, Shinners et al. (2009) showed that when round bales are stored outside without protection, including not being elevated on rock pads or wood pallets, weathering losses can be up to 35% (DM basis). Evidently, protecting bales from rain and soil moisture is critical to avoid reintroducing moisture into the bale, which can sustain extensive aerobic spoilage (Rotz et al., 2020).

For bales stored indoors, it is also important to facilitate moisture loss from hay bales during storage because bale DM values <88% will sustain increasing microbial and plant enzymatic activity that will generate water vapor (i.e., “hay sweat”) that needs to be removed rapidly by an optimal ventilation design (Hancock, 2020). Hay barn features that are known to be beneficial include (Hancock, 2020): 1) ventilated roof gable ends and ridge, 2) ventilated wall bottoms, 3) a layered floor with gravel, pallets, or gratting covered with a layer of loose hay, 4) two doors, one facing south and another one east. Side walls reduce UV light damage to hay pigments (Hancock, 2020), and provide extra protection from rain during severe storms. Pallets have the advantage of facilitating ventilation at the bottom of the bale (Hancock, 2020).

Certain bale types are more susceptible to storage nutrient losses and require more protection from environmental moisture. For instance, rectangular bales tend to be

stored indoors or protected from precipitation because their flat surfaces do not shed water (Collins and Moore, 2017). Round bales shed water readily, particularly in the initial stages of storing when the outer layer of the bale forms a thatch that prevents the water from penetrating the bale (Ball et al., 1998). Bales that are uniformly packaged and dense thatch well, especially if they are produced from fine-stemmed, leafy, weed-free crops such as bermudagrass or tall fescue (Ball et al., 1998). However, once the outer layer is weathered, the bale is more easily penetrated by rain and will not dry as rapidly thereafter (Rotz et al., 2020). Due to the cylindrical shape of round bales, outer layer weathering will lead to a substantial percentage of bale damage. Collins et al. (1997) reported that even a 5 cm weathering layer on a 1.2 x 1.2 m bale represents 16% of the bale volume weathered, even though less than 7% of the bale diameter is affected, and if the weathering process continues, the bale volume affected can reach up to 56% (Supplemental Fig. S2).

In weathered bales, storage DM losses of up to 40% can occur at the bottom if it is in direct contact with the ground, especially if the soil is poorly drained (Collins et al., 1997). Moreover, weathered hay is much lower in nonstructural carbohydrates and higher in fiber than unweathered bales (Collins et al., 1987). For net-wrapped bales covered with a tarp, Lemus (2009) reported that moisture permeates to a greater extent when placed on the ground compared to pallets. It was reported that, after 11 mo of storage on pallets, less than 50% of the biomass had a DM concentration <80%. Conversely, when round bales were stored on the ground, ~80% of the bale biomass had a DM concentration of <80%, with the bottom half of the bales reaching 70% DM. Therefore, storing round bales on pallets helps to reduce storage DM losses by up to 40% (Lemus, 2009).

An alternative outdoor storage method is stacking round bales in a “pyramid”, which allows bales to be covered with tarps for protection against precipitation (Collins and Moore, 2017). Although covering stacked round bales with heavy plastic tarps restricts heat and moisture loss from the top of the stack, more importantly, it protects bales from rain damage (Collins and Moore, 2017). According to Collins et al. (1995), using a plastic cover reduces storage DM losses to about 8%, similar to hay storage in a barn (6%). This type of protection is sometimes implemented for square and rectangular bales, especially when they are stacked outdoors (Rotz et al., 2020).

Equilibrium moisture

Equilibrium moisture is the final moisture concentration at which no transfer occurs between the crop and the environment (Moore et al., 2020). During storage, hay DM concentration stabilizes at about 90% in arid climates and about 85% in humid climates during storage (Shewmaker, 2013). The time it takes the bales to reach equilibrium moisture during storage will be affected by forage family (Coblentz, 2020), storage factors such as ventilation and coverage, environmental factors such as RH and temperature (Pitt, 1990), and bale characteristics such as density and size (Collins and Moore, 2017). Under wet conditions, the RH is the most influential factor affecting bale moisture re-absorption (Rotz, 1995a). According to Hill et al. (1977), alfalfa hay moisture re-absorption will occur to a greater extent in humid conditions (RH >70%) than in conditions where RH is <60%. The authors reported that the equilibrium moisture for alfalfa was ~20% when the RH was 70%, but it increased rapidly to >40% for RH values >90%. Atwal (1987) compared large round alfalfa bales baled at 82.2%, 76.4%, and 68.9% DM (high, medium, and low DM, respectively). After 8 wk of storage, the DM levels were 83.6, 80.7, and

83.6%, respectively. However, after 36 weeks, the DM of the three bale groups had all equilibrated at $\bar{x}$ = 84.6%.

Spontaneous combustion

Numerous factors contribute to the self-heating of aerobically-stored hay, including the DM concentration at the beginning of the storage phase, bale type and density, environmental conditions, storage conditions, and the use of preservatives (Rotz et al., 2020). Among these, the DM concentration at the beginning of the storage is critical because bale heating will gradually increase below 80-84% DM (depending on bale type) and reach maximum levels below 75% DM (Collins, 2004). To the best of our knowledge, only two studies have directly studied spontaneous hay combustion. Festenstein (1971) placed 125 g of hay in 1-L Dewar flasks kept in an incubator that tracked hay temperature in real-time to avoid differences between hay and oven temperature. It was reported that when the temperature of moist hay (56.5% DM) rose above 70°C, oxidative chemical reactions started to occur. Above such temperature, Festenstein (1971) speculated that oxidative chemical reactions take over and sustain hay self-heating, with negligible contributions from microbial and residual plant activity due to the inactivation of most enzymatic activity. Similarly, Ramírez et al. (2010) studied self-heating in stored ground feeds and reported that the initial rise in temperature results from biological activity that increases feed temperature up to 75°C, followed by chemical oxidation reactions that sustain self-heating up to 150°C.

The release of soluble fractions (water-soluble carbohydrates) and hydrocarbon radicals when the hay temperature is above 70°C is considered a preliminary step to

spontaneous combustion because when these compounds are chemically oxidized, they release considerable quantities of heat, especially at 90°C (Festenstein, 1971). The author also speculated that at 70°C, hemicellulose and cellulose depolymerization contributed to self-heating. However, more recent research related to biofuels indicates that the thermal degradation of cellulose and hemicellulose occurs above 275 and 180°C (Kim et al., 2006), respectively, at which point these polymers release hydrocarbon radicals (Dietenberger and Hasburgh, 2016; Qin et al., 2018). Thus, it is more likely that above 70°C, most self-heating comes from the chemical oxidation of water-soluble components (e.g., fructans) rather than structural polysaccharides.

Currie and Festenstein (1971) conducted another study under the same experimental conditions as Festenstein (1971) but with a fixed RH (97±1%) and a tube inserted through the bottom of the Dewar flask that allowed the continuous aeration of hay (35 cm^3/min) with humidified air (97±1%). They reported that for hay to self-heat beyond 100°C, the pumped air needed to be changed from humidified to dry (undisclosed RH for the dry air). This, in turn, dehydrated the hay and allowed it to increase from 70°C to beyond 100°C. Similar work investigating corn grain fires also showed that smoldering fire velocity was reduced with increasing levels of moisture (from 0 to 15%), especially as particle sizes increase (Rosa et al., 2020). Thus, spontaneous hay combustion seems to occur only when most of the initial hay moisture has been vaporized at or near 100°C (Currie and Festenstein, 1971). Current extension guidelines on hay fire prevention indicate that the risk of spontaneous combustion is imminent when hay temperature rises above 80°C, at which point the removal of hot hay needs to be assisted by firefighters (Hancock, 2015).

Feeding Phase

Allowing livestock unrestricted access to hay bales results in over-consumption, trampling, soiling, and animals using hay as bedding (Gaebe et al., 2000). Under such inadequate conditions, DM losses during hay feeding can be as high as 45%, especially if combined with rain damage during feeding (Clark et al., 2008; Kallenbach, 2022). In order to reduce feeding losses, multiple hay feeder designs have been developed over the years, including ring, cone, cradle, and trailer feeders (Gaebe et al., 2000). In the case of square bales, feeding racks with solid bottoms or using fences to limit access to the bales can also reduce feeding losses (Kallenbach, 2022). If possible, bales should be protected from rain damage while being fed.

Buskirk et al. (2003) evaluated the effect of hay feeder design on the feeding DM losses of alfalfa round bales fed to beef cows. After 7 d of feeding, the DM losses for cradle and trailer designs were no different from each other ($\overline{x} = 13\%$) but were higher than the ones observed for ring (6.1) and cone (3.5) designs. According to the authors, cattle eating from a cone or ring feeder mimic more closely grazing behavior than those eating from the cradle or trailer feeder, hence lowering the feeding DM losses. Using grass round bales, Comerford et al. (1994) reported that feeding DM losses were higher in ring vs. cone feeders (8 vs. 1.9%, respectively). Thus, it is crucial to consider these differences when selecting a hay feeder so nutrient losses can be minimized during feeding.

Microbiome Changes across the Haymaking Process

Standing forage crops contain various fungi and bacteria that are part of their phyllosphere (Roberts, 1995). According to Magan and Lacey (1987), *Alternaria*, *Cladosporium*, and *Fusarium* are fungi genera generally found in standing forage, while *Aspergillus* and *Fusarium* can tolerate drier conditions and can also grow on wilted forage. Also, actinomycetes and other bacteria are commonly found in fresh and wilted forage, particularly Gram-negative rods (Kaspersson et al., 1984). For instance, Hu et al. (2020) found that members of the *Proteobacteria* phylum were dominant (70-90% relative abundance) in alfalfa wilted to 30-35% DM. More specifically, that study described the dominance of the *Sphingobium* (46.26% relative abundance), *Acinetobacter* (6.89%), and *Enterobacter* (6.24%) genera. Moore-Colyer et al. (2020) reported that after an undisclosed number of days in storage, the most abundant bacterial genera in hay bales made of a grass mixture were *Pseudomonas, Sphingomonas,* and *Curtobacterium*. Unfortunately, that study did not assess the fungal community. In another study, Kennang Ouamba et al. (2022) also evaluated the diversity of bacterial communities and total fungi and bacteria counts of stored hay samples (forage family undisclosed) taken from 24 dairy farms over two yr. At the genus level, *Pantoea*, *Sphingomonas*, *Curtobacterium*, *Methylobacterium*, and *Pseudomonas* were the most dominant bacterial genera across both sampling periods. Total bacterial and fungal loads were not different in the fall ($\bar{x}$= 12 log copy number/fresh g), but in spring, the former was more numerous than the latter (13 vs. 11.9). The authors did not explain these results.

Several environmental factors influence hay microbiota dynamics, including water and nutrient availability, temperature, pH, gaseous composition of the environment, and

interaction among microorganisms (Wittenberg, 1997). Moisture concentration has the greatest influence on the microbiome of hay (Roberts, 1995). As moisture levels increase above the recommended thresholds, plant respiration and microbial activity intensify (Wood and Parker, 1971), which causes bale heating (Coblentz, 2020). Therefore, bale temperature and moisture influence each other, and it is almost impossible to independently assess their effects on hay microbiome dynamics.

Gregory et al. (1963) assessed the microbial count dynamics of timothy and fescue square bales baled at low (70%) and high (85%) DM concentrations. This is one of the few publications that has studied microbial count dynamics across hay production phases. The initial mold count in the fresh stand was 4.5 log CFU/dry g, which decreased below 2 log after two days of wilting. In the hay bales cured to 70% DM, mold counts increased to 7 log CFU/dry g after 7 d and fluctuated between 7 and 6 during the 77 d of storage. At the start of storage (d 0), the mold counts of the hay baled at 85% DM were 4.5 log CFU/dry g, which decreased to 2 and then slowly increased, stabilizing at 5 after 14 d of storage. Total counts of actinomycetes and bacteria started at 5.75 log CFU/dry g in the fresh stand and decreased to 5 during wilting. At the start of the storage period, counts for actinomycetes and bacteria were highest for the high DM hay (7 log CFU/dry g), compared to low DM hay (5.5), but then decreased to ~4.5 after 77 d. The total counts of actinomycetes and bacteria fluctuated between 4 and 7.5 log CFU/dry g during the storage period (77 d) for the low DM hay.

In another experiment, Taffarel et al. (2013) evaluated the effects of wilting bermudagrass under field conditions or in a shed blocked from the sun. A total of 32 h was required to field-wilt to ~85% DM, while the material in a shed took 123 h to reach

~78% DM. Using potato dextrose agar, the authors found that under sun-wilting conditions, total fungal counts were higher after 30 d storage (3.70 log CFU/fresh g) than at cutting (3.17 log), and both did not differ from counts at baling (3.60 log). Moreover, N fertilization did not affect fungal counts across any of the hay production stages ($\overline{x} = 3.57$ log CFU/ fresh g). In a second cut, bermudagrass fertilized at 25 kg of N/ha had a higher count of fungi at baling (4.24 log CFU/ fresh g) compared to the cutting and storage stages ($\overline{x} = 3.57$). Fungal genera were identified and counted only for the first cut. *Fusarium, Penicillium,* and *Aspergillus*. counts remained the same across all stages ($\overline{x} = 3.84$, $\overline{x} = 2.88$, and $\overline{x} = 2.0$ log CFU/ fresh g, respectively), and some colonies of *Cladosporium* and *Rhizopus* were also observed. It is important to note that hay in this study was not baled in traditional bales but kept loosely within raffia braid bags, allowing for good ventilation. It is unlikely that heat accumulated under such conditions, and the weight of hay per raffia bag was not stated.

In a review article, Wittenberg (1997) argued that in hay that is stable during storage due to high DM levels, the fungal taxonomic profile does not change much relative to the one found after wilting. However, hay that undergoes heating during storage due to low DM levels will shift from the microbiome observed at wilting to one that can tolerate the increased bale temperatures observed in wet hay. Moreover, Wittenberg (1997) suggested that fungal counts do not usually increase during storage spoilage, and the shift in taxa is more important in explaining the losses of nutrients. Breton and Zwaenepoel (1991) assessed the effects of baling DM on the fungal taxonomic profile of tall fescue (*Lolium arundinaceum*) hay and observed that the primary fungal isolates of hay baled at 72.8% DM belonged to the genera *Alternaria, Cladosporium, Colletotrichum*,

Fusarium, *Phaeoseptoria*, *Phoma*, and *Ascochyta*. Moreover, they identified some yeast, such as *Metschnikowia pulcherima, Sporobolomyces roseus,* and *Trichosporon beigeli.* After 12 h of storage, the main fungal genera were *Alternaria*, *Penicillium*, *and Coniothyrium*. After 36 h, isolates consisted of the genera *Absidia*, *Rhizopus*, *Aspergillus*, and *Humicola*. When the bale temperature reached 34°C (48 h after baling), the main fungal genera were *Alternaria, Cladosporium*, *Colletotrichum*, *Conythyrium*, and yeast. After 84 h following baling, *Rhizomucor*, *Aspergillus*, and *Humicola* were the main fungal genera observed. Finally, at the end of storage (75 d after baling), xerophilic species predominated, like *Paecilomyces variotii, Emericella nidulans*, and *Eurotium amstelodami.* In hay baled at 54.7% DM, the genera *Pythium*, *Alternaria*, *Cladosporium*, *Colletotrichum*, *Coniothyrium*, and yeast were found at baling. Isolates belonging to the *Absidia*, *Rhizomucor*, and *Aspergillus* genera were observed from 36 h until the storage end (75 d after baling). At the end of storage, the authors also reported the presence of the genera *Humicola*, *Penicillium*, *Emericella*, *Eurotium*, and yeasts. Undi et al. (1997) also investigated the influence of alfalfa hay DM on microbiome dynamics and reported that during the early storage phase (1-8 d), alfalfa hays baled at 55 and 76% DM were characterized by the presence of some yeast, and the genera *Phoma* and *Cladosporium*. However, in 55% DM bales stored for 60 d, the hay microbiome was succeeded by the genera *Absidia* and *Mucor*, and the species *Emericella nidularis, Aspergillus fumigatus,* and an unidentified thermotolerant hyphomycetes. In comparison, the dominant species observed in hays baled at 76% DM was *Aspergillus repens*.

We used a Venn diagram (Figure 4) to summarize the results of previous research identifying hay microbial taxa. The taxonomic profile was compiled from five papers of

grass hay and one from alfalfa hay. It is important to remember that not all these publications used sequencing to identify hay taxa and that this diagram does not consider differences across studies, like location, forage type, etc. However, we believe it is important to organize available taxonomic information across different stages of hay production. The genera *Cryptococcus*, *Pyrenochaera,* and enterobacteria were uniquely identified at mowing. The genera *Pythium*, *Phoma*, *Phaeoseptoria*, *Ascochyta*, and some yeasts were only found in the baling phase. Finally, the genera *Absidia*, *Humicola*, *Rhizomucor*, *Emicerella*, *Eurotium*, *Rhysopus*, *Paecilomyces*, and some bacteria such as Cyanobacteria and Bacteroidota were identified in the storage phase only. Even though each phase has a unique microbiome, it is possible to identify similar fungi and bacteria species between 2 or 3 stages of hay production. For example, the genera *Fusarium* can be found in both the mowing and baling phase, while *Coniothyrium* and *Colletotrichum* may be present in the baling and storage phase. Additionally, it was determined that *Aspergillus*, *Cladosporium*, and *Alternaria* are fungal genera species that tend to be present throughout the haymaking process. It is important to keep in mind that this microbiome distribution is influenced by the moisture and temperature of hay, among other factors (Magan and Lacey, 1987). Understanding the diversity of the phyllosphere and the microbiome dynamics across the haymaking process is essential to comprehend the interaction across microbial taxa and their impact on nutrient losses.

Hay Mycotoxins

The presence of molds in hay can affect livestock health due to the production of spores, which are responsible for many respiratory and digestive problems in horses (Sheats et al., 2019), and mycotoxins, such as aflatoxins, fumonisins, ochratoxins,

trichothecenes, and zearalenone (Smith et al., 2016). Santos Pereira et al. (2019) stated that *Aspergillus*, *Penicillium*, *Alternaria,* and *Fusarium* genera are the most known mycotoxin producers in forage crops. Raymond et al. (2000) evaluated the mycotoxins levels of stored alfalfa-timothy mixed hay across ten horse farms. The bales sampled in this survey had different proportions of alfalfa and timothy. After 11 mo of storage, hay baled with a higher proportion of alfalfa relative to timothy, had higher levels of vomitoxin (2,925 vs. 1,617 µg/kg). The authors suggested that this was due to alfalfa being more susceptible to mold-induced spoilage. In contrast, zearalenone ($\overline{x} = 390$ µg/kg) and T-2 toxin ($\overline{x} =$ 330 µg/kg) were not affected by forage type proportion in that study. Several studies have focused on identifying mycotoxins produced in hay and evaluating their impact on livestock performance (Jovaišienė et al., 2016; Buszewska-Forajta, 2020; Durham, 2022). However, little attention has been given to how management decisions during hay production can affect mycotoxin levels. Taffarel et al. (2013) conducted a study comparing how drying in a field vs. in a shed affects mycotoxin levels in bermudagrass hay after 30 d of storage. The results showed that field drying resulted in a higher concentration of aflatoxin and zearalenone relative to hay dried in the shed (5.38 vs. 3.07 and 79.9 vs. 40.3 µg/kg, respectively). The authors suggested that higher levels of mycotoxins in hay dried on the field were caused by day-night thermal variation, which may have helped to stimulate fungi to produce more mycotoxins than in hay drying in shed conditions (Cervini et al., 2021) before the swath reached a DM concentration >85%.

Conclusions

Interdependent factors determine nutrient losses during harvest and storage, some that cannot be controlled, like the environment, and some that the producer can

manage. Increasing nutrient losses can be expected in the field as DM increases, especially >85%, and during the storage phase, if DM during storage is <80-85%. Thus, management techniques and specialized equipment have been developed to mitigate nutrient losses throughout the haymaking process. For instance, increasing the cutting height improves the nutritive value of hay, but it reduces the yield and consequently needs to be carefully thought of by the producer. Another critical factor is forage type, as legumes are more prone to nutrient losses during field and storage stages than grasses. Mechanical conditioning and swath manipulation can result in significant leaf losses, especially in alfalfa, if not carefully managed. This issue, combined with the fact that legumes are more susceptible to spoilage during storage and less responsive to organic acid-based preservatives, emphasizes the importance of following good management practices to mitigate nutrient losses in legume hay production. Solar radiation, swath temperature, and VPD are among the most important factors influencing the drying rate, which is critical in humid environments. Storage conditions also affect dry matter losses, with outdoor conditions potentially resulting in massive nutrient losses, especially if bales are in direct contact with the soil.

The recommended moisture levels for baling vary depending on bale type, density, and size. However, adequate storage conditions and wrap type should be considered to prevent re-wetting during storage, possibly leading to increased microbial growth, heating, and DM losses. The hay microbiome is stable during the storage of adequately dried hay (>80% DM), with field fungi such as the genera *Cladosporium* and *Alternaria* persisting across phases. However, low DM (<80%) hay undergoes a drastic shift in its microbiome during storage relative to baling, resulting in the dominance of thermotolerant

microorganisms. Fungi genera commonly found in spoiled hay include *Aspergillus*, *Mucor*, and *Penicillium*, among others, which may represent a health risk to livestock and humans through the production of dust (spores), mycotoxins, and allergic reactions. The ultimate goal of optimal hay production is to adequately store hay nutrients by preventing the growth of undesirable microbes during storage without causing high field losses due to excessive wilting and swath manipulation.

Table 1-1. Number of baler models fitted with special features available in the US market, by baler type.

Baler features	Baler type*		Total
	Square baler	Round baler	
Moisture sensor	9	14	23
Additive applicator	9	14	23
Density sensor	0	15	15
Cutting system	2	19	21
Cutting system + additive applicator	0	5	5
Cutting system + weighing sensor	3	0	3
Cutting system + moisture sensor	9	5	14
Moisture sensor + weighing sensor	0	5	5
Moisture sensor + additive applicator	5	10	15
Cutting system + additive applicator	4	0	4
Cutting system + moisture sensor + weighing sensor	9	0	9
Density sensor + moisture sensor + weighing sensor	2	0	2
Moisture sensor + additive applicator + weighing sensor	2	0	2

*Obtained from John Deere, New Holland, Massey Ferguson, CLASS, Case IH, Kuhn, Krone, Vermeer, and Kubota.

Figure 1-1. Reported ranges of bale weights, dimensions, and densities across bale types and recommended moisture thresholds for storage. Data was obtained from John Deere, New Holland, Massey Ferguson, CLASS, Case IH, Kuhn, Krone, Vermeer, and Kubota and combined with a previous report of (Collins and Coblentz, 2007a)**.**

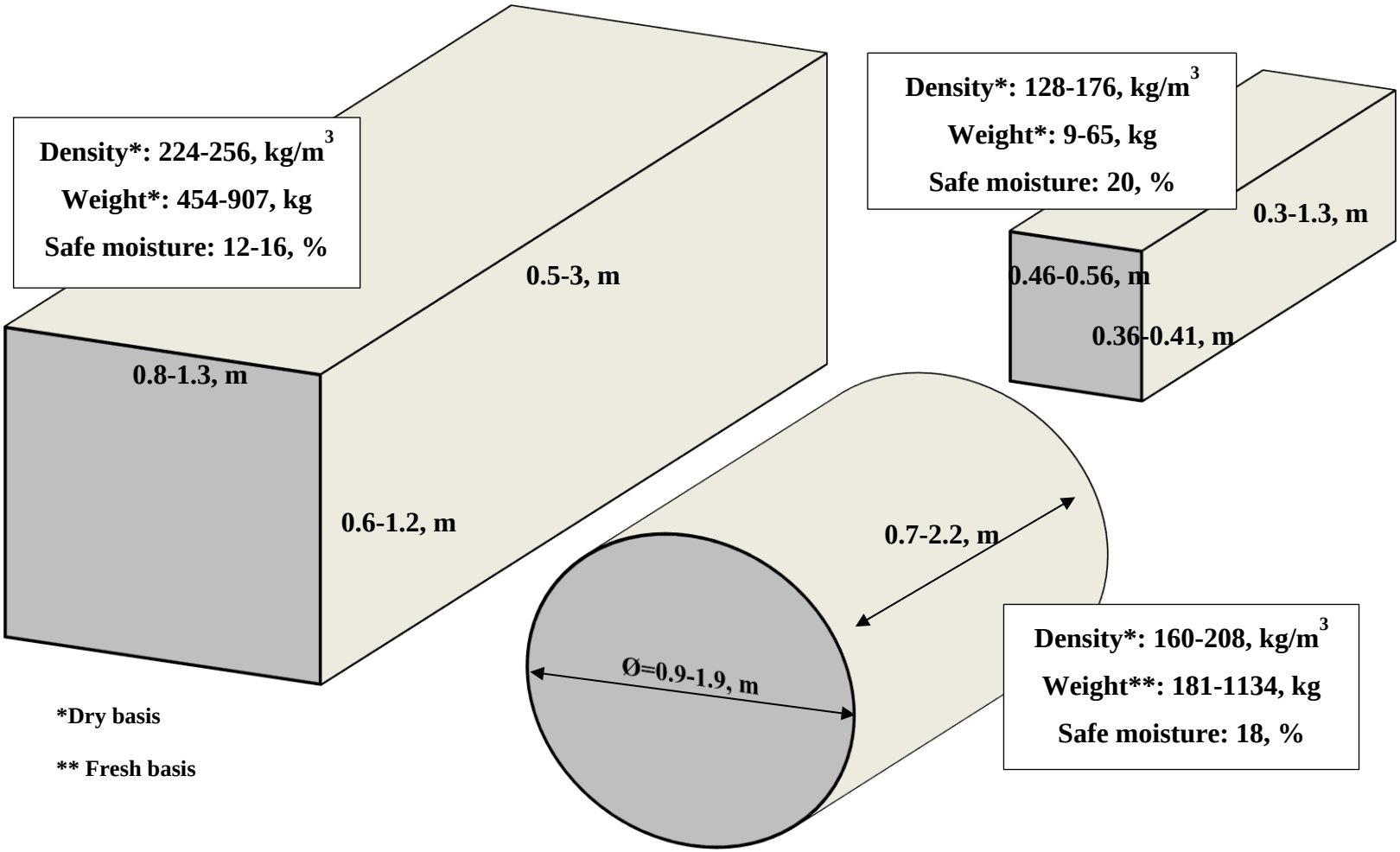


Figure 1-2. Potential storage DM losses (%) of round bales wrapped with twine and net wrap and stored across different conditions. Adapted from (Collins et al., 1997; Coblentz, 2009; Hancock, 2020).

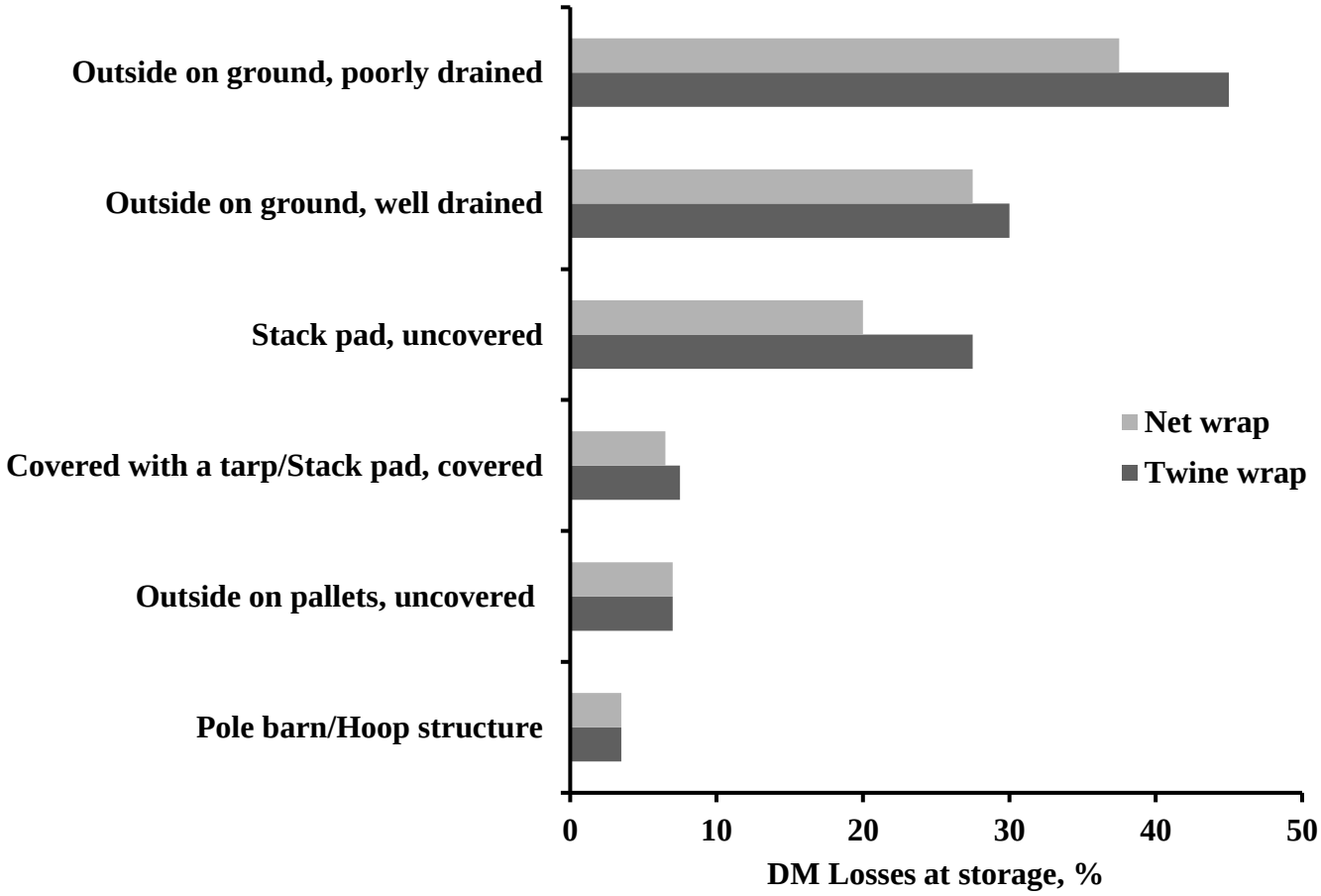


Figure 1-3. Relationship between legume bales moisture (%) and DM loss (%) during storage, according to bale types (round and rectangular).

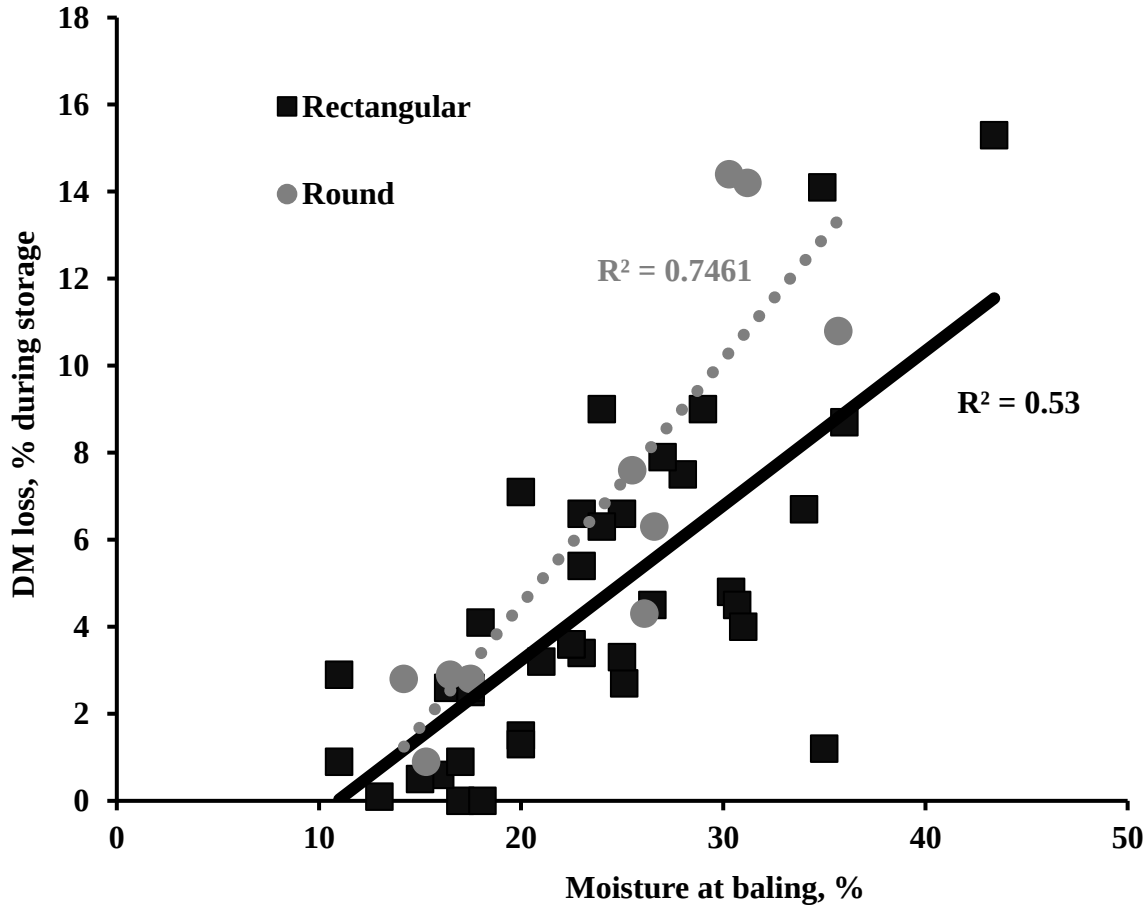


Figure 1-4. Venn diagram analysis of reported hay microbial taxa across mowing, baling, and storage stages of hay production (Gregory et al., 1963; Breton and Zwaenepoel, 1991; Undi et al., 1997; Taffarel et al., 2013; Drouin et al., 2022; Kennang Ouamba et al., 2022). Bacteria= B, Mold= M, and Yeast= Y.

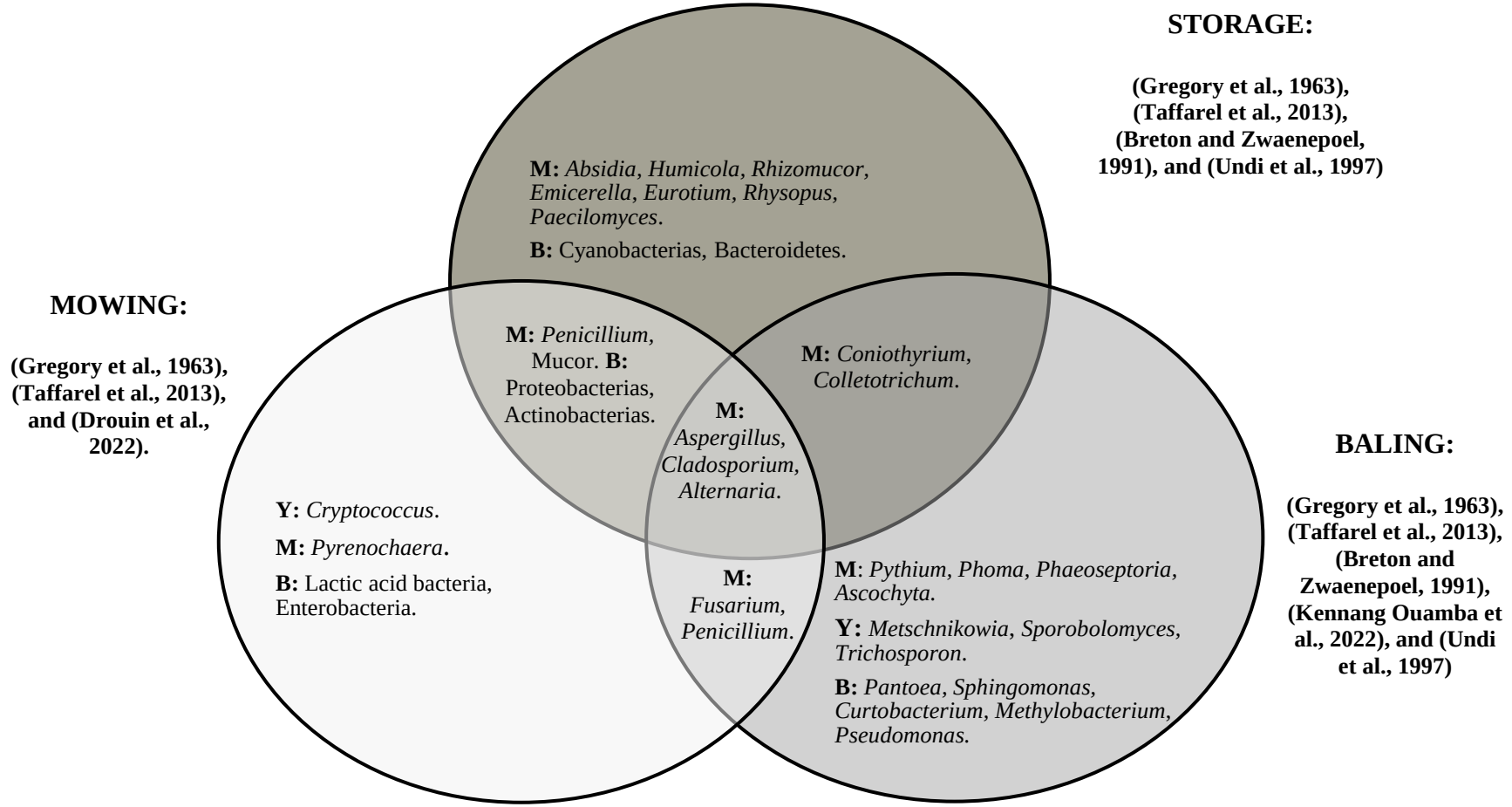
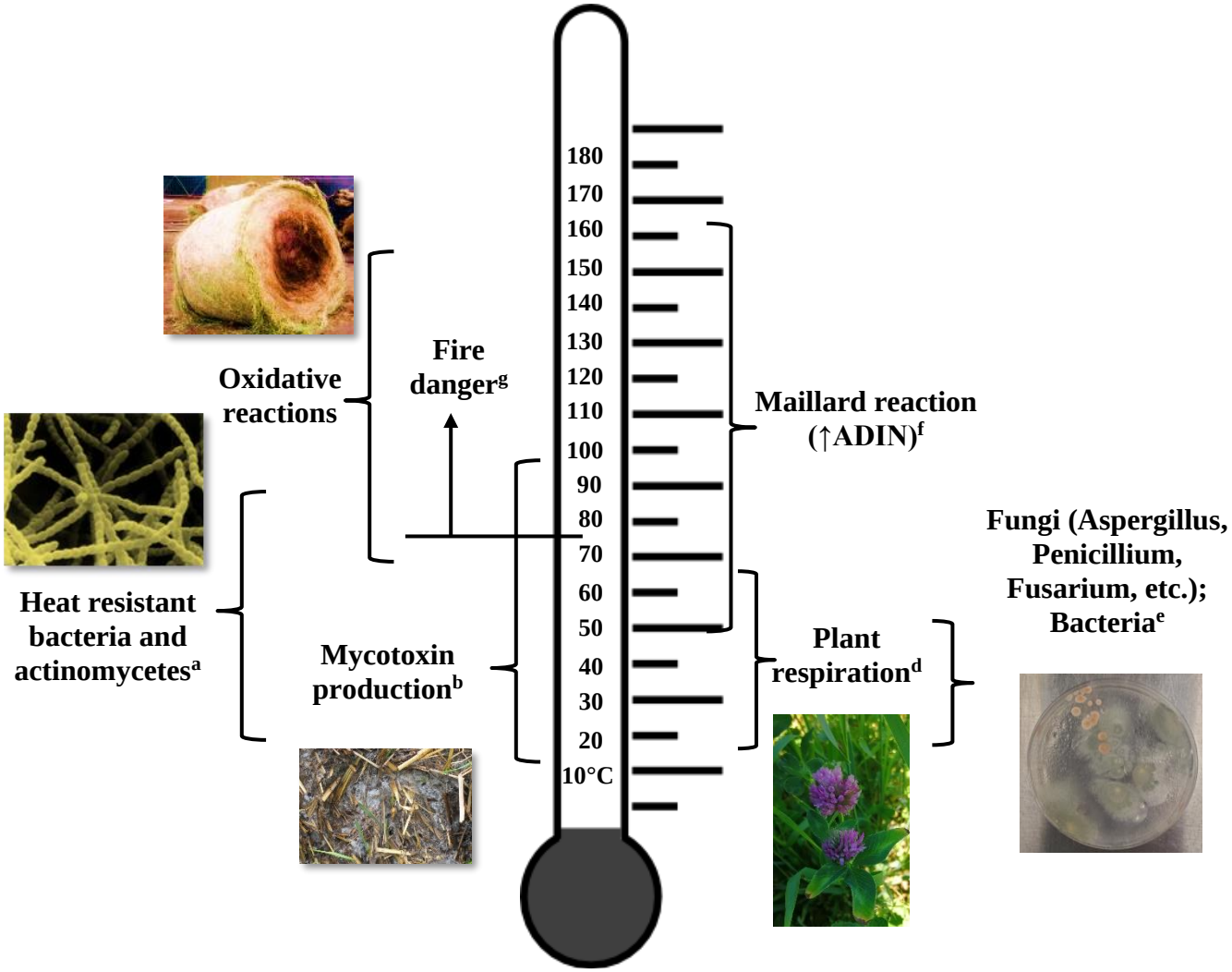
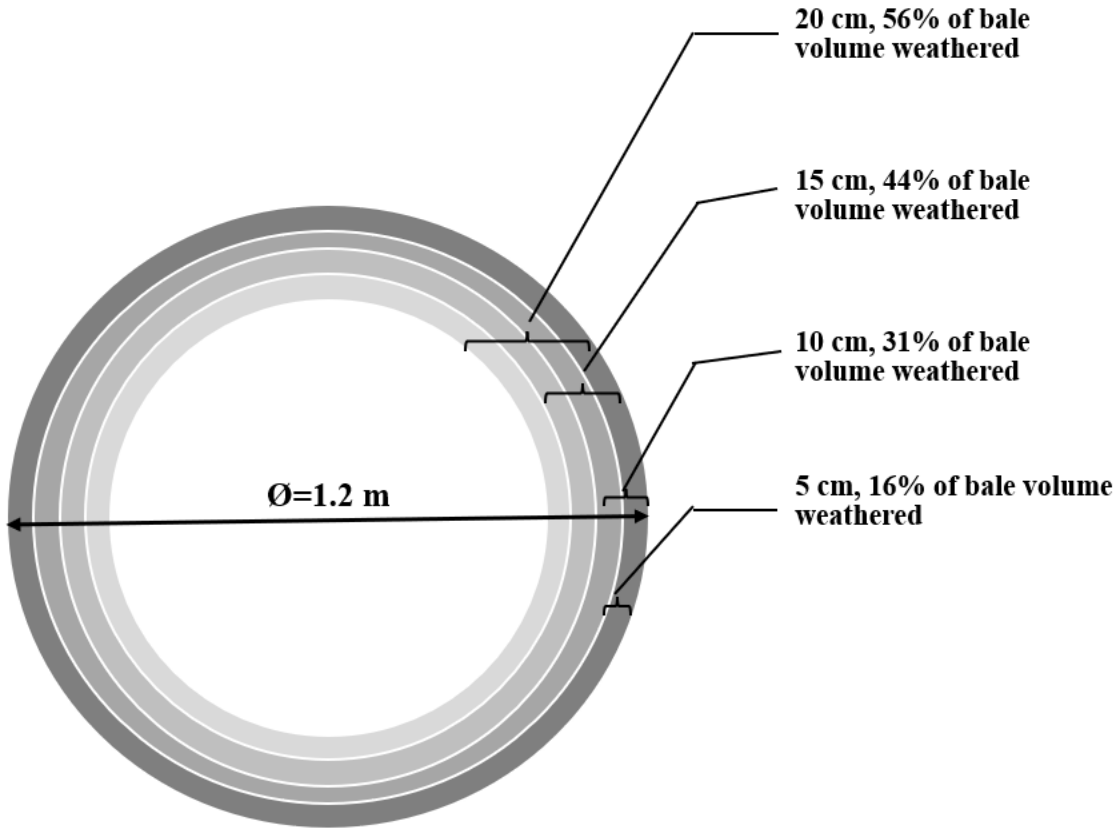


Figure S1. Hay heating causes and potential effects on hay nutritional value, microbial populations, and exothermic reactions.



Taale et al. (2013)[a], Daou et al. (2021)[b], Awuchi et al. (2021)[b,c], Lowell (1995)[d], Mannaa and Kim (2017)[e], Van Soest (1982)[f], Festenstein (1971)[g], and Hancock (2015)[g].

Figure S2. Percentage of round bale volume damaged by 5, 10, 15, and 20 cm of weathered layer depth. Adapted from (Collins et al., 1997).



CHAPTER 2

EFFECTS OF WILTING EXTENT ON THE PHYTOESTROGEN LEVELS, NUTRITIONAL VALUE, MICROBIAL POPULATIONS, AND IN VITRO RUMINAL METHANE EMISSIONS OF RED CLOVER HAY AND SILAGE ACROSS STAGES

Introduction

Relative to grasses, forage legumes fix N and have higher CP values, higher minerals such as calcium (Sturludóttir et al., 2014), increase forage yield, increase voluntary intake, and milk production (Lüscher et al., 2014). Bosworth and Cannella (2007) showed significant positive correlations between legume inclusion in forage stands and quality traits like crude protein and net energy of lactation, and negative correlations with neutral and acid detergent fiber. In fact, Johansen et al. (2018) reported that legume vs. grass-based diets increased the DM intake (DMI, +7%), milk production (+6.5%), milk fat (+3%) and milk protein (+5%) of dairy cattle. While forage legumes can provide many benefits to dairy systems, they can also contain anti-quality components that affect animal performance and health (Mostrom and Evans, 2011), such as phytoestrogens (Hill and Roberts, 2020).

Phytoestrogens are secondary plant metabolites found in legume species with similar biological effects to animal estrogen (Rietjens et al., 2017). The most studied phytoestrogens are isoflavones, isoflavans, and coumestans (Hill and Roberts, 2020). The type of phytoestrogens and their concentration can vary by legume species (Reed, 2016). Daidzein, formononetin, genistein, and biochanin A are the most important

phytoestrogens in red, subterranean (*Trifolium subterraneum* L.), and white clover (Hill and Roberts, 2020). Red clover has a high concentration of biochanin A and formononetin (Sivesind and Seguin, 2005) and at concentrations >500 to 750 mg/kg of DM in the diet, these phytoestrogens can cause fertility issues in ruminants (Mostrom and Evans, 2011). Most studies have been conducted with sheep (Reed, 2016), and the few studies conducted with cows did not report critical thresholds of phytoestrogens concerning adverse effects on cattle (Penagos-Tabares et al., 2022).

When cows are fed with legumes, phytoestrogens (Mackey and Eden, 1998) influence the estrus cycle of ruminants (Mostrom and Evans, 2011) by acting primarily as estradiol-17β (E_2) agonists in cows and ewe (Usui et al., 2002, Reed, 2016). This effect is determined by the ability of phytoestrogens to bind to two main estrogen receptors (ERs), the receptor β (ERβ) and the receptor α (ERα), with the former having a greater affinity to phytoestrogens than the latter (Mostrom and Evans, 2011, Reed, 2016). Therefore, due to the phytoestrogens capability to bind to the ERs, it was observed that they can cause embryonic loss, decreased rate of conception, temporary infertility, anatomical alterations, inhibit estrus sign, and other clinical signs resembling cystic ovaries in ruminants (Wyse et all., 2022)

It has been reported that phytoestrogens act primarily as estradiol-17β (E_2) agonists in cows and ewes, causing an estrogenic effect (Usui et al., 2002; Reed, 2016). When ingested, phytoestrogens can compete for the ERs with E_2, and hence influence the estrus cycle of ruminants (Mostrom and Evans, 2011). This effect is determined by the ability of phytoestrogens to bind to two main estrogen receptors (**ERs**), the receptor β (**ERβ**) and the receptor α (**ERα**), with the former having a greater affinity to

phytoestrogens than the latter (Mostrom and Evans, 2011; Reed, 2016). Therefore, due to the binding effect of phytoestrogens to the ERs, it was observed that they can cause embryonic loss, decreased rate of conception, temporary infertility, anatomical alterations, inhibit estrus sign, and other clinical signs resembling cystic ovaries in ruminants (Mackey and Eden, 1998).

Forage conservation methods like haymaking and ensiling can affect phytoestrogen concentrations in forage legumes (Sarelli et al., 2010; Hloucalová et al., 2016). Sivesind and Seguin (2005) observed decreases in total phytoestrogens of up to 45% when red clover was made into hay. In the case of ensiling, Daems et al. (2016) reported a decrease of up to 73% in total phytoestrogens in unwilted red clover ensiled for as short as two weeks. Similarly, Sarelli et al. (2003) observed that red clover ensiled at 400 g/kg DM had 9% less total phytoestrogens than at 250 g/kg DM. In contrast, Kallela (1975) reported an increase in total phytoestrogens after ensiling red clover and Hloucalová et al. (2016) reported that wilting a mixture of red, Persian (*Trifolium resupinatum* L.), Alexandrian clover (*Trifolium alexandrinum* L.), and alfalfa for 24 h increased the concentration of biochanin A and formononetin by 27.5% compared with the fresh cut. The lack of consistency between studies may be attributed to different factors such as the extent of wilting, ensiling period, phytoestrogens concentration before ensiling, maturity stage at cutting, environmental conditions, genetic material, silage additives (Driehuis et al., 2018), and the purity of stand (Hloucalová et al., 2016). It is important to mention that most of the available research is related to the effects of the ensiling period on phytoestrogens rather than the wilting extent. Further, the impacts of

wilting extent of both red clover hay and silage on phytoestrogens have not yet been compared in the same study.

This study aimed to assess the effects of wilting extent, forage conservation method, and storage stage on the phytoestrogen levels, nutritional value, microbial populations, and in vitro ruminal methane emissions in red clover. We hypothesize that haymaking and ensiling will reduce the phytoestrogens levels in red clover. Also, ample wilting can reduce the phytoestrogen levels without sacrificing the nutritional quality of red clover hay and silage relative to inadequate wilting.

Materials and Methods

Protocol A2021-03-03 involving animal handling in this study was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Maine.

Forage, treatments, and experimental design

An established 0.43-hectare stand of red clover (*Trifolium pratense* L., var. Freedom) located in Old Town (Penobscot, ME) was divided into 5 blocks and then mowed when the stand reached 10% bloom with a New Holland Discbine 210-disc mower (Racine, WI) to a 5 cm cutting height at 11 am on July 27th, 2021 (wide swath, 70% cover). Each block (207 m^2) was further divided into 4 plots, which were randomly assigned to a factorial arrangement of 2 conservation methods (silage or hay; **MTOD**) and 2 wilting extents (below and at recommended levels; **WILT**).

Silage treatments

Current recommendations for legume silage DM at harvest range between 350-400 g/kg for pile, bunker, and ag-bag silos and 450-550 g/kg for baleages (Kung et al., 2018; Muck et al., 2020). For our study, we targeted below 300 g/kg DM but above 250 for the inadequately wilted legume silage treatment (**WET**). For instance, the first cut in the Northeastern US is prone to be ensiled below 300 g/kg DM if rains are prevalent during the spring. For the ample wilting treatment (**CUR**), we targeted between 380-420 g/kg DM. Once swath DM levels were close to the targets for the respective plots (determined with a Koster tester; Canton, OH), windrows of the WET and CUR silage were formed with a CASE IH rotary rake ANDEX 423T (CNH Industrial, Racine, WI) at 2:00 pm on July 27th and at 11:00 am on July 28th, respectively. The swath was chopped immediately after raking with a New Holland 900 forage harvester (New Holland, PA) set to a theoretical length of cut of 1 cm. An actual DM concentration of 294 g/kg (WET) was obtained after 3 h of wilting, and 453 g/kg after 24 h of wilting (CUR), as shown in Table 2-3, following the drying procedure described in the nutritional analysis section.

Immediately after being chopped, a sample of the red clover generated from each of the WET plots was weighed (31 kg, fresh basis), from which 14 kg (fresh basis) was taken to prepare the mini-silo. Similarly, for each of the CUR plots, a sample was weighed (22 kg, fresh basis), from which 9.3 kg (fresh basis) was taken to prepare the mini-silo. In addition, 2 kg (fresh basis) was taken separately from the WET and CUR plots for start of the storage stage (d 0; **STRT**) analyses. The 19.5 L plastic mini-silos were packed using an A-frame 12-ton pneumatic press and sealed with a rubber gasket lid (~214 kg of DM/m^3). Mini-silos were stored for 14 d (**MicA**) and 78 d (**LATE**) for both wilting extents

in a room kept at 22 ± 0.02°C and 67 ± 0.39% RH, after which they were opened, weighed, and aerobically exposed for 7 additional days under the same room conditions.

Hay treatments

Current recommendations for hay DM at harvest range between 800-850 g/kg (Coblentz, 2020). Our study targeted below 750 g/kg DM but above 700 g/kg for the inadequately wilted hay treatment (WET) because of the great variability in DM concentration observed across a hay field during wilting (Collins and Moore, 2017). For the ample wilting treatment (CUR), we targeted between 820-850 g/kg DM. All remainder swaths were tedded with a Kuhn GF5001MH (Kuhn North America INC, Brodhead, WI) right after the CUR silage treatment was chopped on July 28th. Once swath DM levels were close to the harvest DM targets, windrows were formed for the respective plots with the rotary rake. Preliminary square bales, as described in Coblentz et al. (1993), were made with a New Holland 311 square baler (Sperry New Holland, PA) on July 29th at 12 pm for the WET hay treatment and August 1st at 10 am for CUR hay. Immediately after, the preliminary bales were cut into 10 cm segments across the stem axes using a HC-2020 Hedge trimmer (ECHO Inc., Lake Zurich, IL). Subsequently, a sample of cut hay was obtained for the WET treatment (4 kg, fresh basis), from which 0.5 kg (fresh basis) was used to prepare the mini-bale (10.3 x 10.8 x 13 cm; density of ~232 kg DM/m^3). For the CUR treatment, a sample of the cut material was weighed (3.8 kg, fresh basis), from which 0.4 kg (fresh basis) was taken to prepare the mini-bales (~232 kg DM/m^3). An actual DM concentration of 650 and 891 g/kg was observed for WET and CUR hay, respectively (Table 2-3), using the procedure outline in the nutritional analysis section.

The mini-bales were prepared as outlined by Coblentz et al. (1993) using a 12-ton hydraulic bottle jack (Pittsburg Automotive, Camarillo, CA) powered by compressed air. The mini-bales were secured with bale wires and fitted with a temperature sensor (Gemini Data Logger, UK) previously weighed and set to record the bale temperature every 30 min. The mini-bales were placed inside open nylon mesh bags (80 µm pore size, 30 x 30 cm) to avoid particle losses during manipulation while allowing unrestricted airflow. Next, they were placed in open-top insulation boxes made with 5 cm-thick extruded polystyrene foam boards (Kingspan Insulation LLC, GA), following the protocol outlined by Coblentz et al. (1994). Mini bales were stored for 14 (**MicA**) and 50 d (**LATE**) in a temperature-controlled room kept at 22 $\pm$ 0.02°C and 67 $\pm$ 0.39% RH and were inverted every 7 d as described by Coblentz et al. (1994). Mini-bale initial and final weights were recorded to calculate DM losses during storage.

Sampling procedure

Standing red clover

Immediately after mowing, samples of red clover were collected from each block (2 kg, fresh basis). A 20-g sub-sample weighed into strainer Stomacher bags (Seward Ltd., Worthing, UK) using sterile techniques was used to determine lactic acid bacteria (LAB), yeast, and mold counts through plating techniques. Another sub-sample (50 g, fresh basis) was kept at -80°C for phytoestrogen analysis. The rest was frozen at -20°C for other types of analysis.

Silage

At STRT, from the 3 kg left after the preparation of the mini-silos, 20-g (fresh basis) were weighed into strainer Stomacher bags for the enumeration of yeast, mold, and lactic acid bacteria colonies, as stated for the standing red clover samples. Another sub-sample (50 g, fresh basis) was kept at -80°C for phytoestrogen analysis. The rest was frozen at -20°C for other types of analysis. The same procedure was followed for MicA, LATE, and aerobic stability (AES) treatments for sub-samples designated for enumeration of colonies, phytoestrogen analysis, and all other analyses.

Hay

At STRT, from the 2 kg left after the preparation of the mini-bales, 20-g (fresh basis) were weighed into strainer Stomacher bags for the enumeration of yeast and mold colonies, as described for the standing red clover samples. Another sub-sample (50 g, fresh basis) was kept at -80°C for phytoestrogen analysis. The rest was frozen at -20°C for other types of analysis. For MicA and LATE treatments, the same procedure was followed for sub-samples designated for the enumeration of colonies, phytoestrogen analysis, and all other analyses.

Laboratory analysis

Nutritional analysis

For each treatment, a subsample (100 g, fresh basis) of the samples that were stored at -20°C was taken and processed in triplicate to determine the DM concentration by drying at 60°C until they achieved constant weight in a forced-air oven. Dried samples

were ground to pass through a 4 mm screen using a Wiley mill (Arthur H. Thomas Company, Philadelphia, PA). From these ground samples, a subsample was weighed (12 g, fresh basis) and ground to pass through a 2 mm screen in a Cyclone mill (CT 193 Cyclotec; FOSS, Denmark) for subsequent nutritional analysis, and the rest of the 4-mm ground material was used for a ruminal in situ degradability evaluation. The determination of ash, NDF, ADF, and N was conducted as outlined by Leon-Tinoco et al. (2022).

Liquid extracts were obtained by mixing the previously described 20-g samples weighed into Stomacher bags with 180 mL of a 0.9% sterile saline solution and blending them in a 400C Stomacher blender for 3 min (Seward Ltd., Worthing, UK). The filtered liquid was transferred into sterile Nalgene bottles. After the aliquot needed for microbial studies was obtained (see microbiological analysis section), each extract was centrifuged at 8,000 x *g* for 15 min at 5°C, and the supernatant was frozen (−20°C) until further analysis. The pH of the extract was measured with a calibrated Φ34 Beckman pH meter (Beckman, Brea, CA) fitted with an Accumet Universal pH electrode (Thermo Fisher Sci., Waltham, MA). The centrifuge extracts were then acidified to pH 2 with 50% H_2SO_4 (1% v/v) before freezing. The determination of ammonia-N (NH_3-N) and water-soluble carbohydrates (WSC) was conducted as outlined in Killerby et al. (2022a). Only silage extracts were analyzed for lactic, acetic, butyric, and propionic acid, and 1,2-propanediol, and ethanol concentration using an Agilent High Performance Liquid Chromatograph 1200 series system fitted with an Agilent Hi-Plex H column (Agilent Technologies, Santa Clara, Ca) coupled to an Agilent refractive index detector (Siegfried et al., 1984).

Microbiological analysis

For the enumeration of yeast and mold counts, an aliquot of the liquid extracts of hay and silage was taken immediately after filtering and used to perform serial (10-fold) dilutions in 0.9% sterile saline solution (NaCl), which were then spread-plated (100 µL) on Malt Extract Agar (MEA; BD Difco, Franklin Lakes, NJ) with antibiotics (0.1 g/L Penicillin G and 0.1 g/L Streptomycin; Thermo Fisher Sci., Waltham, MA). Plates for yeasts and molds were incubated at 25°C for 72 and 120 h, respectively. Visual moldiness of each CUR and WET hay mini-bale stored for 14 and 50 d was determined on a 0 to 10 subjective scale where 0 represented mold-free hay, and 10 represented very moldy hay, following the criteria outlined by Duchaine et al. (1995a).

For the enumeration of bacterial counts, an aliquot of the liquid extracts of silage was taken immediately after filtering and used to perform serial (10-fold) dilutions in 0.9% sterile saline solution (NaCl), which were then spread-plated (100 µL) on Man, Rogosa and Sharpe agar (MRS; BD Difco, Franklin Lakes, NJ) with 0.01% cycloheximide (Ha et al., 1995) for lactic acid bacteria (LAB) counts. After that, plates were incubated for 24 h at 37°C. The two wilting extents (WET and CUR) of AES samples were only analyzed for yeast and mold counts.

Aerobic stability measures in silage

Aerobic stability was determined by transferring 3 kg of the freshly opened (d 14 and 78) silage into 19.5 L containers. A temperature sensor (Gemini Data Logger, UK) was placed in the middle of the biomass, previously set to record data every 30 min for 7 d. Two additional sensors were placed in the temperature-controlled room (22 $\pm$ 0.02°C

and 67 ± 0.39% RH). The buckets were left open, and silage samples were covered with 2 layers of cheesecloth to avoid excessive drying. Aerobic stability was expressed as the amount of time (h) before the silage heated 2°C above ambient temperature (Kung, 2010)., The maximum temperature and heat degree-days index (°C-day; HDD) above room temperature of the biomass in the buckets were determined according to Coblentz et al. (2013b). Another 3 kg subsample of freshly opened silage was placed in a plastic bucket (19.5 L) and exposed for 7 d to quantify the nutritional and microbial composition changes.

Heating measures in hay

Data recorded by the temperature sensors placed within the mini-bales were used to determine the heat degree-days index (°C-day; HDD) and maximum temperature (MaxT; °C) of mini-bales stored for 14 and 50 d. Two additional sensors were placed in the temperature-controlled room to determine the ambient temperature. Hay HDD was calculated as the sum of the daily temperature increments above ambient temperature (Coblentz et al., 2013b).

Phytoestrogen analysis

The samples taken for phytoestrogen analysis were analyzed as outlined by Payette et al. (2022).

In vitro ruminal digestibility, fermentation, and gas production

In vitro incubations were performed in 250 mL glass bottles using the Ankom Gas Monitoring System (Ankom Technology, Macedon, NY) to determine DM digestibility, and

gas production kinetics of hay and silage samples. The substrate for incubation consisted of 1.4 g of the aforementioned dried and ground samples (2 mm), with one bottle per sample and bottles grouped by block. The ruminal fluid was representatively collected by aspiration 3 h after feeding (11:30 a.m.) from 2 ruminally cannulated Holstein cows in lactation consuming a ration consisting of corn silage (*Zea mays* L., 9.2 kg), timothy and red clover mixed silage (*Phleum pratense* L. and *Trifolium pratense* L., respectively; 5 kg) and concentrate (10.3 kg, DM basis). The collected ruminal fibrous mat was blended with ruminal fluid under a constant CO_2 flush, filtered through 2 layers of cheesecloth (de Assis Lage et al., 2020), and mixed with McDougall's artificial saliva at a buffer:ruminal fluid ratio of 3:1 (McDougall, 1948; Henry et al., 2015). A blank bottle and a zero-module recording were included in each incubation run as outlined by Killerby et al. (2022a). Samples were incubated for 48 h under constant shaking (60 rpm). The cumulative pressure was recorded every 30 min by the Anakom modules, and the global release pressure was set to 2 psi. The total gas produced was collected in gas sampling bags (Supel™-Inert Multi-Layer Foil, Supelco Inc, Bellefonte, PA) that were connected directly to the Ankom modules, following the protocol outlined by Henry et al. (2015). After incubation, the fermentation was stopped by pacing the bottles on ice. The contents of the bottles were centrifuged at 8,000 x *g* for 15 minutes and filtered through previously dried and weighed Whatman No. 541 ashless filter papers (Fisher Scientific, Pittsburgh, PA). Residues were dried at 65°C to constant weight to determine apparent in vitro DM digestibility (IVDMD), and subsequently burned at 600°C in a muffle furnace for apparent in vitro organic matter (OM) digestibility (IVOMD) determination. The filtrate liquid was measured for pH before being acidified with 50% H_2SO_4 (1% v/v) and stored for further

analysis of NH_3-N and total volatile fatty acids (acetate, propionate, butyrate, isobutyrate, isovalerate, and valerate) using the same HPLC as described earlier, but fitted with a diode-array detector (Castillo Vargas et al., 2020). The determination of ruminal methane gas (CH_4) was measured as described by Killerby et al. (2022a). The volume of gas produced was calculated from the pressure results of the ANKOM modules multiplied by the atmospheric pressure (zero-module). Gas production kinetics included asymptotic maximal gas production (M_f), fermentation rate (K_f), and lag phase (h), which were calculated using the modified Gompertz model (Henry et al., 2015):

$$V = M_f \exp\left\{-\exp\left(1 + \frac{K_f e}{M_f}(L - t)\right)\right\},$$

where V is the total gas volume produced during 48 h incubation; M_f is the asymptotic maximal volume of gas (corresponding to complete substrate digestion); K_f is the rate of gas produced; L is the lag time; and t is the incubation time.

In situ ruminal degradability

All treatments per block were analyzed for in situ DM and NDF rumen degradability using 2 ruminally cannulated cows per block (Broderick and Cochran, 1999). Only LATE samples (d 50 and 78 for hay and silage, respectively) were tested for this procedure. The cannulated cows were fed the same diet described in the in vitro digestibility section for 30 d before and during the 4 d of incubation. Two identical sets of bags were weighed per treatment combination within a block, with each set placed in one of the two cows used per block (Broderick and Cochran, 1999). Samples were ground to pass 4-mm screen using a Wiley mill and weighed (5 g DM) into 10 x 20 cm ANKOM R1020 in situ

bags (ANKOM, Macedon, NY). Bags were hooked to a rope and placed in the ventral sac of the rumen of 2 dairy cows for 0, 3, 6, 12, 24, 48, 72, and 96 h (Mustafa and Seguin, 2003). Immediately after removal from the dairy cows, all bags were placed in cold water for 5 min to remove adherent particles and bacteria and then washed in a commercial washing machine (Kenmore 300 series) using a cool-wash cycle (Broderick and Cochran, 1999). Subsequently, washed bags were dried for 48 h at 60°C and weighed. Dried residues were analyzed for DM and NDF. The model of Mertens (1977) was fitted to the in situ DM and NDF degradation data using the NLIN procedure of SAS (SAS Institute, Cary, NC). The DM and NDF input degradation data per treatment combination within a block was the average of the results obtained from the two cannulated cows used per block. The model was:

$$R_{(t)} = D_i \times \left[e^{-k_d \times (t - L)}\right] + I_o,$$

Where $R_{(t)}$ is the total undegradable residue at any time *t,* D_i is the potentially degradable fraction, $e = 2.71828$, k_d is the fractional rate of degradation of D_i, t is the time incubated in the rumen in hours, L is the discrete lag time (h), and I_o is the indigestible fraction. The wash fraction A was the percentage of substrate washed out of the bag at 0 h. The derivative model for k_d is of the form:

$$K_d = A \times [e^{-k \times (t - L)}] \times (L - \text{t}),$$

Where k is the rate of degradation. The derivative model for L is of the form:

$$L = A \times (e^{-k \times (t - L)}) \times k$$

Statistical analysis

Data were analyzed as a randomized complete block design (5 blocks) with a 2 (wilting extents) × 2 (conservation methods) × 3 (storage stages) factorial. The model used to analyze the data was:

$$Y_{ijkl} = \bar{Y} + a_j + b_k + c_l + (ab)_{jk} + (ac)_{jl} + (bc)_{kl} + (abc)_{jkl} + e_{ijkl}$$

where Y_{ijkl} is the dependent variable, Y is the overall mean, a_j is the conservation method effect (MTOD), b_k is the wilting extent effect (WILT), c_l is the storage stage effect (STG), ab_{jk} is the effect of interaction between MTOD x WILT, ac_{jl} is the effect of the interaction between MTOD x STG, abc_{jkl} is the effect of the interaction between STM x WILT x MTOD, and e_{ijkl} is the residual error. The GLIMMIX procedure of SAS 9.4 (SAS Institute Inc., Cary, NC) was used to analyze the data. Means were separated by Fisher's Protected LSD test, and the SLICE option was used to analyze interactions. Significance was declared at $P \leq 0.05$. A model that included only the effects of WILT, STG, and their interaction was used for LAB counts and one that included the effects of WILT, STG (only MicA and LATE), and their interaction was used to analysis the measures obtained from the aerobically exposed silage. In order to facilitate the interpretation of overall results, multiple regression relationships between the chemical composition (DM, WSC, CP, NDF, ADF, hemicellulose, pH, and NH_3-N), microbial composition (mold, yeast, and LAB counts), and formononetin and biochanin A of red clover hay and silage were examined using the stepwise multiple regression procedure of SAS. Predictors were only added to the final model if they increased ($P \leq 0.05$) prediction accuracy. Model overfitting was prevented by keeping the Mallow's C(p) criterion close to the number of regressors plus

1. The CH_4 yield of WET hay and WET silage data from the in vitro digestibility assay were analyzed too using the same approach.

Results

Red clover stand

Right after cutting, the DM was 223 ± 29.3 g/kg (mean ± SD), WSC 6.34 ± 3.6 g/kg of DM, and CP 173 ± 19.7. The concentration of NH_3-N was 4 ± 2 g/kg of total N, and NDF, ADF, and hemicellulose were 452 ± 25.3, 298 ± 22.3, and 154 ± 3.89 g/kg of DM, respectively. Additionally, the pH was 6.02 ± 0.15, yeast counts 7.01 ± 0.33 log cfu/fresh g, mold counts 5 ± 1.59, and LAB counts 6.19 ± 0.7. The total phytoestrogen concentration averaged 1,157 mg/kg of DM, with formononetin and biochanin A accounting for approximately 55 and 40.5% of the total phytoestrogens, respectively. The formononetin and biochanin A concentrations were 5,130 ± 1,692 mg/kg of DM and 3,751 ± 979, respectively. The rest of the phytoestrogens was composed of genistein (195 ± 95.6 mg/kg of DM), prunetin (133 ± 51.3), glycitein (32 ± 20.9), and daidzein (10.9 ± 2.5). S-equol and coumestrol were not detected in our samples.

Nutritional composition

Hay and silage

We found a MTOD x WILT x STG interaction ($P \leq 0.05$) for DM, WSC, NH_3-N, NDF, ADF, and hemicellulose (Table 2-1). The DM of WET hay increased from STRT to MicA and increased again at LATE. For CUR hay, the DM was not different between STRT and MicA, but decreased at LATE (Table 2-2). The DM was not different across

the storage stages for silage. The WSC was lower for WET than CUR at STRT, MicA, and LATE for hay and silage. Regarding the CP values, we observed effects for MTOD x WILT and STG x MTOD ($P \leq 0.01$; Table 2-1). The CP was not different between WET and CUR hay (Table 2-3). However, the CP was higher for WET than CUR silage. Across storage stages, hay had lower CP compared to silage.

At STRT, the NH_3-N was lower for WET vs. CUR hay and higher for WET vs. CUR silage. Within MicA, the NH_3-N was higher for WET vs. CUR for hay and lower for WET vs. CUR for silage. At LATE, the NH_3-N was lower for WET vs. CUR for hay, whereas no differences were observed between WET and CUR silage. In the case of NDF and ADF, the WET and CUR hay were not different for both analytes at the STRT stage. However, at MicA and LATE, the NDF and ADF were higher for WET hay vs. CUR. At STRT and MicA, the WET silage had lower NDF and ADF compared with CUR. At LATE, the WET and CUR silage were not different for NDF, while ADF was lower for WET vs. CUR. For hay, WET and CUR hay were not different in hemicellulose at STRT. However, at MicA and LATE, WET had higher hemicellulose than CUR. For silage, WET and CUR were not different in hemicellulose at STRT, MicA, and LATE stage.

DM losses, pH, and microbial counts

Red clover hay and silage

In our study, an interaction between MTOD x WILT x STG ($P < 0.001$) was found for pH and mold counts. Moreover, we found an interaction between MTOD x WILT and STG x MTOD for yeast counts and an interaction between MTOD x WILT for DM losses ($P \leq 0.03$; Table 2-1). At STRT, the pH was no different between the WET and CUR for

both hay and silage. However, at MicA, hay pH was higher for WET vs. CUR but lower for silage WET vs. CUR. At LATE, hay pH was higher for WET than CUR, whereas no differences were observed between WET and CUR silage (Table 2-4). At STRT, mold counts of WET and CUR were not different for hay and silage. However, at MicA, hay WET had more molds than CUR, while the opposite was observed between silage WET vs. CUR. At LATE, WET hay had more mold counts than CUR, while no differences were observed between WET and CUR silage. The DM losses during storage were higher for hay WET vs. CUR, and no differences were observed for silage WET vs. CUR (Table 2-5). The yeast counts were lower in WET vs. CUR for both hay and silage. Within the STRT, hay and silage were not different in yeast counts. However, within the MicA and LATE stages, hay had more yeast than silage. There were no differences in yeast counts between WET and CUR at STRT for both conservation methods. However, the WET treatment was lower than CUR at MicA and at LATE for both hay and silage.

Lactic acid bacteria (LAB) in silage

An interaction between STG x WILT was found for LAB ($P = 0.015$). At STRT, the WET silage had higher counts vs. CUR (7.26 vs. 6 $\pm$ 0.16 log cfu/fresh g, respectively). The LAB counts were not different for both wilting extents at MicA ($\overline{x} = 8.89 \pm 0.16$ log cfu/fresh g) and at LATE ($\overline{x} = 7.32$).

Moldiness and heating measures of hay

We found an interaction between STG x WILT for visual moldiness and only the main effect of WILT for HDD and MaxT (maximum temperature; $P \leq 0.03$). The visual moldiness score was higher for WET compared to CUR hay at MicA, (9.4 vs. 0 $\pm$ 0.12

units, respectively) and at LATE (10 vs. 0). The HDD was greater for WET hay than CUR (72.5 vs. 13.8 $\pm$ 4.96 °C-day, respectively). A similar trend was observed for MaxT (34.4 vs. 25.8 $\pm$ 0.52 °C, respectively).

Silage fermentation profile

We found the main effect of WILT and STG for both lactic and acetic acid and only a WILT effect for ethanol ($P \leq 0.013$). The WET silage had higher lactic acid (71.7 vs. 31.4 $\pm$ 5.42 g/kg of DM, respectively) and acetic acid (18.2 vs. 12.2) relative to CUR. Also, lactic acid was lower at MicA than at LATE (41.6 vs. 61.5 $\pm$ 5.42 g/kg of DM, respectively), and the same pattern was observed for acetic acid (12.2 vs. 18.2). More ethanol was observed in WET vs. CUR (3.25 vs. 0.80 $\pm$ 0.60 g/kg of DM, respectively). Additionally, the propionic acid was not different across treatments ($\overline{x} = 5.57 \pm 0.81$ g/kg of DM; $P = 0.764$) and the concentration of 1,2-propanediol was below the detection limit ($\overline{x} < 0.2$ g/kg DM).

Aerobically exposed silage

Nutritional composition

An interaction between STG x WILT ($P = 0.041$) was found for WSC and hemicellulose. Also, NH_3-N was affected by STG ($P = 0.005$), and DM and CP by WILT ($P \leq 0.005$; Table 2-1). Additionally, we found the main effect of WILT ($P < 0.001$) and STG ($P < 0.001$) for both NDF and ADF. The DM observed after 7 d of aerobic exposure was lower for WET than for CUR (289 vs. 445 $\pm$ 11.5 g/kg, respectively). Table 2-6 shows that the WSC was not different between CUR and WET at MicA, which differed from the

result observed before aerobic exposure for MicA. At LATE, the WET silage still had lower WSC than CUR after aerobic exposure. The CP after 7 d of aerobic exposure was higher in WET vs. CUR, and the silage NH_3-N concentration was lower at MicA than at LATE. The NDF and ADF values observed after 7 d of aerobic exposure were lower for WET vs. CUR. NDF and ADF levels were higher at MicA than at LATE.

Microbial counts, pH, DM losses, and aerobic stability

The interaction between WILT x STG ($P = 0.018$) for DM losses, MaxT, and HDD are shown in Table 2-1, as well as the main effect of WILT ($P < 0.033$) for pH and yeast counts. The pH levels observed after 7 d of aerobic exposure were lower in WET vs. CUR (Table 2-6). The WET silage had less DM losses after aerobic exposure than CUR at MicA, but no differences were found at LATE. The yeast counts were higher for WET than CUR when aerobically challenged, and mold counts were not different across treatments ($\overline{x} < 2.00$; $P = 0.963$). The WET treatment was aerobically stable for a shorter time relative to CUR across both storage stages. However, at MicA, WET had lower HDD than CUR when aerobically challenged, and no differences were observed at LATE. The MaxT was lower for WET compared to CUR at MicA and LATE. Overall, it seemed that CUR silage was more susceptible to aerobic spoilage, especially if ensiled only for 14 d.

<u>In vitro ruminal digestibility, gas production, and fermentation profile</u>

The interaction between MTOD x WILT x STG ($P = 0.016$) for IVDMD, IVOMD, pH, M_f, and K_f are shown in Table 2-1. In addition, we found an interaction between STG x WILT for CH_4 concentration ($P = 0.02$) and an interaction between STG x WILT ($P = 0.015$) and MTOD x WILT ($P = 0.037$) for CH_4 yield. Table 2-7 shows that the IVDMD

and IVOMD were not different between WET and CUR hay at STRT. However, at MicA and LATE, WET hay had lower IVDMD and IVOMD vs. CUR. At STRT, the IVDMD and IVOMD were not different for WET and CUR silage. The same trend was observed at MicA, but the WET silage had higher IVDMD and IVOMD than CUR at LATE. At STRT, the ruminal pH was not different for CUR and WET hay, but at MicA and LATE, it was higher for WET hay vs. CUR. The ruminal pH of red clover silage was not different across treatments. Regarding gas production kinetics, at STRT, the M_f and K_f were not different for WET and CUR hay, but at MicA and LATE, the WET hay had a lower Mf and K_f vs. CUR. The M_f and K_f were not different for WET and CUR silage at STRT, MicA, and LATE. Table 2-8 shows that the WET main effect mean for CH_4 concentration and yield was lower at MicA and LATE vs. STRT, but the CUR main effect mean did not change across the storage stages evaluated. In addition, the CH_4 yield of WET and CUR hay was not different. However, a lower CH_4 yield was found for silage WET vs. CUR.

Effects of MTOD x WILT x STG interaction on ruminal acetic-to-propionic ratio (**A:P**; $P = 0.019$) and MTOD x STG on ruminal butyric acid ($P = 0.016$) are shown in Table 2-1. The ruminal total volatile fatty acids (**TVFA**) produced in vitro were not different across treatments, as well as ruminal acetic acid and propionic acid levels (Table 2-9). The ruminal A:P was not different for WET and CUR hay at STRT, but it was lower for WET hay vs. CUR at MicA. However, there were no differences at LATE. The ruminal A:P of WET and CUR silage was not different at STRT, but it was lower for WET silage compared with CUR at MicA and LATE. The ruminal butyric acid was not different between hay and silage at STRT and MicA, but hay was lower relative to silage at LATE.

To help us understand the effects of the nutritional (DM, WSC, CP, NDF, ADF, hemicellulose, pH, and NH_3-N) and microbiological variables (mold, yeast, and LAB counts) measured in this study on in vitro CH_4 ruminal production, we did a regression analysis between the chemical composition (DM, WSC, CP, NDF, ADF, hemicellulose, pH, and NH_3-N) and microbial composition (mold, yeast, and LAB counts) with CH_4 yield of both wilting extents (WET and CUR) of hay and silage. We found that pH of WET and CUR silage were positively correlated with CH_4 yield with a partial R^2 of 0.18. In the case of WET and CUR hay, the regression analysis showed that yeast counts and CP were positively correlated with CH_4 yield, with the former having the highest partial R^2 (0.38) followed by CP (R^2= 0.21).

In situ rumen degradation kinetics

Dry matter degradation kinetics

We found an interaction between MTOD x WILT ($P = 0.029$) for the soluble and undegradable fractions. The main effect of MTOD ($P = 0.006$) and WILT ($P = 0.029$) for the potentially degradable fraction and MTOD ($P = 0.003$) and WILT ($P = 0.029$) effect for the degradation rate are shown in Table 2-1. The lag phase was affected by WILT ($P = 0.025$). In situ DM kinetic showed that the lag phase was longer for insufficient wilting compared to ample wilting (Table 2-10). The soluble fraction was lower for the WET hay than CUR, while it was not different between WET and CUR silage. The potentially degradable fraction was higher for hay vs. silage, but the opposite was observed for the degradation rate. For both conservation methods, the potentially degradable fraction was higher and the degradation rate was lower for WET vs. CUR.

Neutral detergent fiber degradation kinetics

Effects of MTOD x WILT on the degradation rate ($P = 0.02$), MTOD main effect on the lag phase ($P = 0.036$), and WILT on the undegradable fraction ($P = 0.006$) are shown in Table 2-1. The lag time was longer for hay vs. silage (Table 2-11). The potentially degradable fraction was not affected by the treatments. The degradation rate was lower for the WET hay vs. silage, but it was not different between CUR hay and silage. For both conservation methods, the undegradable fraction was lower for WET vs. CUR, across storage methods.

Phytoestrogens

Table 2-1 shows the interaction between STG x MTOD ($P < 0.001$) and MTOD x WILT ($P < 0.001$) for formononetin and biochanin A. We found an interaction between STG x MTOD ($P < 0.001$), STG x WILT ($P = 0.015$), and MTOD x WILT ($P < 0.001$) for genistein and the main effect of STG ($P < 0.001$), MTOD ($P < 0.001$), and WILT ($P < 0.001$) for daidzein. Table 2-12 shows that formononetin was not different between WET and CUR hay ($\overline{x} = 2{,}524 \pm 154$ mg/kg of DM). However, the formononetin was higher for WET compared to CUR silage (5,841 vs. 4,608.1 $\pm$ 154 mg/kg of DM, respectively). For both wilting extents, hay had lower formononetin than silage at STRT (2,893 vs. 4,624 $\pm$ 175 mg/kg of DM, respectively), MicA (2,110 vs. 5,383), and LATE (2,569 vs. 5,666). The MTOD main effect showed that daidzein was higher in hay vs. silage (12.8 vs. 6.67 $\pm$ 0.43 mg/kg of DM, respectively). For both conservation methods, the daidzein was lower for WET vs. CUR (7.68 vs. 11.8 $\pm$ 0.43 mg/kg of DM, respectively). Furthermore, the STG main effect showed that for both conservation methods, daidzein was higher at STRT vs.

MicA (12.7 vs. 8.02 ± 0.52 mg/kg of DM, respectively) and then remained stable at LATE (8.46). The biochanin A and genistein levels were lower for WET than CUR hay (1,219.5 vs. 1,644.9 ± 127 and 72.8 vs. 97.3 ± 7.21 mg/kg of DM, respectively), and the opposite was observed for WET vs. CUR silage (3,793 vs. 2,628.9 and 236 vs. 202.3). For both wilting extents, the biochanin A and genistein were lower in hay vs. silage at STRT (2,012 vs. 3,105 ± 139 and 120 vs. 149 ± 8.36 mg/kg of DM, respectively), MicA (1,057 vs. 3,233 and 62.6 vs. 246), and LATE (1,228 vs. 3,295 and 72.9 vs. 264).

To understand the phytoestrogens losses in silage across wilting, we did a regression analysis between the chemical composition and microbial composition with formononetin. The results showed that NDF (partial R^2= 0.66) and DM (partial R^2= 0.06) were negatively correlated with formononetin. Similar results were observed in biochanin A of silage, which was negatively correlated with DM (partial R^2= 0.80) and NDF (partial R^2= 0.03). For hay, yeast counts (partial R^2= 0.49) were positively correlated with biochanin A. The same positive correlation was observed between yeast counts (partial R^2= 0.20) and formononetin levels in hay.

Discussion

Red clover stand

The chemical composition and microbial counts of the freshly cut stand were similar to previously reported values (Gallo et al., 2006; Franco et al., 2022; Wang et al., 2022). As expected for a red clover stand, the major phytoestrogens were formononetin and biochanin A, accounting for 96% of the detected phytoestrogens. Daidzein, genistein, prunetin, and glycitein were detected in minor amounts and represented the remaining

4% of the total detected phytoestrogens in the red clover stand. Phytoestrogens concentrations in our study were consistent with previously reported values (Tsao et al., 2006; Sarelli et al., 2010; Daems et al., 2016).

Nutritional composition of hay and silage

Legume leaf losses rise as the swath is cured more extensively, typically increasing the NDF and WSC and decreasing CP levels because stems contain more NDF and WSC and less CP than leaves in legumes (Lowell, 1995; Orloff and Mueller, 2008). The levels of WSC increased for CUR vs. WET hay and silage at STRT because of this. Hay CUR preserved more WSC during storage because it prevented mold growth, relative to WET, independently of WSC levels at STRT. At LATE, 69.7% of WSC were preserved in CUR hay, while only 24.4% of preservation was observed for WET hay due mold activity (Reyes et al., 2020; Leon-Tinoco et al., 2022). In the case of silage, only 47.4% of WSC were preserved at LATE relative to STRT for WET, but we observed 85.4% preservation in CUR. Van Ranst et al. (2009) reported that after 56 d of ensiling, the WSC of 488 g/kg DM red clover was higher vs. wilting to 263 g/kg DM (22.7 vs. 8 g/kg of DM, respectively). Similarly, Gallo et al. (2006) observed that after 180 d of ensiling, 400 g/kg DM red clover had a higher WSC concentration vs. wilting to 263 g/kg DM (27.8 vs. 14.4 g/kg of DM). This is explained by the higher total microbial activity observed as DM levels decrease in silages (Pahlow et al., 2003). Curing legume silages above 350-400 g/kg DM (Kung et al., 2018) is essential to prevent secondary fermentation caused by enterobacteria and clostridia, which triggers higher DM losses during storage, as observed in this study (Borreani et al., 2018). We did not observe butyric acid in both WET and CUR silages, indicating the absence of clostridial activity. Undesirable

enterobacterial fermentation was likely more prevalent in WET vs. CUR silage, partly explaining the higher DM storage losses and ethanol levels observed in WET, while yeast counts were actually higher in CUR vs. WET silage across all stages.

In our study, the effects of MTOD × WILT on CP can be explained by field leaf losses. Overall, leaf losses were more prevalent in hay vs. silage, but ample curing resulted in higher relative CP losses in silage relative to hay. Our results agree with Jin et al. (2018), who observed that wilting alfalfa hay from 734% to 942 g/kg DM did not affect the CP ($\bar{x} = 200$ g/kg of DM). Similarly, Coblentz et al. (2013a) reported that wilting alfalfa-orchardgrass hay from 762% to 804 g/kg DM did not reduce the CP ($\bar{x} = 176$ g/kg of DM). In the case of silage, Tao et al. (2017) reported that alfalfa wilted to 200 g/kg DM had higher CP levels compared to 546 g/kg DM (230 vs. 214 g/kg of DM, respectively). In contrast, Li et al. (2022) observed that wilting alfalfa from 378 g/kg to 461 g/kg DM did not affect the CP concentration ($\bar{x} = 177$ g/kg of DM). The difference in the CP concentration response between our study and Tao et al. (2017) was because the latter had a smaller DM difference between silage treatments relative to our study.

The extent of CP losses is also affected by microbial plant protein breakdown (Hao et al., 2020), which decreases protein quality significantly (Rotz et al., 2020). In our study, more microbial spoilage in WET hay resulted in more protein breakdown, resulting in NH3-N generation and an increased pH from 6.3 at STRT to 8.11 at the LATE phase. In contrast, CUR hay remained stable during storage, as evidenced by less NH_3-N production and no pH changes. Once pH is above 8.0, a significant portion of the ammonium will change to ammonia, which is volatile and can be lost to the environment (Purwono et al., 2017). Jin et al. (2018) reported that after 180 d of storage, alfalfa hay

baled at 734 g/kg DM had a higher NH_3-N compared to 942 g/kg DM (45.9 vs. 17 g/kg of total N, respectively), which then resulted in a higher pH for hay baled at 734 vs. 942 g/kg DM (7.39 vs. 6.08). Similarly, Nelson et al. (1989) reported that after 39 d of storage, alfalfa hay baled at 643 g/kg DM had more NH3-N than 847 g/kg DM (59.9 vs. 10.4 g/kg of total N, respectively). However, the pH levels were not different between both DM concentrations ($\bar{x} = 5.76$). The difference in the pH response between these two studies could be related to the storage period. Although the NH_3-N was lower for WET hay compared to CUR at LATE (78 d of storage), we suspect that NH_3-N volatilization was predominant in the WET treatment due to its pH levels being >8 (Powlson and Dawson, 2022).

If the red clover were to be ensiled only for 14 d, the WET treatment experienced less proteolysis, measured as NH_3-N, but this difference disappeared after 78 d of ensiling. Acidic conditions, which restrict the extent of proteolysis (Jones et al., 1995), were more prevalent for WET vs. CUR at d 14, but pH levels at d 78 were not. Similar to our findings, Gallo et al. (2006) reported that after 180 d of ensiling, the NH3-N concentration of red clover silage wilted to 263 and 400 g/kg DM was not different ($\bar{x} =$ 8.74% of total N). An experiment with alfalfa also showed that after 120 d of ensiling, wilting alfalfa from 241 to 340 g/kg DM did not affect the production of NH3-N ($\bar{x} = 2.43\%$ of N) (Hartinger et al., 2019). Unlike alfalfa, red clover has the potential to preserve high true protein levels during ensiling due to polyphenol oxidase activity (Sullivan and Hatfield, 2006). This attribute restricts protein degradation during ensiling in red clover relative to alfalfa, which improves nitrogen utilization efficiency by ruminants (Broderick, 2002; Dong et al., 2019).

The metabolization of the digestible fractions in WET hay (e.g. WSC) by microbial spoilage activity increased the NDF and ADF levels as reported elsewhere (Coblentz and Bertram, 2012), which reduces its nutritional value relative to properly cured hay that is stable during storage (Coblentz and Hoffman, 2010). A similar trend was observed by Reyes et al. (2020), who found that high-moisture alfalfa hay (624 g/kg DM) increased its NDF from 478 to 497 g/kg of DM due to fungal spoilage after 15 d of in vitro aerobic conditions. Overall, the nutritional value of spoiled hay is compromised, and that, along with a reduced DM intake (Collins and Coblentz, 2007b), will limit animal performance (Korosteleva et al., 2007). In the case of silage, the NDF of WET and CUR were not different at the end of storage, but WET was lower at STRT and MicA relative to CUR. Leaf losses during wilting can explain the increased fiber for CUR vs. WET silage at STRT. Similar results were observed by Purwin et al. (2014), who found that the NDF concentration of red clover wilted to 204 g/kg DM was lower compared to 423 g/kg DM (43.8 vs. 45.6% of DM, respectively) because of the WSC losses due to plant respiration during wilting. Glenn (1990) also reported that the NDF of alfalfa was lower when wilted to 188 g/kg DM vs. 444 g/kg DM (459 vs. 481 g/kg of DM, respectively). The authors did not explain their results.

We observed that the ethanol concentration was higher for the WET silage than CUR, partially explaining the DM losses during storage results. Although the yeast counts were more predominant for the CUR silage than WET across stages, the ethanol concentration of the CUR treatment was 0.79 g/kg of DM, far below the 30-40 g/kg of DM threshold that signals severe DM losses (Muck et al., 2018). The presence of enterobacteria can explain the higher concentration of ethanol in WET silage relative to

CUR because most ethanol in silages can actually be the result of enterobacteria activity rather than yeast (Heron et al., 1993). The mold counts in WET and CUR silage were reduced rapidly during ensiling, especially in WET silage after 14 d of ensiling, relative to STRT. This is because the WET silage was characterized by a faster drop of pH (<4.5) after 14 d of ensiling, resulting in greater inhibition of microbial growth (Dunière et al., 2013).

In our study, the butyric acid concentration of the WET silage was below the detection limit ($\overline{x}$ <2 g/kg of DM), which indirectly indicates the absence of clostridia fermentation (Leon-Tinoco et al., 2022). High-moisture legume silages (<300 g/kg DM) tend to have higher NH_3-N concentrations than drier silages (>400 g/kg DM) as a result of a higher proteolytic activity from clostridia and enterobacteria (Muck et al., 2020). Even though the WET silage produced in this study did not succumb to clostridial fermentation, the probability of this occurrence is higher when the DM is <300 g/kg (Kung et al., 2018). Thus, it is recommended to wilt legume silages to 350-500 g/kg DM to mitigate losses during ensiling caused by undesirable microbes (Wilkinson and Davies, 2013).

Aerobically exposed silage

After 7 d of aerobic exposure, the yeast counts were higher for the WET vs. CUR silage for both MicA and LATE. However, they did not go above ≥6 log cfu/ fresh g, which is the threshold for zero aerobic stability proposed by Kung et al. (2018). In addition, after being aerobically exposed, the WET silage had higher ethanol levels compared to CUR for the LATE treatment. Although the ethanol concentration of WET silage was not >30-40 g/kg of DM, which is a strong signal of spoilage (Muck et al., 2018), we still observed

that WET silage was aerobically stable for a shorter time than CUR after being exposed to air. Similarly, Knický and Lingvall (2004) reported shorter aerobic stability when a red clover-timothy silage mixture was wilted to 300 compared to 600 g/kg DM (29 vs. 79 h, respectively), and the yeast counts were greater at the former (4.2 vs. 2.6 log cfu/g, respectively). Hamberg (2021) also reported that a red clover-grass silage mixture wilted to 239 g/kg DM had lower aerobic stability compared with 415 g/kg (168 vs. 336 h). However, the ethanol concentration was not different between 239 and 415 g/kg DM ($\bar{x} =$ 5 g/kg of DM) in that study. The difference in the ethanol response between our study and Hamberg (2021) could be related to the number of days the silage was exposed to air (7 vs. 14 d, respectively). In addition, the HDD of CUR silage was higher than WET at MicA, but there were no differences at LATE after exposure to air. This is explained by the low acetic acid levels in CUR silage (7.8 g/kg of DM) relative to WET (16.6) at MicA before being aerobically challenged. Management decisions such as ensiling period also play a role in aerobic stability (Kung et al., 2003). In our study, when the ensiling period of red clover was limited to 14 d, the CUR silage was more susceptible to DM losses and subsequent heating during a 7-d aerobic challenge relative to the WET silage. However, when the ensiling period was extended to 78 d, no differences were observed between CUR and WET silage regarding susceptibility to aerobic spoilage. After 14 d of ensiling, fermentation is not always complete because there is still water in the crop (aw) that allows for bacterial growth, and the supply of available substrate has not been exhausted, limiting the pH decline to the point at which it inhibits microbial growth (Wilkinson and Davies, 2013). Thus, opening the CUR silage before the main fermentation phase is

complete (at 14 d) can cause a severe impact on aerobic stability, resulting in significant economic losses for farmers (Pahlow et al., 2003).

In vitro digestibility and gas production

Ample curing of red clover hay preserved optimal fermentation kinetics relative to insufficient. On the other hand, silage fermentation kinetics were not affected by the wilting extent across storage phases. However, the IVDMD and IVOMD of WET silage were higher than CUR at LATE. To our knowledge, no studies have assessed the effects of wilting extent on the gas kinetics of red clover hay and silage. Regarding ruminal CH_4 production, the regression analysis showed that pH was positively correlated with CH_4 yield of WET and CUR silage. We failed to speculate this correlation due to no studies have assessed the interaction between pH and in vitro ruminal CH_4 production and the R^2 was 0.21.

In the case of WET adn CUR hay, the regression analysis showed that yeast counts and CP were positively correlated with CH_4 yield, with the former having the highest partial R^2 (0.38) followed by CP (R^2= 0.21). Nevertheless, we speculate that mold counts were involved in the correlation between yeast counts and CH_4 yield because molds displaced yeast in the WET hay during storage. The regression analysis did not select mold counts as an explanatory variable to explain CH_4 yield in WET hay because mold counts fail to represent the mold species shift that occurs from field to storage microbiota. The low DM (<80%) hay undergoes a drastic shift in the fungal taxonomic profile during storage relative to baling and wilting (Wittenberg, 1997). Undi et al. (1997) reported that during the early storage phase (1-8 d), alfalfa hay baled at 550 and 760 g/kg

DM was characterized by the presence of the genera *Phoma* and *Cladosporium*. However, after 60 d of storage, the microbiome of hay baled at 550 g/kg DM was succeeded by the genera Absidia and Mucor, and the species *Emicerella nidularis* and *Aspergillus fumigatus*, while the dominant specie in hay baled at 760 g/kg DM was *Aspergillus repens*. Breton and Zwaenepoel (1991) assessed the taxonomic profile of tall fescue (*Lolium arandinaceum*) hay baled at 728 g/kg DM and observed that the primary fungal isolates belong to the genera *Alternaria*, *Cladosporium*, *Colletotrichum*, *Fusarium*, *Phaeoseptoria*, *Phoma*, and *Ascochyta*. At the end of storage, the authors observed the genera *Humicola*, *Peniculium*, *Emericella*, *Eurotium*, and yeast. Understanding the microbiome dynamics across the haymaking process is important to comprehend the impact they and their metabolites may have on enteric methane production and their exploitation as feed additives once isolated and proven safe and consistent.

Mold species associated with spoiled conditions may have influenced the reduction of CH_4 yield in WET hay through the production of secondary metabolites that can inhibit the growth of methanogens (Miller and Wolin, 2001). According to Mohd Azlan et al. (2018), rice straw inoculated with Aspergillus terreus and incubated for 14 d produced 35% less CH_4 in goats fed with inoculated than uninoculated rice straw because A. terreus produces lovastatin. This secondary fungal metabolite inhibits the activity of HMG-CoA reductase in the methanogens and thus interferes with membrane synthesis and inhibits the growth of rumen methanogens (Jahromi et al., 2013). Moreover, the total population of methanogens decreased by 24% in goats fed with the inoculated treatment. Similarly, Morgavi et al. (2013) used secondary fungal metabolites produced by *Monascus* spp., such as Monacolin K, in sheep and reported a 30% reduction in enteric CH4 production

and a decrease in A:P ruminal ratio. The use of molecules produced by fungi and bacteria to decrease methane production has been discussed elsewhere (Chung et al., 2011; Grainger and Beauchemin, 2011; Shen et al., 2017). However, their effectiveness may be dose, strain, and substrate-dependent (Ellis et al., 2016). Thus, further research needs to be conducted to identify fungal metabolites associated with the inhibition of methanogenesis.

In situ rumen degradability

The in situ DM and NDF degradability kinetics were only analyzed at the LATE storage phase for hay and silage. For both conservation methods, inadequate wilting resulted in a longer lag time for ruminal DM degradability compared to ample wilting. Lag time is influenced by substrate hydration rate, microbial attachment, and nutrient availability (López, 2005). The higher concentration of WSC in the CUR hay and CUR silage, relative to WET hay and WET silage, may have increased the microbial attachment to the feed, resulting in a shorter lag phase than their WET counterparts. We also observed that ample wilting reduced the potentially degradable fraction compared to insufficient wilting but increased the degradation rate of DM. To the best of our knowledge, this is the first study assessing the effects of wilting extent on the in situ degradation rate of red clover hay and silage in the same publication.

In our study, the CUR hay had a higher soluble DM fraction than WET hay because the latter went through a spoilage process driven by plant respiration and microbial activity (Collins and Moore, 2017). In the case of silage, the soluble DM fraction of the WET one was not different from CUR. In addition, both the CUR and WET silage had a higher

soluble fraction than CUR and WET hay, likely because of the intensive plant proteolysis that occurs during ensiling, compared to haymaking (Foster et al., 2011). We observed that ensiling decreased the potentially degradable DM fraction by 10.2% relative to haymaking but increased the rate of digestion by 40%. Haymaking reduces the degradation rate of DM in the rumen because the longer the wilting, the more extensive leaf losses are (Orloff and Mueller, 2008). Coblentz et al. (1998) assessed the in situ degradation rate of leave and stem tissues of fresh red clover and alfalfa stand and found that for both legumes, the leaves had a DM higher degradation rate of than stems (15.6 vs. 11.6 and 20 vs. 12.8%/h, respectively). Regarding the potentially degradable fraction, ensiling decreased it, most likely due to a more extensive activation of polyphenol oxidase (PPO) relative to hay. Polyphenol oxidases are activated when cell damage occurs, and these enzymes are mixed with their diphenols substrates, forming protein-bound phenols (Matheis and Whitaker, 1984; Lee et al., 2009). Thus, chopping red clover before ensiling may have been responsible for a more extensive polyphenol oxidase (PPO) activity relative to hay. Seguin and Mustafa (2003) found that ensiled kura clover (*Trifolium ambiguum* M.B.) with 271 g/kg DM had a higher soluble DM fraction (432 vs. 384 g/kg of DM, respectively) and a lower potentially degradable fraction (500 vs. 529 g/kg of DM) compared to fresh kura clover (211 g/kg DM). Even though this study did not compare silage vs. hay, the lower potentially degradable fraction in ensiled kura clover may have been caused by chopping the legume before ensiling. Aufrère et al. (2003) also reported that ensiling cocksfoot (Dactylis glomerata) decreased the potentially degradable fraction by 37.6% compared with haymaking but increased the degradation rate by 94.7%. The authors did not explain the results. To the best of our knowledge, this is the first study

assessing the effects of the conservation method on the in situ ruminal degradability kinetics of red clover.

The more extended lag phase of NDF for hay vs. silage and the lower degradation rate of NDF for CUR silage vs. WET were likely caused by the effects of a longer curing period, which would result in larger leaf losses (Orloff and Mueller, 2008). Stem tissues of legumes are considered low fiber quality with lower exchange cation capacity, slow particle size reduction, and slow rate of hydration (Van Soest, 1988). Within wilting extent, the degradation rate of NDF was not different between CUR hay and CUR silage. In contrast, insufficient wilting (WET) reduced the degradation rate of NDF in hay relative to silage because spoilage of WET hay was much more extensive than WET silage, as seen in the storage DM losses. Reyes et al. (2020) reported that the NDF digestibility of high-moisture alfalfa hay (624 g/kg DM) was 300 g/kg of DM, but after 15-d in vitro aerobic conditions, it reduced to 233 g/kg of DM because of the spoilage process. Murphy et al. (2000) found that a grass mixture of timothy and meadow fescue hay wilted to 905 g/kg DM had a lower degradation rate of NDF compared to ensiling the grass mixture at 292 g/kg DM (3.7 vs. 4.2 %NDF/h, respectively). Contrary to our results, Schulze et al. (2015) reported that the degradation rate of NDF of a glass/clover mixture (ryegrass, red clover, and white clover) hay wilted to 828 g/kg DM was higher to ensiling the mixture at 250 g/kg DM (6.1 vs. 4.2 %NDF/h, respectively). The difference in the degradation rate response between our study and Schulze et al. (2015) could be related to forage species and the in situ incubation time between studies (96 vs. 288 h).

Phytoestrogens

Only three studies have reported phytoestrogens changes during field wilting (Sivesind and Seguin, 2005; Sarelli et al., 2010; Daems et al., 2016). In the hay, we found that wilting from 650 to 891 g/kg DM increased by 34.9% and 33.6% the levels of biochanin A and genistein, respectively. This is likely because of plant respiration during wilting (Jaster, 1995), which results in the disappearance of soluble nutrients that, in turn, increase the concentration of analytes that are not easily metabolized by residual plant enzymatic activity above 700 g/kg DM (Lowell, 1995). In silage, wilting from 294 to 455 g/kg DM decreased biochanin A and formononetin levels by 30.7% and 21.1%, respectively. Based on the regression analysis results, the lower concentration of biochanin A and formononetin in CUR vs. WET silage was most likely due to the result of leaf losses during wilting because it has been reported that leaves have more biochanin A and formononetin than in stems (Tsao et al., 2006; Saviranta et al., 2008). Sarelli et al. (2010) reported that red clover silage harvested at the budding stage and wilted to 400 g/kg DM had 13.1% less biochanin A and 12.9% less formononetin than the one wilted to 250 g/kg DM.

Similarly, Sivesind and Seguin (2005) found that red clover wilted to 400 g/kg DM had 20% and 8.5% less formononetin and biochanin A, respectively, compared with unwilted red clover (280 g/kg DM). In contrast, Daems et al. (2016) reported that red clover harvested at the flowering stage and wilted from 230 to 300 g/kg DM did not affect the formononetin and biochanin A concentration. The difference in the formononetin and biochanin A response between the studies could be related to the maturity stage at harvest, field wilting extent, and DM concentrations. Feeding CUR silage rather than WET

silage could reduce risks associated with red clover because CUR silage reduced the concentration of formononetin. In the rumen, formononetin is metabolized into equol, which is much more estrogenic, while biochanin A is metabolized into nonestrogenic para-ethyl phenol (Reed, 2016). Thus, lowering the formononetin levels in red clover would reduce the estrogenic capacity of red clover.

Microbial degradation is another factor that can affect the concentration of phytoestrogens (Wang et al., 2015). The regression analysis for hay showed that yeast counts (partial R^2= 0.49) were positively correlated with biochanin A. The same positive correlation was observed between yeast counts (partial R^2= 0.20) and formononetin levels in hay. We speculate that mold counts are also involved in biochanin A and formononetin degradation during storage. However, the regression analysis did not select molds as the main explanatory variable due to the reason explained in the in vitro digestibility section. In our study, for both wilting extents, the levels of formononetin, biochanin A, and genistein in hay decreased by 27.1%, 47.5%, and 47.6%, respectively, after 14 d of storage. Since plant enzymatic activity is minimal above 700 g/kg DM (Coblentz, 2020), we suspect that the activity of catabolic enzymes in plants was negligible, and the decrease of formononetin, biochanin A, and genistein in hay was related to microbial degradation. The importance of aerobic microorganisms in the degradation of phytoestrogens has been discussed elsewhere (Shi et al., 2004; Khanal et al., 2006). Kelly et al. (2014) assessed the persistence of genistein in sludge samples collected from a wastewater treatment plant and inoculated with nitrifying bacteria. The results showed that genistein degradation was unaffected by adding a nitrifier inhibitor and persisted until genistein was entirely degraded, suggesting that genistein degraders

are likely aerobic microbes. Although the microorganisms responsible for phytoestrogen degradation are unknown, and the products of degradation have not been assessed for biological activity in animals (Kelly et al., 2014), the biodegradation of phytoestrogens in hay during storage will impact the estrogenic capacity of the feed. Kuiper et al. (1997) reported that genistein has a greater affinity (30-fold) for estrogen receptor β (ERβ) than estrogen receptor α (ERα). In addition, Kuiper et al. (1998) found that the estrogenic effect is ranked differently for both ERs: genistein > daidzein > biochanin A > formononetin for ERα and genistein > daidzein > biochanin A > formononetin for ERβ. Since our study showed that haymaking reduces the phytoestrogen levels in red clover during storage, its practice can reduce the risks associated with phytoestrogens, especially with formononetin.

Unlike haymaking, there was an increment in the concentration of formononetin and genistein during ensiling. This increment may have been related to the disappearance of other carbohydrate compounds (Collins and Coblentz, 2007b), such as WSC and hydrolysis of hemicellulose in both WET and CUR silage during ensiling (Zhao et al., 2018).

Conclusions

Haymaking was the most effective technique for reducing formononetin, biochanin A, and genistein compared with ensiling red clover. The CUR treatment was more beneficial for silage than hay in terms of decreasing the concentration of formononetin, biochanin A, and genistein in red clover. Ample wilting was more critical to preventing storage DM losses in hay than silage. The NDF, ADF, hemicellulose, and pH values of

CUR hay remained stable across the storage phase, while in WET hay they increased due to spoilage. The CUR silage was the best method to preserve sugars across the storage phase and prevent undesirable secondary fermentation. When the ensiling period of red clover was limited to 14 d, CUR silage was more susceptible to nutrient losses and subsequent heating during a 7 d aerobic challenge, relative to WET. However, when the ensiling period was extended to 78 d, no differences were observed between WET and CUR silage in terms of susceptibility to aerobic spoilage. Adequate wilting (CUR) preserved the IVDMD, IVOMD, M_f, and K_f of hay compared to WET across the storage stages, but it did not affect the CH_4 yield. In silage, fermentation kinetics were not affected by wilting extent across the storage phase. However, the WET silage decreased the A:P ruminal ratio across the storage phases. Consequently, for both conservation methods at LATE, the ruminal CH_4 yield of WET was lower than CUR. Ensiling increased the soluble DM fraction compared to haymaking but decreased the potentially degradable DM fraction relative to hay. Haymaking reduced the degradation rate of DM compared to ensiling. The potentially degradable NDF fraction was not affected by the conservation method and wilting extent. In silage, CUR decreased the degradation rate of NDF compared to WET. Overall, ample wilting helps conserve the nutritional quality of hay and silage and decreases the phytoestrogens, especially in silage.

Table 2-1. Statistical analysis (*P*-values) of the interaction effects for conservation method (MTOD), storage stages (STG), and wilting extent (WILT) on the nutritional composition, DM losses, microbial counts, phytoestrogen levels, in vitro ruminal measures, and in situ degradability kinetics of red clover hay and silage and statistical analysis (*P*-values) of the interaction effects for storage stages (STG) and wilting extent (WILT) on the nutritional composition, DM losses, microbial counts, and aerobic stability measures of red clover silage after 7 d of aerobic exposure[1-3].

Item[4]	*P*-value						
	MTOD	WILT	STG	MTOD x WILT	MTOD x STG	WILT x STG	MTOD x WILT x STG
DM, g/kg	<0.001	<0.001	0.01	0.01	<0.001	<0.001	<0.001
WSC, g/kg of DM	<0.001	<0.001	<0.001	0.05	0.82	0.05	0.05
CP, g/kg of DM	<0.001	<0.001	0.64	<0.001	0.01	0.22	0.29
NH3-N, g/kg of total N	<0.001	0.16	<0.001	0.02	<0.001	<0.001	<0.001
NDF, g/kg of DM	<0.001	<0.001	0.01	<0.001	<0.001	<0.001	<0.001
ADF, g/kg of DM	<0.001	0.05	<0.001	<0.001	<0.001	<0.001	<0.001
Hemicellulose, g/kg of DM	<0.001	<0.001	0.08	<0.001	<0.001	<0.001	<0.001
DM Loss, %	0.85	<0.001	0.218	<0.001	0.659	0.672	0.56
pH	<0.001	0.002	0.02	<0.001	<0.001	0.001	<0.001
Yeast, log cfu/g fresh	<0.001	<0.001	<0.001	0.03	<0.001	<0.001	0.156
Mold, log cfu/g fresh	<0.001	0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Aerobically exposed silage							
DM, g/kg	NA[5]	<0.001	0.497	NA	NA	0.39	NA
pH	NA	<0.001	0.132	NA	NA	0.784	NA
WSC, g/kg of DM	NA	0.069	0.317	NA	NA	0.021	NA
NDF, g/kg of DM	NA	<0.001	<0.001	NA	NA	0.172	NA
ADF, g/kg of DM	NA	0.001	<0.001	NA	NA	0.543	NA
Hemicellulose, g/kg of DM	NA	0.069	<0.001	NA	NA	0.041	NA
CP, g/kg of DM	NA	<0.001	0.683	NA	NA	0.381	NA
NH_3-N, g/kg of total N	NA	0.817	0.005	NA	NA	0.714	NA
DM loss, %	NA	0.008	0.016	NA	NA	0.018	NA
Yeast, log cfu/g	NA	0.033	0.476	NA	NA	0.158	NA
Aerobic stability, h	NA	0.016	0.113	NA	NA	0.135	NA
Max Temp., °C	NA	<0.001	<0.001	NA	NA	<0.001	NA
HDD, °C-day	NA	<0.001	<0.001	NA	NA	<0.001	NA
In vitro ruminal digestibility							
IVDMD, g/kg of DM	0.09	0.914	0.229	<0.001	0.476	0.189	0.005
IVOMD, g/kg of OM	0.199	0.421	0.063	<0.001	0.372	0.115	0.003
pH	0.64	0.023	0.005	0.251	0.817	0.01	0.016
NH_3-N, mg/dL	0.488	0.013	0.026	0.143	0.372	0.95	0.335
Total VFA	0.981	0.931	0.229	0.86	0.788	0.712	0.614
Acetic acid, g/kg of DM	0.751	0.612	0.076	0.596	0.855	0.554	0.722
Propionic acid, g/kg of DM	0.981	0.868	0.744	0.94	0.65	0.878	0.527
Isobutyric acid, g/kg of DM	0.142	0.634	0.486	0.818	0.757	0.741	0.588
Butyric acid, g/kg of DM	0.013	0.185	0.606	0.147	0.016	0.916	0.083
Isovaleric acid, g/kg of DM	0.31	0.269	0.249	0.909	0.881	0.689	0.558
Valeric acid, g/kg of DM	0.775	0.706	0.646	0.534	0.111	0.85	0.223
A:P Ratio	0.243	<.001	<.001	0.028	<.001	0.007	0.019
Gas production kinetics							
M_f, ml/g of incubated DM	0.761	<0.001	<0.001	<0.001	0.072	<0.001	0.002

Table 2-1. Continued.

K_f, %/h	<0.001	<0.001	0.002	<0.001	<0.001	<0.001	<0.001
Methane production							
Methane, m*M*	0.195	0.202	0.048	0.386	0.769	0.02	0.358
Methane yield, mmol/g fermented OM	0.691	0.009	<0.001	0.037	0.871	0.015	0.643
In situ degradability kinetics at LATE stage							
DM kinetics							
Lag phase, h	0.322	0.025	0.322	NA	NA	NA	NA
Soluble fraction, g/kg of DM	<0.001	<0.001	<0.001	NA	NA	NA	NA
Potentially degradable fraction, g/kg of DM	0.006	0.029	0.769	NA	NA	NA	NA
Degradation rate, % of DM/h	0.003	0.029	0.065	NA	NA	NA	NA
Undegradable fraction, g/kg of DM	<0.001	0.294	0.029	NA	NA	NA	NA
NDF kinetics							
Lag phase, h	0.036	0.382	0.382	NA	NA	NA	NA
Potentially degradable fraction, g/kg of NDF	0.442	0.724	0.171	NA	NA	NA	NA
Degradation rate, % of NDF/h	0.068	0.618	0.02	NA	NA	NA	NA
Undegradable fraction, g/kg of NDF	0.335	0.006	0.383	NA	NA	NA	NA
Phytoestrogens							
Formononetin, mg/kg of DM	<0.001	<0.001	0.02	<0.001	<0.001	0.568	0.101
Daidzein, mg/kg of DM	<0.001	<0.001	<0.001	0.654	0.053	0.055	0.083
Biochanin A, mg/kg of DM	<0.001	<0.001	0.001	<0.001	<0.001	0.192	0.917
Genistein, mg/kg of DM	<0.001	0.429	<0.001	<0.001	<0.001	0.015	0.607
Prunetin, mg/kg of DM	<0.001	<0.001	<0.001	0.448	<0.001	0.799	0.26
Glycitein, mg/kg of DM	<0.001	<0.001	<0.001	0.629	<0.001	0.266	0.768

[1] DM of hay = WET (651 g/kg) and CUR (891).
[2] DM of silage = WET (294 g/kg) and CUR (453).
[3] STRT = Start of storage; MicA = After 14 d of storage; LATE = 50 d of storage for hay and 78 d for silage
[4] WSC = Water soluble carbohydrates; CP = Crude protein; NH3-N = Ammonia nitrogen; NDF = Neutral detergent fiber; ADF = Acid detergent fiber, Aerobic stability = Aerobic stability was expressed as the amount of time (hours) before the silage heated 2°C above ambient temperature. HDD = Heat-degree days; calculated as the sum of the daily temperatures increments above room temperature; Max Temp = maximum temperature during aerobic exposure; IVDMD = Apparent in vitro DM digestibility; IVOMD = Apparent in vitro OM digestibility; VFA = Volatile fatty acids; A:P = Acetic to Propionic acid ratio; M_f = asymptotic maximal gas production; K_f = rate of gas production.
[5] NA = Not applicable

Table 2-2. Effect of conservation method (MTOD), storage stages (STG), and wilting extent (WILT) on the nutritional composition of red clover hay and silage[1-3].

Item[4]	Storage stages			SEM
	STRT	MicA	LATE	
DM, g/kg				15.4
Hay WET	$651^{B,c}$	$751^{B,b}$	831^{a}	
Hay CUR	$891^{A,a}$	$871^{A,ab}$	844^{b}	
Silage WET	294^{Y}	283^{Y}	282^{Y}	
Silage CUR	453^{X}	447^{X}	443^{X}	
WSC, g/kg of DM				2.38
Hay WET	$19.4^{B,a}$	$10^{B,b}$	$4.74^{B,b}$	
Hay CUR	$46.5^{A,a}$	$36.5^{A,b}$	$32.4^{A,b}$	
Silage WET	$36.7^{Y,x}$	$20.1^{Y,y}$	$17.4^{Y,y}$	
Silage CUR	$43.2^{X,x}$	$40.2^{X,xy}$	$36.9^{X,y}$	
NH3-N, g/kg of total N				0.43
Hay WET	$4.59^{B,b}$	$11.7^{A,a}$	$5.86^{B,b}$	
Hay CUR	$6.06^{A,b}$	$5.86^{B,b}$	$10.3^{A,a}$	
Silage WET	$14.5^{X,z}$	$31.6^{Y,y}$	47.9^{x}	
Silage CUR	$5.52^{Y,y}$	$41.1^{X,x}$	52.9^{x}	
NDF, g/kg of DM				6.29
Hay WET	477^{c}	$570^{A,b}$	$599^{A,a}$	
Hay CUR	482	471^{B}	476^{B}	
Silage WET	$459^{Y,x}$	$435^{Y,y}$	429^{y}	
Silage CUR	$482^{X,x}$	$471^{X,x}$	476^{y}	
ADF, g/kg of DM				5.55
Hay WET	321^{c}	$370^{A,b}$	$389^{A,a}$	
Hay CUR	325	327^{B}	323^{B}	
Silage WET	305^{Y}	302^{Y}	307^{Y}	
Silage CUR	331^{X}	328^{X}	327^{X}	
Hemicellulose, g/kg of DM				3.73
Hay WET	156^{c}	$200^{A,b}$	$210^{A,a}$	
Hay CUR	157	151^{B}	153^{B}	
Silage WET	145^{x}	131^{y}	122^{z}	
Silage CUR	151^{x}	136^{y}	116^{z}	

$^{A,B;\ X,Y}$ Means with different uppercase superscripts within a column are significantly different ($P \leq 0.05$). Uppercase superscripts A,B apply only for hay and X,Y for silage.

$^{a\text{-}c;x\text{-}z}$ Means with different lowercase superscripts within a row are significantly different ($P \leq 0.05$). Lowercase superscripts a-c apply only for hay and x-z for silage.

[1] DM of hay = WET (651 g/kg) and CUR (891).

[2] DM of silage = WET (294 g/kg) and CUR (453).

[3] STRT = Start of storage; MicA = After 14 d of storage; LATE = 50 d of storage for hay and 78 d for silage.

[4] WSC = Water soluble carbohydrates; NH3-N = Ammonia nitrogen; NDF = Neutral detergent fiber; ADF = Acid detergent fiber

Table 2-3. Effect of conservation method (MTOD), storage stages (STG), and wilting extent (WILT) on CP levels of red clover hay and silage[1-3].

Item[4]	Storage stages			MTOD x WILT	SEM
	STRT	MicA	LATE		
	MTOD x STG	MTOD x STG	MTOD x STG		
CP, g/kg of DM					3.35
Hay WET	-	-	-	138	
Hay CUR	-	-	-	136	
Hay mean	143^{B,a}	135^{B,b}	137^{B,b}		
Silage WET	-	-	-	174^{X}	
Silage CUR	-	-	-	149^{Y}	
Silage mean	158^{A}	163^{A}	163^{A}		

$^{A,B; X,Y}$ Means with different uppercase superscripts within a column are significantly different ($P \leq 0.05$). Uppercase superscripts A,B apply only for hay and X,Y for silage.
$^{a\text{-}c;x\text{-}z}$ Means with different lowercase superscripts within a row are significantly different ($P \leq 0.05$). Lowercase superscripts a-c apply only for hay and x-z for silage.
[1] DM of hay = WET (651 g/kg) and CUR (891).
[2] DM of silage = WET (294 g/kg) and CUR (453).
[3] STRT = Start of storage; MicA = After 14 d of storage; LATE = 50 d of storage for hay and 78 d for silage.
[4] CP = Crude protein.

Table 2-4. Effects of conservation method (MTOD), storage stages (STG), and wilting extent (WILT) on pH and mold counts of red clover hay and silage[1-3]

Item[4]	Storage stages			SEM
	STRT	MicA	LATE	
pH				2.68
Hay WET	6.23^{b}	7.92^{A,a}	8.11^{A,a}	
Hay CUR	6.16	6.13^{B}	5.82^{B}	
Silage WET	5.75^{x}	4.42^{Y,y}	4.2^{y}	
Silage CUR	6.23^{x}	5.38^{X,y}	4.74^{z}	
Mold, log cfu/g fresh				2.71
Hay WET	5.47^{b}	7.84^{A,a}	7.92^{A,a}	
Hay CUR	6.09^{a}	5.37^{B,ab}	4.71^{B,b}	
Silage WET	5.64^{x}	0.45^{Y,y}	<2^{y}	
Silage CUR	6^{x}	1.74^{X,y}	<2^{z}	

$^{A,B;\ X,Y}$ Means with different uppercase superscripts within a column are significantly different ($P \leq 0.05$). Uppercase superscripts A,B apply only for hay and X,Y for silage.
$^{a\text{-}c;x\text{-}z}$ Means with different lowercase superscripts within a row are significantly different ($P \leq 0.05$). Lowercase superscripts a-c apply only for hay and x-z for silage.
[1] DM of hay = WET (651 g/kg) and CUR (891).
[2] DM of silage = WET (294 g/kg) and CUR (453).
[3] STRT = Start of storage; MicA = After 14 d of storage; LATE = 50 d of storage for hay and 78 d for silage.

Table 2-5. Effect of conservation method (MTOD), storage stages (STG), and wilting extent (WILT) on DM losses and yeast counts of red clover hay and silage[1-3].

Item[4]	Storage stages						MTOD x WILT	SEM
	STRT		MicA		LATE			
	MTOD x STG	WILT x STG	MTOD x STG	WILT x STG	MTOD x STG	WILT x STG		
DM loss, %								0.82
Hay WET	-	-	-	-	-	-	8.46^{A}	
Hay CUR	-	-	-	-	-	-	-1.47^{B}	
Silage WET	-	-	-	-	-	-	4.34^{X}	
Silage CUR	-	-	-	-	-	-	2.38^{Y}	
Yeast, log cfu/g fresh								3.44
Hay WET	-	7.14^{m}	-	$1.55^{B,n}$	-	$1.33^{B,n}$	4.04^{A}	
Hay CUR	-	7.24^{m}	-	$5.09^{A,n}$	-	$2.69^{A,o}$	6.31^{B}	
Hay mean	7.03^{a}		$4.47^{A,b}$		$4.02^{A,b}$			
Silage WET	-	-	-	-	-	-	2.64^{X}	
Silage CUR	-	-	-	-	-	-	3.7^{Y}	
Silage mean	7.34^{a}		$2.17^{B,b}$		$<2^{B,c}$			

$^{A,B; X,Y}$ Means with different uppercase superscripts within a column are significantly different ($P \leq 0.05$). Uppercase superscripts A,B apply only for hay and X,Y for silage.

$^{a-c;x-z}$ Means with different lowercase superscripts within a row are significantly different ($P \leq 0.05$). Lowercase superscripts a-c apply only for hay and x-z for silage.

$^{m-o}$ Means with different lowercase superscripts within a row are significantly different ($P \leq 0.05$). These lowercase superscripts apply only for WET and CUR effect means across conservation methods.

[1] DM of hay = WET (651 g/kg) and CUR (891).

[2] DM of silage = WET (294 g/kg) and CUR (453).

[3] STRT = Start of storage; MicA = After 14 d of storage; LATE = 50 d of storage for hay and 78 d for silage.

Table 2-6. Effect of storage stages (STG) and wilting extent (WILT) on the nutritional composition, DM losses, microbial counts, and aerobic stability measures of red clover silage after 7 d of aerobic exposure[1,2].

Item[3]	Storage stages		WILT	SEM
	MicA	LATE		
DM, g/kg				11.5
WET	-	-	289^{B}	
CUR	-	-	445^{A}	
pH				0.04
WET	-	-	4.30^{B}	
CUR	-	-	4.74^{A}	
WSC, g/kg of DM				2.01
WET	16.5	13.1^{B}	-	
CUR	15^{b}	$22.7^{A,a}$	-	
NDF, g/kg of DM				2.82
WET	-	-	443^{B}	
CUR	-	-	463^{A}	
Silage mean	472^{x}	433^{y}		
ADF, g/kg of DM				3.18
WET	-	-	317^{B}	
CUR	-	-	333^{A}	
Silage mean	337^{x}	313^{y}		
Hemicellulose, g/kg of DM				2.79
WET	$130^{B,a}$	120^{b}	-	
CUR	$140^{A,a}$	119^{b}	-	
CP, g/kg of DM				3.91
WET	-	-	170^{A}	
CUR	-	-	153^{B}	
NH3-N, g/kg of total N				2.42
WET	-	-	-	
CUR	-	-	-	
Mean	46.9^{y}	56.4^{x}		
DM loss, %				0.74
WET	0.13^{B}	0.08	-	
CUR	$4.53^{A,a}$	0.40^{b}	-	
Yeast, log cfu/g fresh				0.47
WET	-	-	1.66^{A}	
CUR	-	-	0.23^{B}	
Aerobic stability, h				26.6
WET	-	-	339	
CUR	-	-	392	
Max Temp., °C				0.21
WET	22.3^{B}	21.8^{B}	-	
CUR	$25.9^{A,a}$	$23^{A,b}$	-	
HDD, °C-day				0.19
WET	0^{B}	0	-	
CUR	$3.65^{A,a}$	0.04^{b}	-	

^{A,B} Means with different uppercase superscripts within a column are significantly different ($P \leq 0.05$). Uppercase A,B superscripts apply for silage.

$^{a\text{-}b;\ x,y}$ Means with different lowercase superscripts within a row are significantly different ($P \leq 0.05$). Lowercase a,b superscripts apply for WET and CUR silage and x,y for MicA and LATE main effect.

[1] DM of silage = WET (294 g/kg) and CUR (453).

[2] MicA = After 14 d of storage; LATE = After 78 d of storage.

[3] WSC = Water soluble carbohydrates; CP = Crude protein; NH_3-N = Ammonia nitrogen; NDF = Neutral detergent fiber; ADF = Acid detergent fiber. Aerobic stability = Aerobic stability was expressed as the amount of time (hours) before the silage heated 2°C above ambient temperature. HDD = Heat-degree days; calculated as the sum of the daily temperatures increments above room temperature. Max Temp = maximum temperature during aerobic exposure.

Table 2-7. Effect of conservation method (MTOD), wilting extent (WILT), and storage stages (STG) on the digestibility, and gas production kinetics of red clover hay and silage incubated in vitro for 48 h[1-3].

Item[4]	Storage stages			SEM
	STRT	MicA	LATE	
IVDMD, g/kg of DM				15.8
Hay WET	607^{a}	563^{B,b}	552^{B,b}	
Hay CUR	601	600^{A}	625^{A}	
Silage WET	594	583	611^{X}	
Silage CUR	578	573	561^{Y}	
IVOMD, g/kg of OM				14.1
Hay WET	612^{a}	562^{B,b}	547^{B,b}	
Hay CUR	609	608^{A}	626^{A}	
Silage WET	602	587	614^{X}	
Silage CUR	578	573	561^{Y}	
pH				0.03
Hay WET	6.75^{b}	6.85^{A,a}	6.87^{A,a}	
Hay CUR	6.8	6.77^{B}	6.78^{B}	
Silage WET	6.78	6.82	6.81	
Silage CUR	6.77	6.78	6.81	
Gas production kinetics				
M_f, ml/g of incubated DM				7.3
Hay WET	244^{a}	193^{B,b}	198^{B,b}	
Hay CUR	237	223^{A}	238^{A}	
Silage WET	234^{x}	217^{y}	215^{y}	
Silage CUR	233	223	224	
K_f, %/h				0.56
Hay WET	13.3^{a}	9.33^{B,b}	8.36^{B,b}	
Hay CUR	13	13.9^{A}	13^{A}	
Silage WET	14	14.2	14.3	
Silage CUR	14.1^{xy}	13^{y}	15^{x}	
Lag, h	0	0	0	-

$^{A,B; X,Y}$ Means with different uppercase superscripts within a column are significantly different ($P \leq 0.05$). Uppercase superscripts A,B apply only for hay and X,Y for silage.

$^{a\text{-}c;x\text{-}z}$ Means with different lowercase superscripts within a row are significantly different ($P \leq 0.05$). Lowercase superscripts a-c apply only for hay and x-z for silage.

[1] DM of hay = WET (651 g/kg) and CUR (891).

[2] DM of silage = WET (294 g/kg) and CUR (453).

[3] STRT = Start of storage; MicA = After 14 d of storage; LATE = After 50 d of storage for hay and 78 d for silage.

[4] IVDMD = Apparent in vitro DM digestibility; IVOMD = Apparent in vitro OM digestibility; Mf = asymptotic maximal gas production; Kf = rate of gas production.

Table 2-8. Effect of conservation method (MTOD), wilting extent (WILT), and storage stages (STG) on the methane production of red clover hay and silage incubated in vitro for 48 h[1-3].

Item[4]	Storage stages						MTOD x WILT	SEM
	STRT		MicA		LATE			
	MTOD x STG	WILT x STG	MTOD x STG	WILT x STG	MTOD x STG	WILT x STG		
Methane, m*M*								0.06
WET	-	2.34^{m}	-	2.21^{n}	-	2.17^{n}	-	
CUR	-	2.26	-	2.29	-	2.26	-	
Methane yield, mmol/g fermented OM								0.05
Hay WET	-	0.84^{m}	-	0.74^{n}	-	0.71^{B,n}	0.78	
Hay CUR	-	0.82	-	0.79	-	0.81^{A}	0.79	
Silage WET	-		-		-		0.75^{Y}	
Silage CUR	-		-		-		0.83^{X}	

$^{A,B; X,Y}$ Means with different uppercase superscripts within a column are significantly different ($P \leq 0.05$). Uppercase superscripts A,B apply only for hay and X,Y for silage.

$^{m-o}$ Means with different lowercase superscripts within a row are significantly different ($P \leq 0.05$). These lowercase superscripts apply only for WET and CUR effect means across conservation methods.

[1] DM of hay = WET (651 g/kg) and CUR (891).

[2] DM of silage = WET (294 g/kg) and CUR (453).

[3] STRT = Start of storage; MicA = After 14 d of storage; LATE = After 50 d of storage for hay and 78 d for silage.

Table 2-9. Effect of conservation method (MTOD), wilting extent (WILT), and storage stages (STG) on volatile fatty acids of red clover hay and silage fermented in vitro for 48 h[1-3].

Item[4]	Storage stages			Mean	SEM
	STRT	MicA	LATE		
Total VFA	-	-	-	124	11.1
Acetic acid, Mm	-	-	-	74.2	6.58
Propionic acid, Mm	-	-	-	26.5	2.34
Isobutyric acid, Mm	-	-	-	2.65	0.3
Butyric acid, Mm					1.24
Hay mean	14^{a}	13^{ab}	$11.7^{B,b}$		
Silage mean	13.4	14.5	14.7^{A}		
Isovaleric acid, Mm	-	-	-	4.64	0.33
Valeric acid, Mm	-	-	-	2.79	0.24
A:P Ratio	-	-	-		0.12
Hay WET	2.83	2.73^{B}	2.83		
Hay CUR	2.84	2.84^{A}	2.82		
Silage WET	2.97^{x}	$2.67^{Y,y}$	$2.53^{Y,z}$		
Silage CUR	2.94^{x}	$2.85^{X,xy}$	$2.77^{X,y}$		

$^{A,B;\ X,Y}$ Means with different uppercase superscripts within a column are significantly different ($P \leq 0.05$). Uppercase superscripts A,B apply only for hay and X,Y for silage.

$^{a\text{-}c;x\text{-}z}$ Means with different lowercase superscripts within a row are significantly different ($P \leq 0.05$). Lowercase superscripts a-c apply only for hay and x-z for silage.

[1] DM of hay = WET (651 g/kg) and CUR (891).

[2] DM of silage = WET (294 g/kg) and CUR (453).

[3] STRT = Start of storage; MicA = After 14 d of storage; LATE = After 50 d of storage for hay and 78 d for silage.

[4] TVFA = Total volatile fatty acids; A:P = Acetic to Propionic acid ratio.

Table 2-10. Effects of conservation method (MTOD) and wilting extent (WILT) on *in situ* ruminal DM degradation kinetics of red clover hay and silage[1-4].

Item	MTOD x WILT	WILT	SEM
Lag phase, h			0.23
WET	-	0.71^{M}	
CUR	-	0^{N}	
Soluble fraction, % of DM			0.46
Hay WET	199^{B}		
Hay CUR	304^{A}		
Silage WET	363		
Silage CUR	369		
Potentially degradable fraction, % of DM			1.32
Hay WET	-	496^{M}	
Hay CUR	-	457^{N}	
Hay mean	502^{A}		
Silage WET	-		
Silage CUR	-		
Silage mean	451^{B}		
Degradation rate, %of DM/h			0.4
Hay WET	-	5.34^{N}	
Hay CUR	-	6.48^{M}	
Hay mean	5.06^{B}		
Silage WET	-		
Silage CUR	-		
Silage mean	6.75^{A}		
Undegradable fraction, % of DM			0.99
Hay WET	238		
Hay CUR	227		
Silage WET	171^{Y}		
Silage CUR	200^{X}		

$^{A,B;M,N;\ X,Y}$ Means with different uppercase superscripts within a column are significantly different ($P \leq 0.05$). Uppercase superscripts A,B apply only for hay and X,Y for silage, and M,N for WET and CUR effects across conservation methods.

[1] DM of hay = WET (651 g/kg) and CUR (891).

[2] DM of silage = WET (294 g/kg) and CUR (453).

[3] LATE = After 50 d of storage for hay and 78 d for silage.

[4] Only measured at LATE stage

Table 2-11. Effects of conservation method (MTOD) and wilting extent (WILT) on *in situ* ruminal NDF degradation kinetics of red clover hay and silage[1-3].

Item	LATE		Mean	SEM
	MTOD x WILT	WILT		
Lag phase, h				0.14
Hay mean	0.38[A]			
Silage mean	0[B]			
Potentially degradable fraction, % of NDF	-	-	573	3.14
Degradation rate, %of NDF/h				
Hay WET	3.4	-		0.74
Hay CUR	5.03	-		
Silage WET	6.91[X]			
Silage CUR	4.51[Y]			
Undegradable fraction, % of NDF				2.07
WET	-	364[N]		
CUR	-	426[M]		

[A,B;M,N; X,Y] Means with different uppercase superscripts within a column are significantly different ($P \leq 0.05$). Uppercase superscripts A,B apply for hay and X,Y for silage, and M,N for WET and CUR effects across conservation methods.

[1] DM of hay = WET (651 g/kg) and CUR (891).

[2] DM of silage = WET (294 g/kg) and CUR (453).

[3] LATE = After 50 d of storage for hay and 78 d for silage.

Table 2-12. Effect of conservation method (MTOD), wilting extent (WILT), and storage stages (STG) on phytoestrogen levels of red clover hay and silage[1-3].

Item[4]	Storage stages									
	STRT		MicA		LATE		MTOD	MTOD x WILT	WILT	SEM
	MTOD x STG	WILT x STG	MTOD x STG	WILT x STG	MTOD x STG	WILT x STG				
Formononetin, mg/kg of DM										175
Hay WET	-		-		-			2,467		
Hay CUR	-		-		-			2,581		
Hay mean	2,893^{B,a}		2,110^{B,b}		2,569^{B,a}					
Silage WET	-		-		-			5,841^{X}		
Silage CUR	-		-		-			4,608^{Y}		
Silage mean	4,624^{A,b}		5,383^{A,a}		5,666^{A,a}					
Daidzein, mg/kg of DM										0.52
Hay WET	-		-		-				7.68^{B}	
Hay CUR	-		-		-				11.8^{A}	
Hay mean	-		-		-		12.8^{A}			
Silage WET	-		-		-					
Silage CUR	-		-		-					
Silage mean	-		-		-		6.67^{B}			
Total mean	12.7^{a}		8.02^{b}		8.46^{b}					
Biochanin A, mg/kg of DM										139
Hay WET	-		-		-			1,220^{B}		
Hay CUR	-		-		-			1,645^{A}		
Hay mean	2,012^{B,a}		1,057^{B,b}		1,228^{B,b}					
Silage WET	-		-		-			3,793^{X}		
Silage CUR	-		-		-			2,629^{Y}		
Silage mean	3,105^{A}		3,233^{A}		3,295^{A}					
Genistein, mg/kg of DM										8.36
Hay WET	-	147^{A}	-	158	-	159		72.8^{B}		
Hay CUR	-	121^{B,o}	-	151^{n}	-	178^{m}		97.3^{A}		
Hay mean	120^{B,a}		62.6^{B,b}		72.9^{B,b}					
Silage WET	-		-		-			236^{X}		
Silage CUR	-		-		-			202^{Y}		
Silage mean	149^{A,b}		246^{A,a}		264^{A,a}					
Prunetin, mg/kg of DM										11.1
Hay WET	-		-		-				200^{A}	
Hay CUR	-		-		-				175^{B}	
Hay mean	105^{a}		74.4^{B,b}		97.7^{B,a}					
Silage WET	-		-		-					
Silage CUR	-		-		-					
Silage mean	127^{c}		341^{A,b}		382^{A,a}					
Glycitein, mg/kg of DM										2.93
Hay WET	-		-		-				18.7^{B}	
Hay CUR	-		-		-				27.1^{A}	
Hay mean	39.8^{a}		6.87^{B,b}		10.7^{B,b}					
Silage WET	-		-		-					
Silage CUR	-		-		-					
Silage mean	32.3^{a}		16.4^{A,b}		31.4^{A,a}					

$^{A,B;\ X,Y}$ Means with different uppercase superscripts within a column are significantly different ($P \leq 0.05$). Uppercase superscripts A,B apply only for hay and X,Y for silage.

$^{a\text{-}c;x\text{-}z}$ Means with different lowercase superscripts within a row are significantly different ($P \leq 0.05$). Lowercase superscripts a-c apply only for hay and x-z for silage.

$^{m\text{-}o}$ Means with different lowercase superscripts within a row are significantly different ($P \leq 0.05$). These lowercase superscripts apply only for WET and CUR effect means across conservation methods.

[1] DM of hay = WET (651 g/kg) and CUR (891).

[2] DM of silage = WET (294 g/kg) and CUR (453).

[3] STRT = Start of storage; MicA = After 14 d of storage; LATE = After 50 d of storage for hay and 78 d for silage.

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After graduating in 2017, Diego worked as a field supervisor in Agricola Gamuco company, Ica, Peru, where he trained the personnel in agricultural practices related to tangerine production such as tilling, pruning, lime application, and soil sampling. In 2019, he worked as a research assistant on the project titled “Field-based phytohormones phenotyping to select climate resilience cereal varieties” in the Universidad Nacional Agraria La Molina. This project was funded by the National Fund for Scientific, Technological Development, and Technological Innovation (FONDECYT) and it was a collaborative work between the University of Lancaster, Lancaster, UK and Universidad Nacional Agraria La Molina, Lima, Peru. In 2020, he continued improving his research skill through the participation in projects that were selecting wheat, barley, and quinoa genotypes tolerant to abiotic stresses in Universidad Nacional Agraria La Molina.

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