EVALUATION OF FORAGE ENERGETICS AND IMPLICATIONS TO PASTURE-BASED DAIRY CATTLE IN THE NORTHEASTERN UNITED STATES.

A Dissertation Presented

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ABSTRACT

The organic, grassfed (OGF) dairy sector has been growing for the past 10 yr with expansion rapidly outpacing other dairy sectors. Unique management approaches are needed for OGF systems, but research to support said approaches is lacking. The objectives of this dissertation were to 1) survey OGF dairy producers to better understand producer knowledge and practices, as well as identify areas of needed research to support this farmer population, 2) determine *in vitro* rumen performance of energetic supplements commonly utilized on OGF dairy farms in the Northeastern (NE) United States (U.S.), and 3) identify and evaluate forage combinations using *in vitro* continuous culture fermentation, focused on rumen energetic status and environmental output.

While Chapter 1 reviews the published literature relevant to OGF dairy systems with a focus on their nutritional management, Chapter 2 utilized a survey questionnaire that was mailed to 351 OGF dairy farms throughout the U.S. with a 46.8% response rate. This survey confirmed the need for further forage-related research, as producers indicated that agricultural-related challenges, such as forage quality and milk production, were the largest limitations to farm success followed by economic factors.

In Chapter 3, we evaluated rumen fermentation metrics of red clover + orchardgrass harvested at first and second cuttings, and further investigated the impacts of dietary apple cider vinegar (ACV) supplementation in combination with first cut forages on fermentation metrics using two *in vitro* batch culture experiments (Objective 2). In Experiment 1, tubes were supplemented with either 1) 50% red clover + 50% orchardgrass harvested at first cut (CUT 1) or 2) 50% red clover + 50% orchardgrass harvested at second cut (CUT 2). In Experiment 2, tubes received either 1) CUT 1 substrate only or 2) CUT 1 substrate + ACV. Tubes receiving CUT 1 produced more methane (CH_4) and had higher DM (dry matter) disappearance and mean pH compared with those receiving CUT 2. Fermentation metrics of CUT 1 tubes were not affected by ACV. Ultimately, this trial confirmed that forage cut, and chemical profile, is a more impactful variable driving rumen function.

Objective 3 of this dissertation was addressed in Chapters 4 and 5 in which fermentation metrics of mixed forage diets were compared using dual-flow continuous culture fermenters. Chapter 4 focused on forage diversity using cool-season forages. Four diets were mixed (on a DM-basis) to include 40% red clover plus: 1) 60% orchardgrass (OG); 2) 30% orchardgrass + 30% meadow fescue (MF); 3) 20% orchardgrass + 20% meadow fescue + 20% Kentucky bluegrass (KYBG); or 4) 15% orchardgrass + 15% meadow fescue + 15% Kentucky bluegrass + 15% perennial ryegrass (PRG). Diets including 20% Kentucky bluegrass were more digestible but produced higher amounts of CH_4. Other forages, such as perennial ryegrass and meadow fescue, may be just as nutritionally beneficial as Kentucky bluegrass with the additional advantage of decreased CH_4 emissions.

Chapter 5 focused on warm-season annual (WSA) inclusion, complemented by a simple cost analysis. Dietary treatments (on a DM-basis) included 1) 50% orchardgrass + 50% alfalfa (OG-ALF); 2) 25% orchardgrass + 25% alfalfa + 50% red clover (RC); 3) 25% orchardgrass + 25% alfalfa + 50% pearl millet (MIL); and 4) 25% orchardgrass + 25% alfalfa + 50% sorghum x sudangrass (SUD). Diets with WSA showed marked decreases in CH_4 output but were similar in digestibility to other diets.

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LIST OF ABBREVIATIONS

ac	acre(s)
ACV	apple cider vinegar
ACV+	50% immature red clover + 50% immature orchardgrass on a DM-basis + apple cider vinegar
ACV-	50% immature red clover + 50% immature orchardgrass on a DM-basis
ADF	acid detergent fiber
aNDFom	amylase- and sodium sulfite-treated neutral detergent fiber, organic matter basis
AUC	area under the curve
BMR	brown-midrib
BMRSS	brown-midrib sorghum x sudangrass
C	carbon
C_3	cool-season grass
C_4	warm-season grass
Ca	calcium
CH_4	methane
CNCPS	Cornell Net Carbohydrate and Protein System
CO_2	carbon dioxide
CP	crude protein
CRP	Conservation Reserve Program
CSP	cool-season perennials
CUT 1	50% red clover + 50% orchardgrass from first cutting on a DM-basis

CUT 2	50% red clover + 50% orchardgrass from second cutting on a DM-basis
d	day(s)
DE	digestible energy
dL	deciliter(s)
DM	dry matter
DMI	dry matter intake
EQIP	Environmental Quality Incentives Program
ESC	ethanol soluble carbohydrate(s)
FC	Folin-Ciocalteu reagent
FCIP	Federal Crop Insurance Program
g	gram(s)
GDD	growing degree days
GE	gross energy
h	hour(s)
H_2	hydrogen
ha	hectare(s)
HPLC	high-pressure liquid chromatography
IVDMD	*in vitro* dry matter digestibility
K	potassium
kg	kilogram(s)
KYBG	40% red clover + 20% orchardgrass + 20% meadow fescue + 20% Kentucky bluegrass
lb	pound(s)

LSM	least squares means
mcal	megacalorie(s)
ME	metabolizable energy
MF	40% red clover + 30% orchardgrass + 30% meadow fescue
Mg	magnesium
mg	milligram(s)
MIG	management intensive grazing
MIL	25% orchardgrass + 25% alfalfa + 50% millet
min	minute(s)
mL	milliliter(s)
mM	millimole(s)
MPPD	Margin Protection Program for Dairy
MPS	microbial protein synthesis
MW	Midwestern
MUN	milk urea nitrogen
N	nitrogen
Na	sodium
NAN	non-ammonia nitrogen
NDF	neutral detergent fiber
NDFD30	30 h NDF digestibility
NE	Northeastern
NE_L	net energy of lactation

NFC	non-fiber carbohydrates
NH_3-N	ammonia nitrogen
NIRS	near infrared reflectance spectroscopy
NOP	National Organic Program
NRC	National Research Council
NSC	nonstructural carbohydrates
O_2	oxygen
OG	40% red clover + 60% orchardgrass
OG – ALF	50% orchardgrass + 50% alfalfa
OGF	organic, grassfed
OM	organic matter
PLT EXP	experiment 1, batch culture
PPO	polyphenol oxidase
PRG	40% red clover + 15% orchardgrass + 15% meadow fescue + 15% Kentucky bluegrass + 15% perennial ryegrass
RC	25% orchardgrass + 25% alfalfa + 50% red clover
RDP	rumen degradable protein
RUP	rumen undegradable protein
RUSITEC	rumen simulation technique
SAS	statistical analysis system
SD	standard deviation
SEM	standard error of the mean

SUD	25% orchardgrass + 25% alfalfa + 50% sorghum x sudangrass
SUPP EXP	experiment 2, batch culture
TMR	total mixed ration
TRFLP	terminal restriction fragment length polymorphism
U.S.	United States
USDA	United States Department of Agriculture
VFA	volatile fatty acid(s)
WSA	warm-season annual(s)
WSC	water soluble carbohydrate(s)
yr	year(s)

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CHAPTER 1: LITERATURE REVIEW

1.1. Abstract

Research supporting the grassfed dairy sector is becoming increasingly important as producers in the Northeastern (NE) United States (U.S.) are transitioning to organic, pasture-based practices in response to market demands and prices. From a feed management perspective, one of the primary nutritional challenges in maintaining productivity of grassfed dairy cows is providing cattle with a sustained supply of nutrient-dense herbage with high dry matter (DM) and energy content, as metabolizable energy (ME) becomes limiting on pasture-based dairies. While select supplements are available to offset any forage energy deficit, this option often increases the cost of production and may not align with the financial management of the farm or the ethos of the producer. Although pasture-based dairy operations are becoming more commonplace and continually expanding, there is limited scientific information available assessing forage management practices and their efficacy in the NE U.S. This includes utilization of warm-season annuals (WSA) in temperate climates and increasing forage biodiversity for pasture-based diets. The purpose of this literature review is to provide a brief review of U.S. organic, grassfed (OGF) dairy production as well as evaluate current research regarding forage and pasture energetics and outline the effects of forage-based diets on dairy cattle nutrition.

Keywords: pasture-based dairy, forage energetics, grassfed, milk production, grazing management

1.2. Introduction

Dairy farming remains one of the largest agricultural enterprises in the Northeastern (**NE**) United States (**U.S.**). Approximately 44% of U.S. certified organic dairies are located in the Northeast, accounting for 25% of organic milk produced in the country (McBride and Greene, 2013; 2019 Organic Survey, 2020). In New England, the transition to organic production has proven to be crucial to farm sustainability as 75% of organic producers believe their operations would not have survived without transitioning (Parsons, 2010). Although there have been improvements in both efficiency and production (Martin and Mitra, 2001), the dairy industry is under financial strain. Winsten et al. (2010) indicated that dairy producers in the Northeast have responded to financial strain and farm viability issues in three ways: 1) exiting the dairy industry entirely, 2) creating larger herd sizes through confinement feeding, or 3) adopting management-intensive grazing (**MIG**) systems. Although adoption of MIG systems is less common, it can improve farm financial viability compared with other dairy production systems (Winsten et al., 2000; Winsten et al., 2010). To differentiate themselves from other producers using MIG systems, some NE producers have chosen to adhere to organic, grassfed (**OGF**) regulations.

Organic, grassfed operations, are management systems that feed nearly 100% forage diets, both conserved and fresh, to cattle. As defined by the United States Department of Agriculture (**USDA**), pasture is land that is utilized for grazing livestock and that is managed to provide and improve soil and water quality, maintain vegetation, and provide adequate feed value (Coffey and Baier, 2012). While these are important goals, a specific goal of managing pastures for OGF livestock production is to build and maintain soil structure and fertility while also conserving and promoting plant biodiversity and

maximizing forage quality (Hafla et al., 2013). The National Organic Program (**NOP**) of the USDA is responsible for defining U.S. organic standards and transition regulations (USDA National Organic Program). The NOP implemented the "Pasture Rule", which requires certified organic dairy cattle to consume pasture during the grazing season in their geographic location (Rinehart and Baier, 2011). As well as consuming pasture during their grazing season, certified organic dairy cattle must graze on pasture at least 120 days each year, although these days do not have to run consecutively. Additionally, organically produced cattle must obtain an average minimum of 30% of their dry matter intake (**DMI**) from certified organic pasture or rangeland. To comply with OGF standards, this period is extended; dairy cattle that are to be certified as OGF must be on pasture for at least 150 d and must receive 60% or more of their DMI from pasture averaged throughout the grazing season (American Grassfed Association, 2018). Grain and grain-based forages are prohibited in this marketplace.

Adoption of OGF dairy operations has been significant in the NE U.S. over the last decade, partly as a means to maintain economic viability but also to support sustainability of the farm system. More organic dairy farmers are eliminating grains and transitioning to an all-forage system because certified organic grains are costly. Despite the cost of traditional and organic grains, many conventional producers believe that switching to an OGF operation would decrease farm profits, gross income, and net income due to barriers such as access to land and increased labor costs (Winsten et al., 2010). Winsten et al. (2010) surveyed NE MIG producers about perceived barriers before MIG adoption and actual barriers after adoption. Before adopting a pasture-based system, conventional producers indicated that the amount of labor needed to start rotational grazing was the biggest

perceived barrier. However, the lack of land for grazing proved to be the largest actual barrier (Winsten et al., 2010). Regardless of perceived and known barriers, switching to a grassfed production system may be more economically sound for NE dairy producers but it also contributes to environmental sustainability by promoting nutrient cycling and soil carbon sequestration (Provenza et al., 2019). Organic producers have indicated that nutritional and pasture management is a primary challenge (Pereira et al., 2013). Producers are further hindered by the ability to sustain high quality pastures throughout the grazing season that can meet the energetic needs of the cow (Wilkinson et al., 2020; McGilloway and Mayne, 1996).

Pasture ecosystem productivity partially depends on the diversity and functional composition of plant species, with research showing that grassland ecosystems containing a rich diversity are more productive than pastures that are less diverse (Guretzky et al., 2005; Tracy and Faulkner, 2006). A diverse pasture may contain functional plant groups, such as woody plants, legumes, brassicas, forbs, cool-season grasses (**C_3**), and warm-season grasses (**C_4**), which can all be successfully included in ruminant diets to support nutrition of the animal, as well as provide some advantage for consistent forage quality and yield (Dillard et al., 2018). Companion planting of C_3 and C_4 plants also supports soil and grassland health (Negrete, 2021; Dillard et al., 2018). Together, these two strategies can lend themselves to more productive pasture systems, allowing an increasing number of producers to transition from organic to OGF production systems with the goal of improving farm forage productivity and creating a sustainable farm economically (Dalton et al., 2008).

The purpose of this literature review is to explore parameters affecting forage quality, provide insight into the effects of forage profiles on performance and productivity of OGF dairy cows, and finally to assess nutritional management and grazing strategies.

1.3. Pasture Biodiversity and Influence on Animal Production

A strategy that may increase both forage productivity and sustainability is to improve pasture biodiversity. Biodiversity can be easily manipulated in most pastures as typical farm management practices allow for the addition, or removal, of various plant species (Sanderson et al., 2007).

1.3.1. The Role of Legumes in Pasture Systems

Many producers understand the importance and benefits of including legumes (*Fabaceae*) such as alfalfa (*Medicago sativa*), birdsfoot trefoil (*Lotus corniculatus*), and red clover (*Trifolium pratense*) in forage systems but struggle to incorporate them due to problems with persistence (Marten et al., 1989). Stand persistence can be a major concern when establishing legumes, especially for those relying on crown formation (Beuselinck et al., 1994). This is due to the fact that crown-forming legumes are vulnerable to root disease, insects, pathogens, and plant stressors (Taylor and Smith, 1978). Therefore, legume populations do not typically dominate natural grassland ecosystems. However, acceptable levels of legume persistence will depend on the ecosystem, the purpose of forage inclusion, pasture management, legume cultivar, and goals of the producer (Ortega et al., 2019).

Despite drawbacks with persistence, legumes have proven invaluable to forage systems mainly due to their ability to fix nitrogen (**N**) from the soil via its relationship with bacteria belonging to the genus *Rhizobium*, potentially fixing as much as 270 lb N $acre^{-1}$ (**ac**; LaRue and Patterson, 1981). Ultimately, this ability of legumes to fix N results in these

plants typically having a higher concentration of crude protein (**CP**) compared with other forage species (Paulson et al., 2008). Legumes help meet the protein and energetic requirements of grazing dairy cattle by providing both rumen-degradable protein (**RDP**) for microbial protein synthesis (**MPS**) as well as digestible rumen-undegradable protein (**RUP**) to support post-absorptive amino acid profiles (Broderick, 1995). However, rapid degradation of proteins in legumes may lead to decreased protein efficiency (Broderick, 1995).

As well as converting atmospheric N to more usable forms, legumes are generally recognized as valuable pasture components due to their ability to improve forage yield and increase the nutritive value of herbage production (Beuselinck et al., 1994). When legumes and grasses are combined as a binary mixture, they are more productive than grass monocultures, legume monocultures, or grasses fertilized with N (Nyfeler et al., 2011). For example, Sleugh et al. (2000) evaluated the effects of combining legumes such as alfalfa, kura clover (*Trifolium ambiguum*), and birdsfoot trefoil in binary mixtures with orchardgrass (*Dactylis glomerata*), smooth bromegrass (*Bromus inermis*), and wheatgrass (*Thinopyrum intermedium*) on forage yield and quality. Binary grass-legume mixtures were compared with monocultures. After two years (**yr**), the total yield was greatest for alfalfa monocultures, alfalfa-wheatgrass, and alfalfa-smooth bromegrass. Monoculture grasses were lower in yield, CP, and *in vitro* dry matter digestibility (**IVDMD**) than monoculture legumes and legume-grass mixes. Based on these results, it appears that legumes are more productive than grass monocultures (Sleugh et al., 2000). However, other studies have shown that benefits are not evident after 5 yr (Schmid et al., 2008), indicating that legume persistence may be a limitation.

1.3.2. Inclusion of Cool- and Warm-Season Grasses in Pastures

Grasses (*Poaceae*) are typically herbaceous with parallel leaf veins. They are commonly utilized in temperate pasture dairy systems, such as in the upper Midwest and Northeast (Ruh et al., 2018), as they are easier to manage from a whole farm nutrient management perspective (Paulson et al., 2008). While grasses can be divided into three different classifications based on their method of photosynthesis (C_3, C_4, and crassulacean acid metabolism), for the purpose of this literature review only forage plants belonging to the C_3 and C_4 classifications will be discussed.

The C_3, or cool-season, grasses predominantly used for grazing in NE pastures include orchardgrass, Kentucky bluegrass (*Poa pratensis*), smooth bromegrass, timothy (*Phleum pratense*), tall fescue (*Festuca arundinacea*), and reed canarygrass (*Phalaris arundinacea*; Burns and Bagley, 1996). They, along with various legume species, are typically the foundation of dairy pastures in the NE U.S. (Ruh et al., 2018), providing high-quality forage matter in the spring and fall grazing seasons (Dillard et al., 2017). Pastures comprised of C_3 grasses typically exhibit a decrease in growth rate when temperatures are high and precipitation is low, a period known as the "summer slump" (Hudson et al., 2010). Because of this slump in productivity, it is becoming standard practice to include C_4 grasses with C_3 plants in temperate regions of the U.S. as a means to support sustained pasture productivity and yield from spring to fall (Jung et al., 1990).

As well as supporting pasture productivity throughout the grazing season, C_4, or warm-season, forages are becoming increasingly popular in the NE U.S. due to a changing climate (Dillard et al., 2018). A technical report by Kunkel et al. (2013) indicated that the NE U.S. would be impacted by warmer summer temperatures, warmer average winter

temperatures, and higher precipitation. These are all factors that directly and indirectly affect livestock production by impacting quality of pasture conditions and quantity of forages (Hristov et al., 2018). Wolfe et al. (2008) assessed climate projections based on several climate simulations and projected an increase of 5 - 15 d of crop heat stress, indicating that C_3 crops may be negatively impacted. For example, grain crops typically used in dairy cattle diets will have lower yields as summer temperatures increase (Wolfe et al., 2008; Rosenzweig and Hillel, 1998). Hristov et al. (2018) indicated that the projected increase in air temperature would lead to C_3 forage growth earlier in the spring with later fall dormancy. Although the growing season would be longer, forage digestibility would decrease due to increased lignification (Dumont et al., 2015). Additionally, the "summer slump" would be more pronounced with C_3 forages (Hristov et al., 2018). While these conditions are not as favorable for C_3 forage growth, they are for C_4 species (Reyes et al., 2014). Reviews comparatively exploring the nutritive value of C_4 v. C_3 grasses have outlined mixed favorability (Moore et al., 2004); however, if these predictions of climate shifts come to fruition, this could create a more consistent advantage for inclusion of C_4 grasses. Less frequent and intense precipitation would infer a competitive advantage to C_4 annuals over C_3 forages during period of drought (Morgan et al., 2011). Furthermore, plant CP content would increase during drought periods with the caveat that conditions could be favorable for weed encroachment (Dumont et al., 2015). Based on these projections, it is suggested that producers should transition to, or incorporate more, heat tolerant crops and crop varieties such as sorghum (*Sorghum bicolor*), millet (*Pennisetum glaucum*), sudangrass (*Sorghum sudanese*), and sorghum x sudangrass hybrids (*Sorghum x drummondii*). However, a major disadvantage for inclusion of warm-season annuals

(**WSA**) is the need for reestablishment every growing season (Tracy et al., 2010). From an economic perspective, this could prove to be costly (at least 22% higher establishment costs) as WSA establishment requires more input costs as compared to cool-season forage establishment (Tracy et al., 2010).

1.3.3. Potential Benefits of Mixed Pasture Systems

While understanding growth habits of monocultures is important for producers, combining forage types together in a mixed pasture system may have benefits. Many producers believe, based on personal observations and expectations (Wang et al., 2022), that maintaining a diverse pasture increases forage productivity, plant persistence, reduction in toxin consumption, and forage yield stability (Sanderson et al., 2005). Wang et al. (2022) surveyed beef producers across North Dakota, South Dakota, and Texas to quantify and understand producer perspectives regarding grazing practices and pasture management. On a 4-point scale, with 1 being equivalent to no benefit and 4 being a significant benefit, producers ranked grass quality and diversity as a 3, indicating what they perceived to be a medium benefit to cattle and grassland biodiversity. It is speculated that cattle grazing diverse pastures may be more productive as they can choose from a variety of plants (Tracy and Faulkner, 2006). However, few studies have examined the relationships between animal production and mixed swards. Published works show mixed results or only include mixtures of a single legume and grass (Sanderson et al., 2007). Phillips and James (1998) found that cows grazing perennial ryegrass (*Lolium perenne*) - white clover (*Trifolium repens*) pastures had greater milk production than cows on pure ryegrass pastures (22.1 kg cow^{-1} d^{-1} v. 18.9 kg cow^{-1} d^{-1}), although cows expressed a preference for the monoculture-based pasture. In direct contrast, Soder et al. (2006)

examined the effects of diverse pastures on dairy cow nutrition and found that total herbage DMI was similar among forage mixtures. Additionally, milk production and composition were not affected by different forage mixtures. Similarly, Tracy and Faulkner (2006) focused on the use of diverse pastures in a beef grazing system, and reported that although pasture botanical diversity marginally increased herbage mass, cow-calf performance (weight, average daily gain (**ADG**), and gain ha^{-1}) was unaffected although ADG was greater for cow-calf pairs depending on the yr. These researchers suggested that cow-calf weight and gain ha^{-1} was more influenced by stocking rate and climate conditions rather than forage species richness. One relatively consistent observation across studies that assess the impact of diverse pasture mixtures is improved pasture dry matter (**DM**) per unit area (Sanderson et al., 2005; Pembleton et al., 2014). While not universally identified, this observation has been noted across several different trials with differing environmental conditions and livestock enterprises (Tracy and Faulkner, 2006; Nobilly et al., 2013; Woodward et al., 2013).

By increasing species diversity, there is the possibility of a single plant species not being fully capable of utilizing a resource, such as land area, water, or soil nutrients, allowing for niche exploitation by another plant species (Pembleton et al., 2014). Nobilly et al. (2014) showed that higher DM production achieved by diversified pastures were likely due to the deep-rooted species added to the pasture. These researchers added chicory (*Cichorium intybus*), plantain (*Plantago lanceolata*), red clover, and alfalfa, which have deeper root systems capable of adapting to summer water deficits and exploiting the niches of other pasture species. These same species showed decreased herbage yield (kg DM ha^{-1}) and botanical proportions in the study by Sanderson et al. (2005). However, the study by

Sanderson et al. (2005) was conducted in the U.S. while the study by Nobilly et al. (2014) was conducted in New Zealand, and the difference in climate, soil profile, or other parameters may explain this disparity. It could also be argued that despite the potential benefits of management based on pasture diversity, it may not be the most appropriate management plan if the main interest is solely focused on maximum forage production as a long-term solution as certain forage species decline in quantity and quality over time (Sanderson et al., 2007). For example, Belesky et al. (1999) found that chicory in orchardgrass – chicory – birdsfoot trefoil swards decreased from 80% to 20% inclusion in a clipping management study. Sanderson et al. (2003) further found that some forage species, such as chicory, were more susceptible to winterkill, allowing for grass species to exploit resources that would typically be utilized by chicory or other forages. Thus, it can be inferred that while herbage yield in complex forage mixtures may be relatively stable, swards may simplify over time (Sanderson et al., 2005). Furthermore, to maintain pasture diversity, producers may need to reseed legume and forb species every two - three yr which could prove to be expensive. Using additional species will require further modification of management practices, particularly regarding pasture fertilization and grazing to maintain stand productivity and persistence (Pembleton et al., 2014).

1.4. Impact of Plant Classifications on Dietary Carbohydrate Profile and Nutritional Benefits of a Mixed Sward Profile

Forages are primarily composed of carbohydrates, with an abundance of cellulose and hemicellulose in plant cell walls, and sugars, fructans, and starch in the plant cell contents (Moore and Hatfield, 1994; Villalba et al., 2021). Carbohydrates account for 50% – 80% of biomass of forage species, playing key roles in energy storage and transfer,

metabolism, and plant structure (Moore and Hatfield, 1994). As nearly all forages commonly incorporated in ruminant diets belong to the *Poaceae* or *Fabaceae* families, it is easy to make general assumptions about the carbohydrate content of grasses and legumes. However, forage carbohydrate profiles vary greatly within species and among genera (Moore and Hatfield, 1994). Further, carbohydrate profiles are dependent on numerous environmental factors including light intensity, temperature, and nutrient availability (Watts and Chatterton, 2004). The factors impacting forage carbohydrate profiles directly impact forage quality and plant cell component digestibility. From a nutritional perspective, this is important as carbohydrates are the main source of energy in the ruminant diet (particularly nonstructural carbohydrates; **NSC**) and are also important for normal rumen function (particularly the inclusion of structural carbohydrates; Moore and Hatfield, 1994).

1.4.1. Structural Carbohydrates and Cell Wall Phenolics

There is considerable variation among plant species regarding both composition and concentration of structural carbohydrates (Moore and Hatfield, 1994). In terms of total structural carbohydrates, C_4 perennial grasses typically have higher concentrations of structural carbohydrates compared with C_3 grasses, particularly cellulose (220 g kg^{-1} DM v. 150 g kg^{-1} DM) and hemicellulose (250 g kg^{-1} DM v. 120 g kg^{-1} DM; Windham et al., 1983; Moore and Hatfield, 1994). Using high-pressure liquid chromatography (**HPLC**) Windham et al. (1983) compared the fiber composition of three C_3 and 3 C_4 grasses. The C_4 grasses contained higher concentrations of cellulose (319 g kg^{-1} DM) as compared with the C_3 grasses (251 g kg^{-1} DM). However, there were no consistent differences between C_4 and C_3 cellulose: hemicellulose ratios. When compared with legume species, the

hemicellulose content of legumes (40 g kg^{-1} DM) was lower than that of C_3 and C_4 grasses. This is to be expected as cellulose concentrations are generally higher in the cell walls of legumes as opposed to grasses, translating to lower concentrations of hemicellulose (Moore and Hatfield, 1994). Further, cellulose concentrations appear to be similar between grasses and legumes, reflecting a lower concentration in cell wall contents of legumes (Moore and Hatfield, 1994).

Lignin has been recognized as a major limiting factor to digestion in the rumen as other phenolics bind to it, or bind directly to cell wall carbohydrates (Jung and Deetz, 1993). Lignin concentration is negatively correlated with forage digestibility; however, this correlation is not constant across developmental stage or across species (Moore and Jung, 2001). Generally, lignin concentrations of legumes appear comparable to that of grasses when expressed as a proportion of DM (Moore and Jung, 2001). Although plant cell walls have similar architectures, the leaves of legumes are much less lignified than the leaves of grass with plant cell walls containing more pectin (Wilman and Altimimi, 1984). When lignin concentrations are expressed as a proportion of fiber, legumes typically contain a wider range of lignin concentrations that are greater than grasses with most lignin being located in the stems (Buxton and Brasche, 1991). When comparing C_3 and C_4 grasses, Jung and Vogel (1992) demonstrated that fiber and lignin composition were similar between species. However, C_4 grasses typically contained greater concentrations of lignin at growth stages comparable to that of C_3 grasses. This may be due to plant anatomical differences (Moore and Jung, 2001).

1.4.2. Nonstructural Carbohydrates

Nonstructural carbohydrates have a significant impact on forage quality, playing roles in plant metabolism, energy storage, and energy transport (Moore and Hatfield, 1994). Further, they play important roles in ruminant diets as NSC, along with structural carbohydrates, are metabolized to volatile fatty acids (**VFA**). Nonstructural carbohydrates are an all-encompassing term for carbohydrates that do not contribute to plant cell wall structure and integrity. Rather, NSC can be divided into oligosaccharides, monosaccharides, disaccharides, fructans, and starch (Figure 1.1). Analytically, NSC is measured as ethanol soluble carbohydrates (**ESC**), fructans, and starch. Ethanol soluble carbohydrates include simple carbohydrates, such as glucose, sucrose and fructose plus small chained fructans that are soluble in ethanol. Water soluble carbohydrates (**WSC**) include ESC plus larger fructans that are soluble in water. In total, NSC can be defined as WSC plus starch. Concentrations of NSC in plants are influenced by environmental factors such as photosynthesis rates and growth (Kolver, 1997). In vegetative C_3 grasses, NSC concentrations will decrease as the plant matures, and will also do so in response to increased temperature and lower leaf: higher stem ratio (Kolver, 1997). Sucrose and other soluble sugars are generally found in greater amounts in legumes as compared to grasses with starch being the predominant storage polysaccharide (Moore and Hatfield, 1994). Although grasses contain fewer NSC, they are generally higher in C_3 grasses than C_4 grasses with the most common NSC being fructosan, occurring as inulin or levan (Kolver, 1997).

1.5. Factors Affecting Pasture Productivity and Grazing Management

Pasture productivity and composition are heavily influenced by animal, plant, soil, farm, and environmental conditions. In dairy cow systems, these include, but are not limited to, animal factors such as cow breed and plant-animal interactions, pasture factors such as landscape or pasture position, plant composition and quality, and farm and environmental conditions such as climate, grazing management practices, milking technology and herd scale (Matches, 1992; Hennessy et al., 2015). While all important, the focus of this literature review will be on factors that directly affect plant nutrient composition and animal nutrition.

1.5.1. Plant Defenses Against Grazing

Many pasture plants have traits that make them more tolerant to grazing and superior at regrowth after grazing, whether this be physical properties (spines and silica content) or a chemical defense mechanism (secondary metabolites; Hendrickson and Olson, 2006). Structural plant traits serve to reduce the rate at which livestock ingest forages (Laca et al., 2001). These structures, also known as structural anti-nutritive traits, include all characteristics that deter herbivory (Briske, 1996; Laca et al., 2001). This can range from stems, pseudostems, and thorns to characteristics such as fibrousness and tensile strength (Laca et al., 2001). However, pasture plants, primarily grasses, are generally tolerant to grazing and other mechanical stressors due to their potential to form tillers, the presence of underground storage capacity, and their rapid regrowth capabilities after defoliation (Huitu et al., 2014), relying more on chemical defenses.

There are several chemical defenses that have been identified in plants to deter herbivory. For example, many grass species have symbiotic relationships with endophytic

fungi that produce mycotoxins, reducing palatability or making them toxic to grazing livestock (Huitu et al., 2014). The amount of endophyte present and the metabolites they produce may increase in response to herbivory, acting as an induced defense (Bazely et al., 1997). In addition to potentially harboring endophytic fungi, plants typically utilized in pastures may also contain secondary metabolites. Examples of secondary metabolites include tannins, alkaloids, glycosides, and saponins. Secondary metabolites do not play a role in primary plant functions but are thought to have a protective role against herbivory and could potentially be beneficial to the environment by driving soil nutrient cycling and decomposition (Chomel et al., 2016). Traditionally, secondary metabolites have been viewed as anti-nutritive compounds due to adverse effects on nutrient utilization by animals upon ingestion (Patra and Saxena, 2009).

Some phytochemicals may have positive impacts on animal production and nutrition (Patra & Saxena, 2009). For example, Cieslak et al. (2012) reported that dietary tannins decreased rumen protozoal numbers which led to decreased methane (**CH_4**) production and reduced ammonia nitrogen (**NH_3-N)** concentrations. Other studies have attempted to utilize secondary metabolites, such as isoflavones and essential oils, to improve rumen fermentation efficiency by enhancing protein metabolism (essential oil blend; McIntosh et al., 2003), decreasing CH_4 production (anthroquinones; Garcia-Lopez et al., 1996), shifting microbial profiles (anethol and essential oils; Busquet et al., 2006), and reducing nutrition-related health issues such as bloat (tannins; Williams et al., 2011). Other forages, such as red clover, contain high levels of the copper metalloenzyme polyphenol oxidase (**PPO**). Polyphenol oxidase has been implicated in forage browning and, potentially, positively modifying ruminant N metabolism (Lee et al., 2014). For

example, Vanhatalo et al. (2009) found that red clover significantly decreased ruminal NH_3-N as compared to other forages. This response indicates a lower degradation rate of red clover protein due to protein complexing with PPO, reducing rumen digestibility and solubility.

1.6. Rumen Microbial Populations and Carbohydrate Metabolism

Forage-driven differences in ruminal metabolism and microbiota are becoming increasingly characterized. For example, the microbiome of ruminants on predominately forage-based diets is dominated by *Fibrobacter, Ruminococcus, Butyrvibrio,* and *Prevotella* due to greater amounts of cellulose and hemicellulose (Carberry et al., 2012). Using the rumen simulation technique (**RUSITEC**), Belanche et al. (2013) found that a diet composed of perennial ryegrass led to better utilization of N than red clover although red clover promoted greater non-ammonia N (**NAN**) flow and MPS efficiency. Furthermore, perennial ryegrass promoted greater amounts of fungi, methanogens, and cellulolytic bacteria, increasing VFA and gas production more so than the red clover diet. De Menezes et al. (2011) used terminal restriction fragment length polymorphism (**TRFLP**) technology and next generation sequencing to determine that rumen *Fibrobacteraceae* and *Firmicutes* were in greater abundance in dairy cows fed a total mixed ration (**TMR**) diet while *Erysipelotrichaceae, Prevotellaceae,* and *Veillonellaceae* were in greater abundance in dairy cows on a pasture-based diet. The higher abundance of *Prevotellaceae* and *Veillonellaceae* in cattle on pasture-based diets is of noted interest as they produce propionate, which is not as abundant in the rumen of pasture-based cows.

1.6.1. Carbohydrate Metabolism and Volatile Fatty Acid Production

Polysaccharides, which are plentiful in forage-based diets, are hydrolyzed to glucose and simple carbohydrates that are further broken down to VFA and carbon dioxide (**CO_2**). For pasture-based cattle, acetate is produced in greater amounts (Dillard et al., 2017) as compared to cattle on concentrate-based diets (Penner et al., 2009). Higher acetate proportions can be partially attributed to the high amounts of cellulose and low starch content that is typical of a forage-based diet (O'Callaghan et al., 2018). This means that cellulolytic and fibrolytic bacteria are in greater amounts in the rumen, favoring the production of acetate. However, pH also plays a critical role; when rumen pH is higher, it supports an environment that is more hospitable towards CH_4-forming methanogens, increasing acetate and CH_4 production (Kolver, 1998). Although CH_4 formation maintains the hydrogen (**H_2**) balance in the rumen by acting as a H_2 sink, it also constitutes an energetic loss to the animal through enteric emissions. Because of this, acetate production is energetically expensive compared with butyrate and propionate formation as it generates 8 H_2 atoms per glucose molecule rather than four (Beever et al., 1993). Based on theoretical calculations, Beever et al. (1993) concluded that a forage-based diet would produce 1.3 mol of acetate and 0.5 mol CH_4 from a single molecule of hexose as compared to a grain diet that would produce 0.9 mol of acetate and 0.3 mol of CH_4. This indicates that net energy can become limiting to the animal during circumstances of continued high acetate production, impacting production status.

1.6.2. Methanogenesis and Protozoa

Methanogens metabolize H_2 formed during the fermentation process to create CH_4 (Henderson et al., 2015). During monosaccharide metabolism, H_2 is released, which

reduces intracellular cofactors that must be re-oxidized to allow for fermentation to continue (Beauchemin et al., 2020). Methanogens will scavenge H_2 and CO_2, producing CH_4 (Patra et al., 2017). The hydrogenotrophic pathway utilizing CO_2 as a carbon source and H_2 as an electron donor is the predominant pathway in the rumen. To a much lesser extent, CH_4 can be produced through the utilization of methyl groups, formic acid, or acetate (Huws et al., 2018). It is theorized that shifting H_2 away from methanogenesis to fermentation products and microbial synthesis can decrease CH_4 as well as increase productivity through more efficient energy utilization (Beauchemin et al., 2020).

Because diet components differ based on management and dairy production systems, enteric CH_4 production may vary widely (Chagunda et al., 2010). As mentioned previously, dairy cattle on high-forage diets produce more CH_4 as forage diets typically have greater amounts of neutral detergent fiber (**NDF**), resulting in increased proportions of acetate (Thompson and Rowntree, 2020). Increased acetate increases CH_4 production as it increases H_2 concentrations, representing an energy loss to the animal (Thompson and Rowntree, 2020). Therefore, the choice of pasture species and grazing management practices has major impacts on both pasture productivity and the environment, including CH_4 output (Wilson et al., 2020). Wilson et al. (2020) conducted a study in which a forage base composed of grasses (festulolium, tall fescue, and orchardgrass) and white clover was changed to a base comprised of white clover + forbs (chicory and plantain) or legumes (red clover, birdsfoot trefoil, berseem clover (*Trifolium alexandrinum*), and balansa clover (*Trifolium michelianum Savi*)) to determine impacts on CH_4 emissions of Jersey cows. Total dry matter intake (**DMI**) as a percentage of body weight was higher for dairy cows on legume (3.45%) and forb (3.61%) pastures as compared with grass-based (3.19%)

pastures. Additionally, milk solids followed a similar trend with dairy cows grazing on forb (2.00 kg cow^{-1} d^{-1}) and legume (2.10 kg cow^{-1} d^{-1}) based pastures producing more milk solids than cows on grass (1.80 kg cow^{-1} d^{-1}). Furthermore, CH_4 emissions were lower from cows grazing the forb-based pasture, indicating that forb incorporation could potentially support milk production and animal performance while simultaneously reducing environmental impacts. You sw

1.7. Research Hypotheses and Objectives

Although OGF dairy productions are increasing in the U.S., producers need support to make their transition or current production system successful. This includes identifying information and benchmarks that will aid producers in their decision-making processes. In order for these benchmarks to be successful, there is a need for a multidisciplined research approach to characterize and assess farm management, marketing analysis, and forage management practices in the NE U.S., and validate these approaches in terms of their impact on animal nutritional physiology. Currently, there is a paucity of information related to these factors and how they relate to one another. As forage production is the backbone of OGF operations, there is a critical need for research related to increased forage productivity and quality while still maintaining milk production and animal performance.

Based on identified needs and management practices from survey results, we hypothesized that common energetic supplements, such as apple cider vinegar (**ACV**), would have no impact on animal energetic status as supplementation levels of ACV are not enough to induce beneficial physiological changes. Furthermore, the amount of supplementation needed for nutritional benefits would be impractical to feed. We further hypothesized that more diverse forage mixtures would positively impact rumen

performance parameters because increased forage diversity may provide more opportunities for more accessible energy from the diet. Additionally, using a variable cost analysis, we hypothesized that while C_4 forage inclusion in pastures may be beneficial from a nutrition perspective, it may not be economically feasible for producers in the NE. Finally, we hypothesized that increased grass species inclusion with 40% DM red clover would provide more accessible energy and mitigate CH_4 output, potentially due to a more diverse plant nutrient profile. This dissertation aimed to 1) evaluate OGF dairy producer areas of knowledge and expertise and how they relate to forage and animal production, 2) assess the and determine the efficacy of energetic supplements that were identified as being commonly utilized on OGF dairy farms in the NE U.S., and 3) identify and evaluate forage combinations that might be best utilized by NE producers in terms of animal energetic status and environmental output.

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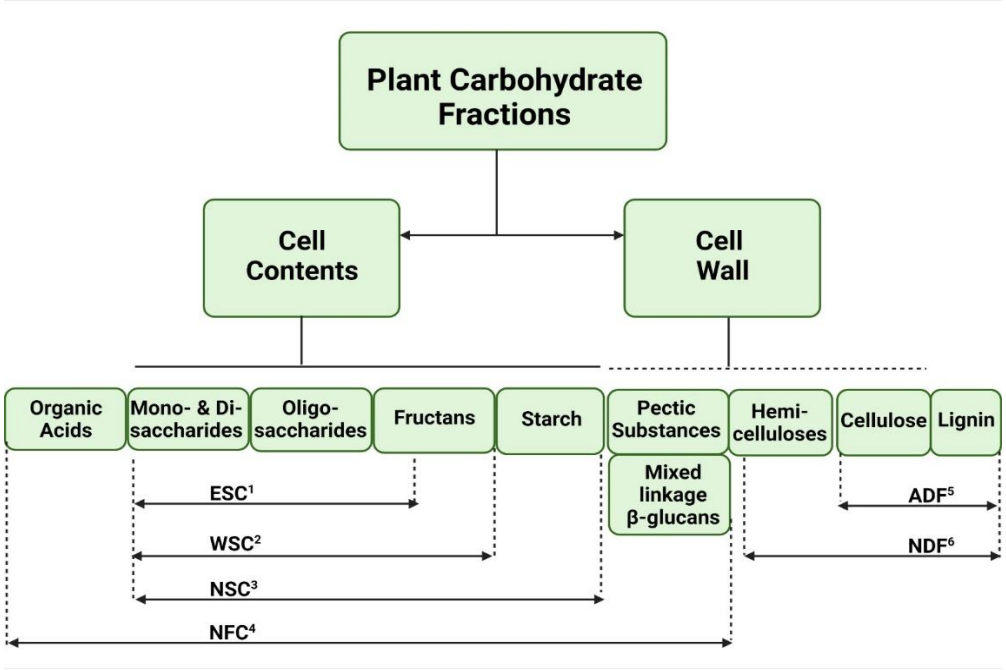
1.9. Figures

Figure 1.1. Plant carbohydrates divided by cell content and cell wall fractions. Cell wall components also includes lignin, a phenolic compound. Cell contents contain non-carbohydrate components such as organic acids.



[1]ESC: Ethanol soluble carbohydrates; mono-, di-, and oligosaccharides, + some fructans.
[2]WSC: Water soluble carbohydrates; ESC + total fructans.
[3]NSC: Nonstructural carbohydrates; WSC + starch.
[4]NFC: Nonfiber carbohydrates; WSC, starch, organic acids, pectic substances, + mixed linkage ß-glucans.
[5]ADF: Acid detergent fiber; cellulose + lignin.
[6]NDF: Neutral detergent fiber; ADF + hemicelluloses.
Adapted from Hall and Eastridge (2014). Image created with Biorender.

CHAPTER 2: AN OVERVIEW OF ORGANIC, GRASSFED DAIRY FARM MANAGEMENT AND FACTORS RELATED TO HIGHER MILK PRODUCTION

2.1. Abstract

Organic, grassfed (OGF) dairy, which requires higher pasture and forage dry matter intake (DMI) compared with standard organic dairy practices, is unique both in its management needs and in production challenges. The OGF dairy sector is rapidly growing, with expansion of this industry outpacing other dairy sectors. There is a lack of research outlining OGF dairy production practices, producer-identified research needs, or social factors that affect OGF systems. The objectives of this study were to, with a group of OGF dairy producers, 1) assess information regarding current production practices and producer knowledge and 2) identify agronomic and social factors that may influence milk production on OGF farms across the United States (U.S.). A mail survey, focused on demographics, forage and animal management, knowledge, and satisfaction of their farm was developed and distributed in 2019, with 167 responses (46.8% response rate). The majority of producers indicated they belonged to the plain, or Amish-Mennonite, community. Milk production was greater on farms that had Holstein cattle, as compared to farms with Jerseys and mixed breeds, and employed intensive pasture rotation. Furthermore, most producers reported the use of supplements such as molasses and kelp meal, which can improve milk production, but also increase feed costs. Producers who indicated that they were at least satisfied with their milk production also reported high levels of knowledge of grazing management and cow reproductive performance. Comparison of response data from plain/ non-plain respondents revealed that those that did not identify as plain were more likely to

utilize certain government programs, had different priorities, and utilized technology more frequently. Based on these results, more research exploring financial and production benchmarks, effective communication strategies to reach OGF producers, and methods to improve cattle production through improved forage quality is needed.

Keywords: grassfed dairy, grazing, milk production, producer perceptions, survey

2.2. Introduction

During the last decade, organic dairy production has increased rapidly in the United States (**U.S.**) with the consumer market for organic milk growing from 1.9% to 5.0% of total milk sales (USDA-AMS, 2020). While organic livestock typically produce less milk than conventionally-raised animals, producers have historically captured higher milk prices to offset increased input costs (Su et al., 2013). Organic dairy producers are now facing declining milk prices due to an oversupply of milk. As of 2019, total organic milk products declined by 2.2% (USDA-AMS, 2020). However, the organic, grassfed (**OGF**) milk market continues to show high demand leading to an increase in grassfed livestock production, particularly in the Northeastern (**NE**) U.S. (USDA-NASS, 2017). Grassfed, for the purposes of this study, is defined as dairy production relying on forages (pasture and conserved forages), without grains, to meet nutritional needs. This specialized system, to be certified OGF, must also comply with organic standards as well as a unique set of regulations specific to grassfed dairy.

Existing research suggests that, to secure the future of their farms, producers are choosing to adopt grassfed feeding systems in part due to economics, but also due to other reasons, including personal philosophy, health/safety, and environmental sustainability (Flaten et al., 2006; Bouttes et al., 2019). In a Norwegian study, Flaten et al. (2006) grouped 161 organic dairy producers in three different categories: early organic farming entrants (certified organic since 1995 or earlier), midconverters (certified organic between 1996 – 1999), and late converters (certified organic since 2000 or later). All groups ranked environment, sustainability, and the production of high-quality food as their main motivators, although those who transitioned later ranked economics as a major motivator

as well. Bouttes et al. (2019) found that organic producers were motivated to switch from conventional management to achieve increased professional satisfaction as competitive organic milk prices enabled them to reduce the number of cows and workload.

Navigating regulatory policies of the transition process is also commonly perceived as a challenge in the OGF dairy industry. Organic and grassfed dairy are similar in management but have different regulations that dictate some aspects of the production system. This includes livestock feeding requirements, which could directly influence milk production. As per regulations set forth by the United States Department of Agriculture (**USDA**) National Organic Program (USDA-NOP, 2020), organic dairy cattle over the age of 6 months must receive 30% or more of dry matter intake (**DMI**) from pasture during the grazing season for a period of at least 120 d. It is further mandated that OGF livestock must be on pasture at least 150 d and receive 60% or more of their DMI from pasture (Organic Trust Plus Inc., 2019). Supplements and feed additives without grain components may also be utilized under strict guidelines. Compliance with these regulations can create challenges on OGF dairies, particularly due to the nutritional reliance on forages and potential limitations in pasture productivity and quality (Wilkinson et al., 2020; Brito and Silva, 2020).

Research supporting grassfed dairy operation needs is currently outpaced by the sector's expansion, creating knowledge gaps for both producers and the allied industry. Demographic (McBride and Green, 2009), management (Stiglbauer et al., 2013), and research and education needs (Pereira et al., 2013) regarding organic dairy production have been previously reported as have perceptions of consumers (Pirog, 2004) and grassfed dairy producers in Europe (Läpple, 2013). There is currently limited research exploring

grassfed dairy industry characteristics and social factors that may influence milk production and management decisions in the U.S. Our research utilized a nationwide survey of OGF dairy producers to improve understanding of this farming demographic and their characteristics. Therefore, the objectives of the current study were to 1) assess information regarding current production practices and producer knowledge and 2) identify agronomic and social factors that may influence milk production on OGF farms across the U.S.

2.3. Materials and Methods

2.3.1. Survey Questionnaire

A survey was developed in 2019 with the aim of recording information about OGF producer characteristics, attitudes, and perceptions. The survey was developed through a multidisciplinary research team in collaboration with a producer advisory group and industry feedback. Institutional Review Board approval was obtained prior to beginning the research from the University of Vermont (IRB # 00000036). We utilized the Dillman tailored design approach to disseminate the survey to 351 farms from a list of producers obtained from dairy cooperatives located in New York and Wisconsin (Dillman et al., 2016). A postcard first announced the survey, followed by the survey instrument, and a reminder postcard. A second survey was sent to any non-respondents one month later. A total of 167 respondents returned the survey, with three undeliverable, three out of sample as their cattle had been sold, and one explicit refusal, resulting in a response rate of 46.8% according to the American Association for Public Opinion Research calculation methodology (AAPOR).

2.3.2. Survey Instrument and Variables

The survey consisted of multiple sections including: 1) general farm information, 2) herd management, and 3) forage and grazing management. The questions utilized in the statistical analysis are included in Supplementary Materials, Table S2.1, including descriptive statistics. All variables included were specific questions from the survey, apart from the Information Scale, which was an aggregated score of all responses for sources of dairy information and frequency (Cronbach alpha = 0.82).

2.3.3. Statistical Analysis

Data were analyzed in Stata 16.0 (Stata 2019), utilizing descriptive statistics and multivariable analysis. Relationships were analyzed using Pearson correlation coefficients with statistical significance declared when $P \leq 0.05$. Results were interpreted as a weak association being 0.1 to 0.3 or -0.1 to -0.3, medium association at 0.3 to 0.5 or -0.3 to -0.5, and strong at 0.5 to 1.0 or -0.5 to -1.0. A multivariable regression analysis was conducted, first utilizing step-wise regression analyses. We conducted 7 separate step-wise regression models focused on characteristics relating to animals, forage management, policy, on-farm management, demographics, producer knowledge, and information (Table 2.1). Factors from individual models were considered significant when $P \leq 0.10$ and used in the final full multivariable regression model to determine influence on milk production. Factors from the multivariable regression model were considered significant at $P \leq 0.05$ and are reported in their respective sections below as it relates to milk production. Model results for all step-wise regression models are included in Supplementary Materials, Tables S2.2, S2.4 - S2.6, S2.8 – S2.9, and S2.11. Post-hoc analysis was also performed to identify any relationship between survey response data and self-identification by respondents belonging

to the plain community. These relationships were analyzed in SAS 9.4 (SAS Institute) using the PROC FREQ procedure with two-sided chi square tests. Values were considered significant at $P \leq 0.05$.

2.4. Results

2.4.1. Producer Demographics and On-Farm Decision Making Influences

Geographically, the largest number of responses (49.7%) were received from OGF dairies located in New York (81 farms). The remaining responses were received from OGF dairies located in 15 states with the majority located in the NE (Pennsylvania (6.1%), Vermont (6.1%), New Hampshire (1.2%), New Jersey (0.6%), and Massachusetts (0.6%)) or Midwestern (**MW**) (Ohio (11.7%), Wisconsin (7.9%), Iowa (3.7%), Minnesota (1.8%), Kansas (0.6%), and Indiana (0.6%)) U.S. Outside of the Northeast and Midwest, remaining responses were received from Maryland (3.7%), Virginia (2.5%), Florida (0.6%), and Oregon (2.5%; Supplementary Materials, Figure S2.1). Demographically, producers predominantly identified as being the farm owner (91.5%), male (91.4%), and as belonging to the plain, or Amish and Mennonite, community (61%; Supplementary Materials, Table S2.2). Respondents indicated that they had been dairy farming for 21.4 ± 15.1 yr with a mean age of 47.6 ± 12.9 yr.

Respondent ranking of factors that influence on-farm decision making is shown in Figure 2.1. When producers compared the self-perceived status of their farm to similar farms on a scale from significantly worse to significantly better, 79.3% indicated that they were at least somewhat better compared to other dairies, whereas 19.3% said that they felt they were doing worse than other farms. Based on responses, autonomy, environment, labor, and future generations were of equal influence regarding on-farm decision making

across the entire sample. This is also reflected by the number of producers that have succession plans; only half of producers stated that they had at least a partial succession plan. However, when assessing farm priorities by respondent who identified as part of the plain community (termed plain producers herein) versus those that did not (termed non-plain producers herein), family and well-being was more influential to non-plain producers ($P = 0.02$). Autonomy was considerably more important to producers identifying as plain producers ($P = 0.03$) whereas economics was considered more important by non-plain producers ($P = 0.03$). Both plain and non-plain producers ranked economics as the greatest influence for decision-making with an average importance of 5.4 ± 2.8, on a scale from 1 to 9. Impacts on society (neighbors, community) and policies and regulations proved to be slightly less influential with averages of 6.4 ± 2.4 and 6.1 ± 2.5 respectively. Non-plain producers indicated that while policy was not as influential on their decision-making, they still held it in higher regard than plain producers ($P = 0.04$; Supplementary Materials, Table S2.3)

2.4.2. Federal Programs and Policy Factors

The questionnaire included questions about participation in nationally recognized programs, including organic and grassfed certifications, and government cost-share programs such as the Conservation Reserve Program (**CRP**) and Federal Crop Insurance Program (**FCIP**). Responses are summarized in Figure 2.2 and Supplementary Materials, Table S2.4. Most producers were either unaware (23.3% of respondents) of these programs or were aware but did not intend to participate (54.2 % of respondents). Non-plain producers were more likely to utilize the Environmental Quality Incentives Program (**EQIP**; $P < 0.0001$) and Margin Protection Program for Dairy (**MPPD**; $P < 0.0001$) than

plain producers. Producers indicated that they utilized both organic certifiers and organic educational organizations, their milk buyer/cooperative, producer publications, and other dairy producers monthly or a few times a yr for sources of dairy information. On average, 96.3% of farms that responded were certified organic for 10.3 ± 7.2 yr or as OGF for 5.1 ± 4.5 yr.

2.4.3. Herd Characteristics and Management

The predominant genetics represented on-farm reported by respondents were crossbred (54.1%), Holstein (21.7%), and Jersey (17.2%). On average, producers had 49 milking cows and an average of 10 dry cows. The calculated average age of cows in a herd was 5.7 ± 1.4 yr old. Results of producer satisfaction are summarized in Table 2.2 and management characteristics in Supplementary Materials, Tables S2.5 – S2.6. Regarding herd health parameters such as body condition score, animal health, young stock quality, and reproduction and calving, most producers were at least somewhat satisfied with body condition, herd health, and young stock quality. Plain producers reported that they were more satisfied with their soil fertility ($P = 0.05$), reproduction and calving ($P = 0.05$), and quality of youngstock ($P = 0.003$) than non-plain producers. Producers indicated that they cull 16.2 ± 8.5% of cows on a yearly basis with four major reasons being reproduction (58.1%), injury or health (35.3%), mastitis (34.7%), and milk quality (30.5%). However, there were no relationships between farmer demographics with culling and age ($P = 0.71$), yr farming ($P = 0.09$), number of milking cows ($P = 0.31$), or total hectares (**ha**) managed ($P = 0.79$) (Supplementary Materials, Table S2.7).

2.4.4. Land and Forage Management

There was a strong positive relationship between the number of total acres (**ac**) managed and number of milking cows (R = 0.57); as the total number of ha managed increased, the number of milking cows increased ($P < 0.001$). Producers managed 108.3 ± 74.8 ha of land and owned 74.7 ± 55.4 ha or rented 49.7 ± 43.7 ha. Of the land being utilized, 88.8 ± 64.5 ha was used for pasture and growing perennial and annual forages. However, most producers did not typically grow annual forage crops such as sorghum sudangrass. Those producers who did utilize annual forages indicated that they grew 7.4 ± 7.9 ha yr^{-1}. Results are further summarized in Supplementary Materials, Table S2.8.

2.4.5. Farmer Knowledge

A total of 93.1% of producers indicated that they believe they have at least an average knowledge regarding grazing management systems (Table 2.3; Supplementary Materials, Table S2.9) and managed their land for species diversity through grazing management strategies. A typical grazing season lasted 196.8 ± 32.2 d on average with animals receiving between 70% and 90% of DMI from pasture. Producers indicated that their grazing seasons lasted at minimum 140 d and 360 d at maximum. In addition to growing their own stored forages, 63.9% of producers also purchased 38.3 ± 29.1% of their total forage needs. Plain producers were more likely to purchase forages ($P < 0.0001$). A moderately negative relationship (R = -0.32) was observed between the amount of forage purchased and total ha managed; as the amount of forage purchased increased, the total amount of ha managed decreased ($P = 0.001$).

In the current study, producers were asked about perceived knowledge in growing forages, understanding forage test results, production costs, and feed supplementation

practices. Approximately 82.9% of respondents reported at least an average knowledge of growing higher-energy forages. Producers also said that they understood the energy requirements of cows according to production levels and were at least somewhat satisfied with their pasture quality and yield (77.3%; $P = 0.01$), stored forage quality (76.9%; $P = 0.003$), and stored forage yield (71.4%; $P = 0.003$; Supplementary Materials, Table S2.10). Respondents (25.6%) indicated that they had a very low or low knowledge regarding forage test result interpretation with similar responses reported about strategies to maximize forage DMI and forage production costs. Plain producers indicated that they were more knowledgeable than their non-plain counterparts regarding interpretation of forage test results ($P = 0.002$), growing high energy forages ($P = 0.03$), grazing management ($P = 0.002$), and cattle energy requirements ($P = 0.02$). However, there were no perceived knowledge differences between plain and non-plain producers regarding forage quality ($P = 0.11$).

2.4.6. Farmer Sources of Information

Producers identified key sources of dairy information that influenced their on-farm decision making. Sources of information included community papers and producer publications, organic certifiers and educational organizations, the USDA, extension groups, feed dealers, consultants, other dairy farmers, and milk cooperatives. On a scale from never to daily, producers were asked to identify which sources they utilized the most and found to be most helpful. Private consultants (72.7%) and Farm Bureau (77.6%) were least likely to be utilized, whereas milk cooperatives (90.3%), producer publications (90.3%), and other dairy producers (97.48%) were used as information sources at least a few times a yr. Overall, the use of outside sources of information was found to influence

milk production in both the step-wise model (P = 0.001; Supplementary Materials, Table S2.11) and the full model (P = 0.001).

2.4.7. Agronomic and Social Influences on Milk Production: Animal and Herd Characteristics

In describing OGF herd milk production and milking seasonality, herds were typically milked at least twice daily (84.9%) with an average annual milk production of 4,220.6 ± 1,044.9 kg cow^{-1}. Results indicate that belonging to the plain community had no bearing on milk production (P = 0.47). Producers (72.0%) indicated that they were at least somewhat satisfied with their current milk production (P = 0.01). A total of 84.8% of respondents reported that their herd produced milk yr-round while 15.2% identified as seasonal milking herds. In this study, if seasonal milk production was utilized, the herd dry-off period most frequently occurred between February and March, followed by July and August, and January and February.

As is typical of pasture-based systems, most producers fed their cattle approved energetic supplements or byproducts. Sugar or molasses were the most commonly utilized feed supplements with 41.4% of surveyed producers stating they utilized energy sources yr-round. Other producers indicated that they supplemented as needed during the winter season (29.9%) or during the grazing season (4.6%). Besides molasses and sugar, producers were also utilizing supplements such as minerals, salt, sodium bicarbonate, clay, yeast, probiotics, and kelp meal, as well as less-commonly utilized products such as apple cider vinegar (**ACV**) and dried carrots.

We completed 7 separate individual step-wise models (Supplementary Materials, Tables S2.2, S2.4 – S2.6, S2.8 – S2.9, and S2.11), which indicated the following significant

relationships to milk production: 1) Holstein breed associated with higher milk production (b = 3357.36, *P* = 0.001), 2) milking twice a d associated with higher production (b = 1577.11, *P* = 0.006), 3) use of energy supplements associated with higher production (b = 964.74, *P* = 0.02), 4) length of grazing season associated with lower milk production (b = 31.11, *P* = 0.03), 5) producers with higher levels of grazing management associated with lower milk production (b = -734.08, *P* = 0.02), 6) producers with higher levels of reproductive performance management associated with higher milk production (b = 534.87, *P* = 0.06), and 7) producers with greater frequency and number of dairy information sources associated with higher milk production (b = 1218.97, *P* = 0.000). The full multivariable model (which incorporated only the statistically significant results from each of the stepwise regression models) indicated that Holstein cow use (b = 1574.95, *P* < 0.001), reproduction knowledge (b = 578.27, *P* = 0.008), and greater frequency and depth of dairy information (Information Scale; b = 1036.45, *P* = 0.001) were all positively associated with per cow milk production (R^2 = 0.31).

2.5. Discussion

We aimed to better understand the characteristics and perspectives of OGF dairy producers across the U.S., and identify the factors related to higher milk production in OGF dairy herds. Overall Holstein cows, producers with higher knowledge of reproduction, and greater frequency and depth of dairy information sources were found to be positively associated with milk production. Given that pasture-based cows typically produce less milk than those fed in confinement due to less control over nutrient intake (Kolver, 2003), our findings provide a guide for the OGF industry to better understand its capacity to increase production. The reported average milk produced in our study was lower than that of

confined and traditional organic (but not grassfed) dairy herds reported in the survey of McBride and Greene (2009), likely because OGF cows are not permitted to be fed supplemental grains. However, other factors may contribute to lower milk production in OGF systems. Responses provided by U.S. OGF dairy producers has for the first time given insight into production and management practices of this production system. Furthermore, there is now a better understanding behind the agronomic and social factors that influence milk production and on-farm decision making on OGF dairies. Below we discuss further demographics, management and knowledge findings, as well as the factors related to higher milk production.

2.5.1. Demographic Factors

The distribution of OGF producers who responded to our survey aligns with data reported by the 2017 USDA-NASS Certified Organic Survey and previous research by McBride and Green (2009), whereby New York represented the state with greatest number of certified organic farms, followed by Wisconsin, Pennsylvania, Indiana, Ohio, and Vermont. Little information is available about the geographic distribution of purely grassfed producers but a study by Weber et al. (2008) indicated that grassfed producers were located in all regions of the U.S. In the current study, OGF farms were most concentrated in the NE and MW U.S. due to proximity to dense human populations, diverse milk markets, infrastructure for handling OGF milk, and OGF milk buyers (Flack, 2016); however, these geographic distributions also likely reflect our sample size based on lists obtained from partner cooperatives.

The large proportion of respondents indicating they belonged to the plain community in this study aligns with reported geographical data as higher densities of

Amish communities are located in the NE and MW U.S. (Cross, 2016), and also may explain the low levels of reported technology use for record keeping and finances. Furthermore, plain producers were less likely to participate in government programs and exhibited different on farm priorities. These findings are important because it suggests that the organic dairy industry needs to communicate and develop benchmarks for the plain community using non-electronic mechanisms and provide technical assistance outside of government programs. McBride and Greene (2009) found similar results with organic dairy producers; based on their survey results, 41% of organic dairy producers utilized information from the internet, whereas only 21% utilized on-farm computer records. In their study, several technologies and practices tended to increase with the size of the organic operation and with higher education (McBride and Greene, 2009); however, we did not account for education level in the current study.

2.5.2. Management Factors

Regarding herd health and culling practices, similar to our results, Rozzi et al. (2007) found that two main reasons organic producers culled animals were poor fertility and mastitis. Additionally, Ahlman et al. (2011) compared dynamics of culling reasons between conventional and organic herds and reported no genotype × environment interactions for longevity. However, it was determined that the main reason for culling in organic herds was due to poor udder health, whereas fertility issues were the main cause in conventional herds. Furthermore, culling occurred at a younger age in organic herds. This directly contradicts the findings of both Mullen et al. (2013) and Stiglbauer et al. (2013). Mullen et al. (2013) found that organic farms and conventional farms utilizing grazing typically kept animals that were older, which are generally associated with mastitis, foot

problems, and milk fever. Stiglbauer et al. (2013) also determined that organic farms kept older cattle, although in their study, there was a trend of younger animals on conventional, non-grazing operations. It should be noted that culling decisions are complex, requiring knowledge of current animal status and predicted performance. In OGF operations, decisions also rely on compliance with grassfed rules and certain ethical standards, meaning that antibiotics cannot be utilized when dealing with mastitis or udder health. Therefore, the reasons provided by producers for culling in our survey may depend on what the producers find acceptable which could be different from conventional producers.

2.5.3. Social Influences

Both plain and non-plain producers ranked economics as their greatest influence for decision-making. Family and well-being were more influential to on-farm decision making to non-plain producers as was economics. This could be related to the number of producers that had succession plans, which, in turn, provides potential security for their families. Of the surveyed producers, 54.8% said they did not have a succession plan. These results align with Brock and Barham (2013) where dairy producers also indicated economics to be one of the primary forces behind decision making. Organic grassfed producers in the current study reported similar drivers for on farm decision-making, with economics, environmental reasons, and health being the major influences. Producers also noted that they were not interested in participating in government sponsored programs, which was similar to results presented by Hanson et al. (2004). Hayden et al. (2018) suggested that organic producers feel at odds with normal farming practices and, therefore, did not feel valued as highly by financing, insurance, and regulatory institutions. This, in turn, could lead to a distrust in government-based programs. Additionally, there is

speculation that producers feel as though some government programs are not adequately promoted and are not adaptive enough (Hayden et al., 2018). For example, a significant proportion of OGF producers in this study indicated they had no knowledge of these programs, which may also be related to the high percentage of producers in OGF dairy that belong to the plain community, who may have an aversion to federal programs based on personal philosophy. One specifically important finding was the extent to which producer frequency and number of dairy information sources positively correlated with milk production, which agrees with previous research (Lubell et al., 2014). These results indicate that dairy producers may be getting a diversity of information across varying sources from other farmers to Extension to industry professionals, all of which may help them identify practices to improve their productivity.

2.5.4. Farmer Satisfaction and Knowledge

The results of this study indicated that the length of the average grazing season was approximately 197 d, which is longer than expected considering the weather conditions of the Northeast and Midwest. Similar to Pereira et al. (2013), despite most producers being located in the Northeast and Midwest, surveyed OGF dairies in this study indicated they operated on a yr-round basis, rather than seasonally. This could potentially be due to the use of annual forages to complement cool-season forages during the warmer months of the grazing season. However, our results indicated that the use of annual forages and yr-round grazing operations did not have a relationship ($P = 0.17$) as most producers did not utilize annuals in this study. The length of grazing season could potentially be related to the amount of forages purchased. As previously stated, approximately 64% of producers purchased forages to supplement grazing diets. Producers supplemented their diets with an

average of 38.3% purchased forages although amounts ranged from 0.33% of the diet to 100%. This is similar to Pereira et al (2013) who reported that 73% of organic farmers purchased feed from off-farm sources. However, in this study there was no relationship between grazing seasonality and whether producers purchased forages or not ($P = 0.65$).

A large portion of OGF producers indicated that they were satisfied with their pasture quality, yield, and milk production. The majority also indicated that they only had an average knowledge about factors (i.e., growing higher-energy forages, forage improvement and quality, forage DMI) that could affect pasture utilization and milk production. This suggests that OGF producers may be prioritizing other management practices and do not see a need to focus on forage quality and milk production. For example, Pereira et al. (2013) assessed research needs and topics of priority of NE organic dairy farmers. Respondents indicated that although obtaining a steady, fair milk price and improving forage production was important to them, there were other priorities, such as integrated pest management, that were just as valuable.

Because OGF producers may be prioritizing factors other than milk production and forage management, based on survey responses, it is possible that they are not supplying cows with high-quality forage to meet energetic demands. Energy intake is dependent on DMI and dry matter (**DM**) energy value (Van Vuuren and Van Den Pol-Van Dasselaar, 2006). Lower DMI in pasture-based systems is one of the main limiting factors for milk production as this affects metabolizable energy (**ME**) supply (Kolver, 2003). In our study, DMI as reported by producers was not a significant factor relating to milk production but this could be due to other on-farm factors such as length of grazing season and management intensity (Weiss and Jeltsch, 2015).

Approved non-grain, energy sources and feed additives are utilized in OGF systems. One such supplement is molasses, as it is palatable, energy dense, and approved for organic dairy production (Soder et al., 2011) and by some grassfed labels (American Grassfed Association, 2020). Producers reported greater milk yield with energy supplements, as well as greater satisfaction with their milk production but still reported using energy-based supplements yr-round, which, depending on the supplement, could prove costly, as compared to using high- energy forages (Soder et al., 2011). Kelp meal was also commonly utilized by respondents (72.5%) in this survey. Although more costly, according to Antaya et al. (2015), organic dairy producers have anecdotally linked kelp meal to improved body condition score and animal appearance, decreased milk somatic cells count, and control of nuisance flies. This may be due to the presence of a wide spectrum of bioactive compounds that are capable of binding proteins and carbohydrates (Antaya et al., 2019). Previous surveys conducted in the Northeast (Antaya et al., 2015) and Midwest (Hardie et al., 2014; Sorge et al., 2016) revealed that between 49% and 83% of organic dairy producers feed kelp meal, thus consistent with our results.

Forage quality and quantity was a common issue as home-grown forages were lacking in either amount or quality. Utilization of energetic supplements and outsourced forages could suggest a forage quality issue, leading to a dietary deficiency. The need to purchase additional forages could also be due to resource constraints or inappropriate stocking rate/density, both of which can affect forage quantity and quality. Economic viability may also play a role as producers in our study indicate they are highly driven by economics; purchasing forages represents a lower initial investment relative to growing forages, which could be economically advantageous.

Reproduction, genetics, and breed type had an influence on milk production. In our study, farms comprised of Holstein cows reported that they had the greatest milk production. These results are similar to Coffey et al. (2016) who found that Holstein herds in seasonal calving systems had a greater milk production than Friesian and Jersey herds. Jersey was found to have greater milk protein and fat concentrations, whereas Holstein had lower concentrations (7.75%), similar to data presented here in which the average concentration of milk fat and protein combined was 7.77%. Apart from breed, reproductive performance knowledge correlated with increased milk production, which agrees with the suggestion that OGF producers are prioritizing factors other than forage management. The economic impact of reproductive performance and knowledge makes management a key priority to producers (Denis-Robichaud et al., 2018), thus consistent with the finding that a large proportion of culling decisions are made based on cow reproductive status. Denis-Robichaud et al. (2018) reported that dairy reproductive performance was one of the most important priorities for research. However, unlike the producers in the current study, almost half of the producers in the study by Denis-Robichaud et al. (2018) were not satisfied with their milk production. This could indicate that because OGF dairy producers prioritize reproductive performance, and possible health and welfare, more so than other factors, they may not encounter the same challenges as conventional producers.

2.6. Conclusions and Implications

Results of this study showed a need for further research to develop financial and production benchmarks that will aid OGF dairy producers in improving management practices thereby enhancing economic sustainability on their operations. However, traditional methods of communication and outreach may not reach a significant portion of

OGF dairy producers from the plain community who do not utilize modern information technologies. This is an important finding, especially because greater frequency and diversity of information sources were found to positively associate with animal production. Future efforts must focus on a wider range of outreach to better meet the educational needs of OGF dairy producers through a broader array of methods to disseminate information. Additionally, results of this study revealed a high level of misunderstanding and disconnect, specifically in the areas of producing high-quality forage, meeting energy needs of OGF dairy cows, feed supplements, and their implications to overall animal productivity and farm profitability.

In addition to creating financial and production benchmarks, our results revealed knowledge gaps in the areas of forage quality, forage energetics, feed supplements, and their implications to grazing nutrition. These identified critical areas indicate that research needs to be developed to determine forage energetics and help producers create pasture plans that will ensure that their animals receive nutrient and energy dense forages. Future research efforts are needed better meet the educational needs of OGF dairy producers through a broader array of methods to disseminate information

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2.9. Tables

Table 2.1. Full multivariable model, following 7 stepwise models, predicting milk production per cow. $R^2 = 0.31$, n =135.

Variable	Coefficient	Standard Error	*P* -Value	95% Confidence Interval	
Holstein	1,574.95	425.95	0.00	732.08	2,417.83
Grazing season	-6.16	5.71	0.28	-17.46	5.15
Twice a d	741.44	513.78	0.15	-275.24	1,758.13
Supplements	504.35	351.41	0.15	-191.03	1,199.74
Grazing knowledge	-283.21	227.79	0.22	-733.96	167.54
Reproduction knowledge	578.27	215.85	0.01	151.13	1,005.40
Information scale	1,036.45	296.48	0.001	449.78	1623.12

Table 2.2. Ranking of satisfaction of production parameters by organic, grassfed (OGF) producers across the United States (U.S.) surveyed during the spring of 2019.

Production Parameter	Rank[1]					
	Very Unsatisfied	Unsatisfied	Somewhat Unsatisfied	Somewhat Satisfied	Satisfied	Very Satisfied
Farm income	3.1	8.8	20.1	37.1	25.2	5.7
Herd health	0.0	1.9	6.2	29.8	43.5	18.6
Cow body condition	0.0	3.7	9.8	31.1	41.5	14.0
Reproduction and calving	0.6	4.3	17.2	30.7	36.2	11.0
Quality of young stock	1.9	2.5	7.4	30.3	41.4	16.7
Pasture quality and yield	0.0	8.0	14.7	46.6	24.5	6.1
Stored forage quality	0.0	6.4	16.7	35.3	32.1	9.6
Stored forage yield	2.0	7.8	18.8	37.7	29.2	4.6
Soil fertility/ health	0.6	7.5	19.4	40.0	28.8	3.8

[1]The results of 167 surveys were analyzed and ranked according to producer satisfaction. Producers were asked to rank their satisfaction with various production parameters on a scale from very unsatisfied to very satisfied. Values are expressed as percentages of total respondents.

Table 2.3. Ranking of self-perceived knowledge of production parameters by organic, grassfed (OGF) producers across the United States (U.S.) surveyed during the spring of 2019.

	Rank[1]				
Production Parameter	**Very Low**	**Low**	**Average**	**High**	**Very High**
Herd Health					
Body condition scoring	1.2	6.2	63.0	20.4	9.3
Reproductive performance	0.6	14.3	49.7	24.8	10.6
Forages and Grazing					
Cow energy requirements	0.6	6.9	62.9	24.5	5.0
Grazing management systems	0.6	6.3	38.4	40.3	14.5
Growing higher energy forages	1.3	15.8	48.7	28.5	5.7
Improving forage quality	0.0	6.3	50.0	36.7	7.0
Forage yields	0.6	15.6	50.0	24.4	9.4
Understanding forage test results	2.6	23.1	44.2	23.7	6.4
Forage DMI[2] strategies	1.9	16.5	47.5	27.9	6.3
Forage production costs	1.9	22.2	55.1	15.8	5.1
Record Keeping & Interpretation					
Farm record keeping	0.0	7.4	53.7	25.3	13.6
Understanding MUN[3] results	3.8	14.5	37.7	33.3	10.7
Understanding soil test results	1.3	22.0	49.1	20.8	6.9

[1]The results of 167 surveys were analyzed and ranked according to self-perceived producer knowledge. Producers were asked to rank what they believed they knew regarding various production parameters on a scale from very low to very high. Values are expressed as percentages of total respondents.
[2]DMI: dry matter intake.
[3]MUN: milk urea nitrogen.

2.10. Figures

Figure 2.1. Respondent ranking of factors that influence on-farm decisions. Responses are based on a scale from 0, being the most influential, to 9, being the least influential. Values placed at a scale value of 10 were written in by surveyed producers. Values are expressed as the mean with the minimum and maximum response range.

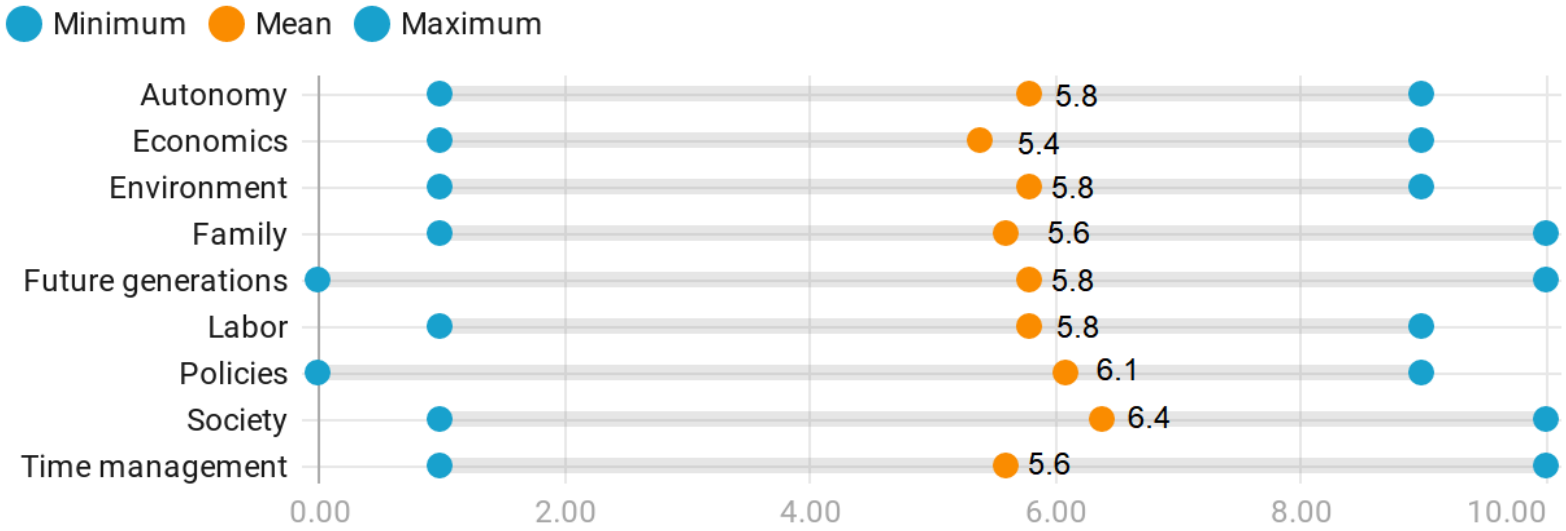
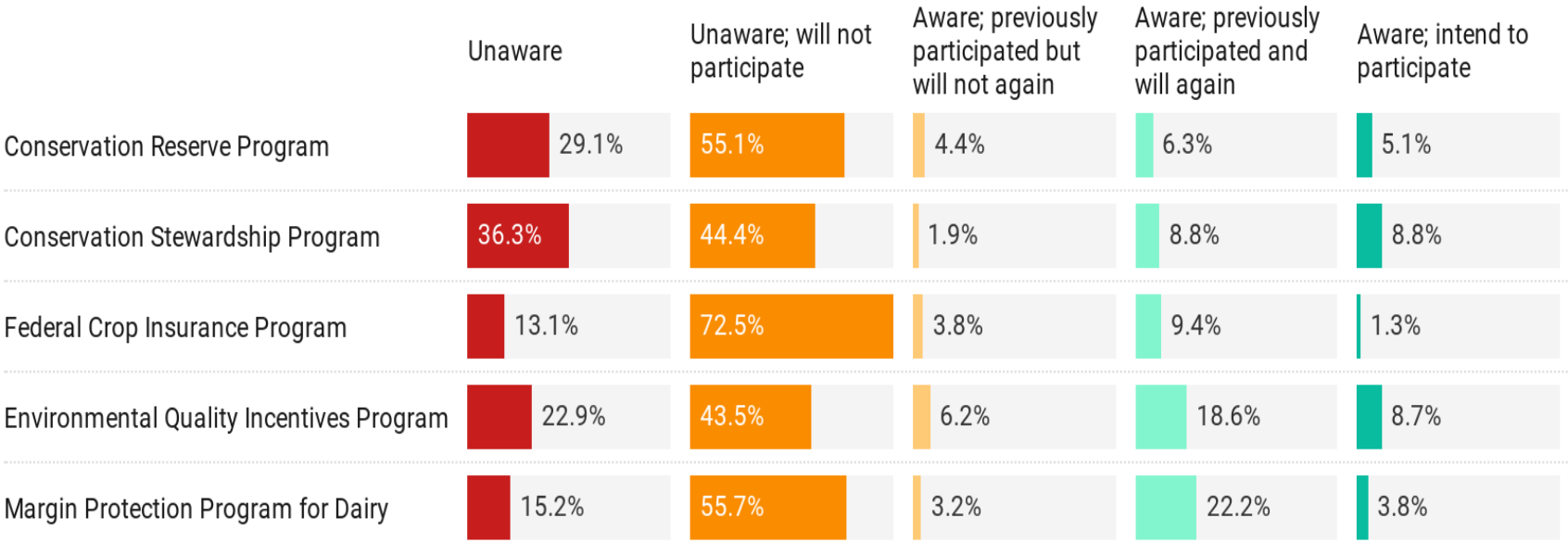


Figure 2.2. Utilization of United States Department of Agriculture (USDA) programs by organic, grassfed (OGF) dairy productions surveyed in spring 2019. Responses are based on a scale from unaware to aware and intending to participate. Values are expressed as a percentage.



2.11. Supplementary Tables

Table S2.1. Variables analyzed in the stepwise model and corresponding survey questions.

Variable Type	Variable	Question
Animals	Breed	What breed predominately makes up your herd?
	Animal age	What is the average age of a cow in your herd? (yr)
	Culling	On average, what percent of cows are culled from your herd each yr? (%) What are the top two most common reasons for culling?
	Herd size	On average, how many livestock do you have?
	Milk fed to calves	How long do you feed milk to your calves? (months) How much milk do you feed your calves? (gal $calf^{-1}$ d^{-1})
	Production per cow	What is your average annual milk production per cow? (lbs cow^{-1})
Forage/feed	Ac[1]	How many total ac do you manage? Of those, how many are owned v. rented?
	Forages bought	Do you typically purchase forage? If yes, estimate what percentage of your total forage needs are purchased?
	Grazing type	What type of grazing do you practice?
	Perennial/annual forages	How many ac are typically in forage production including pasture, perennial, and annual forages? Do you typically grow annual forage crops such as sudangrass? If yes, on average how many ac per yr? (ac yr^{-1})
	DMI[2]	During the grazing season, what is your average percent DMI from pasture?
	Grazing seasons length	In a typical yr, what is the length of your grazing season? (d)
Policy	Federal programs (all)	Does your farm participate in any of the following programs?
	Organic	Is your dairy farm certified organic? If yes, for how many yr?
	Grass fed	How many yr has the farm been 100% grassfed?

Table S2.1. continued. Variables analyzed in the stepwise model and corresponding survey questions.

Variable Type	Variable	Question
Management	Inputs	Do you typically purchase fertilizers, manures, or soil amendments? If yes, what do you use?
	Records and testing	Do you use a service, such as DHIA[3], to keep herd records? Do you use milk testing services?
	Milking per d	How many times a d do you milk?
	Supplements	Do you feed energy supplements such as molasses or sugar? If yes, typically when? What other supplements do you feed?
	Technology	How frequently do you use the following for farm business, record keeping, or information?
Demographics	Born yr	What is your age?
	Sex	What is your sex?
	Plain community	Do you identify as part of the plain community?
	Yr farming	How many yr have you been dairy farming?
	Succession plan	Do you have a farm succession plan for after you retire?
	Income from dairy	Estimate the percentage of your total income that each of the following sources provide.
Producer knowledge		How do you rate your current level of knowledge on the following topics?
Information scale		How frequently do you use the following as source of dairy information?

[1]Ac: acre(s).
[2]DMI: dry matter intake.
[3]DHIA: Dairy Herd Improvement Association.

Table S2.2. Producer demographic relationship to milk production – Stepwise model results. Results are considered significant at a $P \leq 0.10$.

Variable	Coefficient	Standard Error	*P*-Value	95% Confidence Interval	
Yr born	5.31	25.96	0.84	-46.09	56.71
Male	-953.07	692.00	0.17	-2,323.42	417.29
Plain community	75.25	452.77	0.87	-821.35	971.85
Yr farming	6.58	19.83	0.74	-32.69	45.85
Succession plan	-372.21	550.42	0.50	-1,462.19	717.76
Dairy income	6.46	10.46	0.54	-14.25	27.18

Table S2.3. On-farm decision making priorities of plain and non-plain organic, grassfed (OGF) dairy producers. Factors were ranked on a Likert scale of 1 to 9 with 1 being the most influential and 9 being the least influential. Results are considered significant at a $P \leq 0.05$.

Priority	Mean	Standard Deviation	*P*-value
Autonomy	5.77	2.23	0.03
Economics	5.37	2.76	0.03
Environment	5.77	2.36	0.04
Family, livelihood, and well-being	5.61	3.13	0.02
Future generations	5.82	2.71	0.16
Labor	5.78	2.18	0.87
Policies and regulation	6.09	2.44	0.04
Society and community well-being	6.41	2.14	0.75
Time management	5.63	2.22	0.14

Table S2.4. Policy relationship to milk production – Stepwise model results. Results are considered significant at a $P \leq 0.10$.

Variable	Coefficient	Standard Error	*P*-Value	95% Confidence Interval	
Yr organic	32.16	33.34	0.34	-33.85	98.16
Yr grassfed	-42.65	58.95	0.47	-159.34	74.05
CRP[1] awareness	-174.82	268.87	0.52	-707.02	357.38
CSP[2] awareness	-53.79	239.72	0.82	-528.30	420.71
FCIP[3] awareness	-33.26	314.11	0.92	-655.02	588.50
EQIP[4] awareness	-68.55	217.29	0.75	-498.67	361.57
MPPD[5] awareness	150.64	208.28	0.47	-261.64	562.91

[1]CRP: Conservation Reserve Program.
[2]CSP: Conservation Steward's Program.
[3]FCIP: Federal Crop Insurance Program.
[4]EQIP: Environmental Quality Incentives Program.
[5]MPPD: Margin Protection Program for Dairy.

Table S2.5. Farm management relationship to milk production – Stepwise model results. Results are considered significant at a $P \leq 0.10$.

Variable	Coefficient	Standard Error	*P*-Value	95% Confidence Interval	
Inputs	228.47	515.24	0.66	-792.42	1,249.35
Herd record service	43.27	491.09	0.93	-929.78	1,016.32
Milk test	-129.79	547.26	0.81	-1,214.12	954.53
Twice a d	1,577.11	566.55	0.01	454.56	2,699.66
Energy supplements	964.74	416.03	0.02	140.43	1,789.05
Computer use	-225.55	221.68	0.31	-664.78	213.68
Email use	42.79	251.52	0.87	-455.57	541.15
Internet use	289.72	306.43	0.35	-317.42	896.87

Table S2.6. Herd characteristics and management relationship to milk production – Stepwise model results. Results are considered significant at a $P \leq 0.10$.

Variable	Coefficient	Standard Error	*P*-Value	95% Confidence Interval	
Crossbred	1,244.98	878.31	0.16	-495.46	2,985.41
Holstein	3,357.36	946.58	0.001	1,481.66	5,233.07
Jersey	784.08	970.00	0.42	-1,138.05	2,706.20
Cow age	139.13	158.24	0.38	-174.44	452.69
Culling rate	13.14	26.83	0.63	-40.03	66.31
Number of milk cows	3.31	7.97	0.68	-12.49	19.11
Milk fed to calves	89.52	146.09	0.54	-199.96	378.99

Table S2.7. Relationship of culling with farmer demographics. Data was analyzed using Pearson's correlation coefficients. Values were considered significant at a $P \leq 0.05$.

Farmer Demographic	r value	*P*-Value
Age	0.03	0.70
Yr farming	0.13	0.09
Number of milking cows	0.08	0.31
Total ha[1] managed	-0.02	0.79

[1]Ha: hectare(s)

Table S2.8. Forage and land characteristics relationship to milk production – Stepwise model results. Results are considered significant at a $P \leq 0.10$.

Variable	Coefficient	Standard Error	*P*-Value	95% Confidence Interval	
Ha[1] owned	1.59	4.92	0.75	-8.50	11.68
Ha rented	-4.26	5.21	0.42	-14.95	6.44
Buy forage	950.49	787.04	0.24	-664.38	2,565.36
Rotational grazing	-1,374.72	895.29	0.14	-3,211.69	462.26
Forage production	-1.06	5.71	0.85	-12.78	10.65
Annual forage ha yr^{-1}	3.55	20.06	0.86	-37.62	44.71
Grazing season	-31.11	13.14	0.03	-58.07	-4.15
DMI[2]	-226.82	374.74	0.55	-995.72	542.09

[1]Ha: hectare(s).
[2]DMI: dry matter intake.

Table S2.9. Producer knowledge relationship to milk production – Stepwise model results. Results are considered significant at a $P \leq 0.10$.

Variable	Coefficient	Standard Error	*P*-Value	95% Confidence Interval	
Body condition scoring	-286.39	311.83	0.36	-903.78	331.01
Energy requirements	-202.88	439.32	0.65	-1,072.71	666.94
Farm record keeping	-201.16	313.71	0.52	-822.29	419.97
Grazing management	-734.08	304.35	0.02	-1,336.68	-131.49
Growing higher energy forages	215.29	404.07	0.59	-584.73	1,015.32
Improving forage quality	503.59	453.86	0.27	-395.03	1,402.21
Forage yields	-265.14	338.23	0.44	-934.81	404.52
Reproductive performance	534.87	279.43	0.06	-18.38	1,088.12
Forage test results	132.60	301.83	0.66	-464.99	730.19
MUN[1]	404.14	312.34	0.19	-214.26	1,022.54
Soil test results	3.80	283.34	0.99	-557.19	564.79
Maximize forage DMI[2]	81.61	334.84	0.81	-581.35	744.58
Forage production costs	184.51	326.65	0.57	-462.23	831.24

[1]MUN: milk urea nitrogen.
[2]DMI: dry matter intake.

Table S2.10. Producer responses to the question "How do you feel you are doing with…?" regarding various management parameters. Responses were based on a scale of very unsatisfied to very satisfied. Data was analyzed using Pearson's correlation coefficients. Values were considered significant at a $P \leq 0.05$.

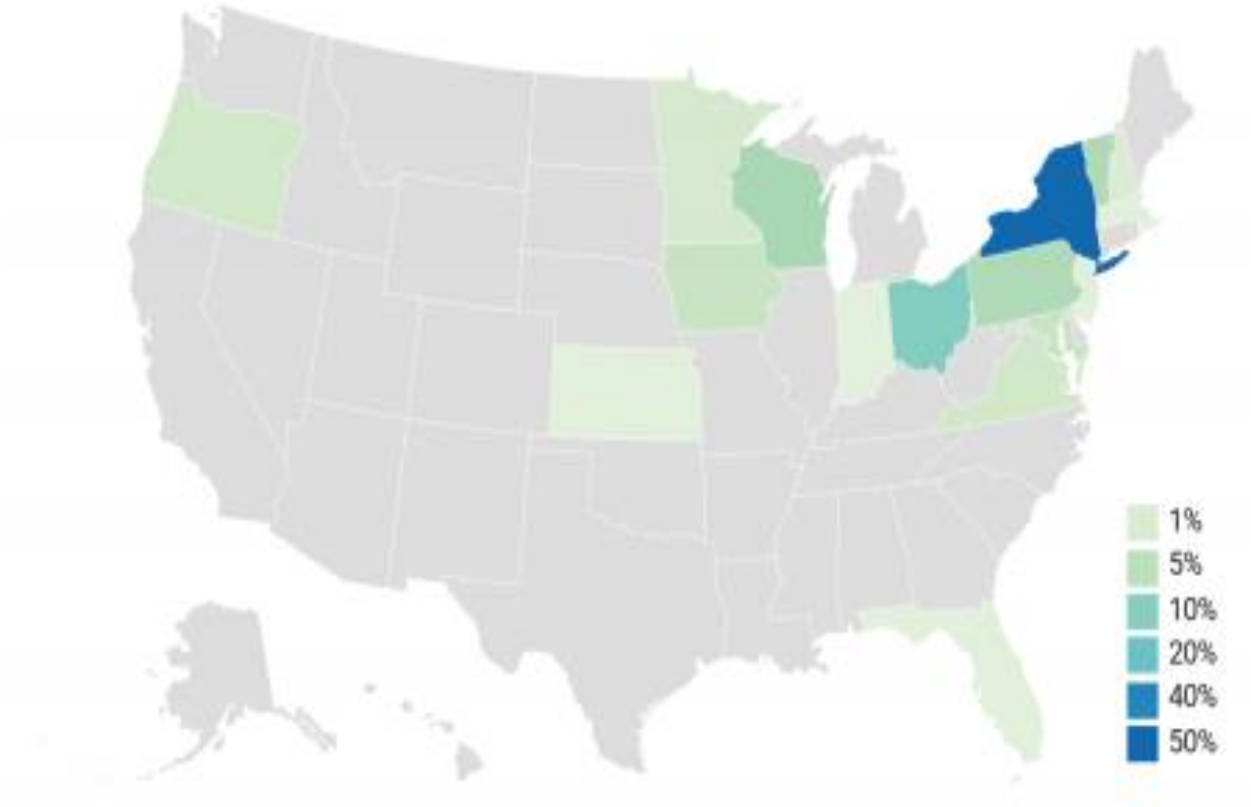
Management parameter	**r value**	***P*-Value**
Pasture quality and yield	-0.19	0.01
Stored forage yield	-0.25	0.003
Stored forage quality	-0.24	0.003
Soil fertility / health	-0.13	0.10
Cow body condition	-0.13	0.10
Milk production	-0.22	0.005
Reproduction and calving	-0.16	0.04
Herd health	-0.20	0.01
Quality of young stock	-0.10	0.20
Farm income	-0.11	0.19

Table S2.11. Farmer information scale relationship to milk production – Stepwise model results. Results are considered significant at a $P \leq 0.10$.

Variable	Coefficient	Standard Error	*P*-Value	95% Confidence Interval	
Information scale	1,218.97	309.17	0.00	608.09	1,829.86

2.12. Supplementary Figures

Figure S2.1. Representation of states from producer respondents.



CHAPTER 3: EVALUATION OF FORAGE CUTTING AND ORGANIC APPLE CIDER VINEGAR SUPPLEMENTATION ON FERMENTATION METRICS USING RUMEN *IN VITRO* BATCH CULTURE

3.1. Abstract

Two *in vitro* batch culture studies were conducted to 1) compare fermentation profiles between forages harvested at two different cuttings of legume and perennial grass mixtures commonly utilized in the Northeastern (NE) United States (U.S.) and 2) to determine the effects of organic apple cider vinegar (ACV) supplementation on these fermentation metrics. Pure *Trifolium pratense* (red clover) and *Dactylis glomerata* (orchardgrass) swards were harvested, frozen at -20ºC, and ground through a 2-mm screen. In experiment 1 (PLT EXP), test tubes were supplemented with 50:50 (DM-basis) red clover: orchardgrass mixture harvested at first cut (CUT 1) or a 50:50 red clover: orchardgrass mixture harvested at second cut (CUT 2). Tubes were inoculated with a 3:1 buffer: rumen fluid mixture and incubated at 39ºC under anerobic conditions for 48 h. Experiment 2 (SUPP EXP) was conducted in a similar manner with all tubes receiving 50:50 red clover: orchardgrass substrate harvested at first cut and half of the tubes being supplemented with 1.5% ACV. Both experiments were repeated for three periods. Samples were collected at 0, 1, 2, 4, 6, 8, 12, 18, 24, and 48 h of incubation. Samples were analyzed for water soluble carbohydrates (WSC), volatile fatty acids (VFA), methane (CH_4), and ammonia nitrogen (NH_3-N) concentrations, as well as pH, and *in vitro* dry matter disappearance (IVDMD). Methane production was not affected by supplementation; however, tubes that received the CUT 1 treatment produced more CH_4 than those that received the CUT 2 treatment. Additionally, tubes that received the CUT 1 treatment had

higher concentrations of effluent WSC and higher IVDMD disappearance, and lower concentrations of NH_3-N compared with tubes that received the CUT 2 treatment. Furthermore, pH was higher for tubes supplemented with CUT 1. Finally, proportions and molar concentrations of VFA did not differ between treatments in the first experiment (PLT EXP) or the second experiment (SUPP EXP). Fermentation parameters were not affected by ACV supplementation. This indicates that forage quality at harvest was the main driver of fermentation metrics, and that 1.5% ACV supplementation does not influence fermentation.

Key Words: high forage diet, orchardgrass, red clover, forage quality, methane

3.2. Introduction

Organic grassfed (**OGF**) dairy systems are reliant on forage quality which is negatively correlated with forage maturity (Cleale and Bull, 1986; Allen, 1996) and timing of cutting (Sheaffer et al., 1988). To support milk production in high forage and OGF systems, critical assessment of forage quality, or use of supplementation strategies to bolster nutrient content of the total diet, is often needed. Strategic grazing or harvesting based on plant maturity and cutting frequency are practical on-farm approaches to increase nutritive value of the diet. Harvest management, especially in terms of cutting intensity, has been a previous area of research focus as forage quality and nutrient content predictability based on harvest number is highly debated. For example, Brink and Marten (1989) evaluated alfalfa quality and nutrient yield harvested during three-cut and four-cut systems. Forage quality and persistence was reduced when cutting interval was decreased, particularly by the final harvest. Bernard and Tao (2015) described the impacts of harvest number of corn and a brachytic dwarf brownrib midrib forage sorghum on production response of lactating Holstein cows. Corn was planted twice: once in March and once in July. Both forages were harvested twice and ensiled. There were no differences in cow dry matter intake (**DMI**) nor milk yield and components. However, milk fat percentage and milk urea nitrogen (**MUN**) values were lower from cows receiving corn silage harvested once in July (summer corn silage) and once in November (fall corn silage) as compared with cows fed the sorghum diets at either harvest. This suggests that forage profile, or perhaps that the time of year in which each forage type was harvested, is of great importance.

Despite grazing and harvesting strategies, additional nutritional support is

sometimes needed to meet the energy requirements of the cow and production targets of the farm. In terms of supplementation strategies, use of an energy-dense supplement is often the target (Bargo et al., 2003). Fieser and Vanzant (2004) investigated the interactions between energetic feed supplements, including pelleted soybean hulls and coarse cracked corn, and tall fescue hay maturity in beef steers. Based on their results, supplementation x forage maturity did not impact digestible organic matter (**OM**) intake. However, the interaction of these variables did affect OM and neutral detergent fiber (**NDF**) digestibilities as well as nitrogen (**N**) retention. Supplementation with either aforementioned supplement increased digestibility whereas diets consisting of forages of greater maturity resulted in greater N retention. Using continuous culture, Soder et al. (2010) evaluated the impacts of corn meal, molasses, or corn meal x molasses supplementation on rumen fermentation metrics of orchardgrass (*Dactylis glomerata*) diets. In this study, the treatment did not impact apparent digestibility or volatile fatty acids (**VFA**), including both proportions and ratios. However, supplementation with molasses did increase mean ruminal pH.

Several studies have documented the effects of forage maturity, forage quality, and energetic supplements on dry matter intake (**DMI**) and digestibility (Huhtanen et al., 2007; Soder et al., 2009); however, many farms may be more restricted in terms of supplement selection due to organic or OGF certification. There is a dearth of information regarding the benefits of supplementing forage diets with alternative, or non-traditional, supplements. Alternative supplements and feedstuffs are those that are used to decrease dependency on feeds that are more commonly utilized on traditional dairy operations (LeValley, 2003). Although not as popular as molasses and kelp meal, certified grassfed producers throughout

the U.S. are utilizing products, such as apple cider vinegar (**ACV**), as energetic supplements (Snider et al., 2021). Dietary supplementation with the acetate derivative, acetic acid, is thought to increase fiber solubility, improve milk quality (Urrutia et al., 2019), improve animal energetic efficiency (Daniel et al., 2013), and raise acetate proportions in the rumen (Russell, 1998; Daniel et al., 2013). As ACV contains 5% acetic acid, it is thought to have similar impacts on animal performance as well as potentially suppressing methanogenesis (Kessel and Russell, 1996). A study by Smith et al. (2013) investigated the effects of three different ACV concentrations on fiber digestion and feed efficiency in non-pregnant, nonlactating Katahdin ewes. Inclusion of ACV did not affect DMI nor acid detergent fiber (**ADF**)/ NDF digestibility, indicating that it was not effective at enhancing fiber digestibility. However, Smith et al. (2013) did not analyze information related to other rumen or animal performance metrics but rather based results exclusively on result of fecal analysis.

When considering the impact of forage quality and use of alternatives in ruminant diets, it appears that these could each provide some benefit. It is hypothesized that a legume-grass mixture harvested at first cutting before heading will have a greater, more digestible carbohydrate fraction compared with a legume-grass mixture harvested at second cut as forages harvested at second cutting are more likely to have greater amounts of undigestible components, such as lignin. Additionally, it is hypothesized that organic ACV supplemented at 1.5% of DMI will not increase the energetic content of the diet or change the fermentative profile of rumen fluid *in vitro*. The aims of the present study were then to 1) evaluate the fermentation profile and *in vitro* energetic profile of two forage mixtures (orchardgrass and red clover) commonly grown in northeast pasture and hay

systems at different cuttings and 2) determine the effects of ACV supplementation on VFA production and other fermentation metrics including CH_4 and NH_3-N production *in vitro*.

3.3. Materials and Methods

3.3.1. Harvesting, Forage Collection, and Processing

Pure red clover and orchardgrass swards were planted at Borderview Research Farm (Alburgh, VT) in the fall of 2017. Monocultures were planted as part of larger small plot trials. Both orchardgrass and red clover were planted using a cone seeder (Great Plains; Salina, KS). Cultivars used in this study are listed in Supplementary Materials, Table S3.1. Orchardgrass was planted on a Benson rocky silt loam over shaly limestone. Red clover was grown on Covington silty clay loam soil. Both crops received organic approved fertility applications based on soil test recommendations. Organic nitrogen sources were applied to the grass swards at a rate of 50 lbs per acre following each harvest. Orchardgrass was harvested four times: May, June, August, and September of 2019. Red clover was harvested three times, once in June 2019, a second cut in July, and again in August 2019. Both orchardgrass and red clover were cut from the same plots at each harvest. Orchardgrass and red clover swards were harvested in the morning using a forage harvester (Carter; 3-in-wide swath; Brookston, IN), set to a 4-in stubble height. Orchardgrass and red clover from the first harvest were combined to create the first cutting (**CUT 1**) treatment. Similarly, orchardgrass and red clover from the second cutting were combined to create the second cutting (**CUT 2**) treatment.

After each harvest, or cutting interval, orchardgrass and red clover cuts were placed on individual plastic tarps for each specie. Forages were placed in plastic bags; bags were then squeezed to remove as much air as possible. Sample bags were then zip tied and

weighed within 30 min of harvest. All forages were transported within 90 min of harvest and frozen within 2.5 h of harvest at -20ºC in a commercial warehouse (Vermont Commercial Warehouse; Williston, VT) until ready for processing. Samples were processed and ground at the UVM Horticulture and Research Education Center (South Burlington, VT). Residual weeds were botanically separated from forage swards before grinding. Forages were flash frozen using liquid N and dry ice and freeze-ground through a mill using a cooled 2-mm mesh screen (Wiley Mill; Thompson Scientific; Philadelphia, PA). Orchardgrass and red clover samples, as well as the different cuttings within species, were each ground separately, with the mill and mesh screen cleaned in between sample grinding. Ground subsamples were collected for dry matter (**DM**) and nutrient content analysis at a commercial laboratory as outlined below (DairyOne Laboratories; Ithaca, NY; Table 3.1).

3.3.2. Forage Nutrient Analysis

Similar to Dillard et al. (2017), samples of ground orchardgrass and red clover at both cuttings were analyzed by wet chemistry (DairyOne Laboratories; Ithaca, NY) according to the following methods: DM was dried in an oven for two h at 135ºC (method 930.15; AOAC International, 2006). Crude protein (**CP**) was analyzed following method 990.03 (AOAC International, 2006). Sample NDF organic matter (**aNDFom**) (ANKOM Technology Method 15, 2020) was analyzed with heat stable α-amylase and sodium sulfite. Samples were then ashed to remove inorganic materials. Acid detergent fiber (ANKOM Technology Method 14, 2020) was analyzed after digestion in ADF solution and an acetone soak. Water soluble carbohydrates (**WSC**) and ethanol soluble carbohydrates (**ESC**) were analyzed using a Thermo Scientific Genesys 10S Vis Spectrophotometer as previously

outlined (Application Note Number 319; YSI Inc. Life Sciences, Yellow Springs, OH). Minerals (Ca, P, Mg, K, Na; Thermo iCAP 6300 Inductively Coupled Plasma spectrometer), and ash (method 942.05; AOAC International, 2006) were also measured by DairyOne Laboratories (Ithaca, NY) using established methods. The non-fiber carbohydrate (**NFC**) content was calculated as described by Dillard et al. (2017) and O'Reilly et al. (2021): NFC (%) = 100% - (CP (%) + aNDFom (%) + ether extract (%) + ash (%)).

3.3.3. Dietary Treatments

Dry matter analysis was performed using unprocessed forage samples to determine inclusion rates of orchardgrass and red clover for experimental diets. Four samples of unprocessed orchardgrass and red clover from both first and second cuttings were weighed and dried at 65ºC for 48 h in a forced air oven (VWR Scientific Products; Radnor, PA). Dry matter values were averaged and treatments were hand mixed to create two treatments: 1) 50% red clover: 50% orchardgrass collected from the first cutting (CUT 1) and 2) 50% red clover: 50% orchardgrass collected from the second cutting (CUT 2) on a DM-basis. Mixed forages were bagged and stored at -20ºC away from light until ready for use.

3.3.4. Rumen Fluid Collection

All experimental procedures involving animal handling and sampling were approved by the University of Vermont Institutional Animal Care and Use Committee (Protocol #16-029). Rumen fluid was collected in the same manner for both experiment 1 (**PLT EXP**) and experiment 2 (**SUPP EXP**). Briefly, rumen fluid was collected from three ruminally fistulated lactating Holstein cows being fed a nutritionally balanced total mixed ration (**TMR**). Animals were housed at the Paul R. Miller Research and Educational Center

(South Burlington, VT) and had ad libitum access to feed and water. Samples of TMR were collected before rumen fluid collection, pooled, frozen at -20ºC, and sent for commercial wet chemistry analysis (DairyOne Laboratories; Ithaca, NY; Supplementary Materials, Table S3.2). Approximately 100 mL of rumen fluid was collected from each cow two h after morning feeding. Similar to the methods of Meale et al. (2012), digesta was hand-collected from the ventral, central, and dorsal sacs of the rumen and squeezed through four layers of cheesecloth to remove larger feed particles. Rumen fluid was then pooled into a prewarmed container maintained at 39ºC and initial pH was measured. Rumen fluid was then transported to the laboratory and further strained through four layers of cheesecloth to separate solid matter from rumen liquor. Strained rumen fluid was maintained at 39ºC in a heated water bath prior to inoculation.

3.3.5. Experimental Design and Batch Culture System Operation

Two experiments were completed. The PLT EXP consisted of a short-term (48 h) *in vitro* ruminal batch culture with two forage mixtures collected from two cuttings as previously described: 1) 50:50 (DM-basis) red clover: orchardgrass from first cutting (CUT 1) or 2) 50:50 (DM-basis) red clover: orchardgrass from second cutting (CUT 2). In the SUPP EXP, the forage mixture with the greatest energetic profile, CUT 1, was supplemented with organic apple cider vinegar (**ACV+**; 1.5% ACV) or received no supplement (**ACV-**). Apple cider vinegar (pH 3.07; Oakton 110; Cole-Parmer; Vernon Hills, IL) was obtained from a local vendor and was certified as organic (Baystate Organic Certifiers; Bloomfield, NJ). *In vitro* incubations were conducted using a completely randomized design in each experiment. Each treatment was repeated in duplicate for three periods (Supplementary Materials, Figure S3.1). Experiments were conducted in 15-mL

test tubes (Fisherbrand; 1495925C; Pittsburgh, PA) fitted with Teflon-lined caps to maintain an anaerobic environment; however, each cap contained a pin-sized hole to relieve gas pressure. Each tube (n = 2 per treatment per period) was inoculated with 1.0 g DM of assigned forage the d before incubation and stored at 4ºC until use. At the beginning of the trial, tubes were placed in an incubator with heating blocks maintained at 39 ± 0.5ºC (Techne Dri-Block; DB-3; Minneapolis, MN). Test tubes were attached to a rotary shaker platform at 120 oscillations/ min (Junior Orbit Shaker; Lab-Line Instruments Inc.; Melrose Park, IL). Tubes with substrate were pre-warmed to 39ºC in the incubator for one h before addition of a 3:1 *in vitro* rumen buffer solution: rumen fluid mixture that contained 0.5 mL cysteine sulfide reducing agent (Meale et al., 2012; Wang et al., 2000). Tubes were inoculated within 20 min of collection. Each tube was continuously gassed with CO_2 through a snugly fit needle. Caps and needles were wrapped with parafilm to prevent gas escape. Tubes were capped and incubated over a period of 48 h with 10 collection timepoints: h 0, 1, 2, 4, 6, 8, 12, 18, 24, and 48. Tubes identified as h 0 were immediately placed on ice at the start of the experiment. All remaining tubes were immediately placed on ice to stop fermentation at the specified timepoint as previously described (Meale et al., 2012).

Based on the results of the PLT EXP, the CUT 1 treatment had the greatest energetic profile and was used as the substrate for the SUPP EXP. The SUPP EXP was conducted in a similar manner to the PLT EXP. In the SUPP EXP, all tubes (40 per period) received the CUT 1 forage mixture. Half of the tubes were supplemented with ACV (ACV+; 125 µL or 1.5% v/v) at the beginning of the SUPP EXP after tubes were inoculated with rumen fluid, while the remaining tubes were not. Dosage volume was calculated based on current

supplementation practices utilized on-farm. Each treatment was duplicated across three periods. All measurements and analytes were measured as above in the PLT EXP.

3.3.6. Sample Collection and Measurements

Methane and pH Collection. Methane measurements were collected from the headspace of the tube immediately after tubes were opened. Measurement of CH_4 were collected in duplicate using a handheld gas analyzer probe (Sewerin Multitec 545; Gütersloh, Germany). Methane concentration (mg dL^{-1}) was calculated using the following equation as previously described (Baker, 2021):

$$\mathrm{CH_4\ (mg\ dL^{-1})} = \frac{(\%\ \mathrm{CH_4}\ \mathrm{Volume\ x\ 554})}{10}.$$

Tube contents were then transferred to 50 mL conical tubes to be subsampled for additional analysis. Sample pH was immediately collected in duplicate after transfer using a handheld pH probe (Oakton 110; Cole-Parmer; Vernon Hills, IL).

Water Soluble Carbohydrate Analysis. Subsamples of fermentation media were collected for WSC analysis. Samples were stored at -20ºC until processing. Water soluble carbohydrate content was determined spectrophotometrically utilizing an anthrone - sulfuric acid assay adapted to a 96-well plate as described by Laurentin and Edwards (2003). Briefly, samples were thawed and incubated at 4ºC for 15 min. An anthrone-sulfuric acid reagent was added to each sample and plates were incubated for three min at 92ºC. After cooling to room temperature, plates were incubated for 15 min at 45ºC. Absorbance was read at 630 nm (BioTek Synergy HT; Winooski, VT). Samples were analyzed in triplicate.

Determination of Ammonia Nitrogen Concentration. Ammonia N concentration was determined according to the methods of Chaney and Marbach (1962) and modified for

use in a 96-well plate. Briefly, a sub-sample (1.0 mL) of fermentation media was collected from each tube and transferred to a micro-centrifuge tube containing 150 µl of 25% *m*-phosphoric acid (Sigma-Aldrich Co.; St. Louis, MO). Samples were stored at -20ºC until processing. Upon processing, samples were thawed on ice and centrifuged at 11,000 x *g* for 20 min at 4 ºC. Supernatant was transferred to a clean tube and the pellet was discarded. Samples were incubated with a phenol – hypochlorite reagent at 37ºC for 10 min in a heated bead bath. Plates were colorimetrically analyzed at an absorbance wavelength of 550 nm (BioTek Synergy HT; Winooski, VT).

Determination of Volatile Fatty Acid Concentration. At the end of the designated incubation length, 1.5-mL subsample was collected from the culture tube and transferred to a two mL microcentrifuge tube for VFA analysis. Samples were centrifuged at 14,000 x *g* for 10 min at 4°C to precipitate a pellet. The pellet was discarded and the supernatant was transferred into a clean two mL microcentrifuge tube. Samples were pooled by treatment (n = 6 per treatment) and stored at -20ºC until processing at the William H. Miner Agricultural Research Institute (Chazy, NY). Samples were analyzed as outlined by Miller et al. (2021). Briefly, individual VFA and total molar proportions were determined through gas chromatography using a Varian CP-3800 gas chromatograph (Varian, Inc.; Palo Alto, CA). The gas chromatograph was fit with a flame-ionization detector and 80/120 Carbopack B-DA/4% Carbowax 20M column (Supelco, Inc.; Bellefonte, PA).

In vitro Dry Matter Disappearance. Following pH, CH_4, and fermentation media collection, contents of 50-mL tubes were frozen at -20ºC until processing. Sample *in vitro* dry matter disappearance (**IVDMD**) was analyzed according to the methods of Meale et al. (2012). Briefly, tube contents were transferred to a new pre-weighed 50-mL conical

tube. Samples were pooled by period within treatment across time points for a total of 6 tubes per treatment (24 tubes total). Tube contents were rinsed with 15 mL distilled water and centrifuged twice for 10 min at 500 x *g* at 4ºC. The supernatant was discarded and the pellet retained. The pellet was weighed and dried for 48 h at 65 ºC. After drying, the residue was weighed again to estimate IVDMD using the following defined equation (Mertens, 1993):

$$\text{in vitro dry matter disappearance} = (1 - \left(\frac{\text{residue weight (g)}}{\text{sample dry matter)}}\right) \text{x } 100.$$

3.3.7. Statistical Analysis

Data were analyzed as a completely randomized design using the PROC GLIMMIX procedure of SAS 9.4 (SAS Institute Inc., Carey, NC) with the following model:

$$Y_{ijk} = \mu + T_i + P_j + TP_{ij} + e_{ijk},$$

where Y_{ijk} = observations for dependent variables, μ = overall mean, T_i = mean of the *i*th treatment, P_j = the mean effect of the *j*th treatment, TP_{ij} = mean *ij*th effect of the interaction, and e_{ijki} = residual error. Methane and pH samples were additionally analyzed using the PROC MIXED procedure with repeated measures using the model:

$$Y_{ijk} = \mu + T_i + H_j + TH_{ij} + e_{ijk},$$

Y_{ijk} = observations for dependent variables, μ = overall mean, T_i = mean of the *i*th treatment, H_j = mean effect of the *j*th h, TH_{ij} = mean *ij*th effect of the interaction, and e_{ijk} = residual error.

Contrasts comparing fermentation metrics from CUT 1 to CUT 2 were performed as were contrasts comparing fermentation metrics of ACV+ to ACV-. Results were reported with least squares means (± SEM) and were considered significant if $P \leq 0.05$.

3.4. Results

3.4.1. Forage Nutrients

Chemical compositions of individual forages and forage treatments are listed in Table 3.1. Organic matter was similar between the CUT 1 (90.2%) and CUT 2 (89.3%) treatments as were CP, aNDFom, ADF, and WSC. The starch content of the CUT 1 treatment was higher than that of the CUT 2 treatment (2.10% v. 0.85%).

3.4.2. Methane and pH

PLT EXP. A treatment effect was observed (Table 3.2) with the CUT 1 treatment producing more CH_4 than the CUT 2 treatment ($P < 0.0001$; 4.98 mg dL^{-1} v. 2.87 mg dL^{-1}; Figure 3.1.a). The CUT 1 treatment produced more CH_4 than the CUT 2 treatment at 4 h (6.46 mg dL^{-1} v. 2.31 mg dL^{-1}) and 6 h (6.93 mg dL^{-1} v. 2.31 mg dL^{-1}) post-incubation. The CH_4 concentration was not affected by time in the CUT 2 treatment; however, CH_4 concentrations were greater at h 6 as compared to h 1 and 24 for the CUT 1 treatment. The mean pH of the CUT 1 treatment was greater than the mean pH of the CUT 2 treatments (6.72 v. 6.60; $P = 0.04$). Further, pH was not affected by time except at h 0 for both CUT 1 and CUT 2; pH at h 0 was lower than all timepoints within each treatment. No period or treatment x period effects were observed.

SUPP EXP. There were no treatment differences in CH_4 between the ACV+ and ACV- treatment (2.51 mg dL^{-1} v. 3.03 mg dL^{-1}; $P = 0.26$). However, there was a period ($P = 0.05$) and treatment x period ($P = 0.05$) effect. Treatment CH_4 concentrations were only different between ACV+ and ACV- at h 1 ($P = 0.003$; 0.46 mg dL^{-1} v. 4.62 mg dL^{-1}; Figure 3.1.b). More CH_4 was produced at h 1 than h 2 and h 4 for tubes that did not receive ACV. Tubes that were supplemented with ACV produced greater amounts of CH_4 from h 12 – h

24 as compared to h 1 and h 2. Mean pH did not differ between ACV+ (pH: 6.59) and ACV- (pH: 6.58). Similar to the PLT EXP, there was no effect of time except at h 0 for both ACV+ and ACV-, with pH being lower at h 0. Additionally, no period or treatment x period effects were observed.

3.4.3. Water Soluble Carbohydrate Concentration

PLT EXP. Treatment ($P < 0.0001$), period ($P < 0.0001$), and treatment x period ($P = 0.0006$) effects impacted WSC concentration, with the CUT 1 treatment containing greater amounts of WSC (2.39 mg mL^{-1}) as compared to the CUT 2 treatment (1.56 mg mL^{-1}; Table 3.2). Greater amounts of WSC were present in period 1 (2.48 mg mL^{-1}) as compared to period 2 (1.39 mg mL^{-1}) and period 3 (2.07 mg mL^{-1}).

SUPP EXP. Similar to the results of the PLT EXP, treatment, period, and treatment x period interaction effects were identified ($P < 0.0001$). Tubes supplemented with ACV had a greater concentration of WSC (2.67 mg mL^{-1}) as compared with the ACV- tubes (2.31 mg mL^{-1}). Water soluble carbohydrate concentrations were greater in period 3 (2.79 mg mL^{-1}) compared with periods 1 (2.47 mg mL^{-1}) and 2 (2.19 mg mL^{-1}).

3.4.4. Ammonia Nitrogen Concentration

PLT EXP. Treatment ($P = 0.007$), period ($P = 0.009$), and treatment x period interactions ($P = 0.0003$) did impact NH_3-N concentration. The CUT 2 treatment tubes had a higher NH_3-N concentration than the CUT 1 treatment (66.9 mg dL^{-1} v. 62.9 mg dL^{-1}; Table 3.2). Further, period 2 tubes had a higher average NH_3-N concentration (67.8 mg dL^{-1}) than period 1 tubes (62.2 mg dL^{-1}). The concentration of NH_3-N from tubes incubated in period 3 (64.8 mg dL^{-1}) did not differ from periods 1 or 2.

SUPP EXP. There was no treatment (P = 0.09) or treatment x period (P = 0.07) effects due to ACV addition. The concentration of NH_3-N was not different across the ACV+ and ACV- tubes (59.7 mg dL^{-1} v. 57.9 mg dL^{-1}). A period effect (P = 0.003) was observed. The concentration of NH_3-N produced in period 3 (61.6 mg dL^{-1}) was greater than that of period 1 (58.3 mg dL^{-1}) and period 2 (56.7 mg dL^{-1}).

3.4.5. Volatile Fatty Acid Concentration

PLT EXP. Treatment effects were not observed for total VFA concentrations (P = 0.86), acetate: propionate ratios (P = 0.38), or acetate + butyrate: propionate ratios (P = 0.29; Table 3.3). Furthermore, no treatment effects were present for individual VFA molar concentrations between CUT 1 and CUT 2. Period effects were observed for individual VFA concentrations including isobutryate (P = 0.02), butyrate (P = 0.04), isovalerate (P = 0.003) and valerate (P = 0.04). No treatment x period effect was observed.

SUPP EXP. Similar to the PLT EXP, no treatment effects were observed for total VFA concentrations (P = 0.91), acetate: propionate ratios (P = 0.15), or acetate + butyrate: propionate ratios (P = 0.14) between ACV+ and ACV-. No impact of ACV supplementation on individual VFA concentrations (Table 3.3) was observed. Further, no period effects or treatment x period interaction effects affected molar proportions or VFA concentrations.

3.4.6. *In vitro* Dry Matter Determination

PLT EXP. Treatment (P = 0.02) and treatment x period (P = 0.003) effects were observed in PLT EXP 1. The DM disappearance of the CUT 1 treatment was greater than the DM disappearance of the CUT 2 treatment (81.7% v. 77.3%).

SUPP EXP. Supplementation with ACV did not affect IVDMD (P = 0.59; Table 3.2). Additionally, there were no differences across treatments due to period (P = 0.84) nor was there an effect of treatment x period (P = 0.63)

3.5. Discussion

Although ACV use in cattle diets is becoming more commonplace on OGF dairies, there is limited research on the impacts of ACV and its effects on rumen fermentation metrics. Nutritional research that is available is either conducted using *in vitro* systems that use different types of vinegar (O'Reilly et al., 2021) or has been studied in non-ruminant animals (Allahdo et al., 2018; Choi et al., 2009). To our knowledge, this is one of the first experiments to characterize the effects of dietary ACV supplementation on rumen fermentation metrics. Furthermore, it is one of the first studies to evaluate the effects of ACV and its potential interactions with forage quality. Additionally, it should be realized that if ACV is used as a supplement, there is the potential for supplement loss due to volatilization (Mitloehner et al., 2009) or microbial oxidation (Spoelstra et al., 1988) during diet mixing and feeding (Daniel et al., 2013), potentially leading to under-supplementation.

3.5.1. Effluent pH

Unlike the SUPP EXP, pH values in the PLT EXP differed between feed substrate with a higher mean pH being reported for the CUT 1 treatment than the CUT 2 treatment. The pH values for both the PLT EXP and SUPP EXP were greater than those reported by O'Reilly et al. (2021) no matter the concentration (0.25%, 0.5%, 1%, or 2%) of wood vinegar utilized in their study. However, O'Reilly et al. (2021) only collected pH values for a single time point at the end of the experiment. Greater pH values may be related to increased acetate content, which was to be expected from the CUT 1 treatment; however,

acetate concentration did not differ between the CUT 1 and CUT 2 treatments in this study. In the PLT EXP, the aNDFom content of the CUT 1 treatment was numerically less than the CUT 2 treatment and also contained greater amounts of NSC, which should reduce pH. Our observation that pH was greater in the CUT 1 treatment could be due to the design of the *in vitro* system as VFA contributing to higher pH would not be absorbed. O'Reilly et al. (2021) indicated that supplementation with 1% and 2% wood vinegar decreased effluent pH for pasture-based diets and 2% wood vinegar supplementation decreased pH for a vegetable mixed-ration substrate.

3.5.2. Methane Concentration

In the PLT EXP, the observation that the CUT 1 treatment resulted in a higher CH_4 concentration could be due to of the high concentrations of CH_4 measured in samples collected at h 4 and h 6; CH_4 output did not differ between treatments at any other timepoint. The CUT 1 diet contained similar amounts of nutrients (OM, NSC, WSC) and structural carbohydrates such as aNDFom and ADF (as a % of DM). Although digestibility was not measured in this study, it could be speculated that the CUT 1 diet was likely more digestible as a result of higher starch content (Philippeau et al., 2017), with the potential for faster nutrient fermentation than the CUT 2 treatment. However, energetic substrates of the CUT 1 diet are potentially not being used as efficiently, with this inefficiency occurring a few h after initial inoculation. This may be related to cutting time or forage maturity. Typically, a more digestible diet would lead to a reduction in the amount of CH_4 produced. When accounting for forage maturity, in a grazing study by Boadi et al. (2002), steers grazing on vegetative pastures produced 29% – 45% less CH_4 than steers that grazed on more mature pastures, indicating that pasture quality and maturity have a large impact

on CH_4. Alternatively, Molano and Clark (2008) compared CH_4 emissions of lambs fed ryegrass harvested at the reproductive phase (seeding and flowering) and the vegetative stage. Molano and Clark (2008) found no differences in CH_4 emissions per unit of DMI based on DMI or diet quality. Additionally, the concentration of CP in the diet has been reported to have a negative correlation with CH_4 production (Yan et al., 2009); this may not fully explain CH_4 production in the current PLT EXP as the CUT 2 treatment contained (numerically) a greater level of CP than the CUT 1 treatment (22.7% v. 18.6%) on a DM-basis. However, these values are only based on a comparison of single composite samples. Although Zhong et al. (2016) evaluated forage maturity of *Medicage ruthenica* and *Leymus chinensis*, they also evaluated forage quality based on timing of harvest. Zhong et al. (2016) indicated that *in vitro* CH_4 production of the aforementioned forages increased with advancing harvest dates. Methane was more impacted by forage type with *L. chinensis* producing CH_4 than *M. ruthenica.*

In the current SUPP EXP, ACV supplementation did not impact average CH_4 output although CH_4 output at h 1 in the treatment supplemented with ACV was lower than the non-supplemented treatment. There was a 12 h period (h 8 – h 24) in which ACV+ produced more CH_4 than ACV+ tubes collected at two h after inoculation. This may indicate that supplementation with ACV may have a brief impact on CH_4 mitigation upon initial intake with the impact lessening over time. However, mean CH_4 did not differ between ACV+ and ACV-, indicating that ACV supplementation has little to no overall impact on CH_4 mitigation. An *in vitro* batch culture study by O'Reilly et al. (2021) recently examined the impacts of biochar and wood vinegar on CH_4 mitigation and other fermentation parameters. In this study, neither biochar or wood vinegar supplementation

reduced CH_4 production, nor were there beneficial impacts on fermentation metrics. Furthermore, similar to the results of this study, there were no impacts on production of VFA, potentially due to interconversion of acetate by microbiota (O'Reilly et al., 2021; Hackmann and Firkins, 2015). O'Reilly et al. (2021) concluded that feed substrate was more impactful on fermentation metrics and CH_4 emissions than the provided supplements. As ACV supplementation in this study did not significantly impact CH_4 output, our results could also support the suggestion that forage maturity and quality may be more impactful than ACV supplementation on CH_4 output. However, it should be noted that CH_4 values in this study were collected after opening tests tubes as compared to the methods of O'Reilly et al. (2021) who measured gas utilizing gas chromatography and a water displacement apparatus. The use of a handheld gas collection meter may have contributed to the loss of fermentation gases.

3.5.3. Water Soluble Carbohydrate Concentration

The type of carbohydrate present in forages is thought to influence the production of CH_4 due to shifts in rumen microbial populations (Johnson and Johnson, 1995; Meale et al., 2012). High soluble carbohydrate content may promote propionate production, lowering rumen pH and inhibiting methanogenesis. In this study, propionate production and acetate: propionate ratios did not differ between treatments in the PLT EXP or the SUPP EXP. However, our measured values aligned with those reported by Meale et al. (2012). Boadi and Wittenberg (2002) reported that CH_4 production was greater in diets of lower quality due to higher fiber content, rather than WSC or ESC, shifting VFA production away from propionate. Results indicated that WSC content from fermentation media was greater in the CUT 1 treatment as compared to the CUT 2 treatment with a

similar trend noted for the treatment supplemented with ACV (ACV+). Although more energy may be available to rumen microbes, the greater amount of WSC present in fermentation media indicates that energy is not being used as efficiently and effectively. This is further supported by the IVDMD results. The CUT 1 treatment had a greater DM disappearance than the CUT 2 treatment, indicating that the CUT 1 treatment was more fermentable than the CUT 2 treatment. Supplementation with ACV may increase WSC content and metabolism; however, the effect noted in this study is more likely due to direct supplementation of ACV rather than actual modification fermentation metrics.

3.5.4. Effluent Ammonia Nitrogen Concentration

While the NH_3-N concentrations in the current study are higher than those reported by Shin et al. (2010), the findings of this current study further align with the findings of Relling et al. (2001) and Lee et al. (2011). In the current SUPP EXP, ACV supplementation did not impact NH_3-N concentration but it was greater in the PLT EXP for tubes receiving the CUT 2 treatment. This indicates that the forage profile is most likely a more influential control of NH_3-N utilization than ACV supplementation. In the current PLT EXP, the CP content of the CUT 2 diet was numerically greater than that of the CUT 1 diet, although no statistical comparisons were made. Relling et al. (2001) indicated that rumen NH_3-N concentrations decreased as pastures matured. A decrease in NH_3-N concentration of pasture-based diets may also be influenced by polyphenol oxidase (**PPO**)-containing plants such as red clover, as PPO in higher quality forages may exhibit a protective function, slowing degradation of dietary protein (Lee et al., 2011). Lee et al. (2011) demonstrated a similar effect when comparing fresh and wilted orchardgrass to fresh and wilted tall fescue in a rumen simulation technique (**RUSITEC**) system. Despite greater N input into systems

with orchardgrass and greater available energy from WSC, systems treated with orchardgrass showed lower concentrations of NH_3-N. This indicates slower degradation of dietary protein which may be occurring in the CUT 1 tubes in this study. However, as PPO and CP were not measured in this study, it cannot be determined whether PPO, or other secondary metabolites, played a role in protein protections and NH_3-N concentrations. Additionally, elevated NH_3-N concentration may be due to the design of the *in vitro* system. As there are no buffer inputs, fluid outflow, or absorption processes in a batch culture system, it is possible that greater concentrations of NH_3-N accumulated within each tube.

3.6. Conclusions

The physiological impacts of ACV supplementation in ruminant diets and its potential impacts when supplemented with different forage maturities is not outlined in the published literature. Data that are available is limited and does not provide a robust justification for use of ACV in ruminant diets. As expected, there was no positive influence of ACV on fermentation metrics and CH_4 output except for an increase in fermentation media WSC. This increase is more likely due to direct supplementation into media rather than changes in fermentation or digestibility. Instead, fermentation metrics were more influenced by forage quality than ACV supplementation. Further research is needed to determine if forage maturity and ACV supplementation impacts rumen fermentation and digestive kinetics. Experiments should expand to larger trials to obtain a more comprehensive assessment of performance and production metrics that are based on more representative *in vitro* and *in vivo* models. Additionally, research should evaluate greater concentrations of ACV supplemented in feed or water as the amount utilized in this study,

and in practice, may not be enough to elicit a response or changes in fermentation metrics and potential animal performance.

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3.9. Tables

Table 3.1. Chemical composition of individual forages and forage mixtures used for batch culture treatments. Forages used in the CUT 1 treatment were also used in the SUPP EXP. Results are listed as a percentage of dry matter (DM) unless stated otherwise.

Item	Ingredient				Treatment[1]	
	First Cut		Second Cut			
	Orchardgrass	Red Clover	Orchardgrass	Red Clover	CUT 1	CUT 2
OM[2]	90.3	90.1	89.7	88.8	90.2	89.3
CP[3]	14.6	22.5	19.1	26.6	18.6	22.7
aNDFom[4]	51.2	41.1	60.7	35.6	46.2	48.2
ADF[5]	33.5	33.6	43.9	29.8	33.6	36.9
NFC[6]	19.2	21.0	4.80	20.2	20.1	12.5
WSC[7]	10.2	4.60	3.40	10.0	7.40	6.70
ESC[8]	7.40	4.40	3.20	9.60	5.90	6.40
Starch	1.40	2.70	0.60	1.10	2.10	0.85
NSC[9]	11.6	7.30	4.00	11.1	9.50	7.60
NE_L, mcal/ kg[10]	1.46	1.38	1.21	1.45	1.42	1.33
Ca	0.59	1.51	0.68	1.72	1.05	1.20
P	0.47	0.35	0.39	0.38	0.41	0.39
Mg	0.23	0.28	0.24	0.30	0.26	0.27
K	2.77	2.85	3.05	3.01	2.81	3.03
Na	0.03	0.003	0.29	0.01	0.02	0.15

[1]Treatment values were calculated using actual nutrient composition and proportion of individual forages on a DM basis; CUT 1: 50% red clover + 50% orchardgrass from first cutting; CUT 2: 50% red clover + 50% orchardgrass from a second cutting.
[2]OM: organic matter.
[3]CP: crude protein.
[4]aNDFom: NDF organic matter after amylase treatment and ashing.
[5]ADF: acid detergent fiber.
[6]NFC: non-fiber carbohydrate. Calculated as described by Dillard et al. (2017); 100 – (CP% + aNDFom% + ether extract% + ash%).
[7]WSC: water soluble carbohydrates.
[8]ESC: ethanol soluble carbohydrates.
[9]NSC: non-structural carbohydrates. Calculated as described by Dillard et al. (2017); starch + water soluble carbohydrates.
[10]Estimated by the NRC (2001) model.

Table 3.2. Methane (CH_4) production, ammonia nitrogen (NH_3-N) concentration, water soluble carbohydrate (WSC) concentration, mean pH, and *in vitro* dry matter disappearance (IVDMD) of 48 h *in vitro* fermentation of forage combinations at different maturities (PLT EXP) and inclusion of the CUT 1 treatment with (+) and without (-) 1.5% supplementation of organic apple cider vinegar (ACV; SUPP EXP).

	CH_4 (mg dL^{-1})	NH_3-N (mg dL^{-1})	WSC (mg mL^{-1})	IVDMD (%)	pH
PLT EXP Treatment					
[1]CUT 1	4.98[a]	62.9[b]	2.39[a]	81.7[a]	6.72[a]
[2]CUT 2	2.87[b]	66.9[a]	1.56[b]	77.3[b]	6.60[b]
SEM	0.47	3.97	0.11	1.58	0.05
SUPP EXP Treatment					
[3]ACV+	2.51[a]	59.7[a]	2.67[a]	74.9[a]	6.59[a]
[4]ACV-	3.03[a]	57.9[a]	2.31[b]	76.9[a]	6.58[a]
SEM	0.39	0.92	0.08	1.45	0.07

[1]CUT 1: 50% red clover + 50% orchardgrass from first cutting on a DM-basis.
[2]CUT 2: 50% red clover + 50% orchardgrass from a second cutting on a DM-basis.
[3]ACV+: 50% red clover + 50% orchardgrass from first cutting on a DM-basis + ACV.
[4]ACV-: 50% red clover + 50% orchardgrass from first cutting on a DM-basis without ACV supplementation.
[a,b]Within column, different letters indicate significant differences ($P \leq 0.05$).

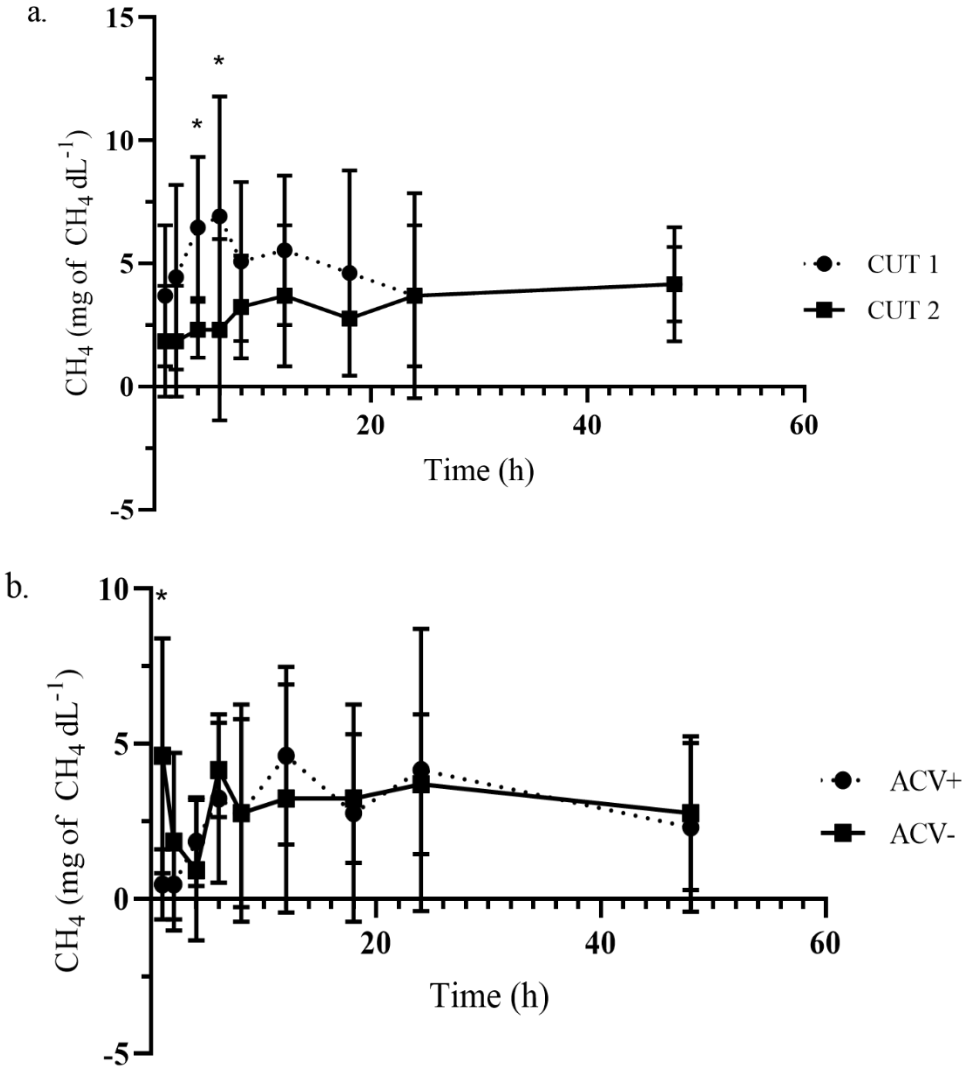
Table 3.3. Volatile fatty acid (VFA) molar proportions and concentrations of a) PLT EXP: red clover + orchardgrass at first cut and red clover + orchardgrass mixtures at second cutting and b) SUPP EXP: first cut red clover + orchardgrass mixtures and first cutting red clover + orchardgrass mixtures supplemented with (+) and without (-) 1.5% apple cider vinegar (ACV) during batch culture fermentation.

	PLT EXP[1]				SUPP EXP[2]			
	Treatment[3]			*P*-value[4]	Treatment[5]			*P*-value
Item	CUT 1	CUT 2	SEM	Treatment	ACV+	ACV-	SEM	Treatment
Total VFA, m*M*	48.5	48.2	1.01	0.86	51.5	52.4	7.86	0.91
Individual VFA, mol/ 100 mol								
Acetate (A)	65.8	66.1	0.68	0.64	65.6	68.7	1.98	0.17
Propionate (P)	19.1	18.5	0.54	0.37	20.8	18.7	1.32	0.15
Butyrate (B)	12.0	12.5	0.40	0.47	9.97	9.32	0.56	0.29
Isobutyrate	0.75	0.73	0.03	0.53	0.64	0.62	0.05	0.58
Valerate	1.59	1.41	0.10	0.11	2.25	2.05	0.20	0.37
Isovalerate	0.81	0.77	0.04	0.22	0.70	0.64	0.05	0.23
A:P	3.46	3.58	0.09	0.38	3.21	3.70	0.29	0.15
A+B:P	4.09	4.26	0.12	0.29	3.69	4.20	0.30	0.14

[1]PLT EXP: Experiment evaluating forage maturity.
[2]SUPP EXP: Experiment evaluating ACV supplementation.
[3]Treatments on a DM-basis: CUT 1: 50% red clover + 50% orchardgrass from first cutting; CUT 2: 50% red clover + 50% orchardgrass from a second cutting.
[4]*P*-values less than or equal to 0.05 are considered significant.
[5]Treatments on a DM-basis: ACV+: 50% red clover + 50% orchardgrass from first cutting + ACV; ACV-: 50% red clover + 50% orchardgrass from first cutting without ACV supplementation.

3.10. Figures

Figure 3.1.a. Methane (CH_4) concentration (mg dL^{-1}) across time (h 1 – 48) of the PLT EXP (CUT 1 and CUT 2). **3.1.b.** Methane (CH_4) concentration (mg dL^{-1}) across time (h 1 – 48) of the SUPP EXP (ACV+ and ACV-).



*Represents significant differences between treatments ($P \leq 0.05$).
Treatments on a DM-basis: CUT 1: 50% red clover + 50% orchardgrass from first cutting; CUT 2: 50% red clover + 50% orchardgrass from a second cutting.; ACV+: 50% red clover + 50% orchardgrass from first cutting + apple cider vinegar (ACV); ACV-: 50% red clover + 50% orchardgrass from first cutting without ACV supplementation.

3.11. Supplementary Tables

Table S3.1. Forage cultivars utilized for both batch culture experiments (PLT EXP and SUPP EXP).

Forage Species and Cultivars	
Orchardgrass (*Dactylis glomerata*)	Red Clover (*Trifolium pratense*)
Echelon	Arlington
Harvestar	Freedom
Husar	Manitoba
Inavale	Milvus
Luxor	Ruby
Niva	
Olathe	
Otello	

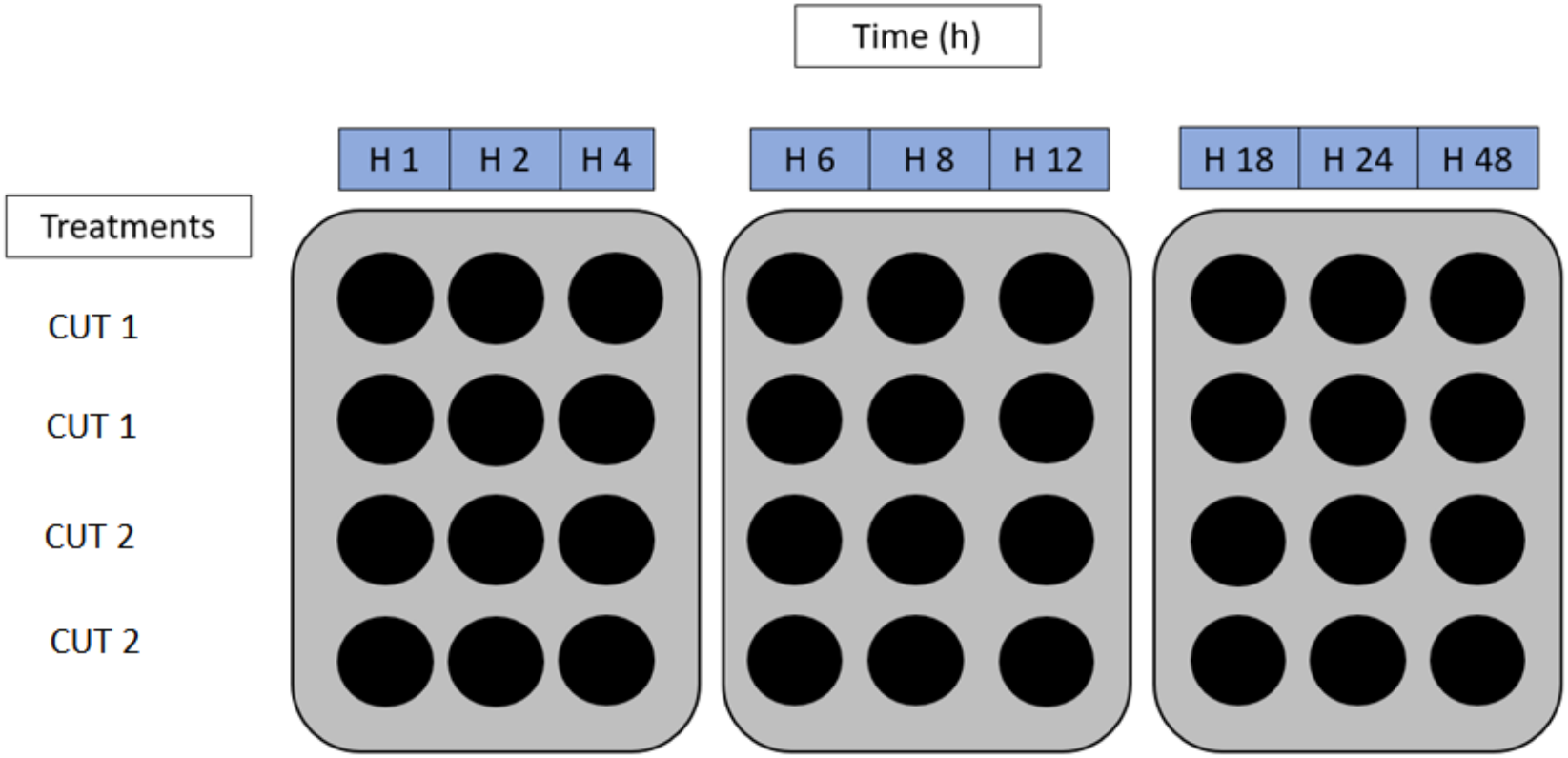
Table S3.2. Chemical composition of basal total mixed ration (TMR) diet from the bunk.

Item (% DM)	Basal Diet (PLT EXP)[1]	Basal Diet (SUPP EXP)[2]
OM	93.1	93.1
CP	14.9	14.8
aNDFom	32.0	32.5
ADF	20.8	19.7
NFC	40.4	40.0
WSC	5.90	5.60
ESC	4.80	4.60
Starch	21.9	22.4
NSC	27.9	28.0
Ca	0.63	0.69
P	0.36	0.36
Mg	0.38	0.32
K	1.07	1.03
Na	0.57	0.58

[1]PLT EXP: Batch culture experiment 1; forages collected from the first harvest v. forages collected from the second cutting.
[2]SUPP EXP: Batch culture experiment 2; supplementation of apple cider vinegar (ACV) to first cut forages v. non supplementation.

3.12. Supplementary Figures

Figure S3.1. Batch culture setup for the PLT EXP. Conditions were the same for the SUPP EXP with the exception of all treatments being 50:50 CUT 1 and the supplementation of apple cider vinegar (ACV). Treatments for h 0 were collected immediately at the beginning of the experiment. Treatments included: CUT 1: 50:50 mixture of red clover and orchardgrass collected from the first cutting; CUT 2: 50:50 mixture of red clover and orchardgrass collected from a second cutting.



CHAPTER 4: EVALUATION OF DIVERSE COOL-SEASON GRASS MIXTURES WITH RED CLOVER ON RUMINAL FERMENTATION IN CONTINUOUS CULTURE FERMENTERS

4.1. Abstract

Pasture diversity is an important feed management consideration in grazing systems, as it impacts rumen fermentation and ultimately animal productivity. Dual-flow continuous culture fermenter systems were used to evaluate the impacts of increasing cool-season perennial (CSP) grass inclusion in the diet on fermentation metrics. Dietary treatments were assigned randomly to fermenters in a 4 x 4 Latin square design. Fermenters were operated for four consecutive 10-d periods with each period comprised of 7 d of adaptation followed by three d of sampling. All diets contained 40% dry matter (DM) red clover which was combined (on a DM-basis) with 1) 60% orchardgrass (OG); 2) 30% orchardgrass + 30% meadow fescue (MF); 3) 20% orchardgrass + 20% meadow fescue + 20% Kentucky bluegrass (KYBG); or 4) 15% orchardgrass + 15% meadow fescue + 15% Kentucky bluegrass + 15% perennial ryegrass (PRG). To simulate pasture rotation, diets were added to fermenters four times per d (33% of the total daily DM at 0700 h and 1600 h; 17% of total daily DM at 0820 h and 1720 h) to simulate grazing intake patterns, totaling 110 g DM total diet per fermenter per d. Fermenter pH was collected every min throughout each period. Methane (CH_4) was measured in triplicate from each fermenter on d 7 – 10 at 0630 h and 1530 h. Effluent samples were collected on d 8, 9, and 10. Collected samples were pooled by fermenter within period and analyzed for volatile fatty acids (VFA), ammonia nitrogen (NH_3-N), and water soluble carbohydrates (WSC). Data were analyzed using the PROC GLIMMIX procedure of SAS and significance declared if $P \leq 0.05$.

Rumen fluid and forage samples were analyzed for DM, organic matter (OM), crude protein (CP), aNDFom (amylase- and sodium sulfite-treated neutral detergent fiber on an organic matter basis), acid detergent fiber (ADF), ethanol soluble carbohydrates (ESC), and WSC. Apparent digestibilities for DM, OM, aNDFom, and ADF were highest for fermenters receiving the KYBG treatment whereas DM and OM digestibilities for the MF treatment were lower by 18.0% and 19.6%, respectively. Fermenters receiving the KYBG treatment spent the most time under a pH of 5.8 (15.5 h/d) as compared with those receiving OG (8.3 h/d). Concentrations of propionate were higher from fermenters receiving the KYBG and PRG treatments. Proportions of acetate: propionate and acetate + butyrate: propionate were higher in fermenters receiving KYBG and PRG. The WSC content was lowest for KYBG, inversely related to CH_4 concentration. Fermenter CH_4 concentrations were lower for OG, MF, and PRG; however, effluent WSC content was highest in PRG-treated fermenters. Although not as digestible as the KYBG treatment, PRG was more digestible than OG with the added benefit of lower CH_4 concentration. Based on these results, there could be potential benefits to inclusion of Kentucky bluegrass in CSP forage mixtures; however, our results indicate that it should be included in smaller proportions (<15% DM) or paired in equivalent amounts with a forage such as perennial ryegrass or meadow fescue to achieve optimal animal performance.

Key Words: pasture-based dairy, botanical composition, perennial ryegrass, high forage diets

4.2. Introduction

In temperate areas of the United States (**U.S.**), high-quality forages, including grasses, legumes, and forbs, are utilized in dairy cattle diets as a low-cost dietary component that can support farm sustainability and viability (Tas et al., 2006). To support animal health and performance, optimizing forage quality and quantity during the growing and grazing season while maximizing herbage dry matter intake (**DMI**) is an important aim for grazing systems (Peyraud et al., 2004). This can prove challenging as finding the correct balance between herbage quality and quantity is difficult, particularly in forage-based systems (Wims et al., 2010). Finding a balance is further complicated when utilizing pastures that are composed completely of a single grass species (Rutter, 2010). Grazing monocultures can be beneficial to producers as the knowledge and experience required for successful monoculture systems is less complex and time-consuming when compared to diverse forage systems. However, there are several disadvantages including decreased soil fertility, increased susceptibility to weeds, and less digestible herbage (Muir et al., 2011; Salaheen and Biswas, 2019). As well as producing less digestible herbage, pure grass stands typically rely on frequent applications of nitrogen (**N**) fertilizers. However, this is not feasible for all grazing systems (Grassfed Dairy Standards, 2018). Therefore, producers must find alternative methods to increase N supply and, thus, increase herbage production and quality.

In the Northeastern (**NE**) U.S., there has been recent interest from grass-based producers to include perennial ryegrass (*Lolium perenne*) in their grazing systems as it is highly palatable and digestible (Hannaway et al., 1999). Perennial ryegrass is commonly utilized in European and New Zealand-based grazing systems and inter-seeded with white

clover (*Trifolium repens*) or red clover (*Trifolium pratense*) as these species complement each other and support animal production (Lee et al., 2004), N fixation, and herbage quantity and quality. Inclusion of legumes with perennial ryegrass in pastures may increase herbage quality while simultaneously fixing atmospheric N that may increase N to growing forage swards (Enriquez-Hidalgo et al., 2014). Aside from the ability to fix atmospheric N, both white clover and red clover offer considerable benefits to pasture-based livestock systems such as high digestibility and protein and promoting animal intake (Black et al., 2009). In a short-term study, Lee et al. (2004) observed that as proportions of dietary white clover were increased, there was a linear increase in DMI. Furthermore, increased proportions of white clover led to marked decreases in methane (**CH_4**) production. In contrast to the findings of Lee et al. (2004), Enriquez-Hidalgo et al. (2014) found that mixed pastures containing white clover and perennial ryegrass, as compared with grass-only pastures, did not differ in regards to herbage production, cow DMI, or CH_4 production.

Although results from grazing studies appear to be mixed, forage legumes such as white clover, red clover, and birdsfoot trefoil (*Lotus corniculatus*) are still utilized in grazing systems. In the NE U.S., red clover is utilized more often than white clover due to greater yields when inter-seeded with other grass or legume stands (Deak et al., 2004; Sanderson et al., 2007), as well as its ability to be used in conserved forages such as silage, hay, and green chop (Black et al., 2009). Red clover also has the added benefit of containing polyphenol oxidase (**PPO**), an enzyme that may reduce proteolysis both *in silo* and in the rumen, increasing animal protein supply (Fraser et al., 2004). Further, when seeded with grass, red clover mixtures have additional benefits of improved N-use efficiency when utilized in ruminant diets (Eriksen, Askegard, and Soegaard, 2012). Eriksen, Askegard, and

Soegaard (2012) reported that inclusion of red clover with white clover – perennial ryegrass created higher yields and led to decreased fertilizer application. However, effects on ruminant nutrition and N-use efficiency were not evaluated.

A potential issue with inclusion of legumes in pastures is increased lignin content which may limit microbial degradation (Smith, Goering, and Gordon, 1972). However, evidence indicates that the chemical composition of lignin may be more important than quantity in relation to plant digestibility (Buxton and Russell, 1988). Jung and Allen (1995) indicated that cross-linkages of cell-wall polysaccharides and lignin by phenolics, such as ferulic acid, may be the cause of decreased forage digestibility. *In vitro* methods indicated that neutral detergent fiber (**NDF**) and acid detergent fiber (**ADF**) digestibility of hay (Mandebvu et al., 1999) and corn silage (Raffrenato et al., 2017) showed a positive correlation with ferulic acid content. However, Casler and Jung (2006) indicated that ferulic acid concentration had pronounced negative impacts on perennial ryegrass degradation after 96 h. There has been interest in identifying the relationship of plant phenolics with NDF and ADF digestibility in order to better predict forage fiber digestibility (Soberon, 2012) although this approach is still debated (Wang et al., 2022). Based on these results, more research is needed to determine if pastures seeded with legumes and perennial ryegrass would be beneficial to producers in the NE U.S.

This study evaluated combinations of red clover with cool-season perennial (**CSP**) grasses, specifically orchardgrass (*Dactylis glomerata*), meadow fescue (*Festuca pratensis*), Kentucky bluegrass (*Poa pratensis*), and perennial ryegrass, on rumen fermentation metrics and energetics using continuous culture fermenter systems. The objectives of this study were to 1) evaluate the impacts of red clover - grass specie

combinations on likely rumen performance using an *in vitro* system and 2) determine which forage or forage combination could potentially be the most beneficial in terms of CH_4 emissions and fermentation metrics in NE pasture systems.

4.3. Materials and Methods

4.3.1. Site Description

All forages were established at Borderview Research farm in Alburgh, VT (45.01º N, -73.31º W) as previously described and outlined in variety trial reports published by the University of Vermont Northwest Crops and Soils Program (Darby et al., 2020; Darby et al., 2021). Forages of interest included orchardgrass, red clover, meadow fescue, Kentucky bluegrass, and perennial ryegrass. Forage cultivars used in this study are listed in Supplementary Materials, Table S4.1. Each grass and legume species were established as monocultures on plots that were 9.3 m^2 and were part of several small-plot experiments. Small-plot experiments were designed as randomized complete blocks with 5 replications. Weather data was recorded with a Davis Instruments Vantage Pro2 weather station (Hayward, CA). This station was equipped with a WeatherLink data logger (Davis Instruments; Hayward, CA). Average growing degree days (**GDD**) for the 2019 – 2020 growing and harvesting season are listed in Supplementary Materials, Table S4.2.

4.3.2. Grass Management and Harvest

Cool-season perennial grass species were planted in August 2019 using a cone seeder (Great Plains; Salina, KS) on Benson rocky silt loam soil. Grass species included orchardgrass, meadow fescue, Kentucky bluegrass, and perennial ryegrass. The 2020 growing season was the first full season after sward establishment. Forages were visually inspected for survival and establishment in early April 2020. Plots with inadequate stands

were reseeded in late April 2020. Soybeans were planted previous to the CSP grasses in the utilized plot areas. Grass plots received fertilizer inputs containing approved organic materials based on soil tests. All grass species were harvested in the morning at the vegetative stage of growth using a flail forage harvester (Carter; 3 -in-wide swath), set to a 4-in stubble height in a 5.8 m^2 area in May, June, August, and September. For the purpose of this experiment, only forages harvested from the first three cuts were used.

4.3.3. Legume Management and Harvest

Red clover was planted and established at a rate of 11.3 kg ac^{-1} in September 2017. Red clover was grown on Benson rocky silt loam soil. Spring barley was planted prior to red clover varieties in the utilized plots. Plots were assessed for yield and quality parameters. Varieties were planted using a cone seeder (Great Plains; Salina, KS) and harvested in a similar manner as the grass stands. Red clover was harvested three times during the summer (June, July, and August) from 5.8 m^2 plots. At the time of harvesting legumes were at pre-bud stage.

4.3.4 Forage Collection and Processing

Individual forages were harvested separately. During harvest, approximately 0.45 kg (wet weight) of harvested material was collected from each forage variety by random grab sample with 5 subsamples collected per variety per cutting. Subsamples were dried at 60ºC for 48 h to calculate dry matter (**DM**) yield and content. Average forage DM yield ac^{-1} was extrapolated to a total of forage samples from the sample plots. Dried subsamples were ground to a grind size of 1-mm using a Wiley Mill (Wiley Mill; Thompson Scientific; Philadelphia, PA) and a cyclone mill (UDY Corporation; Fort Collins, CO). Samples were then analyzed using near infrared reflectance spectroscopy (**NIRS**) methods at the

University of Vermont E.E. Cummings Crop Testing Laboratory (Burlington, VT). The instrument used for NIRS was a FOSS DS2500 Forage and Feed Analyzer (FOSS North America; Eden Prairie, MN) which generates spectral data across a wavelength of 400 – 2500 nm.

For *in vitro* studies, harvested forages were placed on a tarp and transferred to plastic bags within 30 min of harvest. Air was manually forced from bagged forages to prevent further degradation during transportation. Bags were weighed (approximately 11.3 kg bag^{-1}) and zip tied shut. Forages were transported to a commercial warehouse (Vermont Commercial Warehouse; Williston, VT) within 90 min of harvest where they were frozen at -20ºC until further processing. All forages were processed and ground at the UVM Horticulture Research and Education Center (South Burlington, VT). Upon processing, frozen forages were broken apart into smaller portions and allowed to come to room temperature. Before forages were ground, residual weeds and dead material were botanically separated and discarded. Each forage was frozen with liquid N and passed through a 2-mm mesh screen (Wiley Mill; Thompson Scientific; Philadelphia, PA) cooled with liquid N and dry ice. Individual forages were passed through the mill separately, with ground subsamples collected for further analysis at a commercial laboratory (DairyOne Laboratories; Ithaca, NY; Table 4.1). A subsample of each unground forage was held aside and split into two portions. The first portion was dried for 48 h at 65ºC to determine DM percent of each forage. The second portion of each subsample was frozen at -80ºC for enzymatic analysis of total phenolics and ferulic acid.

4.3.5. Plant Phenolics and Ferulic Acid

Total plant phenolics were determined according to the methods of Ainsworth and Gillespie (2007). Briefly, plant materials were homogenized in ice-cold 95% methanol using a bead beater (TissueLyer II; Retsch, Newtown, PA) for 5 min at 30 Hz. Metal beads were removed and samples were incubated in 1.5 mL tubes for 48 h at room temperature protected from light. After 48 h, samples were centrifuged at 13,000 x *g* for 5 min at room temperature. Subsamples of supernatant and gallic acid standards were mixed with 10% Folin-Ciocalteu (**FC**) reagent and 700 mM sodium carbonate. Samples were incubated at room temperature for two h and read using a microplate reader at 765 nm (BioTek Synergy HT; Winooski, VT). Ferulic acid content was determined per the methods of Abdel-Aal et al. (2001) and read at 320 nm (BioTek Synergy HT; Winooski, VT).

4.3.6. Dietary Treatments

Based on vessel volume and inclusion rates of previous studies (Wenner et al., 2017), fermenters received a total of 110 g DM d^{-1} for the duration of each period. Briefly, a DM analysis was conducted to calculate forage inclusion rates for each diet. Four samples of individual forages were randomly collected from harvested materials, weighed, and dried at 65ºC for 48 h in a forced air oven (VWR Scientific Products; Radnor, PA). Calculated DM values were averaged. Diets were weighed, hand-mixed based on DM values, and stored at -20ºC until use. All diets contained, on a DM-basis, 40% red clover. Red clover was combined (on a DM-basis) with 1) 60% orchardgrass (**OG**); 2) 30% orchardgrass + 30% meadow fescue (**MF**); 3) 20% orchardgrass, 20% meadow fescue, + 20% Kentucky bluegrass (**KYBG**); or 4) 15% orchardgrass, 15% meadow fescue, 15% Kentucky bluegrass, + 15% perennial ryegrass (**PRG**). Fermenters received four meals

daily: 33% of the total meal DM at 0700 h and 1600 h, and 17% of the total meal DM at 0820 h and 1720 h (Gregorini, Soder, and Waghorn, 2010). Diets were fed in this manner to simulate pasture intake and ingestion rates during grazing (Gregorini et al., 2008).

4.3.7. Experimental Design and Continuous Culture Operation

The four diets were assessed in a 4 x 4 Latin Square experimental design with complete randomization of treatments. Four dual-flow continuous culture fermenters (Electrolab Biotech, Tewkesbury, UK) similar to that of Hoover et al. (1989) were used. The average fermenter volume was 2.98 ± 0.16 L. Each fermenter vessel was equipped with a heat jacket maintained at 39ºC (160 W, Electrolab Biotech, Tewkesbury, UK), a continuous measurement temperature probe, an indwelling continuous pH probe (Mettler Toledo; Columbus, OH), and an agitator fitted with paddles (200 Stirrer Motor; Electrolab Biotech; Tewkesbury, UK) maintained on a program that continuously agitated samples on a 10 min cycle at 70 rpm for 9 min followed by one min at 200 rpm. Carbon dioxide (**CO_2**) flow, cooling, and air flow were controlled by a service plate (FerMac 360; Electrolab Biotech; Tewkesbury, UK). Similar to Dillard et al. (2019), all probes, tubes, and fittings were sealed with silicone gaskets and parafilm to prevent gas leakage and maintain an anaerobic environment by continuous inflow of CO_2. Each fermenter lid contained a two cm port that was utilized for feeding.

Prior to inoculation with rumen fluid on d 0, fermenter vessels were pre-warmed to 39ºC with a heat jacket and received a continuous flush of CO_2 gas. Buffer lines were primed with 20% clarified rumen fluid as described by Wenner (2016) and modified by Baker (2021). On d 0, rumen fluid was collected from three ruminally fistulated, lactating, multiparous Holstein dairy cows (Paul R. Miller Research and Educational Center; South

Burlington, VT) as approved by The University of Vermont Animal Care and Use Committee (IACUC #16-029) in accordance with the requirements of the Office of Laboratory Animal Welfare. Donor cows had ad libitum access to water and a total mixed ration (**TMR**) diet with a topdress (Supplementary Materials, Table S4.3). Animals were housed in tie-stalls. Random grab samples of TMR and topdress were collected separately and pooled to create a single TMR and single topdress composite sample. Composite samples were frozen at -20ºC, and submitted to DairyOne Laboratories (Ithaca, NY) for a feed nutrient composition analysis (Supplementary Materials, Table S4.3). Approximately 20 L of rumen fluid was collected two h after morning feeding from the ventral, central, and dorsal sacs of the rumen. Rumen digesta was strained through a metal sieve to remove larger feed particles. Strained fluid was collected into plastic buckets maintained in a 39ºC water bath. Strained fluid was then pooled and transferred to a heated (39ºC) container (Powerblanket, Salt Lake City, UT 84119) for transport. Each vessel was inoculated with approximately three L of rumen fluid within 20 min of collection.

Two 3D-printed filters fitted with nylon bags (50 ± 10 µ; ANKOM; Macedon, NY) were used to retain protozoal populations and were fitted to the inoculum end of the liquid outflow tubing. Each filter contained two tubes: one for effluent removal and one for effluent recirculation. Rumen buffer was continuously added to the fermenters by peristaltic pumps. Buffer was prepared according to Hoover et al. (1976) but modified with the inclusion of urea at a rate of 0.4 g L^{-1} to simulate recycled N. For the purposes of a larger trial, urea in saliva buffer was replaced with ^{15}N labeled ammonium sulfate (Sigma-Aldrich Co.; St. Louis, MO) on d 5 at a rate of 0.021 g L^{-1} until the end of each experimental period. However, this data is not presented herein. Peristaltic pumps were used to

continuously pump buffer into the fermenters. Fermenter pH was not regulated but was monitored with an indwelling pH probe. Fermenters were fitted with continuous measurement pH probes connected to the control tower and pH measurements were measured and recorded every min through each period. A gravity-fed outflow spout was utilized to collect effluent samples. Fermenter vessels were maintained at 39ºC for the duration of each period with a continuous flush of CO_2 into the vessel headspace and buffer to maintain an anerobic environment. Agitation, pH, and temperature were continuously recorded with data collected every min. Liquid dilution rates for each fermenter were approximately 5.6% h^{-1} which was maintained by regulation of buffer input and effluent removal.

Fermenters were operated for four consecutive 10 d periods with each period comprised of 7 d of adaptation followed by three d of sampling. Outflow effluent from carboys and collection bottles was collected daily before morning feeding with weight and volume recorded and utilized to determine flow rates. On d 7, outflow collection carboys and bottles were placed and maintained on ice through d 10. Methane measurements (% CH_4 volume) were collected in triplicate on the evenings of d 7, 8, and 9 at 1530 h, and on the mornings of d 8, 9, and 10 at 0630 h from the gas headspace using a gas analyzer probe (Sewerin Multitec 545; Gütersloh, Germany) with a coolant coil (Sewerin; Gütersloh, Germany). Methane concentration (mmol d^{-1}) was calculated using the following equation as outlined by Johnson et al. (2009) assuming atmospheric pressure:

$$\mathrm{CH_4\ (mmol\ d^{-1})} = \frac{(\mathrm{mmol\ x\ CO_2\ gas\ flow}\ \left(\frac{\mathrm{mL}}{\mathrm{min}}\right) \mathrm{x\ 60\ min\ x\ 24\ h}\ )}{1000}.$$

On d 8, 9, and 10, carboys were gently mixed and contents were combined with collection bottle contents within fermenter within d. Samples of outflow (solid and liquid)

for each fermenter were placed in pre-weighed pans and dried at 65ºC for 48 h in a forced air oven. Dry weights were recorded and dried samples were pooled by fermenter with dried rumen residues from d 8, 9, and 10 combined in equal portions. Samples were sent to a commercial laboratory (DairyOne Laboratories; Ithaca, NY) for chemical analysis. Additional samples were collected from carboys for determination of volatile fatty acid (**VFA**), ammonia nitrogen (**NH_3-N**), and WSC concentrations. Samples for VFA and NH_3-N analysis were strained through four layers of cheesecloth. Effluent NH_3-N samples were additionally acidified with two mL of 25% *m*-phosphoric acid (Sigma-Aldrich Co.; St. Louis, MO). Both NH_3-N and WSC samples were stored at -20ºC until further analysis. Samples for VFA analysis were stored at -20ºC until processing at the William H. Miner Institute (Chazy, NY) as outlined below.

4.3.8. Chemical Analysis

Samples were analyzed for VFA concentration as outlined by Miller et al. (2021). Briefly, individual VFA, acetate: propionate ratios, acetate + butyrate: propionate ratios, and total molar proportions were determined through gas chromatography using a Varian CP-3800 gas chromatograph (Varian, Inc.; Palo Alto, CA). The gas chromatograph was fit with a flame-ionization detector and 80/120 Carbopack B-DA/4% Carbowax 20M column (Supelco, Inc.; Bellefonte, PA). Rumen fluid samples used for WSC concentration were analyzed using the colorimetric anthrone-sulfuric acid assay as described by Laurentin and Edwards (2003). Samples collected for NH_3-N concentration were analyzed using the colorimetric phenol-hypochlorite method adapted to a 96-well plate (Chaney and Marbach, 1962).

4.3.9. Forage and Fermenter Effluent Wet Chemistry

Similar to Dillard et al. (2017), samples of orchardgrass, red clover, meadow fescue, Kentucky bluegrass, and perennial ryegrass, as well as pooled, dried fermenter effluent, were analyzed according to the following methods: DM was dried in an oven for two h at 135ºC (method 930.15; AOAC International, 2006). Crude protein was analyzed following method 990.03 (AOAC International, 2006) and aNDFom [ANKOM Technology Method 15, 2020] was analyzed with heat stable α-amylase and sodium sulfite; additionally, samples were ashed to remove organic materials. Acid detergent fiber (**ADF**; ANKOM Technology Method 14, 2020) was analyzed after exposure to ADF solution and acetone. Ethanol soluble carbohydrates and WSC were analyzed using a Thermo Scientific Genesys 10S Vis Spectrophotometer; ESC was analyzed after a phenol – sulfuric acid reaction whereas WSC were determined using a sulfuric acid - potassium ferricyanide colorimetric reaction after incubation at 40ºC for one h. Minerals (Ca, P, Mg, K, Na; Thermo iCAP 6300 Inductively Coupled Plasma spectrometer), starch (Application Note Number 319; YSI Inc. Life Sciences; Yellow Springs, OH), and ash (method 942.05; AOAC International, 2006) were also determined. The non-fiber carbohydrate (**NFC**) content was calculated as described by Dillard et al. (2017): NFC (%) = 100% - (CP (%) + aNDFom (%) + ether extract (%) + ash (%)).

4.3.10. Statistical Analysis and Calculations

In vitro data were analyzed as a 4 x 4 Latin square design using the PROC GLIMMIX procedure of SAS 9.4 (SAS Institute Inc., Cary, NC) using the following model:

$$Y_{ijk} = \mu + F_i + P_j + T_k + e_{ijk},$$

where Y_{ijk} = observations for dependent variables, μ = population mean, F_i = mean of the *i*th fermenter, P_j = mean effect of the *j*th period, T_k = mean effect of the *k*th treatment, and e_{ijk} = residual error. Period was considered a fixed effect and fermenter was considered a random effect. Contrasts comparing each treatment pairing were included. Phenolics were evaluated in a similar manner. Fermenter pH was analyzed for mean, minimum, and maximum values from d 8 to d 10. Further, pH was analyzed for area under the curve (**AUC**) and time (h) spent above a pH of 6.4, below 5.8, and between 5.8 – 6.4. Additionally, CH_4 concentration was analyzed for concentration differences between morning and evening (time) as well as a time x treatment effect. Results were reported with least squares means (**LSM**) and were considered significant when $P \leq 0.05$.

Apparent digestibilities of treatments were calculated as outlined by Soder et al. (2012):

$$\text{Apparent DM digested (\% DM)} = \frac{[\,(\text{g DMI} - \text{g effluent flow DM})}{\text{g DMI}]} \text{ x } 100.$$

4.4. Results

4.4.1. Forage Nutrient Composition

Forage and dietary treatment composition is listed in Table 4.1. No statistical comparisons of individual forages or forage treatments were made as nutrient compositions were based on single, pooled samples. In the current study, most values were similar between individual forages and dietary treatments. However, there were some notable differences in terms of carbohydrate fractions between individual forages including NFC, WSC, ESC, and starch content. The NFC content of red clover was greater, ranging from 6.50% (meadow fescue) to 30.9% (perennial ryegrass) higher when compared with other forages. Additionally, red clover starch content was 26.1% - 60.9% higher than its grass

counterparts. Conversely, the WSC and ESC content of red clover and Kentucky bluegrass was less than the other forages. Lignin content of red clover was 44.1% greater than that of Kentucky bluegrass, which contained the second largest amount of lignin of individual forages. Red clover was greater in total phenolics than all other forages, ranging from 20.0% - 51.9% higher. Ferulic acid was similar for each individual forage. However, when individual forages are combined into their respective diets, differences are diminished.

4.4.2. Treatment Digestibility

Apparent digestibilities for treatments are listed in Table 4.2. Treatment ($P < 0.0001$), period ($P < 0.0001$), and treatment x period effects ($P < 0.0001$) were found for apparent DM, OM, aNDFom, and ADF digestibilities. The apparent digestibilities of DM (56.2%), OM (63.7%), aNDFom (76.8%), and ADF (80.8%) were highest for the KYBG treatment ($P < 0.0001$). The PRG treatment was the second most digestible diet in terms of DM (50.7%), OM (57.6%), and aNDFom (74.2%). The apparent digestibilities of DM and OM did not differ between the OG and MF treatment and were lower than that of both KYBG and PRG. However, the apparent aNDFom (70.0%) and ADF (72.9%) digestibilities were lowest for the OG treatment ($P < 0.0001$). The apparent digestibility for ADF did not differ between the PRG (76.2%) and MF (77.2%) treatments.

4.4.3. pH and Temperature

Fermenter temperature was pre-programmed at 39ºC and did not differ based on fermenter, period, or their interaction. Additionally, pH did not differ between treatments for d 8 - 10 mean ($P = 0.07$), minimum ($P = 0.19$), or maximum ($P = 0.19$; Table 4.3). However, the mean ($P = 0.03$) and minimum ($P = 0.02$) fermenter pH differed based on the interaction of treatment x period. Significant period effects were observed for the mean,

minimum, and maximum pH, and the interaction of treatment x period was also significant for minimum and mean pH parameters.

The duration (h/d) and AUC (pH x min) for each treatment was analyzed; pH was evaluated for the amount of time it spent under 5.8, over 6.4, and between 5.8 and 6.4 (Table 4.3) for the collection period. Treatment with the MF diet correlated to lower pH values, leading to a greater duration of time (15.5 h/d) under a pH of 5.8 as compared with fermenters fed PRG, KYBG, or the binary OG mixture. Additionally, treatment with the MF diet inversely related to the duration of time spent above a pH of 6.4 (1.00 h/d) as compared with fermenters that were fed OG (3.40 h/d) or KYBG (4.4 h/d). Treatment with the OG correlated with higher pH values, leading to less time under or at a pH of 5.8 ($P < 0.0001$; 8.30 h/d). There were no differences between the binary, or two species, diet and the more diverse forage mixtures ($P = 0.06$) in terms of time spent in the optimal pH range. On average, pH values were below a pH of 5.8 for 11.3 h, 3.0 h above a pH of 6.4, and 9.75 h between a pH of 5.8 and 6.4.

4.4.4. Volatile Fatty Acids

Volatile fatty acid data for forage treatments, period, and period x treatment interactions is presented in Table 4.4. Total VFA production did not differ between treatments ($P = 0.14$). Individual molar proportions for acetate ($P = 0.002$), butyrate ($P = 0.022$), propionate ($P < 0.0001$), and valerate ($P = 0.009$) differed based on both treatment and treatment x period interactions. Acetate production was greater for fermenters receiving the KYBG (68.6 mol/ 100 mol) treatment as compared with those receiving OG (67.6 mol/ 100 mmol) and MF (67.1 mol/ 100 mmol). However, acetate production was similar between fermenters receiving KYBG and PRG (68.1 mol/ 100 mmol). Further,

propionate production was the same between KYBG (17.1 mol/ 100 mmol) and PRG (17.0 mol/ 100 mmol) and was also less when compared with propionate production of fermenters treated with OG and MF. This further translates to acetate: propionate and acetate + butyrate: propionate ratios. The acetate: propionate and acetate + butyrate: propionate ratios for fermenters receiving the KYBG and PRG treatments were the same as were ratios for fermenters receiving OG and MF. The acetate: propionate for fermenters receiving KYBG (4.09) and PRG (4.03) were greater than those receiving OG (3.70) and MF (3.69; $P < 0.0001$). Similar treatment relationships were observed for acetate + butyrate: propionate ratios.

4.4.5. Ammonia Nitrogen Concentration of Fermenter Effluent

There was a treatment ($P < 0.0001$), period ($P < 0.0001$), and treatment x period interaction ($P < 0.0001$) effect on NH_3-N production (Table 4.4). Fermenters receiving the PRG treatment had the greatest NH_3-N concentration (23.9 mg dL^{-1}), followed by the OG (18.4 mg dL^{-1}) treatment. Ammonia N concentration between the MF (17.6 mg dL^{-1}) and KYBG (17.8 mg dL^{-1}) treatments did not differ. However, MF and KYBG NH_3-N concentrations were lower than those of PRG and OG treatments.

4.4.6. Water Soluble Carbohydrate of Fermenter Effluent

The WSC of the effluent was impacted by treatment ($P < 0.0001$), period ($P < 0.0001$), and treatment x period ($P < 0.0001$). Fermenters fed the PRG treatment had the highest concentration of WSC (1.82 mg mL^{-1}) compared with all other treatments (Figure 4.1). Effluent from fermenters receiving the KYBG treatment had the lowest concentration of WSC (0.80 mg mL^{-1}) compared with the other treatments.

4.4.7. Fermenter Methane Concentration

Methane concentration of the fermenter headspace was affected by treatment ($P < 0.0001$). Methane concentration, expressed as mmol of CH_4 d^{-1}, was highest ($P < 0.0001$) for the KYBG (186.7 mmol d^{-1}) diet as compared with all other diet combinations (Figure 4.2). The CH_4 concentration of OG did not differ from MF ($P = 0.10$) or PRG ($P = 0.87$). Furthermore, CH_4 concentration did not differ between MF and PRG treatments ($P = 0.07$). Treatment ($P < 0.0001$) and time ($P = 0.005$) effects were observed when CH_4 concentration was analyzed for differences between treatment and time (0630 h v. 1530 h) of collection. There was no treatment x time interaction effect ($P = 0.08$) observed. There was a higher average CH_4 concentration in the evening (96.4 mmol d^{-1}) as compared with the morning (42.8 mmol d^{-1}; $P \leq 0.05$).

4.5. Discussion

4.5.1. Forage and Treatment Nutrient Composition

The nutritive value of orchardgrass was similar to that reported by Dillard et al. (2017) although WSC reported in this study were higher (15.5% DM) than that of Dillard et al. (2017; 9.3% DM). Water soluble carbohydrates are composed of ESC and remaining fructans, effectively measuring the sugar content of feedstuffs (Kramer et al., 2021). As WSC are a primary fermentation substrate for rumen microbes, it is possible that the orchardgrass utilized in this study may provide a higher concentration of energetic substrates to microbes than that of the aforementioned study. This is further supported by the fact that the net energy of lactation ($\mathbf{NE_L}$) of individual orchardgrass and the OG treatment in this study was numerically higher than that of the orchardgrass treatment of Dillard et al. (2017; 1.42 Mcal kg^{-1} v. 1.23 Mcal kg^{-1}). However, it should be noted that

although these values are different, further research is needed to determine the statistical and biological significance.

Crude protein values of individual red clover (21.1% DM) were similar to that of CP values of red clover grown during the spring (20.4% DM) in a continuous culture study conducted by Loor et al. (2003). Crude protein content of the treatments in the current study could be considered moderate quality as CP values were slightly above 18%, similar to that of Dillard et al. (2017; 17.9%) and lower than that of Bargo et al. (2003; 25.3% CP). The OM, CP, and NE_L, were similar between treatments; however, individually, Kentucky bluegrass was lower (14.4%) than the other individual forages in regard to CP and NE_L, indicating that it may not provide as much energy as the other forages. This is similar to the results of Allen et al. (2013) who found that the CP content of perennial ryegrass, quackgrass (*Elytrigia repens*), and smooth bromegrass (*Bromus inermis*) was higher than that of Kentucky bluegrass. Typically, average CP of Kentucky bluegrass is around 12% - 17% (Ball et al., 1996; Rasnake and Lacefield, 1988), indicating that the Kentucky bluegrass utilized in this experiment was also of moderate protein quality.

4.5.2. *In Vitro* Evaluation of Increased Cool-Season Grass Inclusion on Nutrient Digestibility

In a study by de Veth and Kolver (2001), the apparent DM and OM digestibility from ryegrass pastures ranged between 44.7% - 56.4% and 48.1% - 58.7%, respectively, depending on the pH, with greater digestibility occurring at greater pH. Apparent DM and OM from this study was within the aforementioned ranges with the exception of apparent OM digestibility of the KYBG treatment (63.7%) which was above the reported range. Additionally, results from this study aligned with the apparent DM digestibility results

found by Bargo et al. (2003; 47.5% - 52.2%) with the exception of KYBG. Aside from a greater apparent OM digestibility of the KYBG treatment, reported results would be expected as the pH in this study covered the pH range (5.4 – 6.6) evaluated by de Veth and Kolver (2001). However, it should be noted that fiber digestibility may be reduced as the average amount of time fermenters spent under a pH of 5.8 was 11.3 h. de Veth and Kolver (2001) reported large reductions in digestibility as pH dropped below 5.8. In this study, apparent aNDFom digestibilities were lower than that reported by Soder et al. (2013). True OM digestibilities reported by Soder et al. (2013) were close to or greater than 80%, indicating that higher levels of digestion were occurring. In the current study, true digestibility was not calculated but apparent OM digestibilities ranged from 51.5% - 63.7%, indicating a potential reduction in fiber utilization, particularly for the OG (52.8%) and MF (51.5%) treatments. This aligns with our observation that the pH of fermenters receiving the MF treatment spent the most amount of time (15.5 h/d) under a pH of 5.8. Mourino et al. (2001) indicated that low ruminal pH inhibits fiber digestibility with inhibition being particularly strong when pH values are maintained at low values for an extended period of time. This may be due to a washout of cellulolytic bacterial populations, decreasing cellulose digestion. However, Mourino et al. (2001) also found that cellulolytic bacteria continued to ferment cellulose at low pH values. These researchers indicated that the concentration of fermentation end products in their *in vitro* system was lower than that of the rumen, permitting cellulolytic bacteria to retain fermentative capacity at low pH due to reduced accumulation of VFA. This may explain the observed reduction in digestibility below a pH of 5.8 in the current study.

Apparent digestibilities of treatments in this study were lower than that of Dillard et al. (2017) although apparent digestibilities of DM, OM, aNDFom, and ADF of the KYBG treatment were similar to those reported by Dillard et al. (2017). Differences could potentially be due to a) differing dietary complexity or b) differing forage treatment composition. Dillard et al. (2017) utilized treatments composed of pure orchardgrass (single forage) and orchardgrass - warm-season annual (**WSA**; binary and tri- forages) mixtures. The apparent digestibility of pure orchardgrass from the aforementioned study was still greater than that of more complex mixtures, excluding KYBG, of this study, indicating that forage treatment complexity may not be responsible for differing digestibility. A grazing trial by Soder et al. (2006) supports this; Soder et al. (2006) found that when dairy cows were grazing pastures composed of 2, 3, 6, and 9 CSP forage species, there were no differences in DMI, milk production, or blood metabolites. This suggests that, although digestibility was not measured, forage digestibility did not differ between treatments. Further, cows were shown to prefer grasses and legumes over forb species. It could, therefore, be inferred that forage species is more impactful to digestibility and animal production than the complexity of forage diets.

As the dietary treatments utilized by Dillard et al. (2017) contained WSA, it would be expected that apparent digestibilities would be lower than those of treatments containing CSP forages as WSA are generally greater in lignin with lower leaf: stem ratios. This usually means that diets containing WSA will be lower quality and not as digestible (Dillard et al., 2017; Cowan and Lowe, 1998). However, results indicate that diets in this study, excluding KYBG, were not as digestible as diets containing WSA. Aside from WSA inclusion, differences could be related to increased inclusion of orchardgrass. Similar to

Dillard et al. (2017), Soder et al. (2013) utilized dietary treatments that included orchardgrass. Treatments in both studies included at least 50% orchardgrass; in the current study, the greatest inclusion of orchardgrass was 60% and was combined with red clover. Red clover inclusion, although steady across all treatments, may also have an impact on overall digestibility. Legumes generally contain more lignin and indigestible cell wall components. The combination of legumes and decreased inclusion of orchardgrass could explain the differences in digestibility. Further, DM digestibility was similar to apparent digestibility values reported by Ruh et al. (2018), who utilized CSP forages, alfalfa, teff grass (*Eragrostis tef*), and brown mid-rib sorghum x sudangrass (**BMRSS**) in continuous culture. However, apparent digestibility values for OM, aNDFom, and ADF in this study were generally greater than those reported by Ruh et al. (2018). This could be due to greater CP and lower aNDFom content of forage treatments in this study as compared with Ruh et al. (2018).

4.5.3. Impact of Increased Cool-Season Grass Inclusion on Fermentation Variables

pH and Volatile Fatty Acids. Fermenter pH was not regulated nor was it adjusted with HCl or NaOH as done in previous studies (Bach et al., 1999; Ruh et al., 2018). As pH was not regulated in this study, it was expected that there would be considerable variation which could explain the period effects observed for mean, minimum, and maximum pH.

A study by de Veth and Kolver (2001) indicated that the optimal mean rumen pH for cattle on a pasture-based diet was 6.35. Sauvant (1999) found that the optimal mean pH may be lower; decreased ruminal fermentation may occur at pH values that are less than 6.25 but microbial fermentation may not be affected at a pH below 6.0 for as long as four h. Furthermore, Bargo et al. (2003) found that larger, more detrimental decreases in

digestibility occur when ruminal pH is below a threshold of 5.8. In this study, mean pH of d 8 – d 10 ranged from 5.90 to 6.01, which is below the optimal pH suggested by Sauvant (1999) and de Veth and Kolver (2001) but at the optimum range based on the results of Bargo et al. (2003). In the current study, pH was below a threshold of 5.8 for a minimum of 8.3 h for fermenters receiving the OG treatment and a maximum of 15.5 h for fermenters receiving the MF treatment. It is likely that digestibility of all treatments was negatively impacted due to increased time under a pH of 5.8. However, compared with other treatments, the apparent DM and OM digestibilities of the MF and OG treatments were lower than that of KYBG and PRG, indicating fermenters receiving the binary treatment or treatment supplemented with only meadow fescue may be more negatively impacted.

Mean pH values reported in this study were lower than those reported for other forage-based diets (Dillard et al., 2017; Ruh et al., 2018). Higher pH values were expected in this study as forage-based diets tend to producer higher pH values as compared with TMR-based diets as starch content of forage-based diets is not as high (Hafla et al., 2014; Wales et al., 2009). As mentioned previously, differences may be due to differences in dietary components (WSA v. CSP forages). However, it could also potentially be due to lower fiber content and higher plant ESC and WSC of the current treatments, particularly the MF treatment. Van Soest (1994) indicated that low fiber diets lower ruminal pH through increased acid production and increased digestibility. This is in direct contrast with apparent aNDFom digestibilities of MF, which were lower than that of KYBG and PRG. Kolver and de Veth (2002) indicated that lower ruminal pH of animals consuming pasture-based diets increased rumen microbial N flow, DMI, total and individual VFA concentrations, and milk components.

Although apparent digestibilities were lower for the OG and MF treatments, individual proportions of propionate were greater than that KYBG and PRG, which would lower ruminal pH. It should be noted that the study by Kolver and de Veth (2002) had large variations, meaning that the aforementioned production variables may not be reliable for predicating animal performance on pasture-based diets. The increased production of propionate in diets containing meadow fescue as the only supplemental forage may be decreasing pH for prolonged periods of time. The shift from acetate to propionate is potentially due to a shift from fiber digestion to utilization of rapidly fermentable carbohydrates, such as WSC and ESC. This is further supported by the concentration of WSC residue measured from effluent in fermenters receiving the MF diet (1.15 mg mL^{-1}), lower than the WSC residues from PRG and OG treatments. Depending on how low pH values drop below 5.8 could prove to be detrimental to animal health and production. However, inclusions of meadow fescue may be beneficial from an energetics standpoint if included in the correct ratio or combined with another forage.

Compared with the treatment in this study that utilized perennial ryegrass, total VFA concentrations were similar (65.6 mM v. 62.3 mM) to those described by de Veth and Kolver (2001). Results from this study also aligned with those of Dillard et al. (2017) although total VFA amounts (mM) were slightly lower. The acetate: propionate ratios of this study were greater than those of Dillard et al (2017). This could potentially be due to differing diet composition; CSP forages are usually more digestible than the warm-season forages used by Dillard et al. (2017). However, digestibility data presented in this study indicated that diets in this study were not as digestible as those used by Dillard et al. (2017). Ruh et al. (2018) also reported higher total VFA (mM) in diets that contained CSP forages

or WSA. Individual acetate, propionate, and butyrate concentrations were also higher than the values determined in this study. However, the BMRSS treatment utilized by Ruh et al. (2018) aligned with reported individual VFA in this study. It could be inferred that CSP forage treatments in this study could produce similar digestibility patterns to BMRSS. It could also be due to the amount of DM allotted to each fermenter or "pasture allowance." Values in this study aligned with those of Bargo et al. (2003), particularly with the low-pasture treatment. Using continuous culture, Bargo et al. (2003) compared different pasture allotments and found total VFA (mM) that were similar to those reported in this study. However, individual proportions of VFA reported in this study more closely aligned with those in the medium pasture treatment or pasture and concentrate treatment.

Bargo et al. (2003) indicated that an inverse relationship existed between VFA production and pH patterns for pasture-based diets with pH decreasing as DMI and VFA increased. A relationship was not found in this study which was similar to the findings of de Veth and Kolver (2001). The KYBG and PRG treatments had higher acetate: propionate and acetate + butyrate: propionate ratios than the MF and OG treatments which may be related to apparent digestibilities. As mentioned previously, the apparent DM, OM, and aNDFom digestibilities for KYBG and PRG were higher than that of the other treatments, indicating that these diets are more digestible, therefore producing more VFA. Ratios of acetate: propionate and acetate + butyrate: propionate were all above the typical 3:1 ratio seen in pasture diets, indicating a shift away from propionate production to acetate production. This shows that there was an overall shift towards fiber digestion.

4.5.4. Relationship of Plant and Effluent Carbohydrates with Methane Production

Rapid Fermentation of Non-structural Carbohydrates. As previously mentioned, the WSC concentration from effluent of fermenters fed KYBG was lower (0.80 mg mL^{-1}) than all other treatments. This could indicate that WSC from the KYBG treatment are 1) being utilized more rapidly or 2) entrapped within a fiber matrix. Our CH_4 findings are more aligned with the theory that WSC from the KYBG are being utilized rapidly. As seen in Figure 4.2, the CH_4 concentration of fermenters receiving KYBG was greatest (186.7 mmol d^{-1}) as compared with the other treatments (19.0 mmol d^{-1} – 50.7 mmol d^{-1}), respectively. This shows an inverse relationship; as WSC content decreased, CH_4 production increased. This is in agreement with the results of Purcell et al. (2014). Using a batch culture system, Purcell et al. (2014) evaluated the effects of different types and concentrations of WSC on rumen CH_4 concentration when fed with perennial ryegrass. It was found that the type of WSC (sucrose, inulin, or a combination) did not impact CH_4 production; however, increasing WSC decreased CH_4 production. This trend was mirrored in our results; rumen effluent from fermenters receiving PRG contained the greatest amount of WSC (1.83 mg mL^{-1}) but conversely had the lowest CH_4 output (19.0 mmol d^{-1}). This could indicate that increasing WSC content could decrease enteric CH_4 emissions.

Rumen fluid outflow from fermenters receiving OG and MF had a lower WSC content (1.39 mg mL^{-1} and 1.15 mg mL^{-1} respectively) when compared with the PRG treatment but they were still greater than fermenters receiving KYBG. Fermenters receiving the KYBG diet may potentially produce more CH_4 due to higher proportions of butyrate as compared with OG and MF as butyrate is known to increase available hydrogen

(**H_2**) in the rumen. This is further utilized by methanogens, producing CH_4 (Moss et al., 2000; Hafla et al., 2014).

Entrapment of Nutrients in the Fiber Matrix. Although CH_4 results indicate that WSC may be rapidly utilized by rumen microbes, it is also plausible that WSC are entrapped within fibrous carbohydrates. Grant and Cotanch (2012) proposed that larger NDF particles from orchardgrass reduces rumen passage rate, leading to decreased nutrient availability, including WSC. In this study, the ratio of WSC in the PRG treatment was lower than that of the other treatments (Table 4.2). This could indicate that there may potentially be fewer fructans, which are not as easily enzymatically digested (Villalba et al., 2021). However, in this study, plant fructan content of both PRG and KYBG diets was higher than that of MF and OG. It could be theorized that entrapment of nutrients, such as WSC, is possible. This is further supported by the results of total plant phenolics and ferulic acid content. The PRG treatment had higher total plant phenolics and ferulic acid when compared with the other treatments. This indicates that lignin and lignin bonds may be actively entrapping nutrients in the PRG treatment and limiting energy use in the rumen (Casler, 2001). This is further supported by the findings of Casler and Jung (2006) who found that the content of ferulated cross-linkages were more pronounced for NDF digestibility after 96 h. It is thought that ferulic acid may inhibit fiber digestion by acting as a physical barrier (Wong et al., 2019) or may exhibit toxic effects (Borneman et al., 1986). Additionally, the NH_3-N concentration of the PRG was higher (23.9 mg dL^{-1}) than all other forage treatments, indicating that carbohydrate availability is more likely the limiting factor of this diet.

Methane Concentration Based on Time of Collection. In this study, greater amounts of CH_4 were measured in the evening samples as compared with the morning samples. This is similar to the findings of Dillard et al. (2017), who found that CH_4 concentration was lowest at the 0900 h collection timepoint. Hafla et al. (2014) had similar findings; CH_4 production was lowest when collected at the 0730 h timepoint. This was attributed to depletion of forage substrate within the vessel as feeding occurred 12 h previously. This would likely hold true for the current study as well as there was almost 13.5 h between feedings. Furthermore, it is possible the nutrient digestibilities decreased during the morning as substrate was decreased. Unlike Dillard et al. (2017) and Hafla et al. (2014), samples in this study were only collected once in the morning before feeding and once in the evening before feeding. More collection timepoints would need to be utilized to detect diurnal fluctuations for future studies.

4.6. Conclusions

Feeding diets that consist of orchardgrass and red clover may decrease aNDFom and ADF digestibility. However, inclusion of meadow fescue as the only supplemental forage may also lead to potential issues if not included in the correct ratios. Although treatments supplemented with meadow fescue alone may improve individual VFA proportions, there appears to be a risk for depressed pH, which could potentially decrease animal productivity in an *in vivo* setting. Further, treatments including meadow fescue as the only supplemental forage do not appear to be as digestible in terms of DM, aNDFom, and OM as more diverse forage mixtures. Treatments containing Kentucky bluegrass appear to provide the most energy in an *in vitro* setting and are more digestible than the other treatments; however, it is critical to find the correct balance as 20% inclusion of

Kentucky bluegrass increased CH_4 concentration. Additionally, although our results indicate that inclusions of 15% Kentucky bluegrass may be enough to improve animal energetics when balanced with another forage such as perennial ryegrass, it should be realized that the quality of Kentucky bluegrass may not remain consistent across the grazing season. The quality of Kentucky bluegrass, and all forages utilized in this study, only represents forage quality metrics from a single point in time, which may not be truly representative of pasture quality over the entire grazing season. Our findings indicate that increased CSP grass inclusion could be beneficial to grazing dairy cattle if pastures are managed carefully. Additional research is needed to evaluate the impacts and assess the full potential of increased CSP forage use in diets on performance and productivity of pasture-based dairy cattle. Furthermore, inclusions of perennial ryegrass and meadow fescue should be further evaluated using *in vivo* models to evaluate animal performance. Finally, research should be expanded to evaluate economic and environmental impacts that may occur during grazing.

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4.9. Tables

Table 4.1. Chemical composition, as a percentage of dry matter (DM), of individual forages and forage treatments. Treatments include on a DM-basis: OG: 40% red clover + 60% orchardgrass; MF: 40% red clover + 30% orchardgrass + 30% meadow fescue; KYBG: 40% red clover + 20% orchardgrass + 20% meadow fescue + 20% Kentucky bluegrass; PRG: 40% red clover + 15% orchardgrass + 15% meadow fescue + 15% Kentucky bluegrass + 15% perennial ryegrass.

	Ingredient					Treatment			
Item, (% of DM)	Orchardgrass	Red Clover	Meadow Fescue	Kentucky Bluegrass	Perennial Ryegrass	OG	MF	KYBG	PRG
OM	88.5	90.0	89.5	85.5	87.7	89.1	89.4	88.7	88.7
CP	18.9	21.1	19.8	14.4	17.5	19.8	20.0	19.0	19.0
aNDFom[1]	46.6	41.4	43.7	49.3	49.2	44.6	43.7	44.4	44.8
ADF	30.9	33.4	27.7	35.3	32.2	31.9	30.8	31.9	32.1
Lignin	3.80	8.40	3.10	4.70	3.50	5.62	5.30	5.57	5.51
NFC[2]	18.7	23.3	21.8	18.9	16.1	20.5	21.5	21.2	20.6
WSC[3]	15.5	8.30	14.4	9.80	11.2	12.7	12.4	11.4	11.0
ESC[4]	8.80	5.10	9.90	6.20	9.30	7.34	7.77	7.11	7.25
Fructans[5]	6.70	3.20	4.50	3.60	1.90	5.36	4.63	4.41	3.99
Starch	1.20	2.30	0.90	0.90	1.70	1.63	1.51	1.49	1.60
NSC[6]	16.7	10.6	15.3	10.7	12.9	14.3	13.9	13.5	13.3
NE_L, mcal/ kg[7]	1.46	1.37	1.54	1.19	1.44	1.42	1.45	1.39	1.39
Total Phenolics	1.65	3.43	1.97	1.81	2.75	2.35	2.42	2.43	2.57
Ferulic Acid	0.12	0.17	0.13	0.12	0.17	0.13	0.14	0.14	0.15
P	0.28	0.27	0.30	0.26	0.32	0.28	0.28	0.28	0.38
Mg	0.16	0.24	0.15	0.19	0.21	0.19	0.19	0.19	0.20
K	2.20	2.14	2.74	1.71	2.45	2.18	2.36	2.20	2.23
Na	0.04	0.01	0.03	0.04	0.14	0.03	0.02	0.03	0.04

[1]aNDFom: NDF organic matter after amylase treatment and ashing.
[2]NFC: non-fiber carbohydrate. Calculated as described by Dillard et al. (2017); 100 – (CP% + aNDFom% + ether extract% + ash%).
[3]WSC: water soluble carbohydrates.

[4]ESC: ethanol soluble carbohydrates.
[5]Fructans: Calculated as described by Kagan et al. (2014); WSC – ESC.
[6]NSC: non-structural carbohydrates. Calculated as described by Dillard et al. (2017); starch + water soluble carbohydrates.
[7]Estimated by the NRC (2001) model.

Table 4.2. Apparent nutrient digestibilities of red clover and red clover- cool season grass mixtures fed during continuous culture fermentation. Treatments on a DM-basis included: OG: 40% red clover + 60% orchardgrass; MF: 40% red clover + 30% orchardgrass + 30% meadow fescue; KYBG: 40% red clover + 20% orchardgrass + 20% meadow fescue + 20% Kentucky bluegrass; PRG: 40% red clover + 15% orchardgrass + 15% meadow fescue + 15% Kentucky bluegrass + 15% perennial ryegrass.

Item	Treatment				SEM	*P*-value		
	OG	MF	KYBG	PRG		Treatment	Period	Treatment x Period
Apparent Digestibility								
DM[1], %	47.0[c]	46.1[c]	56.2[a]	50.7[b]	0.99	<0.0001	<0.0001	<0.0001
OM[2], %	52.8[c]	51.5[c]	63.7[a]	57.6[b]	1.13	<0.0001	<0.0001	<0.0001
aNDFom[3], %	58.9[d]	62.4[c]	66.4[a]	64.0[b]	0.88	<0.0001	<0.0001	<0.0001
ADF[4], %	62.9[d]	68.9[b]	72.2[a]	66.7[c]	0.98	<0.0001	<0.0001	<0.0001

[1]DM: dry matter.
[2]OM: organic matter.
[3]aNDFom: NDF organic matter after amylase treatment and ashing.
[4]ADF: acid detergent fiber.
[a-d]Within row, different letters indicate significant differences ($P \leq 0.05$).

Table 4.3. Fermenter pH of orchardgrass-red clover and orchardgrass-red clover mixtures during continuous culture fermentation. Treatments on a DM-basis included: OG: 40% red clover + 60% orchardgrass; MF: 40% red clover + 30% orchardgrass + 30% meadow fescue; KYBG: 40% red clover + 20% orchardgrass + 20% meadow fescue + 20% Kentucky bluegrass; PRG: 40% red clover + 15% orchardgrass + 15% meadow fescue + 15% Kentucky bluegrass + 15% perennial ryegrass.

	Treatment					*P*-value[1]		
Item[2]	OG	MF	KYBG	PRG	SEM	Treatment	Period	Treatment x Period
pH								
Mean	6.01	5.89	5.98	5.90	0.03	0.07	<0.0001	0.03
Minimum	5.60	5.52	5.62	5.58	0.02	0.19	<0.0001	0.02
Maximum	6.67	6.58	6.62	6.79	0.05	0.19	<0.0001	0.22
Range[3]	1.07	1.06	1.00	1.21	0.04	0.10	<0.0001	0.07
pH < 5.8								
Duration, h/d	8.30^{c}	15.5^{a}	9.70^{bc}	11.6^{b}	1.56	0.0004	<0.0001	0.02
Total AUC[4], pH x h	46.5^{b}	86.6^{a}	53.6^{b}	64.1^{b}	8.75	0.0004	<0.0001	0.02
pH > 6.4								
Duration, h/d	3.40^{a}	1.00^{b}	4.40^{a}	3.20^{ab}	0.72	<0.0001	<0.0001	<0.0001
Total area, pH x h	19.5^{a}	4.30^{b}	26.8^{a}	18.6^{ab}	4.71	0.03	<0.0001	0.005
5.8 ≤ pH ≤ 6.4								
Duration, h/d	12.3	7.50	9.90	9.30	0.99	0.07	<0.0001	0.15
Total AUC, pH x h	71.2	41.5	56.6	52.2	6.15	0.06	<0.0001	0.19

[1]*P*-values less than or equal to 0.05 are considered significant.
[2]Values are calculated based on averages from d 8 – d 10.
[3]Range is calculated as maximum pH – minimum pH.
[4]AUC: area under the curve.
[a-c]Within row, different letters indicate significant differences ($P \leq 0.05$).

Table 4.4. Fermenter effluent volatile fatty acid (VFA) molar proportions, concentrations, and ratios and effluent ammonia nitrogen (NH_3-N) concentration of orchardgrass-red clover and orchardgrass-red clover mixtures during continuous culture fermentation. Treatments on a DM-basis included: OG: 40% red clover + 60% orchardgrass; MF: 40% red clover + 30% orchardgrass + 30% meadow fescue; KYBG: 40% red clover + 20% orchardgrass + 20% meadow fescue + 20% Kentucky bluegrass; PRG: 40% red clover + 15% orchardgrass + 15% meadow fescue + 15% Kentucky bluegrass + 15% perennial ryegrass.

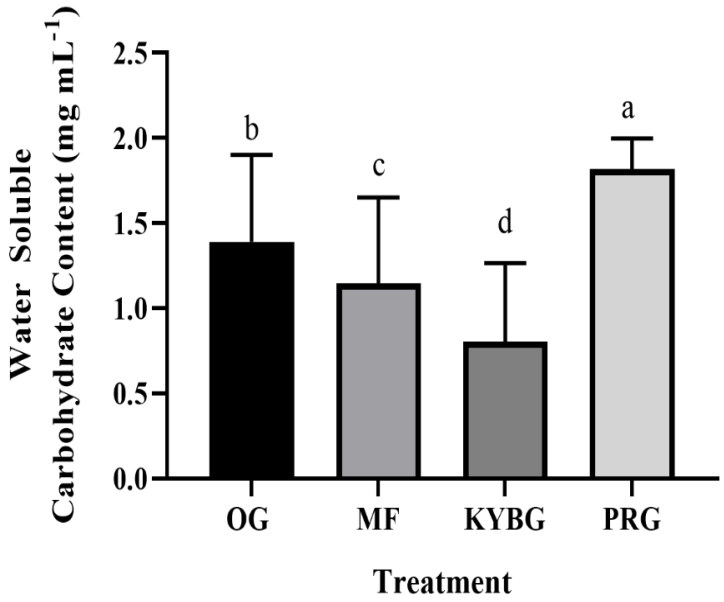
	Treatment					*P*-value[1]		
Item	OG	MF	KYBG	PRG	SEM	Treatment	Period	Treatment x Period
Total VFA, mM	67.5	59.6	59.3	65.6	2.09	0.14	0.08	0.61
Individual VFA, mol/ 100 mol								
Acetate (A)	67.6^{bc}	67.1^{c}	68.6^{a}	68.1^{ab}	0.32	0.002	<0.0001	<0.0001
Propionate (P)	18.4^{a}	18.3^{a}	17.1^{b}	17.0^{b}	0.38	<0.0001	<0.0001	<0.0001
Butyrate (B)	10.0^{b}	10.5^{a}	10.4^{a}	10.6^{a}	0.13	0.022	<0.0001	<0.0001
Isobutyrate	0.79	0.75	0.78	0.79	0.01	0.51	<0.0001	0.009
Valerate	2.42^{b}	2.64^{a}	2.39^{b}	2.71^{a}	0.08	0.009	0.02	0.001
Isovalerate	0.80	0.72	0.79	0.82	0.02	0.21	<0.0001	0.01
A:P	3.70^{b}	3.69^{b}	4.09^{a}	4.03^{a}	0.11	<0.0001	<0.0001	<0.0001
A+B:P	4.25^{b}	4.26^{b}	4.71^{a}	4.65^{a}	0.12	<0.0001	<0.0001	<0.0001
NH_3-N, mg dL^{-1}	18.4^{b}	17.6^{c}	17.8^{c}	23.9^{a}	1.14	<0.0001	<0.0001	<0.0001

[1]*P*-values less than or equal to 0.05 are considered significant.

[a-c]Within row, different letters indicate significant differences ($P \leq 0.05$).

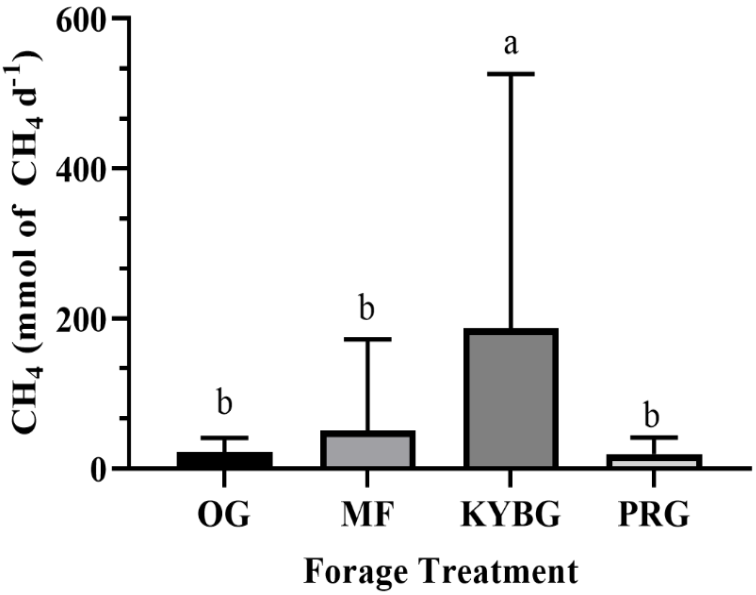
4.10. Figures

Figure 4.1. Fermenter water soluble carbohydrate (WSC) concentration of orchardgrass-red clover and orchardgrass-red clover mixtures during continuous culture fermentation. Units are presented as mg mL^{-1}. Error bars represent SEM. Treatments on a DM-basis: OG: 40% red clover + 60% orchardgrass; MF: 40% red clover + 30% orchardgrass + 30% meadow fescue; KYBG: 40% red clover + 20% orchardgrass + 20% meadow fescue + 20% Kentucky bluegrass; PRG: 40% red clover + 15% orchardgrass + 15% meadow fescue + 15% Kentucky bluegrass + 15% perennial ryegrass.



[a-d]Means with different superscripts are different at $P \leq 0.05$.

Figure 4.2. Fermenter methane (CH_4) concentration of orchardgrass-red clover and orchardgrass-red clover mixtures during continuous culture fermentation. Units are presented as mmol of CH_4 d^{-1}. Error bars represent SEM. Treatments on a DM-basis: OG**:** 40% red clover + 60% orchardgrass; MF**:** 40% red clover + 30% orchardgrass + 30% meadow fescue; KYBG: 40% red clover + 20% orchardgrass + 20% meadow fescue + 20% Kentucky bluegrass; PRG: 40% red clover + 15% orchardgrass + 15% meadow fescue + 15% Kentucky bluegrass + 15% perennial ryegrass.



[a-b]Means with different superscripts are different at $P \leq 0.05$.

4.11. Supplementary Tables

Table S4.1. Forage cultivars used for continuous culture experiment.

Forage Variety				
Orchardgrass (*Dactylis glomerata*)	Red Clover (*Trifolium pratense*)	Meadow Fescue (*Festuca pratensis*)	Kentucky Bluegrass (*Poa pratensis*)	Perennial Ryegrass (*Lolium perenne*)
Echelon	Arlington	Fleet	Balin	Calibra
Harvestar	Freedom	Macbeth	Ginger	Kentaur
Husar	Manitoba	Montana		Remington
Inavale	Milvus			Tivoli
Luxor	Ruby			Tomaso[a]
Niva				Toronto
Olathe				
Otello				

[a]All perennial ryegrass cultivars are tetraploid except Tomaso, a diploid variety.

Table S4.2. Average growing degree days (GDD) for the 2019 – 2020 growing and harvesting season. Historical averages are for 30 yr of data provided by the NOAA (1981 – 2010) for Burlington, VT. Temperature units are expressed in Fahrenheit (ºF) and precipitation units in inches.

Item	**2019**				
Alburgh, VT	August	September	October	November	December
Average temperature (°F)	68.3	60.0	50.4	31.2	26.0
Departure from normal	-0.5	-0.5	2.3	-6.8	0.5
Precipitation (inches)	3.5	3.9	6.3	2.4	1.3
Departure from normal	-0.4	0.2	2.8	-0.7	-1.1
GDD, 50 - 86°F	568.2	335.2	145.9	7.2	1.0
Departure from normal	-13.8	-24.8	13.9	-26.9	-3.1
GDD, 44 - 86°F	753.1	489.6	256.8	22.6	7.5
Departure from normal	-9.9	-19.4	25.8	-52.4	-5.5
GDD, 32 - 90°F	1125.1	839.6	571.0	127.7	66.9
Departure from normal	-16.0	-15.5	58.0	-122.3	-13.1
GDD, 32 - 95°F	1125.1	839.6	571.0	127.7	66.9
Departure from normal	-15.0	-15.5	58.0	-122.3	-13.1
GDD, 41 - 86°F	846.1	571.8	320.3	38.7	14.5
Departure from normal	-8.9	-19.2	28.3	-67.3	-6.5

Table S4.2. continued. Average growing degree days (GDD) for the 2019 – 2020 growing and harvesting season. Historical averages are for 30 yr of data provided by the NOAA (1981 – 2010) for Burlington, VT. Temperature units are expressed in Fahrenheit (ºF) and precipitation units in inches.

Item	**2020**									
Alburgh, VT	January	February	March	April	May	June	July	August	September	October
Average temperature (°F)	23.5	21.8	35.0	41.6	56.1	66.9	74.8	68.8	59.2	48.3
Departure from normal	4.6	0.4	3.9	-3.2	-0.4	1.1	4.2	0.0	-1.3	0.2
Precipitation (inches)	2.6	1.2	2.8	2.1	2.4	1.9	3.9	6.8	2.8	3.6
Departure from normal	0.6	-0.5	0.6	-0.7	-1.0	-1.8	-0.3	2.9	-0.9	0.0
GDD, 50 - 86°F	0.3	0.0	15.7	43.7	297.6	515.7	751.3	583.9	336.0	125.9
Departure from normal	-2.8	-2.0	-6.4	-67.4	5.6	34.7	121.3	1.9	-24.0	-6.1
GDD, 44 - 86°F	3.3	2.8	43.4	101.2	424.4	679.5	937.3	767.4	485.1	224.8
Departure from normal	-4.8	-5.3	-5.6	-83.9	-0.6	31.5	123.3	4.4	-23.9	-6.2
GDD, 32 - 90°F	36.5	48.4	193.1	315.2	745.8	1044.4	1322.7	1141.1	815.8	520.7
Departure from normal	-11.5	-7.7	27.1	-98.8	-13.2	31.4	125.7	0.0	-39.3	7.7
GDD, 32 - 95°F	36.5	48.4	193.1	315.2	746.4	1046.4	1326.0	1141.1	815.8	520.7
Departure from normal	-11.5	-7.7	27.1	-98.8	-12.7	35.4	132.0	1.0	-39.3	7.7
GDD, 41 - 86°F	4.8	6.1	66.3	143.6	497.4	765.9	1030.3	860.4	564.2	290.8
Departure from normal	-7.3	-7.9	-1.8	-88.5	-3.6	28.9	123.3	5.3	-26.8	-1.2

Table S4.3. Chemical composition of basal total mixed ration (TMR) diet from the bunk fed to donor animals. Values are reported as a percentage of DM unless otherwise stated.

Item (% DM)	TMR	Topdress
OM	92.7	91.3
CP	17.4	24.2
aNDFom	29.3	13.0
ADF	20.8	7.60
NFC	40.4	44.8
WSC	5.83	7.13
ESC	2.25	6.38
Starch	25.4	30.7
NSC	31.2	37.9
NE_L, mcal/ kg	0.81	2.18
Ca	0.81	1.82
P	0.36	0.49
Mg	0.36	0.27
K	0.97	0.93
Na	0.40	0.20

CHAPTER 5: *IN VITRO* AND ECONOMIC EVALUATION OF LEGUME AND WARM-SEASON ANNUAL FORAGE MIXTURES FOR USE IN NORTHEASTERN UNITED STATES PASTURE SYSTEMS

5.1. Abstract

The objectives of this study were to 1) evaluate diets composed of warm-season annual (WSA) and legumes on *in vitro* rumen fermentation characteristics and 2) complete a basic cost assessment associated with increased forage inclusion. Forages were harvested from larger experimental plots and included orchardgrass (*Dactylis glomerata*), alfalfa (*Medicago sativa*), pearl millet (*Pennisetum glaucum*), sorghum-sudangrass (*Sorghum x drummondii*), or red clover (*Trifolium pratense*). Warm-season annuals were harvested when at their peak in terms of forage quality whereas perennial species utilized in this study were harvested when at lower quality. For the *in vitro* portion of this study, a 4-unit dual-flow continuous culture fermenter system was utilized. Dietary treatments were assessed in a 4 x 4 Latin square design and fermenters each received one replicate of each treatment across the four periods. Within period, fermenters were adapted to treatments for 7 d followed by three d of sample collection (d 8 - 10). Diets on a dry matter (DM)-basis were 1) 50% orchardgrass + 50% alfalfa (OG-ALF); 2) 25% orchardgrass + 25% alfalfa + 50% millet (MIL); 3) 25% orchardgrass + 25% alfalfa + 50% sorghum-sudangrass (SUD); and 4) 25% orchardgrass + 25% alfalfa + 50% red clover (RC). Feed was added to fermenters four times daily (0600 h, 0720 h, 1800 h, and 1920 h), totaling 105 g DM d^{-1}. Fermenter pH and temperature were measured continuously. Outflow effluent samples and methane (CH_4) samples were collected on d 8, 9, and 10 for analysis of volatile fatty acid (VFA) profiles, ammonia nitrogen (NH_3-N) concentrations, and water soluble carbohydrate

(WSC) content. Rumen fluid and forage samples were analyzed with wet chemistry for DM, organic matter (OM), crude protein (CP), aNDFom (amylase- and sodium sulfite-treated neutral detergent fiber on an organic matter basis), acid detergent fiber (ADF), WSC, and ethanol soluble carbohydrate (ESC) content. A preliminary assessment of cost feasibility was also performed by conducting a simple cost analysis in which variable and fixed costs for establishing red clover and warm-season annuals was evaluated. Results were analyzed by PROC GLIMMIX of SAS. Fermentation parameters did not differ between treatments; however, fermenters receiving the binary OG-ALF treatment had the highest CH_4 concentration. The CH_4 concentration of fermenters receiving RC, MIL, and SUD did not differ from one another but these treatments had lower CH_4 concentrations than OG-ALF. Effluent from OG-ALF contained the highest concentration of WSC. The apparent DM digestibilities of OG-ALF and MIL were higher than those of SUD and RC whereas the inverse was true for apparent OM digestibility. The SUD treatment had the highest aNDFom and ADF apparent digestibility with OG-ALF being the least digestible. Red clover establishment was more expensive than warm-season establishment. However, WSA produced less DM ac^{-1} and were more expensive when standardized for CP content. These results indicate that WSA may be useful to producers in terms of animal production and performance in the Northeast, as they perform in a similar manner to cool-season forages and may even lower CH_4 output. Cost of production may be a disadvantage to producers; however, they are still a viable option for NE producers when considering the impacts of summer slump on perennial forage quantity and quality.

Key Words: pasture-based dairy, forage diversity, fermentation kinetics, cost feasibility

5.2. Introduction

Monoculture production has long been used as a strategy to maximize forage production for grazing animals (Hammond et al., 2014). Many producers choose to utilize monoculture systems as they are relatively simple to manage as compared to more diverse pastures (Muir et al., 2011). Further, from an economic perspective, single-commodity production systems are advantageous as producers can maximize profit through higher throughput and yields using more specialized resources for forage-specific management (pest control, maintenance, and harvest; Soder et al., 2009). However, monoculture pastures can deplete the soil of nutrients that play key roles in soil fertility (Vazquez et al., 2020). Diverse pasture systems, which are gaining in popularity, have known and perceived benefits including improved animal welfare (Washburn et al., 2002), better producer quality of life (Jackson-Smith et al., 1996), and enhanced environmental and grazing system sustainability (Sanderson et al., 2005). Increased forage diversity has been shown to increase herbage yield and improve dry matter (**DM**) production, which, in turn provides a high-quality feed source in greater abundance to grazing animals (Pembleton et al., 2015). However, results have been mixed. Sanderson et al. (2005) indicated that binary forage mixtures produced less herbage (kg ha^{-1} DM) than more diverse mixtures. Regardless of pasture diversity, there was little benefit to DM production. Combined, these results suggest that DM production and herbage yield may be more dependent on the species of forages within a diverse mixture rather than the specie number within a diverse pasture.

Knowing that species identity could potentially be an important factor to support an increase in the productivity of pasture-based dairy systems, it is important to identify species that will help producers meet their production goals (Pembleton et al., 2015). Cool-

season grasses, such as orchardgrass (*Dactylis glomerata*) and timothy (*Phleum pratense*), and legumes, such as red clover (*Trifolium pratens*e*)* and alfalfa (*Medicago sativa*), are commonly utilized pasture species on farms in temperate climates, providing high-quality forage for grazing dairy cattle (Dillard et al., 2017; Ribeiro et al., 2005). Due to their growth physiology (C_3 photosynthesis), cool-season grasses and legumes decrease in quality from June until September in the Northeastern (**NE**) United States (**U.S.**) as temperatures increase and precipitation decreases with this period being known as "summer slump" (Tracy et al., 2010; Billman et al., 2022). Cool-season annuals, such as oat (*Avena sativa*) have traditionally filled this gap in forage quality and quantity (Darby, 2009; Billman et al., 2022). However, cool-season annuals are also susceptible to summer slump, indicating that there is a need for alternative forages. Additionally, the decline in quality and quantity of cool-season forages could prove detrimental to organic, grassfed (**OGF**) dairy cattle as OGF certified dairy cattle must receive 60% of their DM from forages and pastures and must be on pasture for a minimum of 150 d. Pastures based entirely on cool-season forages may not be enough to sustain OGF dairy cattle DMI, which could prove to be detrimental of producers unless other feeding strategies are utilized. Therefore, NE dairy producers are beginning to utilize warm-season annuals (**WSA**), such as sorghum (*Sorghum bicolor*), sudangrass (*Sorghum x drummondii*), sorghum x sudangrass hybrids, pearl millet (*Pennisetum glaucum*) and Japanese millet (*Echinochloa esculenta*). These WSA may be able to maintain pasture production (Ruh et al., 2018) and add flexibility to pasture systems when forage quality decreases during the summer months (Moore et al., 2004; Berdahl et al., 2001).

While there has been research conducted examining tropical legume incorporation and adaptation, little research has been conducted that focuses on WSA (Dillard et al., 2018). Despite the high cost of annual forage establishment and increased risk of stand failure, it has been common practice to include WSA in pasture-based systems in other regions of the U.S. (Birnk and Fairbrother, 1988). Warm-season annuals possess greater drought tolerance and grow better in warmer temperatures than their cool-season counterparts due to their different photosynthetic classifications (C_4; Tracy et al., 2010). An advantage of utilizing WSA, aside from the ability to promote forage growth during the summer, is their rapid establishment and greater nutritive value compared with perennial warm-season forages (Cheeke, 1995; Reid et al., 1988). Further, warm-season forages complement cool-season forages in grazing systems. Research in the Great Plains region has shown that warm-season species (corn, pearl millet, forage sorghum, sudangrass, and sorghum x sudangrass) interseeded with tall fescue (*Festuca arundinacea*) and smooth bromegrass *(Bromis inermis*) doubled forage availability (Guretzky et al., 2020). This indicates that WSA productivity is comparable to that of cool-season grasses should growing conditions be favorable. This could prove to be an invaluable strategy to organic, OGF dairy producers. However, WSA grasses need to be reestablished yearly, which can prove to be costly (Comerford et al., 2005). There may be added benefit to including WSA in a grazing operation but the tradeoffs in the forms of land and milk production would need to be further evaluated (Ritz et al., 2021).

Apart from select few studies that have evaluated the effects of forage and pasture complexity on grazing animal performance in the NE U.S. (Soder et al., 2006), there is little information available on animal production, performance, and forage digestibility of

ruminants diets that include WSA. This is partially due to the lack of research about the compatibility and establishment of WSA in NE grazing systems (Dillard et al., 2018). The studies that are available are limited to experiments that utilize a single legume and single grass mixture (Rutter et al., 2004; Ruh et al., 2018), do not incorporate legumes (Dillard et al., 2017), or produce contradictory results. Additionally, forages utilized in the aforementioned studies were harvested at peak quality and quantity, dissimilar from the perennial forages utilized in this study. We hypothesized that inclusion of WSA in forage-based diets assessed using *in vitro* continuous culture systems would maintain a high digestibility, supporting energy availability in the rumen while decreasing energy (methane; **CH_4**) losses compared with perennial forages mixtures that begin to "slump" in quality and quantity in the summer months. These benefits were hypothesized to be worth the additional cost of WSA planting and land use. The primary objectives of this experiment were to 1) evaluate rumen fermentation kinetics in response to WSA and legume inclusion using *in vitro* continuous culture systems and 2) complete a basic assessment to determine what forage combinations would be most beneficial to producers when considering economic, production and environmental perspectives.

5.3. Materials and Methods

5.3.1. Site Description

All forages were established at Borderview Research farm in Alburgh, VT (45.01º N, -73.31º W) as previously described and outlined in variety trial reports published by the University of Vermont Northwest Crops and Soils Program (Darby et al., 2020a; Darby et al., 2020b). Forages of interest included orchardgrass, alfalfa, red clover, pearl millet, and sorghum x sudangrass. Forage cultivars used in this study are listed in Supplementary

Materials, Table S5.1. Each grass and legume species were established as monocultures on plots that were 9.3 m^2 and were part of other small-plot experiments. All plots were moldboard plowed, disked, and spike tooth harrowed prior to planting. Weather data was recorded with a Davis Instruments Vantage Pro2 weather station (Hayward, CA). This station was equipped with a WeatherLink data logger (Davis Instruments; Hayward, CA). Average growing degree days (**GDD**) for the 2018 – 2019 growing and harvesting season are listed in Supplementary Materials, Table S5.2.

5.3.2. Grass Management and Harvest

Orchardgrass was planted in the late summer of 2017 using a cone seeder (Great Plains; Salina, KS). Warm-season annual species, including sorghum x sudangrass and pearl millet, were planted and established in June 2019 using a grain drill (Sunflower; Duluth, GA). Millet was seeded at a rate of 9.1 kg ac^{-1} and sorghum x sudangrass at 22.7 kg ac^{-1}. Soil for WSA and orchardgrass was a Benson rocky silt loam over shaly limestone. Crops planted previous to the WSA in the utilized plot areas were soybeans and hemp. Warm-season annuals were fertilized with 22.7 kg N ac^{-1} via Probooster 10-0-0 (North Country Organics) while orchardgrass plots received approved organic fertilizer inputs based on prior soil testing. All grass species were harvested in the morning at the vegetative stage of growth using a flail forage harvester (Carter; 3-in-wide swath), set to a 4-in stubble height. Orchardgrass was harvested in June, July, and August 2019; ideal cutting time for orchardgrass is generally earlier in the growing season, which could potentially impact the nutrient content of orchardgrass used in this study. Pearl millet and sorghum x sudangrass were harvested twice with the first cut in July 2019 and the second cut in August 2019 at

ideal quality parameters. Warm-season annual quality by cutting is described in Supplementary Materials, Table S5.3.

5.3.3. Legume Management and Harvest

Red clover and alfalfa were planted and established at a rate of 11.3 kg ac^{-1} in September 2017. Legumes were grown on Benson rocky silt loam soil; soils were not fertilized. Crops planted prior to legume varieties in the utilized plots were spring barley. Alfalfa and red clover were planted using a cone seeder (Great Plains; Salina, KS) and harvested in a similar manner as the grass stands. Similar to orchardgrass, the legumes were harvested in June, July, and August 2019. At the time of harvesting legumes were at pre-bud stage. Similar to perennial grass species, legume species utilized in this study were harvested at ideal maturity, and may not be as impacted by "summer slump" as the grass species.

5.3.4. Forage Collection and Processing

Individual grasses and legumes were harvested separately. Legumes and orchardgrass were harvested at similar times as the WSA during the warmer summer months. During harvest, approximately 0.45 kg (wet weight) of harvested material was collected from each forage variety and each separate cutting by random grab sample with 5 subsamples collected per variety per cutting. Forage subsamples from each separate cutting were then combined once all forages were collected. Subsamples were dried at 60ºC for 48 h to calculate DM yield and content. Average forage DM yield ac^{-1} was extrapolated to a total of forage samples from the sample plots. Dried subsamples were then ground to 1-mm using a Wiley Mill (Wiley Mill; Thompson Scientific; Philadelphia, PA) and a cyclone mill (UDY Corporation; Fort Collins, CO). Samples were analyzed using near

infrared reflectance spectroscopy (**NIRS**) methods at the University of Vermont E.E. Cummings Crop Testing Laboratory (Burlington, VT). The instrument used for NIRS was a FOSS DS2500 Forage and Feed Analyzer (FOSS North America; Eden Prairie, MN) which generates spectral data across a wavelength of 400 – 2500 nm. as well as crude protein (**CP**), acid detergent fiber (**ADF**), neutral detergent fiber (**NDF**), and lignin.

For *in vitro* studies, harvested forages were placed on a tarp and transferred to plastic bags within 30 min of harvest. Air was then manually forced from the bagged forages to prevent further forage degradation during transportation. Bags were weighed (11.3 kg bag^{-1}) and zip tied shut. Forages were transported to a commercial warehouse (Vermont Commercial Warehouse; Williston, VT) within 90 min of harvest where they were frozen at -20ºC until processed. All forages were processed and ground at the UVM Horticulture Research and Education Center (South Burlington, VT). Before forages were ground, residual weeds and dead material were botanically separated and discarded. Each forage was frozen with liquid nitrogen (**N**) and passed through a 2-mm mesh screen (Wiley Mill; Thompson Scientific; Philadelphia, PA) cooled with liquid N and dry ice. Legumes and grasses were passed through the mill separately, with ground subsamples collected for further analysis at a commercial laboratory (DairyOne Laboratories; Ithaca, NY; Table 5.1).

5.3.5. Dietary Treatments and Experimental Design

Dry matter analysis was conducted to calculate forage inclusion rates within each experimental diet, whereby four samples of individual whole forages were collected at random from harvested material. Samples were weighed and dried for 48 h at 65ºC in a forced air oven (VWR Scientific Products; Radnor, PA). Calculated DM values were

averaged; diets were then weighed and hand-mixed based on DM values. Dietary treatments were stored at -20ºC until use. Fermenters were each fed a total of 105 g DM d^{-1} for the duration of each period. Diets consisted of the following forage inclusions on a DM basis: 1) 50% orchardgrass + 50% alfalfa (**OG-ALF**), 2) 25% orchardgrass + 25% alfalfa + 50% red clover (**RC**), 3) 25% orchardgrass + 25% alfalfa + 50% millet (**MIL**), or 4) 25% orchardgrass + 25% alfalfa + 50% sorghum x sudangrass (**SUD**). Herbage was added to fermenters in four fractions: 33% of the total herbage was added at 0600 h and 1800 h and 17% at 0720 h and 1920 h. Diets were added in this manner to replicate diurnal pasture intake during grazing (Gregorini et al., 2008).

This experiment was designed as a replicated 4 x 4 Latin Square including four 10 d periods. Each experimental period consisted of a 7 d of adaptation followed by three d of sampling. Dietary treatments were assigned to fermenters at random at the beginning of period 1. A modified 4-unit dual-flow continuous culture system (Electrolab Biotech; Tewkesbury, UK) was utilized, with each unit having a 2.98 L ± 0.16 capacity.

5.3.6. Rumen Fluid Collection and Continuous Culture Operation

Rumen buffer was prepared according to Hoover et al. (1976) with the inclusion of urea at a rate of 0.4 g L^{-1} to simulate recycled N. For the purposes of a larger trial, urea in saliva buffer was replaced with ^{15}N labeled ammonium sulfate (Sigma-Aldrich Co.; St. Louis, MO) on d 5 at a rate of 0.021 g L^{-1} until the end of each experimental period. However, this data is not presented herein. Fermenters received clarified rumen fluid for the first 24 h of the experiment. Clarified rumen fluid was prepared according to the methods of Wenner (2016) with modifications by Baker (2021). Briefly, clarified rumen fluid was prepared the d before fermenters were inoculated. Rumen fluid was collected

from three ruminally fistulated dairy cows, strained, and pooled. Inoculum was transferred to two L containers and transported back to the lab. There, rumen fluid was further strained through four layers of cheesecloth and centrifuged twice at 900 x *g* for 10 min. The pellet was discarded and supernatant retained. The rumen fluid supernatant was combined with buffer in a 5:1 ratio of buffer: rumen fluid supernatant. Clarified rumen fluid was then autoclaved and allowed to cool to room temperature before use.

The morning of inoculation, fermenters were pre-warmed to 39ºC and were flushed with carbon dioxide (**CO_2**) gas. Clarified rumen fluid was continuously flushed with CO_2 gas. Buffer lines were primed with 20% clarified rumen fluid. Rumen fluid was collected from three ruminally fistulated, lactating, multiparous Holstein dairy cows (Paul R. Miller Research and Educational Center; South Burlington, VT) as approved by The University of Vermont Institutional Animal Care and Use Committee (IACUC #16-029) in accordance with the requirements of the Office of Laboratory Animal Welfare. Donor fistulated dairy cows were housed in tie stalls with ad libitum access to water and their typical total mixed ration (**TMR**) diet. Diet samples were collected immediately prior to rumen fluid collection. Random grab samples of TMR were collected and pooled to create a single composite sample. Samples were frozen at -20ºC upon arrival to the lab until processing. Frozen TMR samples were submitted to DairyOne Laboratories (Ithaca, NY) for a feed composition analysis (Supplementary Materials, Table S5.4). Rumen fluid (target: 20 L) was collected approximately two h after feeding. Briefly, rumen digesta was collected by hand from the ventral, central, and dorsal sacs. Rumen digesta was strained through a warmed metal sieve to remove larger solid digesta. Strained fluid flowed through the sieve into plastic buckets that were continuously warmed in a heated bath (39ºC). Once strained,

rumen fluid was pooled and maintained at 39ºC in a heated container (Powerblanket; Salt Lake City, UT) until the time of fermenter inoculation. Fermenters were inoculated with approximately three L of rumen fluid within 20 min of collection and maintained at 39ºC for the duration of each period. Carbon dioxide was continuously flushed into fermenter headspace and rumen buffer to maintain an anerobic environment. Rumen fluid inoculum was continuously agitated at 70 rpm with an upcycle to 200 rpm for one min every 10 min. Inoculum was agitated via an external motor controlled by software inputs. To retain protozoal populations and help regulate fermenter effluent outflow, 3D-printed filters with filter bags (ANKOM; Macedon, NY) were attached to the fermenter end of the liquid outflow tubing. A customized gravity-fed outflow spout was utilized to collect solid + liquid effluent. Fermenter pH was not regulated and was continuously monitored with an indwelling pH probe (Mettler Toledo; Columbus, OH). Fermenter pH, temperature, and agitation were continuously recorded with data collected every min. Liquid dilution rates for each fermenter were approximately 5.6% h^{-1}.

Effluent from filters was collected and combined with effluent in carboys. Combined carboy and filter effluent was collected daily before morning feeding (0530 h) with weight and volume recorded and utilized to determine flow rates. On d 7, carboys and filter outflow bottles were placed on ice after the second morning feeding (0800 h) and maintained on ice through d 10. Methane (% vol) was measured using a gas analyzer probe (Sewerin Multitec 545; Gütersloh, Germany). Methane measurements were collected in triplicate twice daily on d 7, 8, 9, and 10 from the gas headspace at 0530 h and 1730 h. Estimates of CH_4 were calculated using the following equation as outlined by Johnson et al. (2009) assuming atmospheric pressure:

$$CH_4\ (\text{mmol d}^{-1}) = \frac{(\text{mmol x } CO_2 \text{ gas flow } \left(\frac{\text{mL}}{\text{min}}\right) \text{x 60 min x 24 h })}{1000}.$$

During the collection period, subsamples of solid and liquid outflow collected in carboys from each fermenter were combined within fermenter within d and gently mixed. Samples were placed in a pre-weighed pan and dried at 65ºC for 48 h in a forced air oven. Dried materials were pooled by fermenter with dried rumen residue from d 8, 9, and 10 combined in equal portions. Subsamples of pooled dried effluent were sent to a commercial laboratory (DairyOne Laboratories; Ithaca, NY) for nutrient digestibility estimation and the remainder of the sample was stored for further analysis. Further samples were collected from collection carboys for determination of volatile fatty acids (**VFA**), ammonia nitrogen (**NH_3-N**), and water soluble carbohydrate (**WSC**) concentrations. Samples for VFA and NH_3-N analysis were strained through four layers of cheesecloth. Ammonia N and WSC samples were stored at -20ºC until further analysis. Volatile fatty acid samples were stored at -20ºC until analysis at the William H. Miner Institute (Chazy, NY) as outlined below. Effluent NH_3-N samples were acidified with two mL of 25% *m*-phosphoric acid (Sigma-Aldrich Co.; St. Louis, MO) and stored at -20ºC until further analysis.

5.3.7. Chemical Analysis

Samples used for VFA analysis were analyzed as described by Miller et al. (2021). Briefly, total molar proportions, individual VFA, acetate: propionate ratios, and acetate + butyrate: propionate ratios were determined through gas chromatography using a Varian CP-3800 gas chromatograph (Varian, Inc.; Palo Alto, CA). The gas chromatograph was fit with a flame-ionization detector and 80/120 Carbopack B-DA/4% Carbowax 20M column (Supelco, Inc.; Bellefonte, PA). Rumen fluid samples used for WSC analysis were analyzed using the colorimetric anthrone-sulfuric acid assay as described by Laurentin and

Edwards (2003). Samples collected for NH_3-N concentration determination were analyzed using the colorimetric phenol-hypochlorite method adapted to a 96-well plate (Chaney and Marbach, 1962).

5.3.8. Forage and Fermenter Effluent Wet Chemistry

Frozen samples of harvested orchardgrass, alfalfa, red clover, pearl millet and sorghum x sudangrass, and dried, pooled fermenter effluent were analyzed by commercial wet chemistry methods (DairyOne Laboratories; Ithaca, NY).

5.3.9. Cost Assessment

Variable costs of red clover and WSA establishment and management were calculated similar to the methods of Tracy et al. (2010). Variable costs are costs that will vary or change depending on the level of output. The model included both fixed and variable costs: variable costs included in the model were seed and establishment, fertilizers and application, soil cultivation, and forage harvesting. Variable costs for forage operations included in this model were from the New York Farm Custom Rates and Fees Survey (Cornell, 2019). Other variable costs, including seeding costs, were based on actual seed prices as of 2022 (Supplementary Materials, Table S5.1). No new construction, such as fencing, or equipment purchases and repairs were included in the model; plot rental – land use was the only fixed cost included in the model. Additionally, establishment cost per plant nutrient, including DM, CP, ADF, and NDF, was calculated on a per lb basis as per methods described by Tracy et al. (2010). Costs are based on the assumption that orchardgrass and alfalfa are already established within a pasture.

5.3.10. Statistical Analysis, and Calculations

In vitro data were analyzed as a 4 x 4 Latin square design using the PROC GLIMMIX procedure of SAS 9.4 (SAS Institute Inc., Cary, NC). The following statistical model was utilized:

$$Y_{ijk} = \mu + F_i + P_j + T_k + e_{ijk},$$

where Y_{ijk} = observations for dependent variables, μ = population mean, F_i = mean of the *i*th fermenter, P_j = mean effect of the *j*th period, T_k = mean effect of the *k*th treatment, and e_{ijk} = residual error. Fermenter was considered a random effect and period was considered a fixed effect. Contrasts were conducted comparing OG-ALF to RC, MIL, and SUD as were contrasts comparing RC to MIL and SUD, and MIL to SUD. Fermenter pH was analyzed for mean, minimum, and maximum values on d 7 - 10 as well as amount of time (min) spent between pH 5.8 to 6.4 and time spent above pH 6.4. Additionally, area under the curve (**AUC**) corresponding to duration was analyzed. Methane output differences between morning and evening collections was also included in the model when analyzing the CH_4 variable. Results were reported with least squares means (**LSM**) and were considered significant if $P \leq 0.05$.

Apparent digestibilities of treatments were calculated as outlined by Soder et al. (2012):

$$\text{Apparent DM digested (\% DM)} = \frac{[\text{ (g DMI} - \text{g effluent flow DM)}}{\text{g DMI]}} \text{ x } 100.$$

5.4. Results

5.4.1. Forage Nutrient Composition

Forage and dietary treatment nutrient composition is listed in Table 5.1. No statistical comparisons of individual forages or forage treatments were made as nutrient

compositions were calculated on single, pooled samples. In this study, most values were similar between treatments but differences were noted within carbohydrate fractions including non-fiber carbohydrates (**NFC**), starch, non-structural carbohydrates (**NSC**), WSC, and ethanol soluble carbohydrates (**ESC**). Water soluble carbohydrate residues were 5.3% - 10.8% higher for the SUD treatment than any other treatment. The NFC content of the MIL treatment was lower than the other treatments, approximately 17.6% lower than the next lowest treatment (SUD, 18.2%). Similarly, NSC in MIL was approximately 3.8% lower than the next lowest NSC content (RC, 8.36%). The starch content of the OG-ALF treatment (2.37%) was approximately 38.0% greater in starch than that of the lowest treatment (SUD, 1.47%).

5.4.2. Treatment Digestibility

Apparent digestibilities for treatments are listed in Table 5.2. Treatment ($P < 0.0001$), period ($P < 0.0001$), and treatment x period effects ($P < 0.0001$) were found for apparent DM, OM, aNDFom, and ADF digestibilities. The apparent DM digestibility of OG-ALF (52.8%) and MIL (50.2%) were the same but were higher than the RC (44.7%) and SUD (44.9%) treatments. Inversely, the OM digestibility of RC (55.3%) and SUD (57.9%) were the same but were higher than the apparent OM digestibilities of OG-ALF (46.4%) and MIL (49.2%). The apparent digestibilities of aNDFom and ADF were highest for the SUD treatment (89.6% and 88.2%, respectively). This is followed by the RC treatment. The OG-ALF treatment yielded the lowest aNDFom (80.3%) and ADF (78.5%) digestibilities.

5.4.3. Temperature, pH, and Volatile Fatty Acids

Total VFA (Mm) did not differ as a result of treatment ($P = 0.08$) or as a result of a treatment x period ($P = 0.48$; Table 5.3). Treatment x period interaction effects were observed for individual proportions of isobutyrate ($P = 0.03$), valerate ($P = 0.02$), and isovalerate ($P = 0.05$). Acetate: propionate and acetate + butyrate: propionate ratios did not differ between treatments in this study. The average ratio of acetate: propionate was 4.49 whereas the average acetate + butyrate: propionate ratio was 4.92.

As programmed, fermenter temperature averaged 39.0ºC and did not differ based on fermenter, period or their interaction. Unlike temperature, pH was not regulated; however, pH did not differ based on treatment for the mean, minimum, or maximum (Table 5.4). Period effects were observed for the mean, minimum, and maximum pH. No treatment effects were observed for duration of time spent above a pH of 6.4 or for amount of time spent between a pH of 5.8 and 6.4. Additionally, treatment did not impact AUC, duration over a pH of 6.4, or duration between a pH of 5.8 and 6.4. On average, fermenters spent 191 min/d between a pH of 5.8 and 6.4 and 1,214 min/d above a pH of 6.4.

5.4.4. Methane Concentration

When analyzed for variation between morning and evening CH_4 concentration, there were no differences between the average CH_4 concentration in the morning (33.6 mmol d^{-1}) and the evening (34.4 mmol d^{-1}; $P = 0.95$). A treatment effect was observed ($P < 0.0001$) as were treatment x period ($P < 0.0001$) and period ($P < 0.0001$) effects. Methane concentration was highest ($P < 0.0001$) from fermenters receiving the binary OG-ALF forage mixture compared to all other diet combinations (81.2 mmol d^{-1}; Figure 5.1). The CH_4 concentration of fermenters receiving RC (33.9 mmol d^{-1}) did not differ from MIL

(9.83 mmol d^{-1}; P = 0.09) nor SUD (11.1 mmol d^{-1}; P = 0.10). Furthermore, CH_4 concentration did not differ between MIL and SUD (P = 0.93).

5.4.5. Ammonia Nitrogen and Water Soluble Carbohydrate Content from Outflow Effluent

Treatment (P < 0.0001), treatment x period (P < 0.0001), and period (P < 0.0001) effects were observed for both NH_3-N and WSC (Table 5.3). Outflow effluent from MIL had the highest concentration of NH_3-N (19.9 mg dL^{-1}) while OG-ALF had lower concentrations (12.2 mg dL^{-1}). The RC treatment showed less NH_3-N (16.5 mg dL^{-1}) than the MIL treatment and higher concentrations than the SUD treatment (15.2 mg dL^{-1}). Outflow effluent from fermenters receiving OG-ALF contained the highest amount of WSC (1.15 mg mL^{-1}). Fermenters receiving MIL showed a lower concentration of WSC than OG-ALF (1.15 mg mL^{-1} v. 1.03 mg mL^{-1}); however, the WSC concentration of MIL was higher than that of SUD (0.92 mg mL^{-1}) and RC (0.90 mg mL^{-1}) which did not differ from one another.

5.4.6. Costs and Forage Production

There were more costs associated with establishing a red clover crop in 2017 (Table 5.5). As the WSA took less than a year to establish, there were fewer costs associated with establishment and harvest. Although red clover cost more to establish, it was cheaper to produce in terms of both DM yield ac^{-1} and CP as compared with the WSA (Table 5.6). Furthermore, red clover (4.43 tons ac^{-1}) outproduced both pearl millet (2.73 tons ac^{-1}) and sorghum x sudangrass (3.24 tons ac^{-1}) in total DM yield (P = 0.0002). On a DM-basis, red clover produced more CP (23.0%) than pearl millet (20.1%) and sorghum x sudangrass (17.8%; P < 0.0001). Additionally, sorghum x sudangrass produced less CP than pearl

millet. Cost for ADF production was similar between WSA but NDF production was more expensive for red clover ($0.58 per ac). Sorghum x sudangrass had greater NDF (59.7%; $P = 0.0002$) and ADF (33.9%; $P < 0.0001$) than both pearl millet (ADF:31.5%; NDF: 57.6% and red clover (ADF:30.2%; NDF: 45.3%).

5.5. Discussion

In the summer months, cool season perennial (**CSP**) quality and quantity rapidly decline in what is known as "summer slump." The impacts of summer slump are further exacerbated by changes in the climate, leading to longer, droughty periods in the summer that have become harder to predict, leading to an even greater decline in CSP forages (Ritz et al., 2020). Producers in the NE have shown an increased interest in utilizing WSA in their pasture systems as a means to provide high-quality forage for grazing dairy cattle during this time period when CSP quality declines. Compared with cool-season forages, warm-season crops have low leaf: stem ratios and are more often highly lignified. Dillard et al. (2017) evaluated a monoculture diet of orchardgrass, binary mixtures that included orchardgrass and Japanese millet or sorghum x sudangrass, and a diet of all three grasses in continuous culture to determine digestibility and rumen fermentation kinetics. Dillard et al. (2017) indicate that when perennial forages were harvested individually at highest quality, orchardgrass was slightly more digestible than the WSA which is to be expected. However, the WSA outperformed orchardgrass in regard to bacterial N efficiency. Based on these continuous culture results, Dillard et al. (2017) determined that certain warm-season forage varieties could potentially replace orchardgrass pastures in the summer months in temperate regions. In this current trial, we used *in vitro* continuous culture systems to assess further diversified forage treatments by utilizing a binary combination of

cool-season forages commonly utilized in the NE U.S. as the control treatment to assess the inclusion of different WSA on likely rumen fermentation dynamics and energetic capture.

5.5.1. Forage Nutrient Composition and Digestibility

The nutritive values of orchardgrass, pearl millet and sorghum x sudangrass were similar in nutrient composition to those utilized by Dillard et al. (2017) although the CP of pearl millet in this study (25%) was numerically greater than the CP (17.8%) of their treatment. The CP of pearl millet in this study was greater than the average range reported by Lee et al. (2009; 12 - 14%) which could potentially be due to forage management. However, the Japanese millet utilized in the study by Dillard et al. (2017) may be more digestible than the pearl millet in this study based on NFC (19.9% v. 7.0%) and NSC (10.1% v. 7.0%) content. The dietary composition of combined treatments was similar to values reported by Dillard et al. (2017).

Dry matter and OM digestibilities were numerically lower than those reported by Dillard et al. (2017; 55.5% - 58.8% DM and 62.3% - 71.1% OM, respectively). This may be due to treatment composition as three treatments in this study contained lower ratios of orchardgrass and WSA. Additionally, Japanese millet used by Dillard et al. (2017) may be slightly more digestible than the pearl millet used in this study as previously referenced. This may also be related to the fact that orchardgrass utilized by Dillard et al. (2017) in the aforementioned study was harvested at a higher quality than orchardgrass utilized in this study. Although apparent digestibilities reported in the current study were generally lower than those reported by Dillard et al. (2017), digestibility values generally aligned with those reported by de Veth and Kolver (2001b) and Ruh et al. (2018). Dry matter digestibility

values of perennial ryegrass (*Lolium perenne*) diets at reported by de Veth and Kolver (2001b) ranged from 44.7% - 56.4%. This is similar to the range observed in this study (46.1% - 56.2%) although it should be noted that de Veth and Kolver (2001b) were evaluating pH in relation to digestibility rather than evaluating pasture diversity. Using a continuous culture system, Ruh et al. (2018) compared the digestibilities of mixed cool-season pasture grasses, 100% alfalfa, 100% brown-midrib sorghum x sudangrass (**BMRSS**), and 100% teff (*Eragrostis tef*). Although the apparent digestibilities of Ruh et al. (2018) were similar to results reported in this study, there was a noted difference in relation to the alfalfa treatment of the aforementioned study. The apparent DM digestibility of the pure alfalfa treatment (69.4%) was greater than the DM digestibilities of all treatments in the current study, which contained lower ratios of alfalfa. This is similar to *in vivo* studies that indicate pure alfalfa disappears more rapidly from the rumen than grasses or grass mixtures due to faster rates of digestion and particle size reduction (Waghorn et al., 1989; Chaves et al., 2006). It is possible that although the treatments in this study contained alfalfa, the higher ratios of WSA led to a decrease in digestibility. Additionally, differences may also relate to time of harvest in the current study as alfalfa in this study was harvested during warmer, summer months when quality is generally lower.

The aNDFom and ADF contents of all treatments in this study were higher than the OG-ALF treatment, resulting in a lower apparent DM digestibility of the RC and SUD treatments. For the RC diet, this may be due to higher indigestible fiber fractions, particularly in relation to higher lignin content (9.41% DM-basis) compared to the other treatments. This higher lignin content may interfere with microbial degradation. The same

cannot be said for the SUD treatment which had a lower lignin content (5.82% DM-basis). The DM digestibility of the MIL treatment was the same as the OG-ALF treatment even though the aNDFom and ADF of this treatment was numerically lower than that of the OG-ALF diet. Other studies have reported that apparent digestible DM for sorghum x sudangrass and Japanese millet ranged from 48.6% - 51.6% (Darby et al. 2016; Muldoon, 1985; Dillard et al., 2017). The MIL treatment from this study (50.2%) was within this range but the SUD treatment was slightly less digestible (44.9%) than previously reported.

Values for apparent aNDFom and ADF digestibilities are higher than those reported by Dillard et al. (2017) and Ruh et al. (2018), although aNDFom and ADF values more closely align with those presented by Dillard et al. (2017), most likely due to similarities in diet composition. However, it should be noted that although ranges were similar, the pure orchardgrass diet of Dillard et al. (2017) was found to be more digestible in terms of NDF and ADF than the WSA utilized in that study. This is the opposite of our results which may be due to treatment composition differences i.e., legume inclusion. The aNDFom and ADF digestibility of sorghum x sudangrass was greater than all other treatments (89.6% and 88.2% DM-basis), potentially due to sorghum x sudangrass varieties being brown-midrib (**BMR**) varieties. Brown-midrib forage varieties tend to be more digestible than non-BMR forages (Ketterings et al., 2005). Results of this study tended to align with those presented by Ketterings et al. (2005). Ketterings et al. (2005) evaluated the use of BMR sorghum x sudangrass hybrids as a replacement for corn silage in the NE U.S. Digestibility of NDF averaged 77.2% – 78.0% for first and second cuts of BMR sorghum x sudangrass, slightly lower than aNDFom values reported here which ranged from 80.3% - 89.6%). Cumulatively, comparison of our nutritive value and digestibility results with other

published research suggests that there are tradeoffs depending on forage specie inclusions. Depressed aNDFom and ADF digestibilities were not expected for the OG – ALF diet. As mentioned previously, alfalfa has typically been shown to have greater digestibility and faster passage rates. However, in this study, 50% inclusion may have depressed fiber digestibility. The OG – ALF treatment had the second highest lignin content (7.40%) and may not be as digestible as diets with greater lignin content due to forage variety choice. Diets with greater inclusions of sorghum x sudangrass or red clover may not be as digestible in terms of DM due to increased amounts of indigestible nutrient components such as lignin. However, further research must be conducted as data regarding true digestibilities of WSA in combination with legumes and cool-season forages is still lacking.

5.5.2. Temperature, pH, and Volatile Fatty Acids

Fermenter pH values from this study align with previous continuous culture and *in vivo* studies (Ruh et al., 2018; Dillard et al., 2017; Bargo et al., 2003; de Veth and Kolver, 2001). The average pH of fermenters in this study was 6.71, which was slightly lower than the average pH reported by Dillard et al. (2017; 6.76) who also evaluated WSA inclusion using an *in vitro* model. The average pH of the SUD and MIL treatments were slightly lower than pH values reported by Dillard et al. (2017) for treatments that contained millet and sorghum x sudangrass (6.76 and 6.74, respectively). This may be due to dietary differences; the study by Dillard et al. (2017) utilized treatments comprised of 50% WSA + 50% orchardgrass whereas this study utilized diets that included at least 25% alfalfa in addition to orchardgrass and WSA inclusion.

While these dietary differences may lead to slightly different fermentation patterns, effective digestion was likely still achieved. Kolver and de Veth (2002) reported that the optimal pH for pasture digestion was 6.35. They also predicted that nutrient digestion and microbial protein synthesis (**MPS**) were largely unchanged between a pH range of 5.8 and 6.8. Although there are numerous studies evaluating lower to normal pH range, there is little information available assessing high rumen pH and its impacts on animal performance. Bargo et al. (2003) indicated that there is an inverse correlation between pH and production of VFA from diets composed of smooth bromegrass and orchardgrass. In this instance, higher pH values correlated to lower VFA totals which aligns with the results of this study. This is in direct contrast to the findings of Ruh et al. (2018). However, the lack of correlation between pH and total VFA is potentially due to fermenter pH being regulated throughout the study conducted by Ruh et al (2018). In the current study, pH was not regulated which may have large impacts on VFA ratios and concentrations as this mimics true diet and pH effects that are normally seen in the rumen (Calsamiglia, Ferret, and Devant, 2002).

The concentrations of VFA in the current study were lower than those reported by Dillard et al. (2017). This may be due to a depletion in both bacterial and protozoal populations. The acetate: propionate ratio of this study was higher than the typical 3:1 acetate: propionate ratio that is typical of forage-based diets but is in line with ratios reported by Ruh et al (2018; 4.40) and Bach et al. (1999; 4.00). Volatile fatty acid proportions for each individual treatment were roughly in ratios of 70% acetate: 20% propionate: 10% butyrate (Table 5.3). This was to be expected as high forage diets typically produced acetate: propionate: butyrate ratios in the proportion of 70:20:10 (Ellis et al.,

2008). Volatile fatty acid ratios are largely dependent on diet and the type of carbohydrate being fermented, particularly WSC and NSC. Diets composed of a large proportion of grain that contain a large amount of WSC and NSC typically produce a VFA ratio that favors more propionate production (50:40:10; Ellis et al., 2008). This is due to rapid fermentation and breakdown by rumen microbes, shifting away from fiber digestion to fermentation of readily fermentable carbohydrates such as WSC (Lee et al., 2003). Although pH and VFA proportions, concentrations, and molar proportions did not differ based on treatment, noted fluctuations after feeding may potentially be due to forage WSC concentrations. Using a rumen simulation technique (**RUSITEC**) apparatus, Lee et al. (2003) evaluated the pH and VFA profiles of vessels receiving 80 g DM^{-1} perennial ryegrass and varying levels of WSC (sucrose, fructans, and inulin infusate). Results showed that the lowest pH occurred at the highest level of WSC inclusion. This decline was most likely due to rapid fermentation of WSC, which also resulted in a rapid increase in VFA concentrations. In the current study, diet WSC ranged from 6.36% - 7.13% (DM-basis). In this study, it was thought that a large range of WSC would induce significant VFA changes. While the typical changes after feedings were observed, no changes due to treatment were noted, indicating that WSC was not metabolized as rapidly as shown in previous studies. This is further reflected in the greater proportions of acetate observed in individual VFA concentrations, acetate: propionate, and acetate + propionate: butyrate ratios indicating that there was not a in favor. of readily fermentable carbohydrates. This may potentially be due to entrapment of WSC by the forage fiber matrix (Grant and Cotanch, 2012).

5.5.3. Methane Concentration

In high forage diets, larger proportions of acetate and butyrate lead to greater production of hydrogen (**H_2**) gas (Bannink et al., 2006) and higher CH_4 production. Methane values from this study were greater than those reported by Dillard et al. (2017). The CH_4 concentration produced from addition of 100% orchardgrass by Dillard et al. (2017) was lower than that produced by addition of the binary OG-ALF treatment from this study (81.2 mmol d^{-1} v. 6.80 mmol d^{-1}). Similar to the OG-ALF treatment, the CH_4 output of the SUD treatment was greater than that of pure sorghum x sudangrass (11.1 mmol d^{-1} v. 6.74 mmol d^{-1}) as reported by Dillard et al. (2017). This could indicate that forage diversity may not be as important as the individual forage component.

In this study, the binary OG-ALF mixture followed similar trends in pH and VFA production as the RC, SUD, and MIL treatments, and do not follow the difference in, CH_4 production due to treatment. This could indicate a lack of secondary metabolites that help inhibit CH_4 output, such as tannins (Roca-Fernandez et al., 2020). Loza et al. (2021) found that increased forage species richness, especially those rich in secondary metabolites, reduced CH_4 emissions. Reductions in forage digestibility were also reported. In this current study, decreased DM digestibility was calculated for two of the diverse treatments (RC and SUD) while decreased OM digestibility was only calculated for one of the diverse treatments (MIL). Chaugool et al. (2013) found that feeding BMR sorghum x sudangrass reduced production of CH_4 by 66% and that CH_4 per g of NDF decreased by 50%. However, millet did not cause any decreases in CH_4 and, in fact, produced more CH_4 than its cool season counterparts. This is further supported by Harper et al. (2018) who found that replacing corn silage with pearl millet silage increased CH_4 production of lactating

dairy cows. These researchers suggested that diet digestibility was a major contributing factor to CH_4 increases as pearl millet silage decreased apparent OM digestibility as well as being highly fibrous. This is in line with and supported by our results. This indicates that WSA inclusion could help diversify pastures and provide some reduction in terms of CH_4 output, but should be managed to ensure digestibility, and ultimately productivity, are not significantly reduced.

5.5.4. Ammonia Nitrogen Concentration and Water Soluble Carbohydrate Content from Outflow Effluent

Effluent from the OG-ALF treatment had the highest residual concentration of WSC. Additionally, the NH_3-N concentration of effluent from the OG-ALF-treated fermenters was lower than all other treatments. While differences could be due to substrate specificity of rumen microbes and may also be attributed to changes in rumen microbial populations (Kim et al., 2011), other possibilities are also plausible. The higher residual WSC concentration and lower NH_3-N concentration, coupled with the lower NDF and ADF digestibility, and higher CH_4 concentration, also suggest that 1) the WSC may be entrapped within the fiber matrix, and be unavailable for microbial use, and 2) the NH_3-N concentration available from this diet may be a limiting factor for rumen function, and not carbohydrate availability. The entrapment effect of fibrous carbohydrates in orchardgrass has previously been proposed by Grant and Cotanch (2012) and Mertens (2011). Within this scenario, the larger NDF particles of orchardgrass reduced passage rate for this grass, potentially leading to decreased availability of other nutrients, including WSC, that are entrapped within the matrix. Additionally, Kammes and Allen (2012) compared limiting factors of rumen passage of cows fed orchardgrass and alfalfa silage. It was found that

particle size was not the limiting factor of passage rate. However, digestible NDF decreased for orchardgrass. Furthermore, a model developed by Seo et al. (2009) indicated that the rumen pool size of larger NDF particles of orchardgrass increased, likely entrapping nutrient particles, such as NDF and WSC, and slowing passage rate

The latter suggestion of N limitations has been previously reported in pasture-based diets, including NE pastures that include forages similar to those used in the current trial. Rivero et al. (2020) have previously highlighted that pasture management plays a significant role on plant WSC concentration and of the ratio of WSC:CP, which is important for efficient energetic and nutrient capture and use in the rumen. Typically, when WSC is limiting and not adequately available to rumen microbes, a buildup of NH_3-N occurs. The use of slower-releasing energy sources results in low incorporation of microbial protein, decreasing MPS (Lee et al., 2018). Our observation of higher WSC and low NH_3-N concentration would suggest the inverse is occurring in OG-ALF-treated fermenters. It could be suggested that protein is the limiting factor in the binary OG-ALF diet as CP is often a limiting nutrient for pasture-based dairy cattle (Hafla et al., 2016). For example, compared with the other individual forages in this study, orchardgrass was the lowest in CP (16.9%), although this effect was mitigated when combined with alfalfa. Ayers et al. (2022) evaluated the effects of supplementation of CP on milk urea nitrogen (**MUN**), milk yield, and composition of organic grazing dairy herds. Dietary CP values reported in the study by Ayers et al. (2022) ranged from 16.1% - 19.2%, and consistent with our hypothesis, Ayers et al. (2022) observed that CP supplementation aided in maintaining a higher milk yield during the summer grazing period, suggesting that protein, not WSC, could be a limiting factor in pasture-based systems.

5.5.5. Costs and Forage Production

Although total costs to establish and harvest red clover were higher than establishment and harvest of the WSA (43% - 65% higher based on accrued 3-yr costs), this was to be expected as seed costs, accrued land rental needs, and harvest cost were all higher. However, the individual cost to produce an ac worth of DM and CP was less than that of pearl millet and sorghum x sudangrass as red clover outproduced both summer annuals in terms of DM and CP. It should be noted that price based on DM yield was only $0.01 less for red clover than pearl millet. Furthermore, sorghum x sudangrass had higher (on a DM-basis) NDF (59.7%) and ADF (30.2%) than both pearl millet (57.6%; 31%) and red clover (45.3%; 30.2%), indicating decreased forage quality. The higher establishment costs for the WSA were mainly due to labor and machinery costs, accounting for almost 40% of total costs. Labor and machinery costs also accounted for the majority of total costs for red clover establishment, accounting for 26% of total costs. Tracy et al. (2010) also used a variable costs comparison to determine cost differences between WSA pastures and perennial warm-season grass pastures. Similar to the results of this study, WSA pastures were initially cheaper to establish than their perennial season counterparts. However, costs to maintain and harvest annual pastures eventually exceeded the perennial season pastures with the main costs being field operations and N fertilizer.

A 3-yr grazing study by Comerford et al. (2005) evaluated grazing systems that incorporated orchardgrass and Kentucky bluegrass, both cool-season perennials (**CSP**), with either annual or perennial forages. Annuals included sudangrass, small grains, rape, and corn stalks. Perennial forages included stockpiled fescue and alfalfa-grass combinations. Similar to Tracy et al. (2010), Comerford et al. (2005) calculated that

interseeding annual forages was more expensive than perennial forage establishment. Basweti et al. (2009) went as far as to conclude that interseeding annuals, such as sudangrass, with permanent cool-season grasslands was not economically justified as permanent grassland systems yielded more than those supplement with annual season grasses. However, due to organic, grassfed (**OGF**) grazing standards and regulations, OGF producers may have no other alternative than to utilize WSA to meet animal DM requirements and meet market standards. Organic, grassfed regulations dictate that certified OGF dairy cattle must be on pasture for at least 150 d and receive 60% of their DM intake from pasture. During the summer months when CSP productivity and quality decline, a lack of available forage would prove to be more of an economic hardship than utilizing WSA. Our results indicate that if a WSA were to be utilized, pearl millet may be more beneficial in NE pasture systems compared with sorghum x sudangrass, as pearl millet has a more advantageous nutrient profile if being used for ruminant grazing and nutrition. Additionally, it should be realized that although we were able to make comparisons between WSA and red clover, WSA in this study were not harvested during the same time period as red clover. Warm-season annual species were harvested during their peak quality while red clover was not.

5.6. Conclusions

Inclusion of WSA can provide access to high quality forage for pasture-based dairy cattle during the warmer months of the grazing season. Fermentation metrics indicate that inclusions of pearl millet and sorghum x sudangrass may be useful to producers in terms of animal production and performance. Both WSA performed in a similar manner to diets that included red clover. Binary forage mixtures composed of orchardgrass and alfalfa were

not as digestible as diets including WSA and red clover in terms of ADF and aNDFom. Further, binary forage mixtures produced more CH_4 than more diverse forage mixtures and had higher residual WSC, which indicates some potential issues with NDF passage and carbohydrate entrapment. Red clover quality did not decrease over the summer months nor did DM yield. The price reduction for establishing red clover based on CP may be more economical for producers depending on their production goals. However, this may not be a realistic approach for producers or pasture systems considering the production of red clover during warmer months. It may be more advantageous to integrate WSA in grazing systems based on productivity and OGF marketing requirements. The basic cost assessment completed as part of this trial suggests that WSA incorporation may prove to be a challenge to producers. However, it may still be a viable strategy to OGF producers when taking summer slump of perennial forages into account. Future research should incorporate total farm factors, such as investments and net income, thereby evaluating a return on investments over multiple years. If WSA were to be included in NE pasture systems, pearl millet may be beneficial as it contained less ADF and aNDFom than sorghum x sudangrass. Additional research is needed to fully assess the potential of WSA integration in cool-season pastures in the NE U.S. for both costs and animal productivity.

5.7. Acknowledgments

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5.9. Tables

Table 5.1. Chemical composition as a percentage of dry matter (DM) of individual forages and forage mixtures used during continuous culture fermentation.

Item (% of DM)[2]	Ingredient					Treatment[1]			
	Orchardgrass	Alfalfa	Red Clover	Sorghum x Sudangrass	Pearl Millet	OG - ALF	RC	SUD	MIL
OM	90.0	89.6	89.8	88.3	86.0	89.8	89.8	89.0	88.1
CP	16.9	25.4	23.5	20.0	29.1	21.4	22.4	20.7	25.0
aNDFom[3]	55.5	29.4	38.9	50.4	45.0	41.8	40.4	46.2	42.8
ADF	35.4	25.6	33.5	34.0	30.5	30.3	32.0	32.2	30.2
NFC[4]	13.8	31.6	23.4	14.0	7.01	23.1	23.3	18.5	16.1
WSC[5]	6.80	6.70	6.10	7.50	5.90	6.75	6.44	7.13	6.36
ESC[6]	4.80	5.80	5.30	6.70	5.70	5.32	5.32	6.03	5.51
Starch	0.80	3.80	1.60	0.60	1.10	2.37	2.00	1.47	1.84
Lignin	7.30	7.50	11.5	4.30	6.00	7.40	9.41	5.82	6.77
NSC[7]	7.60	10.5	7.70	8.10	7.00	9.03	8.36	8.56	8.05
NE_L, mcal/ kg[8]	1.20	1.50	1.28	1.38	1.40	1.36	1.32	1..37	1.38
Ca	0.64	1.49	1.49	0.90	0.80	0.90	1.19	0.90	0.88
P	0.43	0.35	0.34	0.40	0.47	0.39	0.36	0.39	0.42
Mg	0.24	0.22	0.27	0.40	0.32	0.23	0.25	0.32	0.27
K	2.85	2.55	2.76	3.05	4.49	2.69	2.73	2.88	3.50
Na	0.14	0.05	0.01	0.01	0.02	0.09	0.05	0.05	0.06

[1]Treatment values were calculated using actual nutrient composition and proportion of individual forages on a DM basis; OG-ALF: 50% orchardgrass + 50% alfalfa; RC: 25% orchardgrass + 25% alfalfa + 50% red clover; SUD: 25% orchardgrass + 25% alfalfa + 50% sorghum x sudangrass; MIL: 25% orchardgrass + 25% alfalfa + 50% pearl millet.
[2]Chemical composition results from DairyOne Laboratories; Ithaca, NY.
[3]aNDFom: NDF organic matter after amylase treatment and ashing.
[4]NFC: non-fiber carbohydrates. Calculated as described by Dillard et al. (2017); 100 – (CP% + aNDFom% + ether extract% + ash%)
[5]WSC: water soluble carbohydrates.
[6]ESC: ethanol soluble carbohydrates.
[7]NSC: non-structural carbohydrates. Calculated as described by Dillard et al. (2017); starch + water soluble carbohydrates.

[8]Estimated by the NRC (2001) model.

Table 5.2. Apparent nutrient digestibilities of orchardgrass-alfalfa and orchardgrass-alfalfa forage mixtures (105 g DM d^{-1}) used during continuous culture fermentation.

Item	Treatment[1]				SEM	*P*-value		
	OG-ALF	RC	SUD	MIL		Treatment	Period	Treatment x Period
Apparent Digestibility								
DM[2], %	52.8[a]	44.7[b]	44.9[b]	50.2[a]	1.07	<0.0001	<0.0001	<0.0001
OM[3], %	46.4[b]	55.3[a]	57.9[a]	49.2[b]	1.34	<0.0001	<0.0001	<0.0001
aNDFom[4], %	80.3[d]	84.6[b]	89.6[a]	83.2[c]	0.54	<0.0001	<0.0001	<0.0001
ADF[5], %	78.5[d]	82.9[b]	88.2[a]	81.0[c]	0.37	<0.0001	<0.0001	<0.0001

[1]Treatments on a DM-basis: OG-ALF: 50% orchardgrass + 50% alfalfa; RC: 25% orchardgrass + 25% alfalfa + 50% red clover; SUD: 25% orchardgrass + 25% alfalfa + 50% sorghum x sudangrass; MIL: 25% orchardgrass + 25% alfalfa + 50% pearl millet.
[2]DM: dry matter.
[3]OM: organic matter
[4]aNDFom: NDF organic matter after amylase treatment and ashing.
[5]ADF: acid detergent fiber.
[a-d]Within row, different letters indicate significant differences ($P \leq 0.05$).

Table 5.3. Fermenter effluent ammonia nitrogen (NH_3-N), water soluble carbohydrate (WSC) content, volatile fatty acid (VFA) molar proportions, concentrations, and ratios of orchardgrass-alfalfa and orchardgrass-alfalfa mixtures during continuous culture fermentation.

	Treatment[1,2]					*P*-value		
Item	OG-ALF	RC	SUD	MIL	SEM	Treatment	Period	Treatment x Period
Total VFA, m*M*	38.6	35.0	30.4	35.2	1.63	0.08	0.14	0.48
Individual VFA, mol/ 100 mol								
Acetate (A)	73.6	73.4	72.9	72.8	0.16	0.22	<0.0001	0.07
Propionate (P)	16.3	16.3	16.9	16.6	0.13	0.52	0.0003	0.41
Butyrate (B)	6.87	7.02	6.70	7.06	0.09	0.32	0.06	0.51
Isobutyrate	0.89	0.85	0.95	0.97	0.02	0.13	0.64	0.03
Valerate	1.30	1.38	1.36	1.44	0.03	0.06	0.19	0.02
Isovalerate	1.02	1.01	1.16	1.12	0.03	0.15	0.38	0.05
A: P	4.56	4.57	4.43	4.41	0.04	0.49	<0.0001	0.31
A + B: P	4.99	5.00	4.83	4.84	0.05	0.46	0.0002	0.36
WSC, mg mL^{-1}	1.57[a]	0.90[c]	0.92[c]	1.03[b]	0.02	<0.0001	<0.0001	<0.0001
NH_3-N, mg dL^{-1}	12.2[d]	16.5[b]	15.2[c]	17.9[a]	0.24	<0.0001	<0.0001	<0.0001

[1] Treatments on a DM-basis: OG-ALF: 50% orchardgrass + 50% alfalfa; RC: 25% orchardgrass + 25% alfalfa + 50% red clover; SUD: 25% orchardgrass + 25% alfalfa + 50% sorghum x sudangrass; MIL: 25% orchardgrass + 25% alfalfa + 50% pearl millet.

[2]Values are calculated based on averages from d 8 – 10.

[a-d]Within row, different letters indicate significant differences ($P \leq 0.05$).

Table 5.4. Fermenter pH and duration of time spent over a pH of 6.4 or between 5.8 and 6.4 of fermenters receiving orchardgrass-alfalfa and orchardgrass-alfalfa mixtures. Fermenters received 105 g DM d^{-1} total of diet.

	Treatment[1]					*P*-value[2]		
Item[3]	OG-ALF	RC	SUD	MIL	SEM	Treatment	Period	Treatment x Period
pH								
Mean	6.64	6.77	6.70	6.71	0.02	0.83	0.0002	0.36
Minimum	5.92	6.02	6.25	6.31	0.09	0.73	<0.0001	0.02
Maximum	7.58	7.64	7.41	7.65	0.13	0.51	0.002	0.63
Range[3]	1.66	1.62	1.16	1.34	0.12	-	-	-
pH > 6.4								
Duration, min/d	1117	1197	1247	1295	38.0	0.39	<0.0001	0.01
Total AUC[4], pH x min	125	135	139	144	4.03	0.46	<0.0001	0.02
5.8 ≤ pH ≤ 6.4								
Duration, min/d	302	173	173	114	39.7	0.28	<0.0001	0.006
Total AUC, pH x min	31	18	12	18	4.01	0.31	<0.0001	0.008

[1]Treatments on a DM-basis: OG-ALF: 50% orchardgrass + 50% alfalfa; RC: 25% orchardgrass + 25% alfalfa + 50% red clover; SUD: 25% orchardgrass + 25% alfalfa + 50% sorghum x sudangrass; MIL: 25% orchardgrass + 25% alfalfa + 50% pearl millet.
[2]Values are calculated based on averages from d 8 – 10.
[3]Range is calculated as maximum pH – minimum pH.
[4]AUC: area under the curve.

Table 5.5. Variable and fixed costs (USD) for establishment and management of pearl millet, sorghum x sudangrass, and red clover for the 2019 growing season. Red clover was established in September 2017 and harvested during the 2019 growing season. The warm-season annuals (WSA) were established and harvested in 2019.

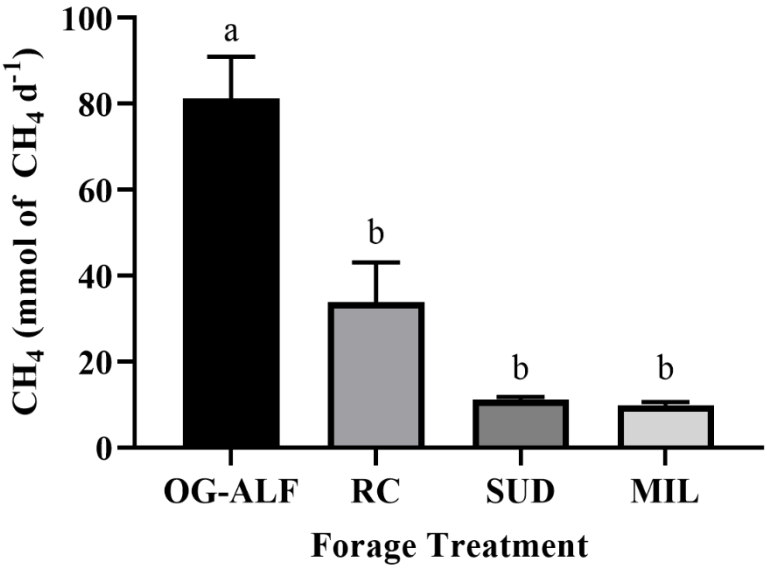
	Pearl Millet	Sorghum x Sudangrass	Red Clover		
Yr	2019	2019	2017	2018	2019
			Dollars ac^{-1}		
Input Costs					
Seed	41.20	103.50	108.50	0.00	0.00
Fertilizer	51.99	51.99	0.00	0.00	0.00
Labor & Machinery	118.00	101.00	74.00	0.00	51.00
Total Variable Costs	211.19	256.49	182.00	0.00	51.00
Fixed Costs					
Land Use	85.00	85.00	85.00	85.00	85.00
Total Fixed Costs	85.00	85.00	85.00	85.00	85.00
Total Costs	296.19	341.49	267.50	85.00	136.00

Table 5.6. Average cost per plant nutrient of pearl millet and sorghum x sudangrass for the 2019 growing season and for red clover from 2017 - 2019. Costs are calculated on a dollars (USD) pound^{-1} basis across two cuttings for the warm-season annuals (WSA) and across three cuttings for red clover.

	Cost ton DM^{-1}	DM	CP	ADF	NDF
			% DM		
Pearl Millet	544.00	0.27	1.36	0.88	0.47
Sorghum x Sudangrass	588.26	0.29	1.66	0.87	0.49
Red Clover	525.10	0.26	1.14	0.87	0.58

5.10. Figures

Figure 5.1. Fermenter methane (CH_4) output of orchardgrass-alfalfa and orchardgrass-alfalfa forage mixtures during *in vitro* continuous culture fermentation. Units are expressed as mmol of CH_4 d^{-1}. Error bars represent SEM.



Treatments on a DM-basis: OG-ALF: 50% orchardgrass + 50% alfalfa; RC: 25% orchardgrass + 25% alfalfa + 50% red clover; SUD: 25% orchardgrass + 25% alfalfa + 50% sorghum x sudangrass; MIL: 25% orchardgrass + 25% alfalfa + 50% pearl millet.
[a-b]Means with different superscripts are different ($P \leq 0.05$).

5.11. Supplementary Tables

Table S5.1. Forage cultivars used for continuous culture experiment.

Forage Species and Cultivars				
Orchardgrass (*Dactylis glomerata*)	Alfalfa (*Medicago sativa*)	Red Clover (*Trifolium pratense*)	Pearl Millet[1] (*Pennisetum glaucum*)	Sorghum x Sudangrass[2] (*Sorghum x drummondii*)
Endurance	FSG420	Arlington[a]	Wonderleaf[c]	KF Sugar Pro 55 SS[e]
Extend	Profusion	Freedom[b]	Exceed[d]	Green Grazer V[f]
Echelon	Road Runner	Manitoba[a]	KF Prime 180[e]	AS 9302[c]
Niva	Secure	Milvus[b]	KF Prime 360[e]	FSG 214[f]
	Traffic Pro	Ruby[a]		FSG 215[f]
	Viking 340			
	Viking 370			
	Viking 542			

[1]All pearl millet varieties, except Wonderleaf, are brown mid-rib (BMR) varieties.
[2]All sorghum x sudangrass varieties, except Green Grazer V, are brown mid-rib (BMR) varieties.
[a-f]Seed companies of forages used for cost analysis.
[a]Albert Lea Seed; Albert Lea, MN.
[b]King's Agriseed Inc.; Lancaster, PA.
[c]Alta Seeds; Amarillo, TX.
[d]Exceed; East Hartford, CT.
[e]KingFisher Seeds; Lancaster, PA.
[f]Seedway, LLC; Hall, NY.

Table S5.2. Average growing degree days (GDD) for the 2018 – 2019 growing and harvesting season. Historical averages are for 30 yr of data provided by the NOAA (1981 – 2010) for Burlington, VT. Temperature units are expressed in Fahrenheit (ºF) and precipitation units in inches.

	2018				
Alburgh, VT	August	September	October	November	December
Average temperature (°F)	72.8	63.4	45.8	32.2	25.4
Departure from normal	4.0	2.9	-2.3	-5.8	-0.1
Precipitation (inches)	3.0	3.5	3.5	4.5	3.0
Departure from normal	-1.0	-0.2	0.0	1.4	0.6
GDD, 50 - 86°F	696.5	427.0	81.3	3.3	5.1
Departure from normal	114.5	67.0	-50.8	-30.8	1.1
GDD, 44 - 86°F	881.5	584.5	161.1	13.2	12.0
Departure from normal	118.5	75.5	-70.0	-61.8	-1.0
GDD, 32 - 90°F	1263.7	940.9	435.2	135.5	72.1
Departure from normal	122.7	85.9	-77.9	-114.5	-7.9
GDD, 32 - 95°F	1260.4	940.9	435.2	135.5	72.1
Departure from normal	120.4	85.9	-77.9	-114.5	-7.9
GDD, 41 - 86°F	974.5	670.8	214.2	27.8	19.8
Departure from normal	119.5	79.8	-77.8	-78.3	-1.2

Table S5.2. continued. Average growing degree days (GDD) for the 2018 – 2019 growing and harvesting season. Historical averages are for 30 yr of data provided by the NOAA (1981 – 2010) for Burlington, VT. Temperature units are expressed in Fahrenheit (ºF) and precipitation units in inches.

	2019									
Alburgh, VT	January	February	March	April	May	June	July	August	September	October
Average temperature (°F)	15.0	18.9	28.3	42.7	53.3	64.3	73.5	68.3	60.0	50.4
Departure from normal	-3.9	-2.5	-2.8	-2.1	-3.2	-1.5	2.9	-0.5	-0.5	2.3
Precipitation (inches)	1.5	1.7	1.4	3.7	4.9	3.1	2.3	3.5	3.9	6.3
Departure from normal	-0.5	0.0	-0.9	0.8	1.5	-0.6	-1.9	-0.4	0.2	2.8
GDD, 50 - 86°F	0.0	0.0	9.3	59.4	188.9	445.5	716.0	568.2	335.2	145.9
Departure from normal	-3.0	-2.0	-12.7	-51.7	-103.1	-35.5	85.9	-13.9	-24.8	13.9
GDD, 44 - 86°F	0.0	1.6	25.2	119.7	313.2	611.0	902.0	753.1	489.6	256.8
Departure from normal	-8.0	-6.4	-23.8	-65.4	-111.8	-37.1	87.9	-10.0	-19.4	25.8
GDD, 32 - 90°F	22.5	38.1	107.9	345.6	659.9	970.3	1285.6	1125.1	839.6	571.0
Departure from normal	-25.5	-18.0	-58.2	-68.5	-99.1	-42.7	88.6	-16.0	-15.4	58.0
GDD, 32 - 95°F	22.5	38.1	107.9	345.6	659.9	970.3	1284.7	1125.1	839.6	571.0
Departure from normal	-25.5	-18.0	-58.2	-68.5	-99.1	-40.7	90.7	-15.0	-15.4	58.0
GDD, 41 - 86°F	0.1	4.0	40.7	163.1	391.4	700.3	995.0	846.1	571.8	320.3
Departure from normal	-11.9	-10.0	-27.4	-68.9	-109.6	-36.7	87.9	-9.0	-19.3	28.3

Table S5.3. Warm-seasonal annual (WSA) quality and chemical composition after first and second cut. The first cut of each forage was made in July 2019 and the second in August 2019. Values are expressed as the mean of combined varieties.

	Pearl Millet		Sorghum x Sudangrass	
Item	1st Cut	2nd Cut	1st Cut	2nd Cut
DM[1] (%)	24.6	17.4	22.7	15.8
DM yield (tons ac^{-1})	0.87	1.84	1.70	1.56
CP[2] (% DM)	19.7	20.5	15.9	19.6
ADF[3] (% DM)	28.9	33.1	33.4	34.5
NDF[4] (% DM)	55.0	60.2	58.7	60.6
Lignin	5.64	4.84	4.78	5.41
NDFD30[5] (% NDF)	79.7	76.8	73.9	72.9

[1]DM: dry matter.
[2]CP: crude protein.
[3]ADF: acid detergent fiber.
[4]NDF: neutral detergent fiber.
[5]NDFD30: 30 h NDF digestibility.

Table S5.4. Chemical composition of basal total mixed ration (TMR) diet fed to donor fistulated Holstein Dairy cows used for inoculum collection.

Item (% DM)[1]	TMR
OM	93.1
CP	16.4
aNDFom	34.2
ADF	23.3
NFC	37.8
WSC	5.90
ESC	5.20
Starch	19.9
NSC	25.8
NE_L, mcal/ kg[2]	0.77
Ca	0.69
P	0.45
Mg	0.37
K	1.30
Na	0.60

[1]Chemical composition results from DairyOne Laboratories; Ithaca, NY.
[2]Calculated using NRC (2001).

CHAPTER 6: GENERAL DISCUSSION, SUMMARY, AND CONCLUSIONS

This dissertation focused on advancing our understanding of organic, grassfed (**OGF**) dairy production utilizing survey metrics, *in vitro* studies, and a variable cost analysis for forage production on OGF dairies. As an initial step, a survey was developed and distributed to OGF producers across the United States (**U.S.**). Producers were asked questions ranging from demographics and herd characteristics to management practices, knowledge, and satisfaction with their operation. Results showed that although OGF producers were generally satisfied and felt knowledgeable about their forage and animal management practices, there was a high level of disconnect and misunderstanding regarding production of high-quality forages and implications to their herds. This indicated a need to evaluate forage nutritional content and identify high energy forages that could be utilized on Northeastern (**NE**) pastures. Additionally, this survey identified supplement types used on OGF farms. Based on survey responses, three *in vitro* studies were developed to determine the usefulness of supplement inclusion, such as organic apple cider vinegar (**ACV**), and evaluate forage biodiversity and forage combinations on rumen fermentation parameters and kinetics. Additionally, we evaluated variable costs of inclusion of warm-season annuals (**WSA**) in NE pasture systems as establishment can prove to be costly to producers and may not outweight the benefits. Using batch culture methods, supplementation of first cut forage mixtures with organic ACV did not impact rumen fermentation; however, parameters were altered by harvest cutting. A continuous culture study that evaluated increased cool-season grass inclusion indicated that inclusion of Kentucky bluegrass (*Poa pratensis*) may be useful for grazing operations as digestibility is increased. Additionally, inclusion of meadow fescue (*Festuca pratensis*) or perennial

ryegrass (*Lolium perenne*) may be beneficial as results showed decreased concentrations of methane (**CH_4**). Finally, results from a second continuous culture study indicated that inclusion of WSA could be a viable option for grazing systems in the Northeast as apparent digestibility increased while CH_4 concentrations were diminished. When accounting for variable costs, WSA inclusion may prove to be costly. However, WSA inclusion in temperate pasture systems will need to be considered as perennial forage quality and quantity will decrease during summer months that are hot and dry. The results of these studies were designed with the aim of providing applicable results that could prove useful to producers as well as consultants, researchers, and extension agents. Additionally, the results of these studies have the potential to shape OGF producer management practices in areas outside of the NE U.S. Survey results have allowed us to develop a deeper understanding of the characteristics, needs, and opportunities of OGF producers across the U.S. These results may allow for creation of production and financial benchmarks to allow OGF producers to compare themselves to one another in terms of management and production practices. Additionally, these benchmarks could be critical for expansion of the OGF market.

6.1. Identification of Producer Needs and Challenges

Although organic milk production has emerged as one of the fastest growing segments of organic agriculture in the NE U.S., formal needs assessments, including parameters relating to producer knowledge, satisfaction, and understanding have been lacking. Previous research has focused on organic dairy producers primarily focused in the NE U.S. (Pereira et al., 2013). Consumer demand has allowed for new opportunities for organic dairy farmers located in the NE U.S. As interest in OGF production is expanding,

there has been growing demand for information and education to support farmers entering the market. A preliminary survey by Benson et al. (2019) was sent to 140 grass-based dairies across New York and Vermont with a 60% response rate. From this preliminary survey and a secondary survey, 21 producers were chosen to participate and contacted. Each producer was asked to participate in monthly surveys, known as the Grassfed Monitor, to create financial and production benchmarks to allow producers to compare themselves to other grassfed productions (Benson et al., 2019). Preliminary results indicated that the average grazing season was 184 d, longer than the mandated 120 d for organic operations and the mandated 150 d for grassfed productions. Average pasture dry matter (**DM**) was 80.4% in 2017 and 78.7% in 2018 with average milk urea nitrogen (**MUN**) values ranging from 12.1 (2017) to 12.4 (2018). Although these values are, on average, within an acceptable range (10 – 15), the range of values was widely variable (3.3 – 23.8), indicating that animals may not be receiving enough energy from a forage-based diet or are receiving an excess of protein.

Based on these results, we sought to understand the population of producers that have operations certified as organic and grassfed. Further, we aimed to understand what motivated producers to utilize pasture-based operations. The results of our survey are an important first step in providing a foundation for future research, educational outreach, determining grassfed producer needs. To improve OGF dairy producer management practices, there is a need for further research to develop production and financial benchmarks. Additionally, the results of this survey highlight challenges to the OGF community that indicate a need to develop alternative methods of information dissemination as a subsection of OGF producers belong to the Amish and Mennonite

communities. Future research must focus on a wider range of outreach to better meet the educational needs of OGF dairy producers. Finally, the results of this survey indicated discrepancies and misunderstandings in the areas of high-quality forage production and how it relates to OGF dairy production and farm sustainability.

6.2. Forage Energetic Supplementation, Quality, Biodiversity, and Management

Forages are the primary component of OGF dairy cattle diets. It is, therefore, important that forages be of the highest quality. Forage species and quality and pasture diversity vary across the U.S. depending on climate, previous land use, and availability of forages (Sanderson et al., 2005). Because of variable forage quantity and quality, producers typically rely on supplemental energy, forages, and other supplements. Certified OGF farms are restricted in what they may use as supplemental feed and supplements and are, therefore, relying on less traditional supplements to meet animal energetic demands. Results from the aforementioned survey (Snider et al., 2021) indicated that 54.3% of OGF producers were relying on energetic supplements such as dried carrots, yeast, kelp, and ACV. Although there is evidence that kelp may be beneficial in dairy diets (Antaya et al., 2015), there is little research related to alternative supplements such as ACV. Results from the current batch culture study indicate that ACV supplementation does not improve or alter rumen fermentation kinetics. Further, the timing of cutting may have a bigger impact than ACV. However, research related to ACV supplementation is lacking, especially outside of *in vitro* models. Therefore, further research is needed and should utilize *in vivo* models for more accurate results.

Pasture diversity has previously been a focus of researchers as some producers believe that diversity is beneficial to the sustainability of their grazing systems and to

animal production (Sanderson et al., 2005). A grazing study by Sanderson et al. (2005) evaluated pastures composed of 2, 3, 6, and 9 forage species on herbage yield and pasture productivity, finding that more complex mixtures were more productive than simple mixtures during periods of drought. Further, more complex mixtures exhibited greater weed suppression. However, legume species and chicory (*Cichorium intybus*) proportions decreased after two yr in which pastures became dominated by orchardgrass (*Dactylis glomerata*). This indicates that while diversity does play an important role in pasture productivity, the species composition may play a more important role. A study by Pembleton et al. (2015) indicated that increasing pasture diversity extended the grazing season as well as improving forage nutritive characteristics. In turn, this increased milk production per cow and may potentially mitigate environmental consequences of intensive pasture-dairy systems. However, Pembleton et al. (2015) came to similar conclusions as Sanderson et al. (2005), indicating that diverse pastures are only as productive as the individual species comprising the pasture, which can be species specific. The results of our studies are in agreeance indicating that individual species may be more important than the number of species available. For example, in the NE U.S., cool-season perennials (**CSP**) and legumes dominate pastures. However, results from continuous culture studies indicate that WSA inclusion may be beneficial as inclusion decreases CH_4 production without altering fermentation metrics negatively (Dillard et al., 2017; Dillard et al., 2019). This further aligns with our results that showed decreases in CH_4 in diets including sorghum x sudangrass (*Sorghum x drummondii*) and millet (*Pennisetum glaucum*).

Future research may potentially want to focus on inclusions of less commonly used WSA, brassica, legume, or forb species that may be beneficial in terms of animal

performance and CH_4 reduction. For example, Roca-Fernandez et al. (2020) evaluated orchardgrass combined with various legume species, including alfalfa (*Medicago sativa*), birdsfoot trefoil (*Lotus corniculatus*), crown vetch (*Securigera varia*), and sericea lespedeza (*Lespedeza cuneata*) in continuous culture. Legume species were chosen based on their secondary metabolite profile as these metabolites may reduce CH_4 production while simultaneously decreasing the risk of metabolic disease. It was found that diets incorporating sericea lespedeza decreased CH_4 emissions. However, this diet also showed decreased fiber digestibility and VFA concentrations. Diets including crown vetch also decreased CH_4 emissions, but not at the rate that diets with sericea lespedeza did. In contrast to diets with sericea lespedeza, diets including crown vetch were more digestible and exhibited fewer negative effects on rumen fermentation metrics. Further research incorporating alternative legumes should be evaluated but it should be noted that some alternative legumes, such as crown vetch and sericea lespedeza, may be harder to establish in NE climates (Tracy et al., 2022).

Aside from alternative legume species, brassica species may be of use in NE pasture systems. Brassica species are more readily adapted to NE climates and produce a large biomass rather quickly (Dillard et al., 2018). However, brassicas do contain a secondary metabolite known as glucosinolates which may reduce dry matter intake (**DMI**) and cause mineral deficiencies (Gustine and Jung, 1985). Dillard et al. (2018) evaluated fermentation metrics of diets composed of canola (*Brassica napus*), rapeseed (*Brassica napus*), turnip (*Brassica rapa*), and annual ryegrass (*Lolium multiflorum*) combined with orchardgrass in continuous culture. It was found that there was a highly negative correlation between CH_4 production and glucosinolate content. Diets containing canola also showed the additional

benefit of increased ruminal bacteria efficiency. Additionally, brassica species proved to be similar to annual ryegrass in terms of nutrient digestibility, indicating that brassicas may be an alternative to some cool-season forages in pasture-based diets. However, further research is needed to evaluate inclusion rates of brassicas when interseeded in cool-season pastures and their impacts on pasture-based dairy cattle.

6.3. Limitations

Limitations and challenges related to the survey portion of this dissertation have been previously addressed in Chapter 2. However, it is important to highlight limitations related to *in vitro* methods encompassed within this dissertation.

The advantages to using batch and continuous culture are 1) the ability to evaluate a large number of treatments with sufficient replication, 2) the ability to evaluate greater inclusion levels of feed additives that could, in some cases, be toxic to animals if fed at the same level *in vivo*, and 3) that *in vitro* experiments are less expensive as compared to an animal trial (Hristov et al., 2012). Further, as compared to the batch culture system used in one of the aforementioned studies, continuous culture has the ability to remove fermentation media containing end-products while maintaining generally stable fermentation environments (Hristov et al., 2021; Czerkawaski and Breckenridge, 1977). Despite the benefits of using continuous and batch culture systems there are some limitations that should be acknowledged and taken into consideration. Two challenges for both batch and continuous culture systems are inoculation and maintenance of an anaerobic environment. During the inoculation process, rumen contents are removed from fistulated cattle, allowing for exposure of rumen microbes to light, oxygen ($\mathbf{O_2}$), and decreased external temperatures which may impede microbe survival. As previously described,

methods were utilized to overcome these obstacles, such as using a container pre-warmed to 39ºC, collecting inoculant in buckets in heated water baths, and inoculating fermenters or test tubes within 20 min of collection. Other studies utilizing continuous culture have negated these effects by controlling fermenter pH (Ruh et al., 2018), regulating temperature (Dillard et al., 2018), or by increasing infusion of carbon dioxide (**CO_2**) or nitrogen (**N**) to increase anaerobic conditions (Paula et al., 2017). Of these mechanisms, we controlled temperature at 39ºC, maintained a continuous flow of CO_2 into the buffer, and optimized the amount of time spent during and between collections to ensure successful operation for both continuous and batch culture studies (Baker, 2021). However, time of year of collection may have had an impact, creating period effects, as rumen inoculum was sometimes collected during periods of variable temperatures. Despite these challenges, a benefit of using *in vitro* systems is the ability for quick turnaround time should a period or inoculation fail.

Aside from the differences between continuous culture operation and *in vivo* trials (differences in passage rates and lack of absorption; Hristov et al., 2012) another challenge that arises in continuous culture studies is the ability to maintain microbial populations, specifically protozoal populations (Karnati et al., 2009). Differing from single-flow systems, dual-flow continuous culture systems utilize outflow spouts and filters, controlling solid and liquid passage rates. However, protozoal populations tend to decline in dual-flow systems due to the presence of an outflow spout. This is due to protozoa generation time being slower than the turnover time of the continuous culture system (Teather and Sauer, 1988). Other continuous culture studies have utilized filters to improve protozoa retention (Karnati et al., 2009) or sponges (Cabeza-Luna et al., 2013; Cabeza-

Luna et al., 2018). However, the dual-filter design utilized in this study may be inefficient as filters were susceptible to blockage by feed. Further, flow rates between filters and fermenters were unequal, requiring frequent line priming.

It is possible that CH_4 measurements from the current *in vitro* studies may be lower than previously thought as protozoa play important roles in CH_4 metabolism. As mentioned, protozoal populations have been shown to decline in continuous culture systems due to generation time lag and the design of continuous culture systems. Protozoa, particularly entodiniomorphids and isotrichids, have significant impacts on ruminal methanogenesis (Belanche et al., 2015). Isotrichids may have more significant effects due to ruminal O_2 consumption (Firkins et al., 2020). The role of rumen protozoa in methanogenesis is potentially due to their ability to produce H_2 in their hydrogenosomes as well as their symbiotic relationship with methanogens (Dai et al., 2022). It is possible that CH_4 measurements in this study is inaccurate due to lower populations of isotrichids and entodinomorphids, particularly in regard to the isotrichids. Impacts on CH_4 are further exacerbated due to the fact that neither the batch culture or continuous culture systems are entirely closed systems. Steps were taken to create an anaerobic environment, including wrapping openings and seals with parafilm and greasing gaskets to ensure tight sealing of equipment pieces. However, this would not prevent gas escape or oxygen ($\mathbf{O_2}$) introduction entirely. This could influence microbe populations as well as leading to unreliable readings as it pertains to CH_4 concentration. Aside from utilizing a completely closed system, future research should focus on evaluating rumen protozoa populations in continuous culture systems to address potential discrepancies in CH_4 production. This could be accomplished

by conducting a microbial diversity analysis or by collecting samples of fermenter effluent to evaluate protozoa numbers and species.

For continuous culture studies, fermentation vessels were larger than those used in other studies (Bennett et al., 2021; Dillard et al., 2017). An advantage of utilizing larger vessels is the capability to use feed at a more representative particle length (2-mm) as well as feed a more representative diet composed of fresh feed as opposed to freeze dried (Dillard et al., 2017) or pelleted (Ruh, 2017) diets. A disadvantage of this study relates to the source of rumen fluid inoculum for the trials outlined in this dissertation. Ideally, rumen fluid would be obtained from OGF dairy cows or those on a high forage diet. However, inoculum was only available from cows on a standard dairy total mixed ration (**TMR**). As continuous culture periods were only 10 d and bath culture periods 48 h, rumen microbes may not have had enough time to adapt to high forage diets. For continuous culture studies, this was potentially controlled by a 7 d adaptation period but this was not possible for batch culture. However, 7 d may not be enough time for microbial shifts. Further, generation time of various fibrolytic and cellulolytic microbes may be greater than the turnaround time in a dual-flow continuous culture system.

A final limitation of the *in vitro* studies outlined in this dissertation was related to our collection methods. Samples for continuous culture were collected from large carboys and glass outflow bottles that were maintained on ice from d 7 – 10. Samples were collected for proteomic analysis from combined outflow once daily and frozen at -80ºC. The proteomic methods attempted as part of this research were similar to those of Honan and Greenwood (2020), who investigated the rumen metaproteome from live fistulated lactating Holstein dairy cows. However, protein from fermenter effluent collected in the

current trials was too degraded to conduct proteomic analysis. Despite attempts to halt microbial metabolism by placing outflow containers on ice, metabolism was most likely still occurring leading to protein degradation. One explanation is that microbes within the collection containers were not adequately cold-shocked. Future studies should either use smaller collection containers that can be maintained at a temperature that halts microbial action or effluent should be collected from the fermenter vessel. A limitation of collecting fluid from the fermenter vessel at a single timepoint is that it would be representative of fermenter conditions at that time rather than as a collective from the past 24 h.

6.4. Final Perspectives and Future Directions

From this work, several areas of future research focus can be recommended. First, future research should be focused on developing alternative methods of communication and information dissemination for grassfed farmers that do not use traditional means of communication. Further, the findings in the current dissertation agree with previous literature suggesting that forage quality, not quantity, is the most important factor relating to rumen fermentation metrics. Additionally, botanical composition of forages may play an important role in rumen fermentation and metabolism as diets including WSA led to a reduction in CH_4 and increased apparent digestibility. However, a more thorough analysis of costs and benefits or an all-encompassing economic model should be developed to determine if inclusion of alternative supplements or forages would contribute to farm economic viability. Cool-season perennials that are gaining popularity in the Northeast, such as perennial ryegrass, also influenced fermentation with increased proportions of acetate. However, if utilized, greater inclusions of Kentucky bluegrass should be balanced with perennial ryegrass or meadow fescue as Kentucky bluegrass may lead to increased

CH_4 concentrations. To support OGF dairy nutrition, supplementation options should be more closely examined, as supplementation with ACV did not appear to alter fermentation metrics and suggests current commercial use of this supplementation may have limited benefits from a rumen performance perspective. Future research should focus on increased inclusions of ACV in combination with diets of different forage qualities utilizing an *in vivo* model. Finally, additional research focused on alternative forages, such as those with increased plant secondary metabolites, may prove to be useful in terms of animal production, including positive impacts on CH_4 output and protein digestion. The findings in this thesis, including inclusion of WSA in temperate pastures, further evaluation of dietary inclusion of ACV, and increasing pasture biodiversity, have immediate applicability to OGF dairies, and further research as described above will further support the OGF industry.

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