Improving Grazing Capacity Through Introduction of Bloat Free Legumes in Existing Pasture Stands

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ABSTRACT

Three experiments were conducted over 5 yr to evaluate the inclusion of bloat-free legumes through sod-seeding in existing pasture stands, with respect to plant persistence under grazing pressure, animal performance, rumen fermentation, enteric methane production and economic impact. Experiment 1 was separated into two parts, the first which assessed 2 legumes (CMV; cicer milkvetch [*Astragalus cicer* L] and sainfoin [SAIN; *Onobrychis viciifolia* Scop.]) at 2 stages (vegetative and late flower) incubated with alfalfa (ALF; *Medicago sativa*) at 5 inclusion rates 0:100; 25:75, 50:50, 75:25 and 100:0 (as DM) incubated in batch culture. The second part of experiment 1 assessed CMV and ALF at the vegetative physiological stage (16-30 cm stem length, no buds, flowers or seed pods) were incubated in ratios of 25:75, 50:50, 75:25 and 100:0 (as DM) in RUSITEC. In batch culture, dry matter disappearance (DMD) increased linearly ($P \leq 0.01$), propionate concentration (mol 100mol^{-1}) increased linearly ($P = 0.02$), and methane (mg g^{-1} DMD) decreased linearly ($P \leq 0.01$) with vegetative CMV inclusion. In RUSITEC, DMD, ADF and NDF digestibility were increased quadratically ($P \leq 0.01$) with increased vegetative CMV inclusion. Total ruminal SCFA production (mmol d^{-1}) did not change (P=0.59); however, acetate to propionate ratio and NH_3-N (mmol d^{-1}) were linearly decreased (P<0.01), and total microbial protein synthesis and efficiency of microbial protein synthesis were linearly increased (P<0.05) with increased vegetative CMV inclusion. In experiment 2, a two-year (2017, 2018) replicated (n=6) experiment was conducted to evaluate the effect of bloat-free legumes on grazing animal ruminal fermentation, ruminal microbial populations, and enteric methane production. Each yr, 15 ruminally cannulated cows (average 739 kg ± 40kg) were randomly allocated to 1 of 3 replicated (n=6) treatments: grazing sod-seeded SAIN; grazing sod-seeded CMV; or grazing non-sod-seeded control (CONT). Total short chain fatty acid production (mmol d^{-1}; $P = 0.59$) and acetate (mol 100mol^{-1}; $P = 0.26$) did not differ between treatments. Propionate increased linearly ($P \leq 0.01$) and butyrate decreased linearly ($P \leq 0.01$) with increased CMV inclusion. Acetate to propionate ratio decreased linearly (P=0.01) in cattle grazing CMV paddocks compared CONT or SAIN. Ruminal ammonia (NH_3-N) concentration and plasma urea nitrogen were linearly increased ($P \leq 0.01$) in cattle grazing CMV paddocks compared to SAIN and CONT. Enteric methane (g kg^{-1} DMI) and ruminal microbial populations were not impacted by treatment. In experiment 3, a five-year experiment evaluated the effects of

sod-seeding SAIN and CMV into mixed alfalfa-meadow bromegrass (Lanigan, SK) (CONT) or monoculture alfalfa (Lethbridge, AB) (CONT) stands on pasture productivity, steer performance, and economics. At Lanigan, SAIN decreased (treatment × year, $P = 0.01$) from 13% in yr 1 to 2% in yr 2 (% plant population) and did not differ thereafter, while CMV, maintained a proportion of 16% in the stand. Forage yield was greater (treatment × year; $P < 0.01$) in yr 1 in the SAIN and CMV milkvetch treatments compared to CONT. Dry matter intake (DMI) of steers was greater only in yr 5 and ADG was greater ($P < 0.01$) in SAIN and CMV treatments compared to CONT. At Lethbridge, SAIN decreased (treatment × year; $P = 0.01$) from 46 to 17% (% DM yield), while CMV maintained its proportion at 11% over 5 yr. Forage yield increased (treatment × year; $P < 0.01$) only in yr 2 and 3 of SAIN, compared to CMV or CONT. Average daily gain (kg d^{-1}) was not affected by treatment (treatment × year; $P = 0.12$). At Lanigan, SAIN and CMV generated greater gross returns compared to control; however, once establishment costs were applied there were no differences in present value of net returns.

Throughout the three experiments outlined in this thesis, the data suggests possible increases in energetic efficiency and microbial protein synthesis may be observed in cattle grazing mixed CMV pasture compared to ALF when grazed at equal maturity. Results also suggests CMV may be more persistent in mixed pasture under variable environmental conditions, when compared to SAIN; however, risk associated with increased undesirable species in sod-seeded paddocks were observed and cannot be overlooked. Costs associated with sod-seeding CMV and SAIN, such as fertilizer and seed price, combined with variable forage yield and animal performance, reduced the economic feasibility of sod-seeding these species which may limit producer uptake of this type of pasture rejuvenation. The results of these experiments highlight the dynamic environment of mixed species pastures, as results varied depending on level of legume inclusion in pasture, species sod-seeded, and existing pasture species. More research is required to determine the agronomic best management practices for pasture rejuvenation, the impact of pre-existing pasture species on newly introduced species, and the impact of non-tanniferous legumes, such as CMV, on livestock production.

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DEDICATION

I dedicate this thesis to my husband Greg and son Emmett, as they inspire me daily and have both made innumerable sacrifices which have allowed me to complete this project.

Greg, you are my biggest supporter, and I am incredibly fortunate and blessed to be your partner in life.

Emmett, you are my greatest accomplishment.

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ABBREVIATIONS

ADF	Acid detergent fiber
ADG	Average daily gain
ALF	Alfalfa
BW	Body weight
Ca	Calcium
CMV	Cicer Milkvetch
CP	Crude protein
d	Day
dL	Deciliter
DM	Dry matter
DMD	Dry matter disapperance
DMI	Dry matter intake
g	Gram
kg	Kilogram
mL	Millilitre
mm	Milimeter
N	Nitrogen
NDF	Neutral detergent fibre
NFC	Non-fibre carbohydrates
SAIN	Sainfoin
SAS	Statistical Analysis Systems
SCFA	Short-chain fatty acid
SEM	Standard error of mean

1 GENERAL INTRODUCTION

Advancement of the beef industry in western Canada is contingent on the development and growth of the cow-calf, and backgrounding industries. Since the quality and quantity of the forage consumed will directly impact productivity of the cattle (Popp et al. 1997a), the Canadian beef industry requires increased productivity of perennial pasture areas during spring, summer and fall periods. Canadian forage production was reported to have declined over a 30 yr period (Jefferson and Selles 2007), with total hay and pasture acreage declining by 1.1 million hectares over a 5 yr period (2011-2016) (Statistics Canada, 2017). A combination of factors has culminated in the decline of forage acres, including the outbreak of Bovine Spongiform Encephalopathy (BSE) in 2003, high feed prices, unfavourable currency exchange rates, high input costs, and low commodity returns, all contributing to reduced margins for cattle producers (Jefferson and Selles 2007; McGeough et al. 2017). As such, Canadian cattle producers have attempted to remain competitive and increase animal productivity through the adoption of extended grazing practices and alternative winter-feeding systems to reduce overall input costs and maintain positive margins (McGeough et al. 2017; Kelln et al. 2011). Including alfalfa in pasture or hay blends is another way producers can increase animal productivity, however there is a risk of frothy bloat associated with grazing monoculture or high levels of alfalfa which prevents producers from maximising the full economic potential of this legume (Popp et al. 2000). Therefore, producers have started to include bloat-free legumes into pasture blends to increase animal productivity on pasture, while reducing the risk of frothy bloat (Sottie et al. 2013).

Rumen microbial populations are crucial to the health of the host animal, contributing to the digestion of feed particles and production of microbial protein and short chain fatty acids (Li et al. 2009). The community structure and population of the rumen microflora is diverse, and populations can be impacted by diet (Petri et al. 2012; Kocherginskaya et al. 2001), leading to changes in rumen fermentation and rumen methanogensis (Beauchemin et al. 2008). Enteric methane (CH_4) emissions are the largest source of greenhouse gas (GHG) emission from Canadian beef farms, accounting for 63% of total emissions, compared to nitrous oxide (N_2O) emissions of 27%, and CH_4 emission from manure and CO_2 energy emission making up the remaining 10 percent (Beauchemin et al. 2010). The bulk (84%) of enteric CH_4 emissions in the

Canadian beef industry are produced from the cow-calf sector, therefore mitigation strategies could be focused on this sector of the beef industry for high impact (Beauchemin et al. 2010). However, it must be recognized that this sector also contributes greatly to carbon sequestration through forage and grassland production (Beauchemin et al. 2010). Thus, with the introduction of new legumes into a grazing system, studying the effects of novel forages on rumen fermentation dynamics, nutrient metabolism, enteric methane production, and animal performance is critical.

Past research has reported conflicting results regarding the compatibility and sustainability of seeding polyculture legume crops (Jefferson et al. 1994; Sottie et al. 2013). For example, Sottie et al. (2013), reported that novel sainfoin varieties were compatible when seeded in pasture blends with alfalfa, but only at certain geographical locations and under a low stress, minimal-cut environment. Jefferson et al. (2013) reported that alfalfa and sainfoin seeded together may not be stable or sustainable for the western Canadian environment, with alfalfa displacing sainfoin over a 2 yr period (Jefferson et al. 1994). Therefore, it is equally important to understand the best management practices for pasture rejuvenation, how plant species interact with each other, the impact of plant species on soil microbiome, and the plant response to grazing management to allow for long term successful rejuvenation practices (Khatiwada et al. 2021).

The objectives of the following literature review are to provide an overview of pasture rejuvenation in western Canada. This review will focus on relevant forage species for pasture use, discuss techniques available for measuring forage biomass and animal performance on pasture, review ruminant metabolism and methanogenesis, and discuss techniques available for quantifying enteric methane emissions, microbial nitrogen production and the rumen microbiome in a grazing ruminant.

2 LITERATURE REVIEW

2.1 Pasture Rejuvenation

Pastures of western Canada are prone to depletion over time, in which their productivity, nutritional value, regrowth ability, and species composition declines (Khatiwada et al. 2021). Pasture depletion can be caused by over grazing, environment, invasion of unpalatable species, and poor soil fertility (Lardner et al. 2000; Khatiwada et al. 2021). In addition, surveys of western Canadian cattle and forage producers have reported that majority of pastures are over 11 years old before a rejuvenation technique is implemented (WCCCS 2014). Therefore, methods of rejuvenation need to be long lasting and persistent.

Past research has analyzed methods of rejuvenation, which include, but are not limited to, specialized grazing systems, fertilization, burning, mechanical aeration, sod-seeding, and break-reseed (Lardner et al. 2000; Omokanye et al. 2018; Khatiwada et al. 2021). The traditional method of break and re-seed can be expensive and leave land prone to soil erosion (Khatiwada et al. 2021). Mechanical aeration may help increase forage production caused by soil compaction; however, forage yield and quality results after mechanical aeration have been variable in western Canada (Lardner et al. 2000; Khatiwada et al. 2021). Pasture dry matter yield has been reported to increase, but only in the short term (1-2 yr), when fertilization or burning has been used as a rejuvenation technique (Lardner et al. 2000). Continuous grazing involves grazing pasture throughout the grazing season, which can be problematic since livestock will preferentially graze plants and specific locations leading to overgrazing (Holechek 1983). Continuous grazing can have reliable results if pastures are managed for no more than 50% defoliation, and use of salt, fence and water development to promote even livestock distribution (Holechek 1983). Alternatives to continuous grazing include specialized grazing systems such as; (1) deferred grazing in which grazing is delayed until seed maturity of important species is complete; (2) rest rotation grazing in which one pasture receives 12 months of rest, while other pastures carry the grazing load; (3) short duration grazing, in which paddocks are created and grazed for short, intensive periods, with a long rest period; (4) high intensity-low frequency grazing in which stocking rates are light to moderate, grazing occurs for periods longer than 2 weeks, and land is rested for longer than 60 days (Holechek 1983). Sod-seeding (direct-seeding of forage into existing pasture) is a rejuvenation technique that allows a producer to introduce new species into

existing pasture (Khatiwada et al. 2021). Although this technique may appear desirable to producers due to the minimal soil disturbance and low cost, sod-seeding has marginal producer uptake due to its requirement for specialized equipment, and risk of allelopathy and autotoxicity, by existing species, such as alfalfa (Hedge and Miller 1990). In certain circumstances sod seeding has shown to improve dry matter yield and pasture productivity (Khatiwada et al. 2021); however, it may also have variable results depending on species seeded, pre-existing plant species, soil conditions and environment (Khatiwada et al. 2020; Kelln et al. 2022).

2.1.1 Economics of Pasture Rejuvenation

Increased productivity may not lead to increased profitability for cow-calf, stocker, and backgrounding producers, and thus understanding the economic impact of pasture rejuvenation strategies is critical to allow producers to make decisions which may positively impact their profitability. Canadian cattle producers are resilient and innovative, attempting to lower winter feed costs through use of extended grazing practices, use of by-product feeds, and alternative winter feed systems (McGeough et al. 2017; Kelln et al. 2011). Reduction of perennial hay and pasture production has been a lingering problem for decades (Jefferson and Selles 2007); however, there is still a knowledge gap regarding the best management practices, longevity, and economic benefit of rejuvenation (Khatiwada et al. 2020).

Increases in fertilizer costs have led to a reduction in fertilization of perennial hay and pasture and thus production (Jefferson and Selles 2007), but also have played a contributing role in the reduction of net returns of fertilized pastures (Watson et al. 2012). Producers have many rejuvenation strategies available to them; however, choosing a strategy that is economically profitable may be challenging. In a 5 yr study, Watson et al. (2012), compared forage production, stocking rates, and profitability between fertilized (90 kg N ha^{-1}), supplemented (calves grazing pastures were supplemented with dried distillers grains plus soluble at 0.6% BW), and control (no fertilization or supplementation) smooth bromegrass pasture. Their research reported that although forage production was greatest in fields fertilized with N, stocking rates and net returns were greater in the supplemented fields. The relationship between price of land, fertilizer price, supplement cost will play a role in net returns and highlight the intricacies in determining economically profitable pasture rejuvenation and producer profitability (Watson et al. 2012). It is well known that inclusion of legumes into grass pastures will increase the quality and quantity of

forage, and thus animal production (Popp et al. 2000); however, research regarding economic analysis of this type of rejuvenation strategies is limited (Khatiwada et al. 2020). Although forage production was increased in yr 1 after sod-seeding legumes into an existing grass pasture stand, Kelln et al. (2022) reported forage production and animal gains remained unchanged over the 5 yr experiment. This resulted in negative economic returns when compared to control treatment, which may have been attributed to high seed cost associated with the legumes, and poor persistence due to pre-existing plant species (Kelln et al. 2022).

Ineffective rejuvenation results, high feed prices, unfavourable exchange rates, and high input costs all contribute to conflicting economic outcomes from pasture rejuvenation. This underscores the complexity of sustainable, persistent, and economical pasture rejuvenation, and highlights the need for pasture rejuvenation research to allow producers to make informed decisions regarding their operation.

2.2 Perennial Forage Species for Western Canada Pastures

Perennial legumes, such as alfalfa (*Medicago sativa* L.), can increase forage yield and quality of pastures, thus improving pasture productivity (Popp et al. 2000). However, the risk of frothy bloat associated with grazing alfalfa, has led to the recommendation of including at least 50% grass, such as meadow brome grass (*Bromus riparius Rehm*) in pasture blends (Popp et al. 2000). Although this reduces risk of frothy bloat, it limits the productivity of the pasture and thus economic returns for the producer (Acharya et al. 2013). To increase legumes in pasture, while at the same time limiting bloat-risk, producers have started to incorporate bloat-free legumes such as sainfoin (*Onobrychis vicifolia*) and cicer milkvetch (*Astragalus cicer*) into their pasture mixtures. Sainfoin contains condensed tannins (CT) that bind to protein in the rumen and provide protection from ruminal microbial degradation, reducing bloat risk, while still allowing for intestinal digestion (Dahlberg et al. 1988). Alternatively, cicer milkvetch is bloat safe due to its reticulate veining leaf structure which slows digestion in rumen (Howarth et al. 1979). Unfortunately, research surrounding the introduction of bloat-free legumes into existing pasture stands, specifically the cultivars ability to persist in existing stands, the impact on pasture and grazing livestock productivity, the impact on rumen fermentation, and the economic viability of seeding bloat-free legumes, is negligible (Acharya et al. 2013).

2.2.1 Meadow Bromegrass

Meadow bromegrass (*Bromus riparius Rehm*) is a perennial, cool season grass that was introduced into North America in the 1960's and is a prevalent grass species grown for livestock forage in western Canada (de Araujo et al. 2002; Biligetu and Coulman 2010). Meadow bromegrass is a bunch-type, creeping root perennial grass that is known for its leafy production and rapid regrowth following defoliation (de Araujo et al. 2002).

When compared to other bromegrass species, such as smooth bromegrass (*Bromus inermis)*, meadow bromegrass has been reported to have greater stored carbohydrate reserves and thus energy reserves (Lardner et al. 2003). Due to its shorter rhizomes, meadow brome is less aggressive than smooth bromegrass making it more suited for grass-legume pasture mixtures, as this grass generally will not out compete legumes (Ferdinandez and Coulman 2000). The leaves of meadow bromegrass are concentrated at the base of the plant and the species is characterized by its rapid tiller growth and greater tiller density after defoliation, compared to smooth bromegrass or hybrid bromegrass (Ferdinandez and Coulman 2000; Biligetu and Coulman 2010). Meadow bromegrass has rapid regrowth ability following defoliation, particularly during the initial stages of regrowth (0-20 days) when compared to smooth bromegrass or hybrid bromegrass (*B. riparius* × *B. inermis*) (Knowles et al. 1993; Biligetu and Coulman 2010). Meadow bromegrass regrowth ability is attributed to its greater tiller density, rapid leaf area index development and ability to maintain a greater below ground biomass (Biligetu and Coulman 2010).

2.2.2 Alfalfa

Alfalfa (*Medicago sativa* L.), fondly labelled the "queen of the forage crops", belongs to the sub-family of Papilionoideae, and has a historical record of providing highly nutritious livestock forage dating back more than 3300 years ago origins in Persia and Turkey. It is an important perennial legume grown globally due to its longevity, high nutritional value, capacity to persist in a variety of soil and environmental conditions and its ability to increase the yield and quality of pastures (Goplen et al. 1982; Popp et al. 2000; Bhattarai et al. 2020). Alfalfa was first introduced into Canada in 1871 and is a commonly grown perennial legume in western Canadian for pasture, hay, silage, seed production, and dehydrated product production (Goplen et al. 1982; Bhattarai et al. 2020).

Alfalfa consists of leaves containing 3 leaflets, slender, solid, or hollow stems, blue, purple, yellow or white flowers, and tap, branch, rhizomatous or creeping roots that penetrate deep into the soil (Goplen et al. 1982). Alfalfa adapted to a wide variety of soil and environmental conditions and is tolerant of moderately saline soils (Goplen et al. 1982; Bhattarai et al. 2020); however, it is best suited for soils with neutral pH and good drainage, and ambient temperatures of 15-25 ˚C (Goplen et al. 1982).

To mitigate the risk of bloat in livestock grazing alfalfa, selection of reduced bloat alfalfa cultivars began in Canada in the 1970's and the first reduced bloat alfalfa was released in 1997 named AC Grazeland BR (Coulman et al. 2000). Over the past number of decades, researchers have attempted to select varieties with low initial rate of ruminal degradation or varieties with increased level of proanthocyanidins (Wang et al. 2006; Jonker and Yu 2016). Alfalfa contains very low levels of proanthocyanidins, and they accumulate in the seed coat of alfalfa rendering them non-functional for bloat reduction (Koupai-Abyasani et al. 1993). Anthocyanidins share the early and middle stage of the biosynthetic pathway with proanthocyanidins (polymers of anthocyanidins) (Wang et al. 2006) and accumulate as pigments in red/blue flowers and leaves of alfalfa during senescence and stressed conditions (Wang et al. 2006). Recent molecular biology research efforts have attempted to manipulate the flavonoid pathway through transformation of transcription factors to increase the level of proanthocyanidins in alfalfa forage (Wang et al. 2006; Jonker and Yu 2016). Although this strategy is promising, it has had variation in its success, regarding the levels of proanthocyanidins concentration and more research is needed to understand the effects of gene transformation on chemical profiles, ruminal fermentation, and nutrient availability (Heendeniya et al. 2019).

2.2.3 Sainfoin

Sainfoin (*Onobrychis viciifolia* Scop.) is a perennial legume belonging to the Fabaceae family, *Hedysareae* tribe and *Onobrychis* genus (Bhattarai et al. 2016). Sainfoin has been documented in literature for at least 2000 years and is grown as livestock forage throughout North America, Europe and the Middle East (Waghorn et al. 1998). This species is known to be extremely palatable, and tolerant of drought, cold and low nutrient status. However, producer usage of sainfoin (*Onobrychis viciifolia*) (Figure 2.1) has been nominal in the past due to its low

productivity, difficult establishment, and variability in polyphenol concentration (Waghorn et al. 1998; Carbonero et al. 2011).

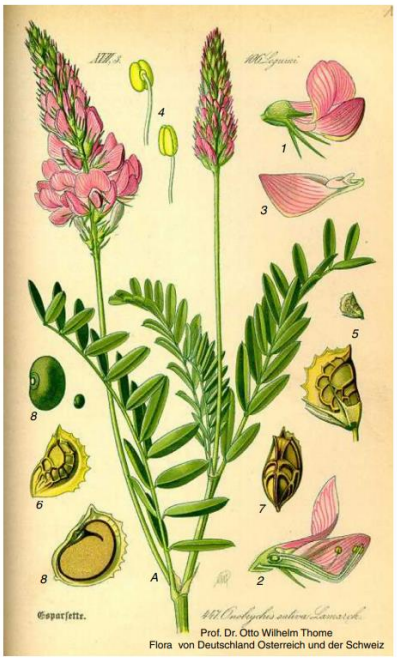

Figure 2.1. *Onobrychis viciifolia*
(Adapted from Carbonero et al. 2011).

Sainfoin persists in well drained, neutral to alkaline soil, and has been reported suited to drought because of its deep tap root (Waghorn et al. 1998; Bhattarai et al. 2016.). Due to its non-aggressive nature, sainfoin can be slow to establish and requires minimal weed competition during establishment (Waghorn et al. 1998). Sainfoin can be seeded as a monoculture or in a mixture with other perennial legumes and grasses; however, it tends to compete better with caespitose vs rhizomatous or creeping rooted grasses if seeded in a forage blend (Bhattarai et al. 2016). Due to slow regrowth after cutting or harvesting, post flower grazing is recommended, and limiting harvest to no more than twice yearly allows for replenishment of root reserves and stand survivability (Waghorn et al. 1998; Carbonero et al. 2011). New cultivars of sainfoin have been developed in recent years and have been observed to persist in mixed stands at a greater rate than older varieties. Sottie et al. (2014) reported that new sainfoin variety (LRC_3519) persisted at >20% of stand compared to older varieties (Nova) and could potentially provide 25-30% of pasture stand biomass over a 3-year period.

2.2.4 Cicer Milkvetch

Cicer milkvetch (*Astragulus cicer* L.) is a long-lived perennial native to Europe, that was introduced to North America from the USSR in 1931 (Johnston et al. 1976; Acharya et al. 2006). Cicer milkvetch has a rhizomatous, creeping root system (Figure 2.2). The stems of cicer milkvetch are hollow and succulent, growing upwards during juvenile plant stages and trailing as the plant ages (Johnston et al. 1976). Cicer milkvetch is adapted to the black and dark brown soil zones in western Canada and provides excellent winter hardiness (Acharya 2001). It has been reported to tolerate a wide range of soil types, including saline or acidic soils, and inclement environmental conditions such as drought (Johnston et al. 1976; Acharya et al. 2006). Cicer milkvetch has pale yellow or white flowers and a seedpod that turns black and leathery as the seeds mature. The seeds of cicer milkvetch are hard, making them resistant to abrasion, water, and air, thus seed scarification is recommended prior to proper seed inoculation (Johnston et al. 1976; Acharya 2001).

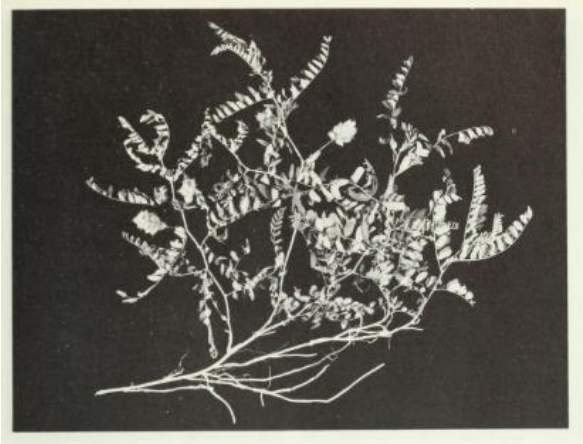

Figure 2.2. Creeping roots, trailing stems and seedpods of *Astragulus cicer* L.
(Adapted from Johnston et al. 1976)

The leaves of cicer milkvetch are pinnately-compound and have a thick epidermal layer (Lees et al. 1982; Acharya 2001). In contrast to sainfoin, cicer milkvetch is not bloat safe due to condensed tannins, but rather due to the mechanical strength, veining pattern and structure of the leaves (Lees et al 1982). The leaves of cicer milkvetch have a reticulate veining pattern, with collenchyma cells extending from the vascular tissue to both epidermal layers in the primary,

secondary and tertiary veins. This creates an “I” beam leaf structure which acts as a physical or mechanical barrier to microorganisms and slows microbial digestion in the rumen (Figure 2.3).

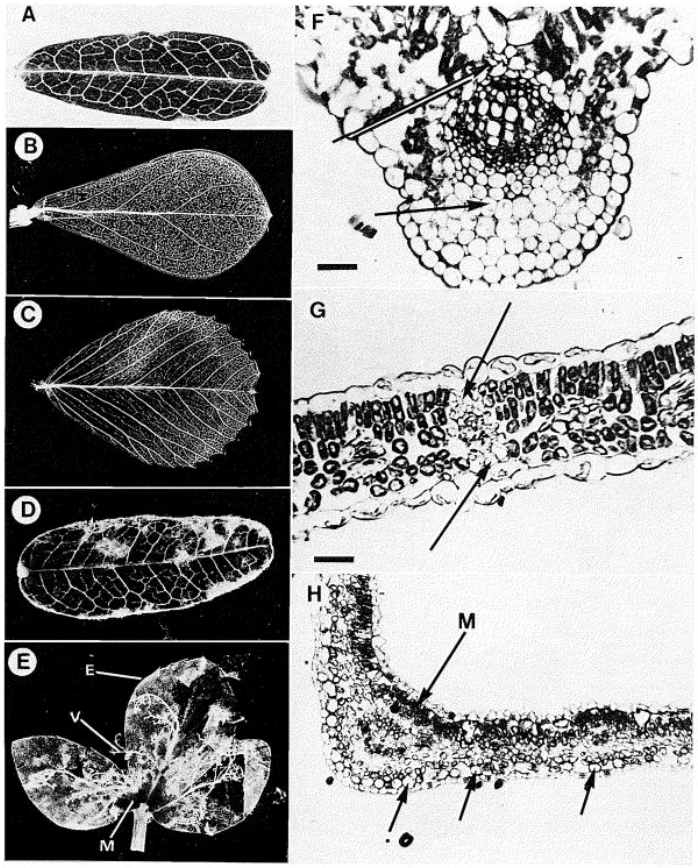

Figure 2.3. Leaf structure of common plants.

(A) Milk-vetch leaflet (X3.25) with reticulate venation pattern. (B) Trefoil leaflet (X4) showing cladodromous venation with few secondary veins. (C) Alfalfa leaflet (X4) showing simple craspedodromous venation typical of sainfoin, white clover, and red clover leaflets. (D) Milk-vetch leaf showing glass-bead damage. Note that the secondary venation pattern and leaf shape are maintained. All dark areas within the leaflet correspond to remaining mesophyll tissue (X4). (E) Trefoil leaf treated as in Fig. 1.3D. The primary and secondary veins (V) have been displaced within the epidermal layers (E). The only mesophyll tissue (M) remaining is found at the base of the leaf near the petiole (~3). (F) Cross section of a midrib from milk-vetch showing collenchyma cells (arrows) extending from the vascular tissue to both epidermal layers. Scale bar = 50 pm. (G) Cross section of a milk-vetch leaflet showing a secondary vein. Arrows denote bundle sheath extension cells extending from vascular tissue to both epidermal layers. Scale bar = 50 pm. (H) Cross section of a sainfoin leaflet (X 140) showing palisade mesophyll cells (M) between vascular tissue and the adaxial epidermis. Arrows indicate the intermittent layer of subepidermal cells adjacent to the abaxial epidermis. Adapted from Lees et al. (1982)

Cicer milkvetch maintains high quality throughout the growing season and has been shown to have higher energy levels into late fall compared to AC Grazeland alfalfa, due to its ability to retain leaves after flowering (Acharya 2001; Lardner et al. 2018). Although some native species of *Astragalus*, like two grooved milkvetch and narrow-leaved milkvetch, accumulate selenium at toxic levels (1000-2500 ppm) cicer milkvetch accumulates Se at levels slightly higher than alfalfa (8.8 ppm) (Johnston et al. 1976).

There have been reports of cattle becoming photosensitive after grazing cicer milkvetch, due to the presence of phylloerythrin, a photodynamic agent and chlorophyll metabolite found in the blood stream of livestock after grazing cicer milkvetch (Marten et al 1987). Increased phylloerythrin concentrations typically result due to liver damage, as the damaged liver is unable to adequately remove the phylloerythrin from the blood for excretion in the bile. Thus, secondary photosensitization may occur and can be most severe when large amounts of lush, green forage are ingested, which produces a large chlorophyll intake (Cheeke 1995).

2.3 Forage Evaluation Techniques in Grazing Systems

2.3.1 Forage Biomass

Although agricultural practices have developed over centuries, George Sinclair was the first to report yields of herbage in 1816 book titled *Hortus Gramineus Woburnensis* (Campbell 1969). Forage biomass (forage yield) is defined as the amount of dried biomass above a specified reference level (ground) at a given time (Allen et al. 2011). Costs associated with grazing and pasture production were estimated to be 20% of total cost of production for Saskatchewan cow-calf and backgrounding operations (Larson 2013). Thus, increasing the yield of perennial forage crops is a critical component to maximize profitability of operations, which rely on perennial forage for grazing, and is a primary focus for plant breeders (Gebremedhin et al. 2019).

Methods of assessing forage biomass on grazed paddocks is more difficult than on clipped (small plot) paddocks due to variability in forage sites, forage mass, selective grazing by livestock, the requirement of forage to remain for grazing, and non-uniform growth from feces and urine deposition (Sollenberger and Cherney 1995). Evaluation techniques can be grouped into direct methods, such as the clip and weigh or mow and weigh (Cooke 1969; Sanderson et al. 2001) or indirect methods, such as pasture rulers, rising plate meters, or more recently ground and aerial based sensor platform technologies (Sanderson et al. 2001; Gebremedhin et al. 2019).

The direct method of quadrate clipping or mowing plots is an accurate and universally used method to assess forage mass; however, this method can be expensive, time consuming and requires a high labour input to collect enough representative samples (Sanderson et al. 2001). Quadrate clipping or mowing of forage samples also is destructive possibly limiting the number of seasons, or the number of samples collected per site (Gebremedhin et al. 2019).

Indirect methods rely on the development of experimental relationship between indirectly measured and actual sample values, and are often easy to use, inexpensive and non-destructive; however, may result in inconsistent results (Gebremedhin et al. 2019). Pasture rulers relay on a positive relationship between forage yield and canopy height (Sanderson et al. 2001), where as a rising plate meter relies on the relationship between sward height and density (Michalk and Herbert 1977). Other methods of biomass estimation could include exclusion cage comparison and ocular estimates (Campbell 1969). Recently, new ground and aerial sensor platforms have been developed that allow for fast, non-destructive phenotyping of not only plant yield, but plant growth, and development (Gebremedhin et al. 2019). Although promising, this technology is still in infancy and requires more research to understand and analyze the "big data" that comes from the sensors and determine its research potential (Gebremedhin et al. 2019).

2.3.2 Forage Quality

Forage quality is a relative term defined as the degree to which forage meets the nutritional requirements of a specific kind and class of animal, and the nutritive value associated with the herbage is dependent on the type of animal production it supports (Pigden 1969; Allen et al. 2011). Forage nutritive value can be analyzed through chemical composition and *in vitro* digestibility of the sample, and nutrient composition is dependent on such factors as plant species, soil fertility, weather conditions, and growth stage (Sollenberger and Cherney 1995; Kopp et al. 2003; Newman et al. 2009). Harvest and storage conditions can also play a role in the quality of forage (Newman et al. 2009). The term forage quality is used synonymously with the term nutritive value; however, forage quality is a broader term considering not only the nutritive value of the forage, but also forage intake (Newman et al. 2009).

Due to variability in plant characteristics as mentioned above, laboratory analysis of forage chemical properties is required to understand whether the forage meets animal requirement (Newman et al. 2009; NASEM 2016). Laboratory analysis of forage may be

performed through wet chemistry or near-infrared spectrometry and includes measurements of forage digestibility, moisture, protein, carbohydrates, minerals, and vitamins (Newman et al. 2009; NASEM 2016).

2.3.2.1 Energy

Gross energy (GE) is related to chemical composition and is the heat released when a substance is combusted to carbon dioxide, water and ash (NASEM 2016). Although GE can be measured in a laboratory, it does not provide information regarding the digestibility or energy available to the animal or take into account losses associated with digestion or metabolism (NASEM 2016). Digestible energy (DE) provides slightly more information than GE as it reflects the diet digestibility, however it does not account for loss of energy associated with digestion and may overestimate energy values for high fiber feedstuffs, such as hay (NASEM 2016). Total digestible nutrients (TDN) can be calculated from DE values and provides no advantage with respect to energy predictions over DE estimates (NASEM 2016). Metabolizable energy (ME) is the GE minus associated urinary and gaseous loss, and although ME provides further progression in accounting for energy requirements, it does not account for the loss of heat energy (HE) produced in ruminal fermentation (NASEM 2016). With respect to energy flow, ME is represented as either HE, or retained energy which is net energy for gain (NEg), or net energy for maintenance (NEm). Therefore, ME is the starting point for much of the net energy (NE) system (NASEM 2016). The advantage to using a NE system is that animal requirements are independent from diet, and as such require feeds to be assigned multiple NE values (NASEM 2016). As such, commercial laboratories estimate forage energy values, such as total digestible nutrients (TDN, %DM), metabolizable energy (ME, Mcal kg^{-1}), net energy for maintenance (NE_M, Mcal kg^{-1}) net energy for lactation (NE_L, Mcal kg^{-1}) net energy for gain (NE_G, Mcal kg^{-1}) from equations based on chemical properties such as carbohydrates, protein, and lipids (NASEM 2016; Newman et al. 2019). These calculations provide insight into the forage value; however, caution should be used as they may vary based on intake, rate of digestion, passage rate, and composition of ingredients.

2.3.2.2 Carbohydrates

Carbohydrates are the main source of energy for ruminants. Plant carbohydrates consist of cell contents (non-fiber carbohydrates), such as organic acids, simple sugars, starch, water-

soluble carbohydrates, and pectin (Hall et al. 1999). Cell wall carbohydrates (fiber carbohydrates), include hemicellulose, cellulose and lignin (NASEM 2016). Structural carbohydrates are typically measured as neutral detergent fiber (NDF) and acid detergent fiber (ADF) as per the Van Soest (1991) detergent method. Non NDF carbohydrates, are typically measured as soluble fiber, sugar and starch (Hall et al. 1999). The energy content of forage is impacted by the digestibility of the fiber carbohydrate fraction, specifically neutral detergent fiber, (NDF) which is the main source of digestible energy (NASEM 2016). Therefore, the proportion of NDF and its rate of degradation has profound influence on the energy content of forage and impacted by such factors as plant species, leaf to stem ratio, and plant maturity making NDF highly variable between forages (NASEM 2016).

2.3.2.3 Neutral Detergent Fiber Digestibility

Neutral detergent fiber digestibility can be determined through *in situ*, *in vivo,* or *in vitro* experiments. The *in situ* bag technique involves the suspension of feed into the rumen using a nylon bag (Nocek 1988). Although this method does not allow for mastication and passage, and results may be variable depending on bag porosity and sample particle size, it provides ideal simulation of the ruminal environment (Nocek 1988). *In vivo* experiments compare feed offered to post-ruminal samples, and often involve use of chemical components, such as protein, fiber or lignin, as markers; however, these chemical components are not always equal across forage species, thus increase variability of results (Cochran et al. 1986). Although *in vivo* experiments allow for physiological context, like *in situ* experiments, can be expensive, laborious, subject to sample contamination, and are not practical for routine analysis (Nocek 1988). *In vitro* digestibility may be determined by single or two stage batch culture experiments, or in semi-continuous culture systems (Tilly and Terry 1963; Czerkawski and Breckenridge 1977; Nocek 1988).

Neutral detergent fiber can be separated into fast, slow, and indigestible pools based on the undigestible portions at a given time point (Raffrenato et al. 2019). Raffrenato et al. (2019) found most forages have fast pools that are completely digested by either 48 or 72h. However, there may be variability as some forages, such as immature grasses or alfalfa forage, which may have NDF fast pools that is digested at by 36 or 48h (Raffrenato et al. 2019). Raffrenato et al. (2018) compared undigested NDF (uNDF) at 12, 24, 48, 72, 96, 144, 240, and 504h and

determined that 240h was sufficient length of fermentation to determine uNDF of forage. Based on Raffrenato et al. (2019), the best combination of timepoints to capture NDF digestibility in most forages were 48, 120, and 240 hr, and if utilizing only two time points, the optimal combination could be 36 or 48 hr and 120 hr.

2.3.2.4 Protein

In addition to energy, proteins are a crucial nutrient for livestock (Newman et al. 2019). Crude protein (CP) is indirectly measured by determining the amount of nitrogen in the forage and multiplying by 6.25, with the assumption that nitrogen constitutes about 16% of the protein in the tissue of forage (ie. 100/16) (Newman et al. 2019). The Kjeldahl method is a validated and standardized method for determining the N content of substances, and involves the digestion, distillation, and ammonia determination of samples (Saez-Plaza et al. 2013). Alternatively, crude protein can be determined through the combustion method (AOAC 990.3) and has been accepted for use by AOAC International for feedstuffs containing 0.2-20% nitrogen (Bicsak 1993)

Digestion of protein and nitrogenous compounds is complex due to pregastric, ruminal fermentation, as rumen microbes utilize dietary nitrogen prior to intestinal absorption (NASEM 2016). NASEM (2016) changed from the CP system to the metabolizable protein (MP) system in 1989 to delineate dietary protein as rumen degradable protein (RDP) or rumen undegradable protein (RUP) and to separate protein requirements into animal requirement and rumen microorganism requirement. Rumen degradable protein provides peptides, amino acids, and ammonia for microbial metabolism, while the protein that enters the duodenum provides nutrients for host metabolism and includes microbial protein, RUP, endogenous protein (NASEM 2016). Metabolizable protein is protein which is digested and absorbed as amino acids in the intestine (NASEM 2016).

2.3.3 Botanical Composition

Determining botanical composition of a grazed sward can provide insight regarding stand persistence, species competitiveness, past management, and animal performance (Sollenberger and Cherney 1995; Whalley and Hardy 2000). Technical methods for measuring botanical composition can include clipping and hand separation of species, visual estimation such as the Daubenmire (1959) method, step-point sampling and recently remote sensing (Sollenberger and Cherney 1995; Whalley and Hardy 2000; Wachendorf et al. 2018). The technique chosen

depends on the research objective, level of detail and scale required, and the number, size and location of the samples (Whalley and Hardy 2000).

Clipping of quadrats and hand separation of individual species involves quadrat samples clipped at a defined height, the species separated by hand, dried, and their mass is recorded (VanKeuren and Ahlgren 1957). Although this method is the most direct and accurate method, it is time consuming, difficult to perform with large sample size, and logistically challenging (Whalley and Hardy 2000). Visual estimation, such as the Daubenmire (1959) method is useful for binary or simple mixtures; however, it requires technician training and can be subjective (Sollenberger and Cherney 1995; Bonham et al. 2004). This method relies on the use of a quadrat, typically, 20 cm × 50 cm, to estimate percent cover, based on 5 to 20% coverage (Daubenmire 1959; Bonham et al. 2004). The step-point method is a rapid and accurate, although tedious, technique, by which a sampling pin is lowered to the ground guided by a notch in the boot of the technician, and the first plant to be touched by the pin is recorded (Evans and Love 1957; Sollenberger and Cherney 1995). Recently, technological advances have allowed remote sensing to be used at a tool to assess grassland vegetation, allowing for a less invasive, simple technique to measure botanical composition; however, this technique is still limited due to its prohibitive cost and low spatial resolution (Wachendorf et al. 2018).

2.4 Measuring Beef Cattle Performance

2.4.1 Body Weight and Body Composition

Measurement of live weight gains (or loss) is a useful took indicative of an animal's response to their environment and overall energy balance (Clark and Campbell 1969; NASEM 2016). Accurate measurements of live bodyweight can be difficult to attain, since bodyweight is a relative number that may be influenced by factors such as gut fill, environmental conditions, or variation between animals (Clark and Campbell 1696; Stock et al. 1983; Watson et al. 2013). Between animal variation can be minimized by using animals of similar age, breed, and size (Watson et al. 2013). Minimizing between animal variation and measuring weights accurately becomes increasingly important when evaluating different feeding systems, such as pasture compared to feedlot (Watson et al. 2013). To reduce variation in live bodyweight measurements, researchers attempt to standardize time of weighing, ambient temperature, cattle handling, feed

consumption and include multiple-day weights (2 or 3 d) to reduce error (Stock et al. 1983; Watson et al. 2013).

Ultrasound has been since the 1950's; however, early techniques were slow and labour intensive (Robinson et al. 1992). Technological development of real-time ultrasound in the 1970's allowed for accurate measurement of 12^{th}-rib fat thickness, longissimus muscle, and rump fat, providing insight into the weight and carcass quality of livestock (Greiner et al. 2003). This technique is quick, portable, and accurate; however, it requires the development technical skills and training of technicians (Robinson et al. 1992).

Body condition scoring is another technique to assess animal body composition (Lowman et al. 1976). Body condition scoring is a subjective technique that involves palpation of the lumbar region, behind the last rib (Zulu et al. 2001; Mulliniks et al. 2015). This is a useful technique because it is not influenced by gut fill, skeletal size, breed, or physiological state (Zulu et al. 2001; Mulliniks et al. 2015). This technique requires training; however, it quick and easy to perform on farm to assess animal body reserves (Zulu et al. 2001; Mulliniks et al. 2015).

2.4.2 Dry Matter Intake

Grazing animal performance depends on multiple factors, but one of the most crucial elements is dry matter intake (DMI). To improve forage utilization and DMI, it is necessary to first understand and measure grazing animal DMI; however, it is exceedingly difficult to measure DMI from grazing animals (Pigden and Minson 1969; Cordova et al. 1978). Dry matter intake on pasture can be determined with indirect measurements such as measurement of forage disappearance, ratio techniques utilizing internal and external markers, or prediction equations (Sollenberger and Cherney 1995).

Measurement of forage disappearance is a measurement of dry matter disappearance over a designated time (Undi et al. 2008). This technique is advantageous because it requires limited laboratory analysis, minimal equipment, and results can be acquired quickly (Pigden and Minson 1969; Undi et al. 2008). However, this method has limitations as individual animal intake can not be estimated, it is labour intensive, and relies on high sampling frequency to reduce error (Undi et al. 2008)

Ratio techniques use fecal output estimation from either total fecal collection or fecal markers and forage digestibility to estimate dry matter intake (Sollenberger and Cherney 1995). Use of fecal markers must be unaltered as they pass through the animal and must be able to be measured in feed and feces accurately (Sollenberger and Cherney 1995). Common fecal markers used in past research include chromic oxide, n-alkanes, lignin, and nitrogen; however, fecal markers have limitations as they are difficult to utilize for lengthy periods of time, may be inaccurate if fecal samples are not representative of total excretion, and are subject to diurnal variation (Cordova et al. 1978; Sollenberger and Cherney 1995; Greenwood et al. 2014). For example, the lignin ratio technique has been used in the past, it may generate biased results, since the digestibility of lignin is dependent on the maturity of the forage (Cordova et al 1978).

Prediction equations typically estimate DMI as a function of body weight, energy requirements, forage quality, and production status (Undi et al. 2008; NASEM 2016). Although prediction equations allow for quick, low input estimation of DMI, they have limitations if a wide range of pasture and environmental conditions are experienced (Undi et al. 2008). Dry matter intake of growing and finishing cattle can be predicted using dietary NEm concentration (Mcal kg^{-1} DM) and average shrunk body weight during a feeding period (NASEM 2016). Although this prediction equation may have variable results depending on the quality of the diet, it is still a recommended prediction equation for dry matter intake (NASEM 2016). Neutral detergent fiber (%, DM) can also be used to predict DMI, with a range of 1.1-1.3% of BW suggested depending on the forage quality (Mertens 1987; NASEM 2016). As with all prediction equations there is some level of variability, and their accuracy is dependent on the precision of measurement of feed quality parameters (NASEM 2016).

Recently, advancement of sensor and wireless sensor technology has allowed for estimation of individual livestock DMI on largescale grazing pastures (Greenwood et al. 2014). This technology relies on animals to wear a small, lightweight sensing device which records specified traits and behaviors (Greenwood et al. 2014). The sensor generated data is used to develop algorithms and prediction equations for use in individual animal intake prediction (Greenwood et al. 2017). As an emerging technology, more research is required to develop, validate, and refine the algorithms used in this technique (Greenwood et al. 2017). Near-infrared spectrometry (NIR) is a technology that can be used to determine chemical composition of feed

and feces, such as total nitrogen, fibre, tannins, digestibility of nutrients, and more recently dry matter intake (Dixon and Coates 2009; Janceqicz et al. 2016; Johnson et al. 2017). This technique allows for rapid, inexpensive estimation of the dietary composition and diet digestibility of grazing livestock; however, results have been inconsistent in predicting DMI and require further research to generate accurate prediction equations (Landau et al. 2015; Johnson et al. 2017).

2.5 Ruminant Metabolism and Methanogenesis

2.5.1 Carbohydrate Metabolism

Carbohydrates consumed by ruminants are degraded and fermented into short chain fatty acids (SCFA) (van Lingren, et al. 2016), mainly acetic, propionic, and butyric acids, which are the principal energy sources used by the ruminant host. The most common pathway of hexose metabolism is glycolysis, which yields per mole of hexose; 2 moles of pyruvate, ATP and NADH (Baldwin and Allison 1983; Hegarty and Gerdes 1999; van Lingen et al. 2016). During the process of glycolysis, there is a net production of hydrogen which is disposed of by methanogens through reducing equivalents that are attached to electron carriers and used to reduce carbon dioxide to methane (Czerkawski 1969). After glycolysis, pyruvate may be coupled with the end products to oxidize NADH. For example, fermentative microbes may convert pyruvate into ethanol, lactate, or succinate, which also results in oxidation of NADH (Stams and Plugge 2009). Alternatively, pyruvate may be used to form acetate; however, the production of acetate is not directly coupled with oxidation of NADH, rather NADH is oxidized through H_2 production, allowing for oxidation of NADH to reducing equivalent NAD+ to allow glycolosis to continue. Therefore, utilization of H_2 by methanogenic archaea, results in a lower hydrogen partial pressure, thus enabling NADH to be oxidized in bacteria that are not able to directly couple NADH oxidation to reduction of metabolites (Stams and Plugge 2009; van Lingen et al. 2016).

2.5.2 Protein Metabolism and Microbial Protein Synthesis

Ruminal degradation of protein plays a pivotal role influencing the availability of ammonia nitrogen, amino acids, peptides, and branched chain volatile fatty acids and microbial growth rates in the rumen. Pre-gastric fermentation of protein and other N compounds impact amino acid supply to the intestines, making ruminant protein metabolism complex (NASEM

2016). Depending on the solubility of the protein, the amount degraded in the rumen may differ. The fraction of protein that is not degraded in the rumen is termed rumen undegradable protein (RUP) and the portion that is degraded in the rumen is termed rumen degradable protein (RDP) with end-products of degradation being amino acids, peptides, branched chain short-chain fatty acids and ammonia, the latter of which may become an energetic expense the animal must dispose of through conversion to urea in the liver excretion (NASEM 2016; Reynolds and Kristensen 2008). Ammonia released in the rumen may be combined with carbon skeletons that can be used for microbial protein synthesis, or it may be absorbed across the rumen wall to be removed by the liver as urea in urine or milk. Once in the liver, urea can be recycled back to the rumen for use by the host through transfer across the gastrointestinal tract (GIT) through either saliva or transfer across the rumen epithelial tissue and other GIT sections (NASEM 2016; Reynolds and Kristensen 2008). Roughly 40 to 80% of urea synthesized in the liver is returned to the gut, showing the importance urea recycling to N balance within ruminants (Lapierre and Lobley 2001).

Urea recycling provides ruminants with an evolutionary advantage, by which during times of low protein, or asynchronous carbohydrate and protein supply, a ruminant can sustain themselves through microbial capture of recycled urea nitrogen (NASEM 2016; Reynolds and Kristensen 2008). The microbial protein that flows from the rumen to the small intestine, is the most important supply of essential amino acids and is estimated to account for close to three quarters of the absorbed amino acid supply for the ruminant (NASEM 2016). Since this microbial protein can supply the host ruminant with most of their AA requirements, urea recycling is very important to the maintenance and supply of microbial protein and ultimately the ruminant host. The factors that influence the amount of microbial protein supply that leaves the rumen can be complex; however, in brief it is dependent on the extent of ruminal degradation, nutrient availability, efficiency of the ruminal bacteria to utilize these nutrients, and rate of outflow from the rumen (Hespell and Bryant 1979; Mangan 1988; NASEM 2016).

The efficiency of microbial protein synthesis is a function of the energy used by the microbial population for maintenance vs growth (Hespell and Bryant 1979; Hackmann and Firkins 2015; NASEM 2016). It has been estimated that microbes use as little as 1/3 of ATP on growth, therefore, increasing utilization of ATP for microbial growth instead of maintenance,

would be beneficial for the host animal (Hackmann and Firkins 2015). Energy used by the microbial population for maintenance could include such functions as motility, production of enzymes, transport of nutrients into the cell, and recycling of cells (NASEM 2016). Energy may also be required if there is asynchronous fermentation, or "uncoupled fermentation" which is the result of energy being released faster than the microbial population can utilize (NASEM 2016). This results in synthesis of reserve carbohydrates (storage of glycogen for later use) and/or energy spilling, wherein energy is dissipated as heat (Hackmann and Firkins 2015; NASEM 2016). Therefore, efficiency of fermentation results when N and energy release in the rumen are in balance with each other (NASEM 2016). Animals fed a high forage diet likely would not experience energy spilling; however, reserve carbohydrate synthesis may still occur (Van Kessel and Russell 1997). Energy spilling and reserve carbohydrate synthesis occur primarily when carbohydrates are in excess in the rumen, such as in a grain-fed animal (Hackmann and Firkins 2015). However, energy spilling may also occur in animals where NH_3-N is the main source of nitrogen since rumen microbes grow slower with NH_3-N vs amino-N (Van Kessel and Russell 1996).

2.5.3 Enteric Methane Production

Methanogens are strict anaerobes (Janssen and Kirs 2008) and diverse members of the archaea domain differing in their composition from prokaryote-bacteria with the absence of a cell wall peptidoglycan polymer. (Wolin and Miller 1987). Although phylogenetically diverse, methanogens share a common feature as they are the only organisms to use fermentation end products to create methane as a sole source of energy or adenosine triphosphate (ATP) (Wolin and Miller 1987; Wolin et al. 1997; Morgavi et al. 2010).

Highly selective in nature (Patra et al. 2017), archaea are estimated to comprise 6.8% of total rumen subunit rRNA (Ziemer et al. 2000), which is small in comparison to populations of protozoa, fungi, and bacteria (Yang et al. 2016). Jannsen and Kirs (2008) reviewed a global data set and concluded that most of the rumen archaea could be classified into three genera: *Methanobrevibacter* (61.6%), *Methanomicrobium* (14.9%), and an uncultured group known as rumen cluster C (15.8%). In contrast, other studies differed greatly in their proportional ranking of archaea (Tajima et al. 2001; Shin et al. 2004), differences which may be attributed to differences in research methods, animals, and diet across studies (Morgavi et al. 2010).

Hydrogen is the central element that drives methanogenesis and ultimately fermentation in the rumen (Hegarty and Gerdes 1999; Morgavi et al. 2010). During the process of rumen fermentation, enteric methane is produced due to the need to dispose of hydrogen (H_2) within the rumen (Newbold et al. 2005). Without disposal, the partial pressure of H_2 could inhibit the normal function of microbial enzymes that are involved in electron transfer, leading to an increase in NADH and reducing rumen fermentation (Hegarty and Gerdes 1999; Morgavi et al. 2010; Leng 2014). Since H_2 is non-polar it may pass freely through microbial membranes, possibly achieving equilibrium, unlike the hydrogen ion concentration (H^+) which must remain in tight control within the cytoplasm (Hegarty and Gerdes 1999). The balance of hydrogen ions (H^+) and dissolved hydrogen gas (H_2) determines the redox potential of the rumen and therefore the ability of the rumen to oxidize and degrade feedstuffs (Hegarty and Gerdes 1999).

Carbohydrate fermenting bacteria do not produce CH_4, but they produce formate, CO_2, and reducing equivalents as products that can be used as substrates by the methanogens to convert H_2 and CO_2 to methane. A select few methanogens can utilize other substrates such as acetate, formate, or methyl compounds (Thauer 1998). For example, formate can be converted to H_2 and CO_2, for the further reduction to CH_4; however, it is estimated that this pathway accounts for a small amount of the methane produced in the rumen (Carrol and Hungate 1955; Hungate 1967; Wolin and Miller 1987). The capture of H_2 by the methanogens involves a symbiotic relationship between methanogens and fermentative hydrogen producers that is referred to as interspecies H_2 transfer (Wolin et al. 1997). In contrast to bacteria, fungi and protozoa contain cellular organelles called hydrogenosomes, which are responsible for producing hydrogenase enzymes and generating hydrogen (Müller 1993). The hydrogenase enzymes act on ferredoxin arising from either phosphoroclastic reactions or as part of a redox couple which allows re-oxidation of reducing equivalents, such as NADH. However, these reduced cofactors, such as NADH, need to be oxidized to NAD+ for the fermentation process to continue (Hegarty and Gerdes 1999). Most methanogens colonize in the biofilm on the surface of feed particles, possibly due to less anti-methanogenic properties at this location (Patra et al. 2017) and play an important role in ruminal digestion (McAllister et al. 1994a). These microbes are found in a consortium embedded in the biofilm, where end products of fermentation produced by one colony can be used by another (Leng 2014). Since the process of fermentation occurs in the rumen, the majority (95%) of CH_4 produced is eructed through the mouth, with the

remainder produced from hind gut fermentation expired through the rectum (Murray et al. 1976).

2.6 Techniques For Measuring Enteric Methane Production In Ruminants

There are several methods available to measure methane (CH_4) emission from ruminants, and they include both indirect and direct methods (Goopy et al. 2016). Accurate and reliable methods of CH_4 measurements are required to allow for establishment of greenhouse gas (GHG) inventories and the development of mitigation strategies (Goopy et al. 2016; Hammond et al. 2016). Indirect methods include *in vitro* incubations, such as the rumen simulation technique (RUSITEC), estimations from diet, and computer modelling. Direct methods rely on the measurement of CH_4 concentration in the air and include, sulphur hexafluoride (SF_6) tracer technique, respiration chambers, and short-term measurement techniques like the Greenfeed® emission monitoring system (C-Lock Inc., Rapid City, South Dakota, USA) (Goopy et al. 2016; Hammond et al. 2016; Hill et al. 2016). After an extensive review of available techniques, Hammond et al. (2016) concluded that all methods will have random error and experimental variation. The decision on which method to use should be based on experimental objectives and resources available as these techniques will vary in their application, cost, accuracy and precision (Hammond et al. 2016). For the purposes of this review, *in vitro* gas production, *in vivo* SF_6 tracer technique, and the Greenfeed® emission monitoring system are discussed.

2.6.1 Short-term Measurements (Greenfeed®)

Short term measurement techniques, like the Greenfeed® emission monitoring system, have arisen due to the need to estimate large numbers of animals in their natural environment (Goopy et al. 2016). The Greenfeed® emission monitoring system (Figure 2.4) is a patented, static, short-term measurement device that measures CH_4 (and other gases including CO_2) emission every 3-6 minutes from individual cattle, while integrating measurements of airflow, gas concentration, and the position of the animal's head during the measurement (Zimmerman and Zimmerman 2012; Goopy et al. 2016; Hammond et al. 2016).

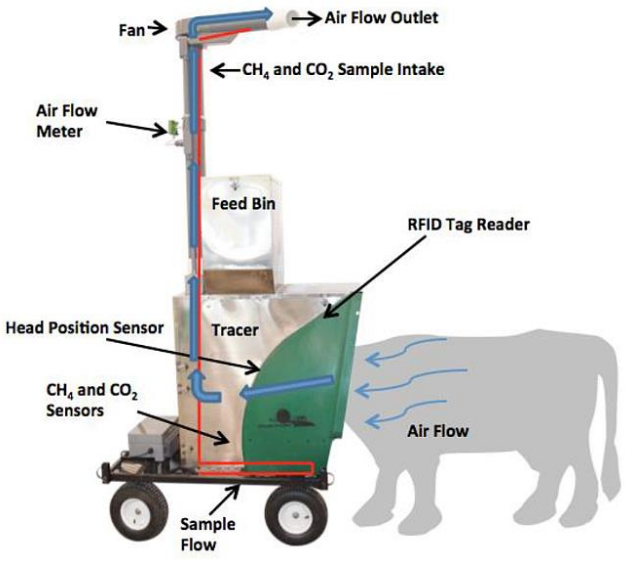

Figure 2.4. Components of the Greenfeed® emission monitoring system. Adapted from: Hristov et al. (2015).

The device measures individual cattle repeatedly over 24-hr, through a Radio-Frequency Identification (RFID) system, as the animals voluntarily visit the device, baited into the system using a pelleted concentrate (Goopy et al. 2016). Limitations to this system include the need for the pelleted energy source as an attractant, as this will impact the digestibility of the diet, short chain fatty acid profiles and the daily methane production, in addition to the reliance on voluntary visits to the unit by the animal (Hristov et al. 2015; Goopy et al. 2016). However, benefits of the Greenfeed® include its non-invasive nature allowing for animals to move freely in their natural environment, its suitability when comparing the effects of different feeds or supplements, its ease of transportation from field to field, and its lower cost compared to other techniques, such as respiration chambers (Hristov et al. 2015; Goopy et al. 2016).

Length of duration of sampling and number of samplings per day are both critical to ensure repeatability of the measurement relative to diurnal patterns of CH_4 emission (Hammond et al. 2016). Manafiazar et al. (2017), determined that 7 to 14 d sampling, with a minimum of 20 spot samples per day, were required for repeatable and reliable data collection. Renand and Maupetit (2016) determined that 50 spot measurements over a two-week period would be sufficient for measurement of CH_4 emission only, but a longer test duration (5 weeks) is required if simultaneous measurement of CH_4 emission and dry matter intake is needed. Included in the prerequisite for accurate measurements is the requirement for a proper training of animals and an

adaptation period of upward of 6 weeks, to allow the animals to become accustomed to the unit. (Renand and Maupetit 2016).

2.6.2 Sulphur Hexafluoride Tracer Technique

The sulphur hexafluoride (SF_6) tracer technique is an *in vivo* technique that can be used for penned animals, but also is a very valuable research technique because it can be used to measure methane emissions from animals in their natural environment while free range grazing (Hammond et al. 2016). A small permeation tube containing a premeasured release rate of SF_6 is placed in the cows rumen and samples of exhaled air (CO_2, CH_4, and SF_6) are continuously collected from the animals mouth using an evacuated container (yoke) connected to a halter attached to the animal's neck (Figure 2.5) (Johnson et al. 1994; Hammond et al. 2016). The halter contains a capillary tube with flow restrictors and regulation of the sampling rate is controlled by the length of the capillary tube (Johnson et al. 1994; Hammond et al. 2016).

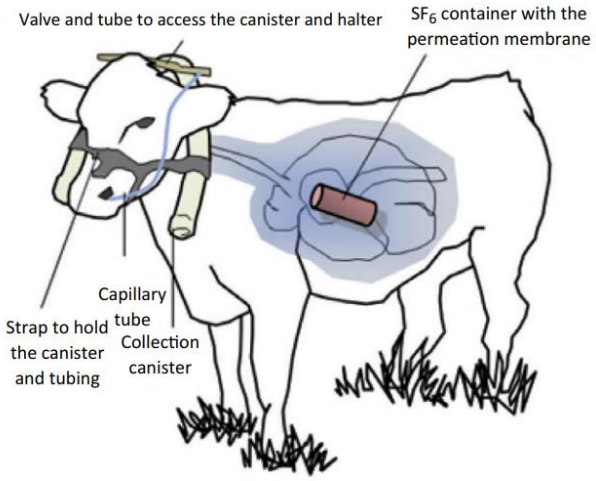

Figure 2.5. Sulphur hexafluoride (SF_6) technique.
Adapted from: Hill et al. 2016.

As described by Johnson et al. (1994), the technique uses the predetermined release ratio of SF_6 to account for dilution as gases exiting the cow's mouth mix with ambient air. At sample collection time, the collection canisters are pressurized with N_2 gas prior to gas chromatography analysis. The SF_6 emission rate is presumed to be identical to the CH_4 emission rate and daily

enteric CH_4 emission is estimated by multiplying the ratio of CH_4: SF_6 concentrations by the known permeation tube release rate and correcting for duration of sample collection and the background SF_6 and CH_4 concentration (Grainger et al. 2009).

Benefits to the SF_6 technique include its ability to monitor animals while they graze normally on pastures; however, it is a costly method, may be less precise, can have high equipment failure and is more labour intensive than other methods (Goopy et al. 2016). It also does not account for CH_4 emission from cannulas (if cannulated animals are used) or the rectum (8% of total emission), and the animals must be trained to wear the halter and collection canister (Grainger et al. 2009; Goopy et al. 2016). Yonker et al. (2020) provides a robust review for proper use of the SF_6 technique including strategies for using cannulated animals. If properly managed using tight fitting cannulas and rumen fluid sampler syringes, difference in emission collection between cannulated and non-cannulated animals is reported to be minimal (Yonker et al. 2020).

Although the SF_6 technique provides a mean CH_4 emission rate, the variability within days and among animals can account for a 5-10% difference (lower or higher) in the CH_4 emission rates observed in a respiration chamber. Therefore, multiple animals and sampling days must be conducted to ensure representative sampling (Hammond et al. 2016). For example, Grainger et al. (2009) reported that the coefficient of variation (CV) among animals was 19.6 vs 17.8%, and within animal was 6.1 vs 4.3% for SF_6 and respiration chamber, respectively. However, if these limitations are considered when utilizing this technique, the SF_6 technique can be reasonably accurate for CH_4 inventory purposes and evaluating mitigation strategies (Grainger et al. 2009).

2.6.3 *In Vitro* Techniques

Simplistically, *in vitro* techniques rely on the anaerobic incubation of rumen inoculant with a feed substrate in gastight culture bottles, and the measurement of cumulative gas volume, providing a powerful tool for feed evaluation (Dijkstra et al. 2005; Goopy et al. 2016). Many *in vitro* techniques are designed for short-term (batch) measurements (Figure 2.6), and although they may be less expensive to perform and allow for high reproducibility, standardization and steady-state conditions may be difficult to achieve (Hill et al. 2016). *In vitro* techniques also do not account for animal intake or rumen retention time which could change the amount of CH_4, and short chain fatty acids produced and the extent of DM disappearance (Aboagye 2019).

The rumen simulation technique (RUSITEC) technique (Figure 2.7) is one example of a semi-continuous culture technique designed for longer-term measurements (4-8 weeks) (Czerkawski and Breckenridge 1977). The RUSITEC allows for a solid phase and liquid phase that are mixed allowing no dead spaces. Since the inhibition of methane production can significantly impact ruminal metabolism gradually over a period of weeks not just days, the RUSITEC aids in reducing variability due to methane inhibition over longer periods of time (Czerkawski and Breckenridge 1977).

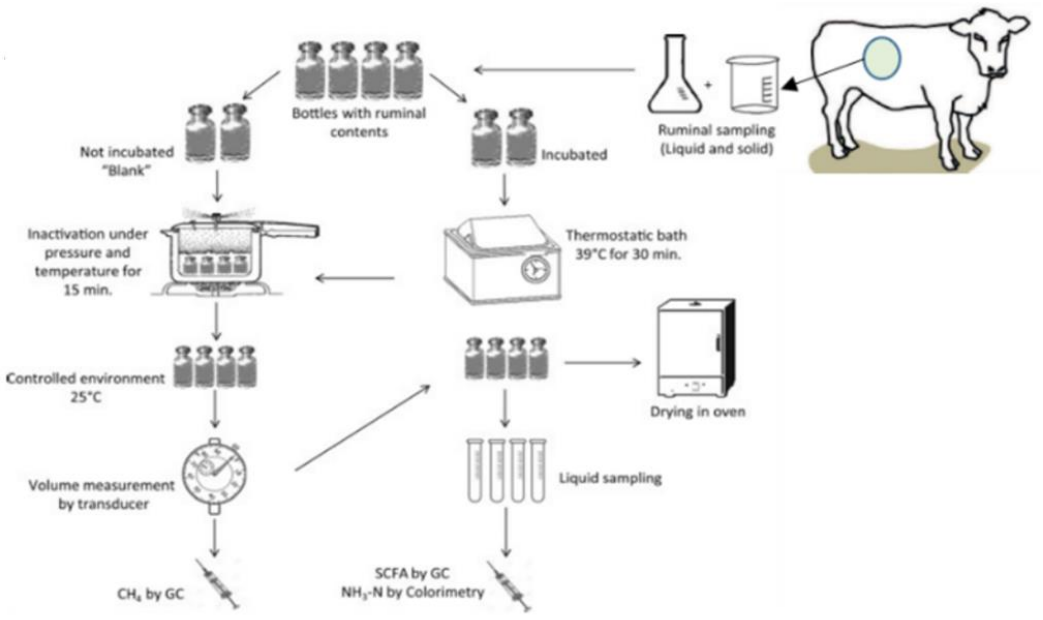

Figure 2.6. In vitro gas technique.
Adapted from: Aboagye 2019.

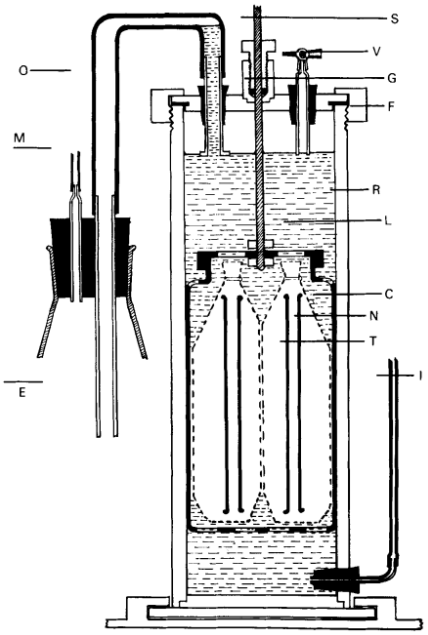

Figure 2.7. Rumen simulation technique.
Schematic example diagram of one unit of a long-term artificial rumen. The driving shaft (S) is made of stainless steel. V, Sampling valve; G, gland (gas-tight); F, flange; R, main reaction vessel; L, rumen fluid; C, perforated food container; N, nylon gauze bag; T, rigid tube; I, inlet of artificial saliva; O, outlet through overflow; M, line to gas-collection bag; E, vessel for collection of effluent. Adapted from: Czerkawski and Breckenridge 1977

2.7 Techniques for Measuring Microbial Protein Synthesis and Rumen Microbiome

Microbial protein can supply 50% of beef cattle metabolizable protein requirement, and thus measuring microbial protein is an important nutritional factor to account for when formulating rations (Broderick and Merchen 1992; NASEM 2016). Total microbial nitrogen (N) flow depends on the amount of available energy in the rumen (fermentable energy) and the efficiency of the rumen microbial population to synthesize protein (g bacterial N kg^{-1} of fermentable energy) (Calsamiglia et al. 2010). Measurement of microbial N flow relies on the premise that the efficiency of microbial protein synthesis is indicative of the efficiency of rumen microbial fermentation, that ruminal ammonia-N is an indicator of microbial available N, and that microbial N flow to the small intestine is indicative of available microbial protein to the host animal (Calsamiglia et al. 2010)

Microbial markers can be used to quantify ruminal protein yield and include internal markers, such as diaminopimelic acid and nucleic acids (RNA, DNA, individual purines, total purines and pyrimidines), or external isotopic markers such as ^{15}N or ^{35}S (Broderick and Merchen 1992). Microbial markers must be able to account for both bacterial and protozoal pools in fluid and solid digesta, and the marker must be easily quantified, unique to bacterial protein (not present in feed), present at a constant ratio, and biologically stable so as not to be an environmental hazard (Broderick and Merchen 1992). This method has limitations because it assumes that protein to marker ratios are standard across rumen microbial populations; however, bacterial and protozoa populations will have different protein to marker ratios (protozoal protein would be labelled indirectly through predation of ^{15}N labelled bacteria) (Broderick and Merchen 1992). In addition, bacteria (fluid associated and particulate associated) and protozoa, have different growth rates and duodenal flow rates resulting in different protein to marker ratios (Broderick and Merchen 1992; Perez et al. 1996).

The desire to better understand the complex microbial ecosystem and dietary impacts on rumen fermentation, microbial protein synthesis, and microbial populations has challenged researchers for centuries (Hungate 1966). Traditional characterization of the rumen microbial ecosystem, through isolation, purification and cultivation of bacteria was challenging (Petri 2013). However, recent advances in molecular technology, have enhanced the ability to quantify the rumen environment and its microbial populations (Petri 2013). Using this technology

bacterial genes (ie. 16S r RNA gene) can be used as markers, extracted from ruminal genomic DNA, and purified, amplified, and sequenced for taxonomic identification (Petri 2013).

2.8 Summary of Literature Review

Growth and profitability of the western Canadian cow-calf, grasser, and backgrounding industries are reliant on perennial pasture production. Declining pasture productivity has been one contributing factor in reduced producer margins, which has resulted in conversion of pasture acreage to annual crop production and a reduction in the Canadian beef herd (Statistics Canada 2017). Perennial legume forage can increase the quality and quantity of pastures (Popp et al. 2000). However, improved knowledge is required to understand the best management practices for legume introduction into existing pasture and the impact of mixed perennial forage on ruminal fermentation, animal production, production economics, and the environment. Focused research on perennial forage management will provide greater understanding of challenges, risks, and opportunities available to producers, whilst helping producers manage their operation's profitability and economic sustainability.

2.9 Hypothesis and Objectives

Sod seeded bloat-free legumes will persist in existing monoculture legume and monoculture grass pastures under grazing pressure and will increase forage biomass, forage quality, steer performance, soil nutrient status, and show a positive financial return as a pasture rejuvenation strategy. Mature cows grazing mixed bloat-free legume pasture will experience change in short chain fatty acid concentration, ammonia production, and enteric methane production when compared to cows grazing monoculture grass pastures. Increasing bloat-free legumes will improve ruminal microbial protein synthesis, short chain fatty acid concentration, ammonia production and reduce methane production.

The objectives of this research are to determine the following:

1. To evaluate the *in vitro* effects of mixtures of legumes on ruminal fermentation characteristics, methane production, and microbial protein synthesis.
2. To evaluate the *in vivo* effects of sod-seeded legumes on ruminal fermentation characteristics, rumen microbial populations, and enteric methane emissions from grazing cattle.

3. To evaluate the effects of sod-seeding bloat-free legumes into existing pastures on pasture productivity, steer performance and production economics.

3 IMPACT OF CONDENSED TANNIN CONTAINING LEGUMES ON RUMINAL FERMENTATION, NUTRITION AND PERFORMANCE IN RUMINANTS: A REVIEW[1]

3.1 Abstract

Legume forages, such as sainfoin, and birdsfoot trefoil can increase the forage quality and quantity of western Canadian pastures, thus increasing producer profitability due to increased gains in grazing ruminants, while reducing risk of bloat in legume pastures due to the presence of proanthocyanidins. Proanthocyanidins or condensed tannins (CT) are secondary plant polyphenol compounds that have been regarded as anti-nutritional due to their ability to bind protein in feeds, enzymes, and microbial cells, therefore disrupting microbial digestion and slowing ruminal protein and dry matter digestion. Research has shown that at high concentrations (>50 g kg^{-1} DM), CT can disrupt microbial digestion. However, at low dietary inclusion rates (5-10 g kg^{-1} DM) they reduce bloat risk, and increase ruminal undegradable protein (RUP), reduce enteric methane production, and confer anthelmintic activity. Yet, research gaps still exist regarding grazing persistence and forage yield of novel CT containing forages and their biological activity due to their vast differences in CT stereochemistry, polymer size, and intermolecular linkages. The objectives of this review are to summarize information regarding the impact of CT on ruminal fermentation, carbohydrate and protein metabolism, and the potential to identify and select for forages that contain condensed tannins for ruminant production.

3.2 Introduction

Alfalfa (*Medicago sativa* L.) is one of the most important legumes grown for forage in western Canada and elsewhere (Sottie et al. 2014) due to its winter hardiness, high yield, persistence, and nutritional quality (Majak et al. 2001; Popp et al. 2000). Use of alfalfa in gazing systems results in greater yield of beef per hectare than grass pastures (Popp et al. 2000). However, concerns over the development of frothy bloat has limited producers from gaining the full benefits of cattle grazing pure alfalfa or mixed alfalfa-grass pastures (Popp et al. 2000,

[1]A version of this chapter has been published. Kelln, B.M., Penner, G.B., Acharya, S.N., McAllister, T.A., and Lardner, H.A. 2020. Impact of condensed tannin containing legumes on ruminal fermentation, nutrition and performance in ruminants: a review. *Can. J. Anim. Sci.* 101:210-223.

Wang 2012). This barrier has been estimated to cost Canadian producers upwards of $50 million per year in lost productivity (Majak et al. 2001; Acharya et al. 2013).

Interest in condensed tannin-containing legumes has grown due the fact that they reduce the risk of bloat, possess anthelminthic properties, improve protein utilization and potentially lower enteric methane emissions (McAllister 1994a; Min and Solaiman 2018; Mueller-Harvey et al. 2019). Forages such as sainfoin (*Onobrychis vicifolia*) and birdsfoot trefoil (*Lotus corniculatus*) contain condensed tannins (CT) that bind to proteins and provide protection from ruminal microbial degradation, limiting bloat, while still allowing for intestinal digestion and absorption of nutrients when included in small amounts (5 g CT kg^{-1} DM) of the diet (Dahlberg et al. 1988; Wang 2015). In addition to bloat reduction, CT-containing forages have the potential to benefit the growth and production of ruminants, through increased ruminal bypass protein, potentially enhancing the efficiency of protein utilization (Wang 2015). However, if concentrations of CT become too high (> 50 g CT kg^{-1} DM), there can be adverse effects on production, including enzymatic function inhibiting fiber digestion and the creation of insoluble protein complexes, which can increase fecal N excretion (Berard et al. 2011; Refat et al. 2015; Lagrange and Villalba. 2019).

The objectives of this review are to summarize information regarding the impact of condensed tannins on ruminal fermentation, carbohydrate and protein metabolism, and the potential to identify and select for forages that contain condensed tannins for ruminant production.

3.3 Overview of Tannins

Toxins and compounds that impair digestibility represent a form of plant defense that can affect digestive and metabolic processes in herbivores. Plant defense mechanisms include tannins, alkaloids, saponins, and cyano-glycosides and often account for less than 2% of the plant dry matter weight (Cates and Rhoades 1977). Tannins are secondary plant compounds that are naturally occurring and may be uniformly or non-uniformly distributed in the bark, leaves, flowers, and seeds of plants (Wang 2015). Since they are energetically expensive to produce, they were originally thought to be of benefit to the plant due to their ability to ward off herbivory (Bate-Smith 1972). However, more recent research has shown that they also play a role in providing defense against microbial pathogens, and insect pests that may harm plant

development (Dixon et al. 2004). Polyphenols have also been reported to provide a competitive advantage to plants by controlling the amount of nitrogen (N) in the organic versus mineral form, impacting N in litter, facilitating N recovery by host plant mycorrhizae, and minimizing N availability of N for competing plants (Northup 1995). The effect of polyphenols on N mineralization and cycling, may be of benefit to plants during times of low N availability by increasing N availability to the plant, but also by reducing losses of mineral N forms (NH_4^+, NO_3^-) through leaching and volatilization (Kraus 2003). In plants that produce tannins, it is estimated that they are the fourth most abundant compounds produced in plant tissues after cellulose, hemicellulose, and lignin (Hernes and Hedges 2000). Interestingly, condensed tannins (CT) are structurally similar to lignin in that they both are composed of phenol monomers (Hagerman and Butler 1991). The name tannin is derived from the French word *tan* meaning the bark of the holm oak (Frutos et al. 2004), as the CT it contained were originally used to tan the hides used to produce leather (McMahon 1999a).

Common forages in Canada that contain tannins include but are not limited to: (1) Sainfoin (*Onobrychis viciifolia Scop*.); (2) Birdsfoot trefoil (*Lotus corniculatus L*.); (3) Purple Prairie Clover (*Dalea purpurea* Vent.); (4) Sulla (*Hedysarum coronarium L*.); (5) Lotus (*Lotus pedunculatus Cav.);* and (6) Dorycnium (*Dorycnium rectum L*) (Waghorn et al. 1998; Berard et al. 2011; Peng et al. 2016). However, tannin extracts from black wattle (*Acacia mearnsii*), quebracho, and chestnut trees have been used in research pertaining to total mixed feed rations (Beauchemin et al. 2007; Grainger et al. 2009; Carrasco et al. 2017). Canadian research has recently characterized purple prairie clover (PPC) as a source of high concentration of CT (87 g kg^{-1} DM), with unique anti-microbial properties and novel research is being conducted to determine the effect of PPC on ruminal fermentation, animal performance and optimal inclusion of PPC in ruminant diet (Wang et al. 2012; Huang et al. 2018; Peng et al. 2020)

3.4 Condensed Tannin Concentrations and Location in Plants

Condensed tannins can occur in leaves, roots, wood, bark, fruit, buds, and flowers (Kraus et al. 2003), and can play an important role in regulating nutrient dynamics in ecosystems (Schweitzer et al. 2008). Condensed tannins are produced by a plant organelle, known as the tannosome, and are stored in vacuoles within the plant (Wang et al. 2015, Barry and McNabb 1999). They can also be found in intercellular spaces and cell walls with the factors that dictate the site of CT deposition within the plant being largely unknown (Kraus et al. 2003). It is known

that CT concentrations and chemical composition varies among environments, species, growth stages and accessions (Wang 2015). Although condensed tannins are found in the seed coat of alfalfa they are absent in vegetative tissues.

3.5 Chemistry of Tannins

To be classified as a tannin, molecules must be oligomeric, consisting of repeating structural units containing phenolic groups (Hagerman and Butler 1991) that are free to complex with proteins, starch, cellulose, hemicellulose, pectin, and minerals (Mangan 1988; Hassanpour et al. 2011). More specifically, tannins can be further classed into either condensed or hydrolysable forms (Figure 3.1; McMahon 1999a) and are differentiated by their molecular structure and size (Min et al. 2003).

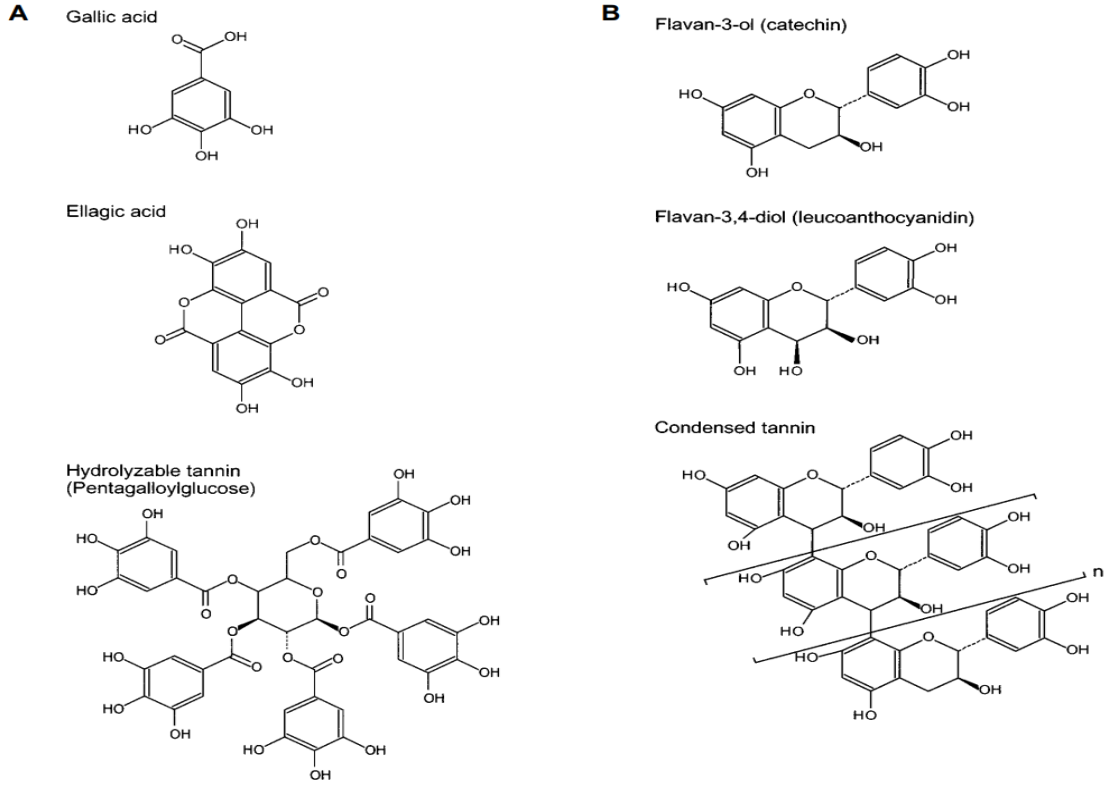

Figure 3.1. Structural components of (A) hydrolysable and (B) condensed tannins. (Reprinted with permission McMahon et al. 1999a. © Canadian Science Publishing or its licensors).

Concentrations of hydrolysable tannins (HT) are generally lower than condensed tannins in most plants (Hassanpour et al. 2011) and HT consist of polyphenols ester-linked to a hexose group. As their name suggests, they can be hydrolyzed enzymatically or by heating with a weak acid (Mangan 1988; McMahon 1999a). Hydrolysable tannins, found in plants such as small burnet (*Sanguisorba minor* Scop.; Stewart et al. 2019) have a carbohydrate (usually D-glucose) as their central core (Min and Hart 2003), and the hydrolysable group is esterified with phenolic groups of a smaller molecular weight (simple monomers), such as ellagic or gallic acid. These simple monomers can be toxic, resulting in liver necrosis in mice following oral administration (2.0-4.6 g kg^{-1} BW), hypersecretion of intestinal mucosa in rats fed diets containing 3% DM and metabolic acidosis and methaemoglobinaemia in sheep following oral administration (8 g kg^{-1} BW) of tannic acid (Mitjavila et al. 1977; Zhu and Filippich 1992; Wang et al. 2015).

Proanthocyanidins (PA), more commonly referred to as condensed tannins, are polymers of flavanol units, linked by carbon-carbon bonds with molecular weights of 2000 to 4000 Da or greater (Hagerman and Butler 1981; Foo et al. 1982; Scholfield et al. 2001). PA differ from HT in that they can only be degraded by hot mineral acid (McMahon, 1999a). The molecular weight of condensed tannins is dependent on the hydroxylation pattern, degree of polymerization, and the number of functional groups (Naumann et al. 2013). The chemistry of condensed tannins is exceedingly complex (Schofield et al. 2001; Barry and McNabb 1999), as they differ greatly in their stereochemistry (2,3-cis vs 2,3-trans of C ring), polymer size (degree of polymerization), and intermolecular (monomeric) linkages (McMahon et al. 1999a; Barry and McNabb 1999; Foo et al. 1997). The flavonoid skeleton, with its standard numbering system and letters to identify the rings is shown in Figure 3.2.

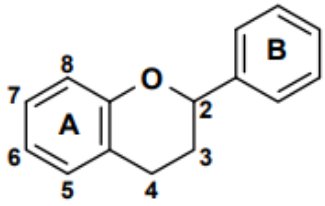

Figure 3.2. Flavanoid skeleton, and numbering system.
(Reprinted with permission Hagerman 2011).

More specifically, CT can be characterized by the hydroxylation of their B ring, where a tri-hydroxylated B ring is termed a prodelphinidin and a di-hydroxylated B ring is a procyanidin (Tharayil et al. 2011). Depending on their stereochemistry, prodelphinidins may be further differentiated as gallocatechin or epigallocatechin, and proanthocyanidin can be differentiated into catechin or epicatechin forms (Figure 3.3).

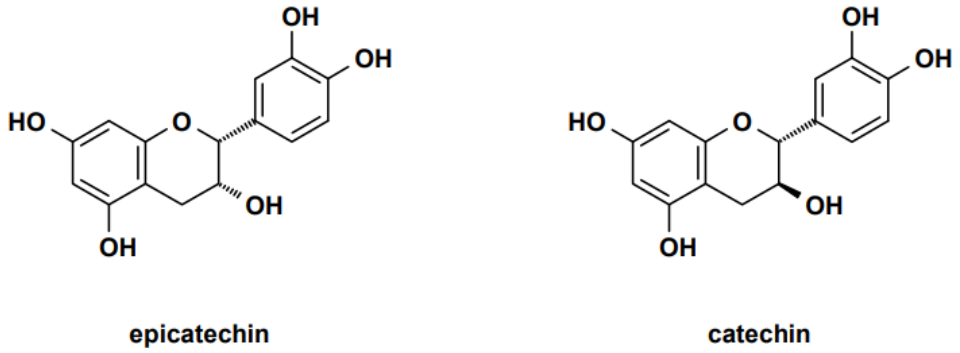

Figure 3.3. Structural components of procyanidin. (Reprinted with permission Hagerman 2011).

In combination, these structural confirmation, size, and linkages allow for numerous differences in chemical structures, each having a different outcome on the biological activity of the condensed tannin. The diversity in chemical structure impacts their binding capacity, with respect to their affinity for proteins, carbohydrates, and minerals, and thus their nutritional impact on digestion and metabolism in ruminants (McMahon et al. 1999a). In addition, there may also be differences observed at differing physiological stages of plant development with respect to the location, concentration, and composition of CT within plant tissues, impacting their molecular weight and degree of polymerization (McMahon et al. 1999a). For example, Koupai-Abyazani (1993) reported a decrease in the composition of cis-isomers (83 to 48%) and an increase in trihydroxylated B-rings (60 to 90%) as sainfoin leaves matured from youngest leaves (leaflets not separated) to oldest mature leaves.

In the past there has been confusion when classifying tannins, due to the analytical method used to quantify them (Schofield et al. 2001). For example, phenols with a smaller molecular weight (eg. chlorogenic acid), which do not precipitate proteins, can react with

reagents such as in the Folin-Dennis assay, resulting in the misidentification of some phenols as tannins (Mangan 1988). Alternatively, as phenols increase in size, their affinity for proteins can decrease and that for cell-wall components can increase, resulting in them being isolated with the lignin portion of the plant cell wall (Mangan 1988). In the past, analytical assays assumed that CT were contained within the soluble fraction, and thus the analysis of the CT in this portion was used to indicate the total CT concentration (Barry and McNabb 1999). However, CT may be contained in the protein and fiber bound portions, which can account for 20 to 30% of CT and may be underestimated depending on the assay chosen (Terrill et al. 1992; Wolfe et al. 2008). For example, inclusion of a cosolvent, such as acetone, to the butanol-HCL spectrophotometric assay, solubilized CT from plant tissue and increased anthocyanidin yields from birdsfoot trefoil and big trefoil species (Grabber et al. 2014). Therefore, the assays selected must be chosen appropriately to ensure that all CT (soluble, protein bound, and fiber bound) are extracted and quantified (Schoefield et al. 2001).

3.6 Evolutionary Role of Condensed Tannins

Co-evolutionary biology is the study of pattern of interactions between plants and animals that lead to selection pressures that alter function over time (Cates and Rhoades 1977). As researchers seek to understand the effect of CT on livestock production, they must also understand the function of plant defensive systems. Since tannins are produced at an energetic cost to the plant, their purpose and evolutionary role has been questioned and has yet to be fully defined. Historically, it was speculated that condensed tannins act as a feeding deterrent or form of herbivory defense, which would benefit the plant and outweigh the metabolic demand required for their synthesis. Herbivory defense may play a role given the reduction in dry matter intake, organic matter digestibility, and weight gain in ruminants consuming forages that contain high concentrations of condensed tannins (>50 g kg^{-1} DM) (Feeny 1970; Barry and Duncan 1984; Dschaak et al. 2011). In addition to herbivory defense, Hernández and Van Breusegem (2010) hypothesized that flavonoid accumulation may play a role as a carbon sink, consuming energy, while sparing nitrogen during times of abiotic stress. Many authors have reported an increase in tannin concentration when plants are stressed, for example by lower soil fertility, increased ambient temperature and low soil moisture (Anuraga et al. 1993; Bussotti et al. 1998; Booker and Maier 2001), possibly resulting in a plant defense strategy aimed at resource conservation and reduced herbivory (Kraus et al. 2003).

Other proposed functions of CT include allelopathic responses, as condensed tannins are thought to alter soil conditions to inhibit the growth of competitors (Haslam 1977). When plants die, it is speculated that the tannins complex with proteins and inhibit N mineralization and nitrification in the soil (Kraus et al. 2003). Condensed tannins may also have a role in the attraction of pollinators in anthocyanidin-containing plants. Like proanthocyanidins, anthocyanidins are flavonoids, and share components of the synthetic pathway with proanthocyanidins. These compounds have been reported to be important attractants for pollinators (Lev-Yadun and Gould 2008). Finally, CT provide a protective function for the leaf surface against plant pathogens. For example, in sainfoin during vegetative growth, CT are located in the leaflets. However, with maturation, they shift from an abaxial to adaxial (lower to upper) position and provide protection to the leaf surface (Lees 1995; McMahon et al. 1999a).

3.7 Condensed Tannins and Rumen Fermentation

An extensive review of CT by Wang et al., (2015) indicated that the optimal concentration of total CT in sainfoin was 50 g kg^{-1} for ruminants to avoid reductions in feed intake and DM digestibility, while still reducing ruminal protein degradability thereby enhancing rumen undegradable protein. Achieving this target concentration of dietary CT, may be difficult under monoculture or polyculture pasture management given that the total tannin concentration in plants can vary considerably with plant phenological stage, accessions, varieties, and cultivars (Wang 2015). This variability in tannin concentration illustrates the important role that forage and grazing management can have in achieving optimal concentrations of condensed tannin in forages for ruminants. Currently, CT-containing forages fed to grazing livestock include: (1) birdsfoot trefoil (*Lotus corniculatus* L.; (2) sainfoin (*Onobrychis viciifolia Scop*.); (3) sulla (*Hedysarum coronarium* L.); (4) purple prairie clover (*Dalea purpurea* Vent.); and (5) white prairie clover (*D.candida* Michx. ex Willd; Table 3.1).

Table 3.1. Condensed tannin concentrations in common forages

Forage Type	Concentration	References
	g kg^{-1} dry matter	
Birdsfoot Trefoil (*Lotus corniculatus* L.)	15-36	Grabber et al. 2014; Grabber 2015
Sainfoin (*Onobrychis viciifolia Scop.*)	23-75	Koupai-Abyazani et al. 1993; Malisch et al. 2015
Sulla (*Hedysarum coronarium* L.)	1-40	Cabiddu et al. 2009; Tibe et al. 2011
Purple prairie clover (*Dalea purpurea* Vent.)	59-94	Jin et al. 2012; Peng et al. 2016
White prairie clover (*D. candida* Michx. ex Willd)	9-62	Huang, et al. 2018; Li et al. 2014.

Evaluation of the effects of these forage species on ruminant production can be difficult and complex due to variation in the chemistry and concentration of CT (Mueller-Harvey et al. 2019). Nutrient availability, specifically N availability to plant roots, leaf carbon:nitrogen ratios, ambient temperature, precipitation, CO_2 and O_2 concentrations all may play a role in the concentration of tannins within plant tissues (Foo et al. 1982; Dixon et al. 2004; Lindroth 2010). For example, increased ambient temperature, reduced precipitation, decreased soil fertility, and increased CO_2 concentrations have all been reported to increase concentrations of CT (Tharayil et al. 2011). In addition to the environmental influences, between-species and within-species differences in tannin types and production can occur depending on the stage of maturity and the plant tissue examined (Kraus et al. 2003; Schweitzer et al. 2008). For example, the rate of proanthocyanidin polymer synthesis and degree of polymerization in polymers isolated from sainfoin leaves differs with stage of development (Figure 3.4). These factors combined can have a significant effect on the production of CT, and thus has prompted researchers to seek to understand the variability in plant CT concentrations and role of CT in plants with respect to ruminant production.

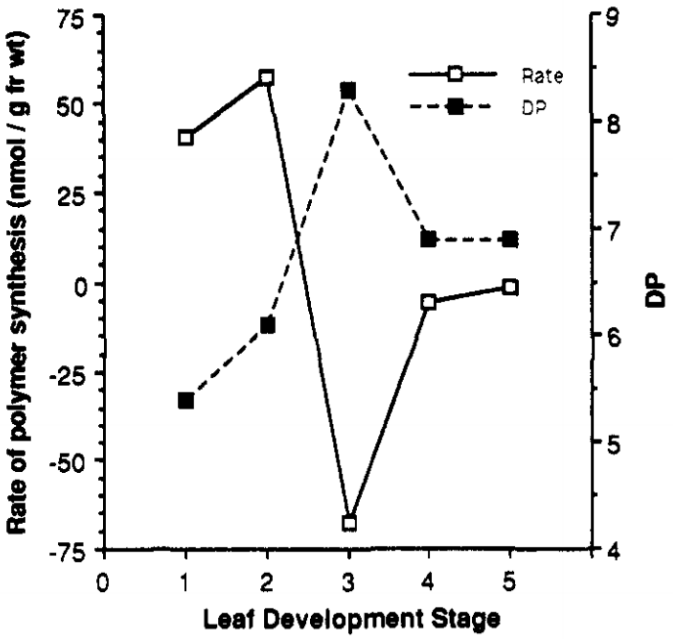

Figure 3.4. Changes in the proanthocyanidin polymer synthesis (nmol/g fr wt) and degree of polymerization (DP) in polymers isolated from sainfoin leaves at different stages of development.

Leaf stages: 1 = youngest leaves; 2 = leaflets separated but folded; 3 = leaflets partially unfolded; 4 = leaflets fully unfolded; 5 = older mature leaves. (Reprinted with permission from Koupai-Abazani et al. 1993. Copyright (1993) American Chemical Society)

3.8 Effect of Condensed Tannins on Carbohydrate and Protein Digestibility

3.8.1 Carbohydrate

Although proanthocyanidins are noted for their ability to bind proteins, they also play an important role in carbohydrate metabolism, due to their ability to complex microbial enzymes and alter microbial populations that can reduce fiber digestion (Bae et al. 1993; Martinez et al. 2006). Barahona et al., (1997) reported that ruminal digestibility of organic matter (OM) was reduced by 20% and total tract digestibility of OM and fiber (NDF and ADF) was decreased by 3.6, 20.3 and 41.7%, respectively, when *Desmodium ovalifolium* and *Flemingia macrophylla* tannin-containing forages were included in the diet (90 g kg^{-1} DM) of sheep compared the same forages treated with polyethylene glycol which binds CT. Differences in the extent of degradation were observed between the two different legume sources, attributed to the astringencies of the CT and level of protein bound CT in the forage source. In other studies,

decreases in the digestion of cellulose and hemicellulose have been reported when *Lotus pedunculatus* containing high (106 g kg^{-1} DM) vs low (46 g kg^{-1} DM) CT was fed (Barry and Manley 1984). The reduced fiber digestibility as a result greater concentration extractable CT may be linked with the inhibitory effect of CT causing an uncoupling of N availability coupled with carbohydrate fermentation or direct inhibition of microbes involved in carbohydrate fermentation (Barahona et al. 1997; Carrasco et al. 2017). Some of the inhibitory effect may arise from the direct binding and inactivation of carbohydrases by CT (McAllister 1994a). Similar results were observed by Martinez et al. (2006), in which tannins were not observed to prevent bacterial attachment to starch granules, but instead starch hydrolysis was slowed leading to a reduction in degradation due to a physical modification of the surrounding protein matrix. Therefore, when considering the use of tannins to aid in the control of digestive disorders such as rumen acidosis, it was proposed that tannins would be more effective in grains that possess a readily degradable protein matrix (Martinez et al. 2006).

As alluded above, CT have been observed to have an inhibitory effect on many species of microorganisms (Carrasco et al. 2017). For example, CT inhibit the ability of *Fibrobacter succinogenes* to digest fiber through inactivation of extracellular enzymes and interference with the adhesion to cellulose fibers (Bae et al. 1993). While there are numerous studies in which a reduction in fiber digestibility has been reported, differences among plant species with regard to the structure and concentration of CT can affect the extent to which fiber digestion is inhibited (McAllister 1994b; McMahon et al. 1999a; McAllister et al. 2005). Beauchemin et al. (2007) reported no effect on DM or fiber digestibility, when cattle were fed low amounts (0, 1, and 2% DM) of a quebracho tannin extract; however, this source of tannin also failed to reduce ruminal methane emissions. Similarly, McMahon et al. (1999b) did not see a clear effect of CT in sainfoin on *in vitro* DM disappearance. Differences in results among studies may be related to variation in quality of the forages, concentration of CT, or the affinity of the CT sources for carbohydrates and proteins. The fractionation of CT within the plant (protein-bound vs., fiber-bound vs., extractable) may also have a significant effect on the biological activity of tannins (Naumann et al. 2013). Perez-Maldonado (1996) suggested that free CT may be metabolized to non-phenolic compounds and excreted, or possibly produce an ‘artifact’ in lignin as a result of tannin-fibre interactions, leading to an overestimation of dietary ADF and NDF digestibility. These differences speak to the complexity of CT regarding livestock species, plant species,

tannin concentration, molecular structure, and environmental effects on the role and severity of CT on ruminant metabolism.

3.8.2 Protein

The ability of proanthocyanidins to precipitate proteins has been of interest for decades as it may be a means of reducing protein degradation in the rumen and increasing the amount of amino acids that flow to the small intestine, possibly improving protein utilization in ruminants (Waghorn et al. 1998). In addition, a reduction in urinary nitrogen excretion, due to reduced ruminal proteolysis, and increased fecal nitrogen may have a beneficial environmental impact as a result of fecal nitrogen being more stable in soils as less mobile and volatile than urinary nitrogen (Mueller-Harvey et al. 2019). The mechanisms by which CT reduce ruminal protein degradation may be a function of their ability to: (1) prevent microbial attachment and reduce substrate availability (McAllister et al. 1994a; Scalbert 1991; Mangan 1988); (2) inhibit enzymes (McAllister et al. 1994b; Makkar et al. 1988); or (3) directly affect the ruminal microorganisms (Scalbert 1991; Frutos et al. 2004).

A reduction in ruminal protein degradation is associated with lower ruminal ammonia production and greater flow of non-ammonia N to the duodenum. This alters the rumen degradable and undegradable protein fractions within feed, shifting the site of N metabolism and absorption from the rumen to the intestine (Frutos et al. 2004; Waghorn et al. 1987; Naumann et al. 2013). Barry and Manley (1984) concluded that the inclusion of CT increased the post-ruminal non-ammonia N digestion compared to non-CT containing forages. The protein–tannin complexes formed in the rumen are insoluble at ruminal pH (pH 6.5); however, they are theoretically released in the more acidic environment of the abomasum (pH 3.5), enabling the protein to be hydrolyzed and the amino acids to be absorbed in the small intestine (Jones and Mangan 1977; Waghorn et al. 1987). Lagrange and Villalba (2019) reported an increase in fecal N and a decrease in blood urea N, when lambs were fed sainfoin (31.2 g CT kg^{-1} DM) compared to alfalfa (2.1 g CT kg^{-1} DM) and birdsfoot trefoil (13.7 g CT kg^{-1} DM), suggesting a decrease in ruminal ammonia concentration and a shift in site of protein digestion.

Shifting the site of N digestion and absorption may also have environmental benefits. As discussed previously, when protein is rapidly degraded in the rumen, a higher amount of ruminal NH_3 may be produced, much of which ultimately is excreted in urine as urea (Dijkstra et al. 2013). The shift in N excretion from urea in the urine to a more stable form of N in feces can

increase soil organic N concentration and reduce GHG emissions caused by nitrous oxide emissions and nitrogen leaching (Mueller-Harvey 2019). Alternatively, increased fecal N excretion is also a reflection of CT reducing the availability of N, and suggests that some of the protein-CT complexes fail to dissociate in the abomasum, contributing to a loss of dietary N (Naumann et al. 2013).

3.9 Effect of Condensed Tannins on Enteric Methane Production

Ruminants utilize and host a vast and diverse microbial population to facilitate the anaerobic digestion of structural carbohydrates to yield utilizable monomers. These monomers are fermented into end products such as short chain fatty acids (SCFA), ammonia, carbon dioxide, and hydrogen (Naumann et al. 2013; Aboagye 2019; Morgavi et al. 2010). During the process of rumen fermentation, methanogens convert H_2 and CO_2 to methane due to the need to dispose of hydrogen (H_2) within the rumen (Newbold et al. 2005). Given the high energy content in CH_4 (13.3 Mcal/kg; Aboagye, 2019), the process of methane production represents an energetic loss to the animal, equivalent to as much as 12% of gross energy intake of the host (Czerkawski 1969; Johnson and Johnson 1995; Beauchemin et al. 2008). Condensed tannins can act to modify the rumen environment and, in some studies, have been reported to linearly decrease enteric CH_4 emissions with increasing dose (Min and Solaiman 2018), while in other studies, no differences in CH_4 emissions have been reported when ruminants are fed condensed tannin containing forages (Chung et al. 2013; Huang et al., 2018; Stewart et al. 2019). The exact mechanism by which CH_4 inhibition occurs is not well understood. However, it has been hypothesized, that depending on the source and concentration of tannin, it may be the result of: (1) tannins acting directly on methanogens to reduce their population in comparison to other species such as fibrolytic, amyolytic, and ureolytic bacterial communities (Patra and Saxena 2010; Williams et al. 2011; Carrasco et al. 2017); (2) tannins act as a H sink reducing the amount of hydrogen available to reduce CO_2 to CH_4 (Naumann et al. 2013; Becker et al. 2014); and (3) indirect inhibition by decreasing available nutrients to other microorganisms that produce the reducing equivalents used by methanogens to reduce CO_2 to CH_4. This may be a result of direct interaction of tannins with plant cell wall carbohydrates, leading to a reduction in the colonizable surface area available for fibrolytic bacteria, through direct inhibition of fibrolytic bacteria, or changes in ruminal pH that in turn impact microbial populations (Carulla, et al. 2005; Naumann et al. 2013; Carrasco et al. 2017).

3.10 Effect of Condensed Tannins on Animal Feed Intake and Health

3.10.1 Feed Intake

The effects of CT on nutrient supply are variable including studies that report increases in nutrient supply and others that report a reduction (Table 3.2). As highlighted previously, interpretation of responses are likely affected by the analytical methods used to determine CT concentrations (Frutos, et al. 2004; Schofield et al. 2001) in addition to the structure and concentration of the CT. Reduction in feed intake has been observed with increasing dietary CT (Min and Solaiman 2018) and has been speculated to be the result of reduced palatability due to the astringent nature of tannins and potentially a slowing of ruminal digestion thereby increasing the filling effect (Kaitho et al. 1997; Frutos et al. 2004). Rationale for reduced feed intake are still speculative since many studies have reported an increase in intake of CT forages (Maugen et al. 2014; Scharenberg 2007). Feed intake can be influenced by the concentration of CT in the diet with > 50 g CT /kg DM consistently decreasing feed intake (Barry and Duncan 1984; Waghorn et al. 1994; Beauchemin et al. 2007), which speaks to the importance of understanding differences in plant species, environmental effects, and CT concentrations in plants.

Differences in feed intake and DM digestibility have also been observed among domesticated ruminants (sheep vs. cow vs. goat) and between ruminant and monogastric livestock (Mangan 1988; Min and Solaiman 2018). For example, Min and Solaiman (2018) reported a linear reduction in DM digestibility in sheep, but not in goats fed S. lespedeza They also reported a slight increase in DMI for goats compared to sheep when grazing S. lespedeza, which was attributed to increased rumen digestion rate and rumen volume. Diet selection, microbial species, and physiological differences between livestock species may play a role in the passage rate of feed particles through the rumen and thus the time available for CT to impact digestibility. Ruminants such as cattle have a longer rumen residence time than small ruminants, such as sheep and goats, due to extensive compartmentalization of the rumen and a relatively small omasal opening. In comparison, goats have a less complex rumen, with an increased omasal opening, and a larger abomasum, all leading to increased passage rate of digesta out of the rumen and a reduced effect of CT on DM digestibility (McSweeney 1988).

Table 3.2. Effect of condensed tannin (CT) on nutrient digestion, ruminal fermentation parameters, and animal performance

Forage Type	Method	Response[a]	References
Birdsfoot Trefoil (*Lotus corniculatus* L.)			
	In vivo, cattle	Reduced milk urea N, ruminal NH_3-N, urinary N excretion, linear reduction in milk yield with increasing CT	Broderick et al. 2017
	In vivo, cattle	Reduction in urinary N, blood urea N, greater retained N	Stewart et al. 2019
	In vitro	Reduced CH_4 production, NH_3-N concentration, no effect on total VFA concentration, increase in A:P ratio	Christensen et al. 2017
	In vivo, cattle	Decrease in apparent CP digestibility, milk urea N, urinary N, increased fecal N	Ghelichkhan et al. 2018
Sainfoin (*Onobrychis viciifolia Scop.*)			
	In vitro	Unaltered microbial production, increased bacterial incorporation of NH_3-N, decrease in VFA concentration, enzyme activity, dry matter disappearance	McMahon et al. 1999b
	In vivo, cattle	Reduction in bloat, soluble protein level in rumen fluid	McMahon et al. 1999b
	In vitro	Reduction in CH4 and total gas production, A:P ratio	Hatew et al. 2015
	In vitro	Decreased DMD, OMD, VFA concentration, no difference in ruminal concentration of peptides or NH_3-N	Refat et al. 2015
Purple prairie clover (*Dalea purpurea* Vent.)			
	In vivo, sheep	Increased DMD, OMD, reduced blood urea N, reduced concentration of total VFA, propionate, NH3-N, no difference in growth, wool growth or carcass characteristics	Peng et al. 2016
	In vitro, In situ, cattle	Increased gas pressure, DMD, total VFA concentration, A:P ratio, NH3-N production	Peng et al. 2020

[a]VFA = volatile fatty acid; DMD = dry matter digestibility; OMD = organic matter digestibility; A:P = acetate:propionate

Another explanation for the difference in effect of CT on DMD and DMI among ruminant species may be due to differences in salivary proteins. Certain mammals, such as deer and goats, have proline rich salivary proteins that can bind to CT in the diet, reducing the detrimental effects that they may have on enzyme activities or ruminal microorganisms (Barry and McNabb 1999; Hagerman and Robbins 1992). For example, after mastication, 90% of CT were recovered from oesophageal extrusa in sheep vs. only 25% in extrusa from deer, a result that likely reflects the affinity of CT for salivary proteins in deer (Barry and McNabb 1999). In addition, there is evidence that some bacterial species in the rumen may be able to degrade tannins. Although they are unlikely to inhabit the rumen of all domestic ruminants, *Streptococcus caprinus,* isolated from feral goats grazing tannin rich *Acacia* species, was shown to be capable of degrading CT (Brooker, et al. 1999) possibly explaining the ability of feral goats to consume high concentrations of CT.

3.10.2 Animal Health

3.10.2.1 Livestock Performance

As CT have potential to reduce feed intake and the digestibility of protein and fiber, CT may impact production parameters such as milk, wool, and meat production. Obviously, at high concentrations of CT (> 50 g kg^{-1} DM), the reduction in feed intake likely explains the reduction in productivity (Wang et al. 2014). However, at low to moderate concentrations (20-45 g kg^{-1} DM) CT have been reported to improve indicators of nutrient use efficiency (Min et al. 2003). For example, Min et al. (1999) reported increased wool production in lambs fed *Lotus corniculatus* (17g CT kg^{-1} DM), and Wang et al. (1996) observed that *Lotus corniculatus* (44g CT kg^{-1} DM) increased milk yield without affecting feed intake in sheep.

High levels of dietary crude protein levels (23.1% DM) and plasma urea nitrogen levels greater than 19 mg dL^{-1} have been reported to decrease first cycle pregnancy rates and over all pregnancy rates in dairy cattle by negatively impacting the uterine environment (Butler et al. 1996; McCormick et al. 1999; Min et al. 2001). Diets containing high levels of rumen undegradable protein (6.8% DM) lower plasma urea nitrogen and have been shown to increase reproductive rates (McCormick et al. 1999). Therefore, forages containing CT may have the ability to increase reproductive efficiency in ruminants. For example, it has been reported that sheep fed CT (17g CT kg^{-1} DM) forage (L. *corniculatus*) experienced an increase in mean ovulation rates leading to an increase in the number of lambs born, without an increase in feed

intake (Min et al. 1999). Ewes grazing pasture containing L. *corniculatus* (18g CT kg^{-1} DM), had an increased lambing percentage (21%) which was attributed to increases in mating and fecundity (number of corpora lutea per ewe ovulating). When compared to diets supplemented with polyethylene glycol to bind CT, it was reported that the 50% of the increase in reproductive rate was due to the condensed tannins in the L. *corniculatus* (Min et al. 2001).

Condensed tannin-containing forages also have the potential to alter the fatty acid composition of meat and milk by reducing ruminal biohydrogenation of unsaturated fatty acids. This has been shown to increase the unsaturated fatty acid profile of both meat and milk, likely due to shifts in the composition of rumen microbial populations. For example, steers finished on birdsfoot trefoil (*Lotus corniculatus* L.) pasture have been shown to have carcass dressing percentages and consumer sensory evaluations similar to grain-finished beef (MacAdam and Villalba 2015).

The positive effects on livestock production are confounded by differences in plant species and CT, with beneficial responses being most likely when protein is limiting the diet (Mueller-Harvey 2019). As the concentration of CT in forages differs, responses in ruminant livestock may be both forage species and CT dependent. For example, birdsfoot trefoil has been shown to increase body weight, milk, and wool growth in sheep, while other species such as sulla and lotus pedunculatus may reduce body weight and wool production (Waghorn et al. 1998).

As previously discussed, hydrolysable tannins can be toxic and result in negative effects on intestinal morphology (Mitjavila et al. 1977; Zhu and Filippich 1992; Wang et al. 2015). However, research regarding the direct effect of condensed tannins on ruminant gastrointestinal morphology is limited.

3.10.2.2 Bloat

Bloat, or ruminal tympany is a digestive disorder resulting in the overdistention of the reticulo-rumen. It can occur as either frothy bloat, where gases formed during the fermentation process are trapped in a stable foam that prevents eructation, or free gas bloat where gas separates from the rumen contents but eructation does not occur (Howarth 1975; The Merck Veterinary Manual 1998; Majak et al. 2001). Bloat is a concern to producers because when untreated it can result in high mortality. However, bloat is difficult to manage in extensive grazing systems, as its onset is rapid and unpredictable. Pasture bloat is believed to be the result

of the rapid release and accumulation of high levels of soluble protein in the rumen that contributes to the coalescence of the stable froth that leads to bloat (Howarth 1975; Majak et al. 2001; Majak et al. 1995). Thus, bloat can be a common disorder in cattle grazing legume pastures, such as alfalfa and clover. Pasture management systems that allow for a continuous and rapid clearance of ruminal contents will reduce the incidence of bloat, due to a reduction in the release of soluble proteins and production of gas in the rumen (Majak et al. 1995). Other strategies to increase utilization of alfalfa pasture have included use of pluronic detergents which are effective in reducing bloat, selection for alfalfa cultivars with reduced bloat risk, grazing at advanced maturity, and allowing adaptation to the forage and ensuring rumen fill before turning cattle into alfalfa pastures (Majak et al. 2001; Acharya 2013). However, the grazing of pure alfalfa stands is still limited as these mitigation strategies are not completely effective in preventing bloat (Acharya et al. 2013). Because of the effect of CT on soluble protein, forages that contain CT reduce the risk of bloat by preventing the formation of stable foam. McMahon et al. (1999b) found that displacing alfalfa with increasing levels of sainfoin in the diet (10 vs. 20% of DMI) of cattle reduced the degradation of ruminal protein and resulted in 26 and 82% reduction in the incidence of bloat, respectively. Similarly, a reduction in bloat in cattle has been observed when alfalfa was displaced by sainfoin at 10% of DMI (McMahon et al. 1999b). Barry and McNabb (1999) suggested that the minimum concentration of CT to prevent bloat was 5 g kg^{-1} of DM. However, Wang et al. (2006) found differences in bloat reduction depending on the phenotypic stage of the forage and suggested that pasture inclusion of 35% sainfoin in alfalfa pasture would reduce, but not eliminate risk for bloat over the grazing season. Optimal concentrations for bloat prevention are likely to vary among forage species, with maturity at the time of grazing and with growing conditions.

3.10.2.3 Antiparasitic Activity

Worldwide, nematode infections of the gastrointestinal tract are a major concern that impact the production, health, welfare, and economics of grazing ruminants (Hoste et al. 2012). The effect of tannins on microorganisms is well documented (Scalbert 1991), and recently there has been interest in the anthelmintic and anticoccidial properties of CT. However, research is limited regarding specific forages, types of CT, and their effect on parasites. Condensed tannins with a higher molar percentage of prodelphinidins, such as those in sainfoin, have exhibited anti-parasitic activity *in vivo* in sheep and goats (Mueller-Harvey et al. 2019). The mechanisms by

which the CT act on gastrointestinal parasites are not well understood, but may be related to their ability to bind with protein and directly inhibit parasite enzymes or an indirect effect due to a more responsive host immune system as a result of improved protein utilization (Hoste et al. 2012; Mueller-Harvey et al. 2019; Min and Hart 2003). In support of the direct effect theory, Chan-Perez et al. (2016) found that extracts of chimay (*Acacia pennatula*) and sainfoin (*Onobrychis viciifolia*) blocked the emergence of L1 larvae, and those larvae that did emerge exhibited altered morphology, with separation of the cuticle from the pharynx, bulb and intestinal cells. Wang et al. (2013) screened tannins from ten western Canadian forages to determine their antimicrobial activity, and found purple prairie clover had unique activity on *E.coli* O157:H7, increasing lag time and reducing the rate of growth of *E.coli* O157:H7. When compared to sainfoin, a forage with similar CT concentrations, Liu et al. (2013), PPC was found to have greater ability to precipitate protein, increasing their ability to alter outer membrane permeability, thus requiring 4-6 times lower dosage of CT to initiate an inhibitory effect on *E.coli* O157:H7.

3.11 Future Plant Breeding and Research on Condensed Tannins

Historical research has focused on plant breeding for increased CT in legumes, such as alfalfa and lower levels of CT in some tropical plants (Gruber et al. 1999). As discussed, there are marked differences in the concentration and composition of CT due to environment, plant species, and harvest time. Therefore, improving the predictability of biological responses to varying CT profiles is a prerequisite to the success of future breeding programs. For this to be achievable, evaluation of not only the variety, but the study geographical location and plant physiological stage must be considered in the selection of optimal types and levels of CT (Azuhnwi et al. 2013). In addition, the ability to relate the responses observed from feeding CT forages to the CT concentration in plants has proven difficult, thus the structure of CT must be defined so as to be able to predict their post-consumption biological activity in livestock (Azuhnwi et al. 2013). This is further emphasized as methods used to quantify CT in plants yield different results and that the extraction process may not fully relate back to biological activity in vivo (Scholfield et al. 2001).

Azuhnwi et al. (2013) determined that certain varieties of sainfoin possessed CT with chemical properties (molecular weight) that remained more consistent over multiple harvests and across geographical locations, thus making them more suitable for future breeding programs

(Mueller-Harvey 2019). Malisch et al. (2016) found that the procyanidin:prodelphinidin ratios in sainfoin were 50:50 in stems and 10:90 in leaves and that high yielding plants had lower concentrations of PA with fewer leaves. In support of this, tannin concentrations have been found to increase when plants are stressed, which could be associated with a reduction in yield (Kraus et al. 2003). This may be problematic since yield is one factor that will be of primary importance for industry adoption of new varieties. Thus, forage breeding programs focusing on varietal persistence and yield will be critical for new varieties to be adopted by industry. Additionally, it will be critical for breeding programs to focus on decreasing variability in the activity of CT in legumes by: (1) refining the composition of PA in the plant; (2) increasing the overall PA concentration; or (3) increasing the proportion of leaf biomass which has the highest concentration of proanthocyanidins's (Malisch et al. 2016). Despite considerable research, genomic tools suitable to manipulate CT levels in forages are lacking, restricting rapid advancements through breeding programs (Hayot Carbonero et al. 2011; Mueller-Harvey et al. 2018). In addition, prominent legume species, such as alfalfa contain low levels of CT levels, and conventional plant breeding approaches have been unsuccessful in increasing CT levels. Research and knowledge are lacking regarding the mechanisms associated with CT polymerization, transport, and storage and thus should be a major focus of CT research going forward (Dixon et al. 2013).

Research regarding the impacts of practical agronomic practices on CT concentration and structure is needed to select for CT types and concentrations that will produce desirable biological responses. Certain species, such as sainfoin, historically had low productivity and grazing persistence. However, focused breeding programs have shown improvement in persistence of cultivars selected under high-intensity grazing conditions (Hayot Carbonero et al. 2011; Sottie et al. 2014; Sheppard et al. 2018).

Finally, research regarding the direct effect of CT on the gastrointestinal morphology of ruminants is required to eliminate knowledge gaps regarding the physiological effects of CT supplementation and condensed tannin-containing forages.

3.12 Conclusion

Condensed tannin-containing forages can benefit livestock production by reducing bloat, increasing nitrogen utilization, increasing reproductive efficiency, and increasing antiparasitic activity. A reduction in greenhouse gas emissions through reductions in methane and nitrous

oxide emissions may occur when ruminants consume CT containing forages. Continued research and increased knowledge have resulted in a switch from a once negative perception of condensed tannins to a more positive viewpoint. However, there is still a knowledge gap that needs to be addressed regarding the agronomic persistence of forages containing CT, and the consistency of concentration and chemical properties of condensed tannins.

4 EFFECT OF MIXTURES OF LEGUME SPECIES ON RUMINAL FERMENTATION, METHANE AND MICROBIAL PROTEIN PRODUCTION IN BATCH AND CONTINUOUS CULTURE (RUSITEC) SYSTEMS

4.1 Abstract

The effects of cicer milkvetch and sainfoin on ruminal fermentation, methane production, and microbial protein synthesis in batch and continuous culture systems (RUSITEC) were assessed. Experiment 1 analyzed 2 legumes, cicer milkvetch (*Astragalus cicer* L.) and sainfoin (*Onobrychis viciifolia* Scop.) at 2 stages (vegetative and late flower) incubated with alfalfa (*Medicago sativa*) at 5 inclusion rates 0:100; 25:75, 50:50, 75:25 and 100:0 (as DM) in batch culture. Experiment 2 analyzed vegetative cicer milkvetch and alfalfa incubated in ratios of 25:75, 50:50, 75:25 and 100:0 (as DM) in RUSITEC. In batch culture, dry matter disappearance (DMD) increased linearly ($P \leq 0.01$), propionate concentration (mol 100mol^{-1}) increased linearly (P =0.02), and methane (mg g^{-1} DMD) decreased linearly ($P \leq 0.01$) with vegetative cicer milkvetch inclusion. In RUSITEC, DM, ADF and NDF disappearance increased quadratically ($P < 0.01$), acetate:propionate ratio decreased quadratically, while NH_3-N concentration (mmol d^{-1}) decreased linearly ($P \leq 0.01$) and butyrate concentration (mol 100mol^{-1}) quadratically decreased ($P \leq 0.01$) with increased cicer milkvetch inclusion. No differences were observed for methane (CH_4) production (mg g^{-1} DMD) (P = 0.38), or short chain fatty acid (SCFA) production (mmol d^{-1}) (P =0.16). Microbial protein synthesis and efficiency of protein synthesis linearly increased (P = 0.01) with increased inclusion of cicer milkvetch. Results suggest cicer milkvetch may provide greater supply of synchronous energy and nitrogen, and enhance diet digestibility.

4.2 Introduction

Hay production in western Canada has declined over the past 30-years as a result of producers converting upwards of 1 million hectares of tame perennial forage to arable hectares (Jefferson and Selles 2007; Statistics Canada 2017). Despite this cropping trend, including perennial forages in cropping rotations is well known to enhance the sustainability of cropping systems through improved soil fertility, reduced soil erosion, suppression of weeds, disruption of plant disease cycles and providing environmental benefits such as carbon sequestration, reduced NO_3 leaching, and provision of wildlife habitat (Entz et al. 1995). In addition, including perennial legumes, such as alfalfa, in pasture mixes can increase animal productivity (Popp et al.

2000). Thus, strategies to increase perennial forage acreage will benefit both the producer and the environment. It is well known that quantity and quality of forage provided can impact livestock performance, through its ability to improve the chemical composition and digestibility of forage (Popp et al. 2000; Petri 2012). The microbial protein that flows from the rumen to the small intestine is the most important supply of essential amino acids and supplies majority of the metabolizable protein needs of the host ruminant (NASEM 2016). Factors that influence the amount of microbial protein supply that leaves the rumen include the extent of ruminal degradation, nutrient availability, efficiency of the ruminal bacteria to utilize these nutrients, and rate of outflow from the rumen (Hespell and Bryant 1979; Mangan 1988; NASEM 2016).

Due to producer adoption of bloat free legumes, there has been increased need to better understand the impact of bloat free legumes on rumen fermentation (McMahon et al. 1999; Williams et al. 2011; Stewart et al. 2019). Previous research has determined the effects of sainfoin inclusion in the diet on rumen fermentation and identified a recommended rate of inclusion to increase livestock performance and reduce bloat (McMahon et al. 1999). However, research surrounding the level of inclusion in the diet and effects of cicer milkvetch on ruminal fermentation and microbial protein synthesis are negligible, leaving producers with limited information to make decisions regarding pasture mixtures. Hence, the objectives of these experiments were to assess the effects of differing proportions of cicer milkvetch (*Astragalus cicer* L) and sainfoin (*Onobrychis viciifolia*), when displacing alfalfa (*Medicago sativa*) in forage mixtures on digestibility, ruminal fermentation, methane production, and microbial protein synthesis in batch and continuous fermentation systems.

4.3 Materials and Methods

All animal procedures and protocols used in this experiment were reviewed and approved by the Lethbridge Research and Development Center Animal Care Committee (ACC number 1501) in accordance with the guidelines of the Canadian Council on Animal Care (CCAC, 2009).

4.3.1 Plant Material and Forage Quality

Cicer milkvetch (AC Oxley), sainfoin (Melrose), and alfalfa (AC Grazeland) were harvested during the summer of 2018 from plots (n = 4) grown at AAFC Saskatoon Research Center, Saskatoon, Canada, and composited until experiment start. To limit seasonal change in forage (McGraw and Marten, 1986), samples were clipped to a 5-cm height at vegetative plant

growth stage (16 to 30 cm stem length, no buds, flowers or seed pods) and at late flower plant growth stage (≥ 2 nodes with open flowers; no seed pods) (Kalu and Fick, 1981). Samples were dried in a forced air oven at 55°C for 72 h, then ground to pass through a 2-mm screen (AOAC 2000) and stored until initiation of the experiment. All samples were analyzed for CP, ADF, and NDF at Cumberland Valley Analytical Services (Waynesboro, PA, USA) (Table 4.1).

4.4 Batch Culture Experiment

The semi-automated batch culture *in vitro* gas production technique as described by Mauricio et al. (1999) was used to study the effects of inclusion of cicer milkvetch and sainfoin legumes on ruminal fermentation and digestibility of forage mixtures.

4.4.1 Treatments

The experiment was designed as a randomized complete block design with 2 legumes (SAIN, CMV), 2 plant growth stages of maturity (vegetative, late flower), and 5 inclusion rates for each species displacing alfalfa (0:25:50:75:100). The experiment included 3 incubation times (6, 24, 48 h) and 3 replicates for each time point (Table 4.2). Each run also included 3 bottles containing inoculum and medium without substrate for a blank. The two runs were completed on different days using inoculum from the same cows.

4.4.2 Source of Inoculum

Rumen inoculum were obtained from 3 ruminally fistulated cattle fed an alfalfa-meadow bromegrass hay. Mixed digesta contents were collected from four distinct sites within the rumen, 2 h prior to morning feeding and were filtered through 4 layers of cheesecloth to remove particulate matter. Strained fluid was pooled between the three cows and placed in a pre-warmed, air-tight, insulated vessel and transported at 39°C from collection site to the laboratory. The pH of the rumen inoculum (pH 6.43) was measured at the time of collection (B20PI,98 Symphony Benchtop Meters; VWR, Edmonton, AB, Canada). The rumen inoculum was then placed in a glass bottle, flushed with CO_2, fitted with a bottle-top dispenser, and stored in a water bath at 39°C until being added to incubation vials.

Table 4.1. Chemical composition of legumes evaluated in forage mixtures incubated in batch cultures and the RUSITEC

Forage	Stage of Maturity	Chemical composition (% DM)				
		CP	ADF	NDF	Ca	P
Alfalfa	Vegetative	28.3	23.9	37.8	1.1	0.3
	Late flower	20.5	33.1	41.1	1.1	0.3
Sainfoin	Vegetative	21.4	25.1	39.7	1.0	0.3
	Late flower	16.3	35.2	42.4	1.1	0.2
Cicer milkvetch	Vegetative	27.2	22.9	40.3	0.86	0.4
	Late flower	16.9	30.9	46.6	0.93	0.3

Note: DM, dry matter; CP, crude protein; ADF, acid detergent fiber; NDF, neutral detergent fiber.

Table 4.2. Ingredient and chemical composition of experimental treatments incubated in batch culture

	Cicer milkvetch:alfalfa combination (DM) (CMV)				
	0:100	25:75	50:50	75:25	100:0
Nutrient (%, DM)			*Vegetative*		
DM	92.4	92.6	92.9	94.4	94.5
CP	28.3	26.9	26.0	27.2	27.2
ADF	23.9	23.9	23.2	23.0	22.9
NDF	37.8	40.0	41.3	39.7	40.3
			Late Flower		
DM	93.4	93.7	93.4	93.3	93.9
CP	20.5	19.4	18.4	18.1	16.9
ADF	33.1	31.9	31.4	31.6	30.9
NDF	41.1	43.3	44.9	44.9	46.6
	Sainfoin:alfalfa combination (DM) (SAIN)				
	0:100	25:75	50:50	75:25	100:0
			Vegetative		
DM	92.4	92.8	92.1	91.9	92.7
CP	28.3	26.4	24.8	23.5	21.4
ADF	23.9	23.9	25.3	23.7	25.1
NDF	37.8	38.2	38.2	37.8	39.7
			Late Flower		
DM	93.4	92.4	92.7	92.8	92.4
CP	20.5	17.2	18.0	16.7	16.3
ADF	33.1	35.4	33.3	34.4	35.2
NDF	41.1	42.6	42.8	43.3	42.4

Note: DM, dry matter; CP, crude protein; ADF, acid detergent fiber; NDF, neutral detergent fiber

4.4.3 Sample Preparation and *In vitro* Procedure

Forage mixtures at the specified inclusion rates were created and then samples were prepared by placing a 0.5 g (DM basis) sample of each treatment substrate in a dried (55°C), pre-weighed, labelled, and acetone rinsed Ankom F57 filter bag (5.0 × 5.5 cm.; Ankom Technology Corporation, Fairport, NY). Bags were heat sealed prior to placement in serum vials (100 mL) and serum vials were loaded on a tray and placed in an incubator at 39°C for 48 h prior to incubation. In vitro medium (pH 6.71) was prepared according to Goering and Van Soest (1970) and it (45 mL) and rumen fluid (15 mL) were dispensed into serum vials warmed at 39°C, while flushing with CO_2. Serum vials were sealed with ANKOMRF Gas Production System rubber stoppers (Ankom Technology Corporation) and incubated in a rotary shaker (125 rpm) incubator at 39°C.

4.4.4 Measurements and Analyses

Headspace gas pressure in each vial was recorded at 3, 6, 9, 12, 24, and 48 h of incubation by inserting a 23-gauge (0.6 mm) needle attached to a pressure transducer (model PX4200-015GI, Omega Engineering, Inc., Laval, QC, Canada). The needle was left in the septum and the transducer removed to vent the gas. Gas pressure values, corrected for amount of substrate organic matter (OM) and gas produced by the blank, were used to determine the gas volume produced using the equation of Mauricio et al. (1999).

$$\text{Gas volume} = 0.18 + (3.697 \times \text{gas pressure}) + (0.0824 \times \text{gas pressure}^2) \quad \textbf{(4.1)}$$

At the end of each incubation time, vials were removed from the incubator and placed in ice water for 5 min to inhibit fermentation. Three replicate vials were removed at each timepoint for each treatment and serum vials were opened, and pH was measured immediately. Filter bags were removed and washed with cold water until clean. After rinsing, filter bags were dried at 100°C for 12 h and subsequently weighed for DM calculation. Neutral detergent fibre of residue was determined as described by Van Soest et al. (1991) using heat-stable α-amylase and sodium sulfite and corrected for ash. *In vitro* NDF (IVNDF) and dry matter (IVDMD) digestibility was calculated and used as an indicator for digestibility. Ash content was determined by combustion at 550°C for 5 h, and OM content was calculated as 100 minus the proportion of ash (AOAC, 2005; method 942.05).

Incubation liquid (5 mL) was mixed with 1 mL of metaphosphoric acid (0.25; w/v) for determination of short chain fatty acids (SCFA), and a second subsample (5 mL) was mixed with 1 mL of H_2SO_4 (0.01; v/v) for ammonia N(NH_3-N) analysis (Rhine et al.1998). Concentration of SCFA was quantified using a gas chromatograph (Model 6890N, Agilent Technologies Canada Inc., Mississauga, ON) equipped with a capillary column (30 m × 0.32 mm i.d., 1-μm phase thickness, Zebron ZB-FAAP, Phenomenex, Torrance, CA, USA), and a flame ionization detector, with crotonic acid (trans-2-butenoic acid) as an internal standard (Beauchemin et al. 2009).

4.5 RUSITEC Experiment

4.5.1 Experimental Design and Treatments

The experiment was designed as a randomized complete block design with 4 treatments randomly assigned to sixteen replicated (n=4) fermentation vessels. Vegetative cicer milkvetch and alfalfa were incubated in ratios of 25:75, 50:50, 75:25 and 100:0, respectively (DM basis; Table 4.3). The RUSITEC experiment was 15-d in duration and included an 8-d adaptation and 7-d sampling period.

4.5.2 Source of Inoculum

Rumen inoculum were obtained from the 3 ruminally fistulated cattle, collected and stored in the same manner as described in the in vitro batch culture assay. Mixed digesta was squeezed through four layers of cheesecloth to partition into liquid and solid fractions. The pH of the fluid (pH 6.65) was measured at the time of collection (B20PI,98 Symphony Benchtop Meters; VWR, Edmonton, AB, Canada).

Table 4.3. Chemical composition of vegetative cicer milkvetch and alfalfa treatments incubated in the RUSITEC

	Cicer milkvetch:alfalfa combination (DM) (CMV)			
Nutrient (%, DM)	25:75	50:50	75:25	100:0
DM	92.2	94.0	94.7	95.4
CP	27.5	28.5	26.3	26.9
ADF	23.7	24.2	22.9	23.3
NDF	43.8	43.1	46.6	44.9

NOTE: DM, dry matter; CP, crude protein; ADF, acid detergent fiber; NDF, neutral detergent fiber

4.5.3 Experimental Apparatus

The experiment used two rumen simulator technique (RUSITEC) apparatuses (Czerkawski and Breckenridge, 1977) located at Agriculture and Agri-Food Canada (AAFC) Lethbridge Research Center, each equipped with eight 920 mL anaerobic fermenters. An input and output port were attached to each fermenter to infuse buffer and collect effluent, respectively. Each fermenter was housed in a 39ºC circulating water bath. To initiate fermentation, 200 mL of McDougall's buffer (McDougall 1948), 700 mL of strained rumen fluid, 20 g of mixed solid digesta and 5 g of diet (both digesta and diets were supplied in separate bags) were placed into each fermenter while they were flushed with CO_2. After 24 h, the bag containing 20 g of digesta was replaced with a bag containing 5 g of each specific diet. Thereafter, one bag was replaced daily so that each bag remained in the fermenter for 48 h. The buffer was modified according to McDougall (1948) with pH = 8.2, using $NaH_2PO_4 \cdot H_2O$ 3.69 g L^{-1}, and $NaHCO_3$ 9.83 g L^{-1} containing 0.3 g L^{-1} of $(NH_4)_2SO_4$ and was continuously infused into the fermenters via peristaltic pump to achieve a dilution of 2.9% h^{-1}. The effluent was collected daily into a 2.0 L Erlenmeyer flask, while gases were collected in 2.0 L gas tight collection bags (Covidien Dover Drainage Bag #30510).

4.5.4 Measurements and analyses

Gas volume, effluent volume and fermenter pH were measured daily at 0900 h during substrate bag exchange. Fermentation gases were collected daily into reusable urine collection bags. Total gas production was measured daily using a wet-type gas meter (Alexander-Wright and Co. London, UK). Gas was taken from each collection bag using a 20 mL syringe and injected into 6.8 mL exetainers (Labco Ltd., Wycombe, bucks, UK). Methane (CH_4), carbon dioxide (CO_2) and hydrogen (H_2) measurements were analyzed through gas chromatography (Varian 4900 equipped with GS CarbonPLOT 30 m × 0.32 mm × 3µm column and thermal conductivity detector) from Agilent Technologies Canada Inc (Santa Clara, CA, USA). The device was equipped with an isothermal oven at 35ºC with helium as the carrier gas (27cm s^{-1}).

Effluent was sampled on d 8 to 12 for measurement of SCFA and NH_3-N concentration. Effluent subsamples of 5 mL were collected from the effluent flasks at the time of feed-bag exchange. The samples were then placed in screw cap vials, preserved in 1 mL of 25% (wt/wt) metaphosphoric acid and frozen in -20 ºC until further analysis. A second subsample of the

effluent (5mL) was collected and placed in screw cap vials preserved in 1 mL of H_2SO_4 (1% v/v) for determination of NH_3-N. Short chain fatty acid analysis was performed with same procedure as the batch culture assay. The concentrations of SCFA and NH_3-N (mmol L^{-1}) were multiplied by daily effluent production (L d^{-1}) to determine SCFA and NH_3-N production (mmol d^{-1}).

Dry matter, OM, CP, ADF, and NDF disappearance were determined on sampling day 9 to 13 from feed bags incubated for 48 h. Feed bags were collected from each fermenter and were rinsed under cold running water until the rinsate was clear. The bags were then oven dried at 55ºC for 48 h and hot-weighed to determine DM disappearance (DMD). Daily residues collected from each fermenter were pooled to yield a single sample for each fermenter over the 5 d measurement and analyzed for OM, total nitrogen (NA 2100 Carlo Erba Instruments, Milan, Italy), NDF, and ADF content.

Effluent and feed residue solids were sampled on d 8 to determine the background ^{15}N concentration. On day 9, the 0.3 g L^{-1} $(NH_4)_2SO_4$ in the McDougall's buffer solution was replaced with 0.3 g L^{-1} ^{15}N-enriched $(NH_4)_2SO_4$ and infused until the end of the experiment. After day 9, daily effluents were collected and preserved with 3 mL of sodium azide at a final concentration of 0.1% wt/volume. On d 14 and 15, the daily effluents were measured from each fermenter and a 35 mL subsample was collected and centrifuged at 20,000 × *g* for 30 min, at 4º C, to isolate liquid associated bacteria (LAB). Collected microbial pellets were washed with deionized water, and centrifuged three more times (20,000 × *g*, 30 min, 4ºC). Samples were then suspended in distilled water and lyophilized to determine N and ^{15}N concentration.

Microbial fractions bound to feed particles were prepared from the 48 h feed bags. On d 14 and 15, bags were removed from the fermenter, gently squeezed, then added to a stomacher bag filled with 20 mL of McDougall buffer and processed for 60s in a Stomacher 400 laboratory blender (Seward medical Ltd., London, UK). The processed liquid was squeezed out, poured off, and retained. The solid feed residues were washed twice with 10 mL of McDougall's buffer (1948) in each wash. The washed buffer was retained and pooled with the initially expressed fluid, to represent the feed particle associated bacteria (FPA), and the total volume recorded. Washed solid feed residues were considered to represent the feed particle bound (FPB) bacterial fraction. The FPA samples were centrifuged (500 × *g*, 10 min, 4ºC) to remove feed residues and the supernatant was centrifuged (20,000 × *g*, 30 min, 4ºC) to isolate a bacterial pellet which was

washed three times as described above. Washed feed residues (FPB fraction) were oven dried for 55ºC 48 h, weighed for DM determination, ball ground (MM400, RestchInc., Newtown, PA, USA), and analyzed for N and ^{15}N by combustion analysis using a mass spectrophotometer (NA1500, Carlo Erba instruments, Milan, Italy). All samples were frozen at -20ºC until analyzed.

Total microbial protein synthesis (mg d^{-1}) was calculated as (Ribeiro et al. 2015)……..….(**4.2)**

$$Microbial\ Biomass = LAB + FPA + FPB$$

Where,
LAB is the liquid associated bacteria
FPA is the feed particle associated bacteria
FPB is the feed particle bound bacteria

Efficiency of Microbial Protein Synthesis (EMPS) (g bacteria N kg^{-1} DMD) was calculated as (Refat et al. 2015)……...(**4.3)**

$$EMPS = g\ bacteria\ N \div kg\ DMD$$

On d 14 and 15, fermentation liquid was sampled in duplicate (35 mL each) from each fermenter to determine the concentration of dissolved hydrogen (dH_2) and dissolved methane (dCH_4) as described in Wang et al. (2014) and Wang et al. (2016). In brief, a 50 mL syringe containing 35 mL of fermentation liquids was connected to a 20 mL syringe filled with 5 mL of N_2 gas. The N_2 was injected from the small syringe into the large syringe via a T tube and the apparatus was vigorously shaken by hand for 5 min. The entire gas phase was then transferred from the large syringe into the small one to determine the gas volume. Finally, the small syringe was removed from the T tube and 6 mL of gas was sampled for both gas and dissolved gas analyses determined by gas chromatography as previously described.

4.6 Statistical Analysis

Data for the batch culture experiment was analyzed according to a randomized complete block design using the PROC MIXED procedure of SAS (SAS Inst. Inc., Cary, NC) to test for the effect of percentage of legume species in the diet. The model included treatment as fixed effect, and random effects of run (1 to 2). Polynomial contrasts were used to test for linear and quadratic components in the relationships with percentage of legume. Degrees of freedom were

adjusted using the Kenward-Roger option. Results were considered significant when $P \leq 0.05$, and trends were discussed when $P \leq 0.10$.

For the RUSITEC, data were analyzed as repeated measures according to a completely randomized block design using the MIXED procedure of SAS (SAS Inst. Inc., Cary, NC). The MIXED model included treatment as fixed effects and random effects of fermenter, with sampling day as the repeated measure. Polynomial contrasts were used to test for linear and quadratic components in the relationships with percentage of legume. Degrees of freedom were adjusted using the Kenward-Roger option. Results were considered significant when $P \leq 0.05$, and trends were discussed when $P \leq 0.10$.

4.7 Results

4.7.1 Batch Culture

With vegetative legumes, DMD increased linearly ($P < 0.01$) with increasing vegetative CMV inclusion, whereas DMD tended to decrease linearly ($P = 0.07$) with increasing vegetative sainfoin inclusion (Table 4.4). At late flower, DMD remained unchanged by CMV or SAIN inclusion. The NH_3-N concentration (m*M*) was unchanged in vegetative SAIN or CMV inclusion. Likewise, NH_3-N was unchanged by late flower CMV or SAIN inclusion. Methane (CH_4; mg g^{-1} DMD) decreased quadratically ($P = 0.05$) with increasing vegetative CMV inclusion, whereas no change was observed for vegetative SAIN, late flower CMV, or late flower SAIN inclusion.

At vegetative plant stage, total short chain fatty acid production (mmol) and the molar proportion of acetate (mol $100mol^{-1}$) was unchanged by vegetative CMV and SAIN inclusion (Table 4.5). The molar proportion of propionate (mol $100mol^{-1}$) was increased linearly ($P = 0.02$) with increasing vegetative CMV inclusion, with no difference observed for vegetative SAIN inclusion. Molar proportion of butyrate (mol $100mol^{-1}$) tended to decrease linearly ($P = 0.09$) with vegetative CMV inclusion and was reduced quadratically ($P = 0.05$) with vegetative SAIN inclusion. Molar proportion of minor SCFA (mol $100mol^{-1}$ isobutyrate, valerate, isovalerate, caproate) were unchanged by vegetative CMV inclusion, and were reduced linearly ($P < 0.05$) by vegetative SAIN inclusion. The acetate to propionate ratio was unchanged by vegetative CMV or SAIN inclusion.

At late flower, no change was observed for total SCFA production (mmol) or molar proportion of acetate (mol 100mol^{-1}) with either CMV or SAIN inclusion (Table 4.6). Molar proportion of propionate (mol 100mol^{-1}) increased ($P = 0.04$) with increasing late flower CMV inclusion and was unchanged with late flower SAIN inclusion. Molar proportion of butyrate (mol 100mol^{-1}) was unchanged by late flower CMV inclusion but was decreased quadratically ($P = 0.04$) with increasing late flower SAIN inclusion. Molar proportion of isobutyrate (mol 100mol^{-1}) tended to decrease linearly ($P = 0.07$) with increasing late flower CMV inclusion and decreased quadratically ($P < 0.01$) with increasing late flower SAIN inclusion. Molar proportions of valerate and isovalerate (mol 100mol^{-1}) decreased linearly ($P < 0.01$) with increasing late flower CMV and decreased quadratically ($P < 0.05$) with increasing late flower SAIN inclusion. Molar proportion of caproate (mol 100mol^{-1}) was unchanged with increasing late flower SAIN inclusion and decreased linearly ($P < 0.01$) with late flower SAIN inclusion. Acetate to propionate ratio was unchanged by late flower CMV or SAIN inclusion.

Table 4.4. Dry matter disappearance (DMD), NH_3-N and methane (CH_4) production after 48 h *in vitro* incubation

	Cicer milkvetch:alfalfa combination (DM) (CMV)						Contrasts	
	0:100	25:75	50:50	75:25	100:0	SEM	L	Q
			Vegetative					
DMD (%)	62.68	67.00	66.75	71.03	68.34	1.188	<0.01	0.68
NH_3-N (mM)	34.76	35.10	35.8	35.74	35.03	0.627	0.23	0.99
CH^4 (mg g^{-1} DMD)	48.74	43.79	41.31	37.27c	35.34	1.915	<0.01	0.05
			Late Flower					
DMD (%)	54.13	54.41	57.26	58.17	63.24	1.429	0.65	0.39
NH_3-N (mM)	30.92	32.17	31.79	31.14	30.31	0.938	0.47	0.47
CH^4 (mg g^{-1} DMD)	49.47	49.09	44.42	44.16	41.12	1.8869	0.14	0.54
	Sainfoin:alfalfa combination (DM) (SAIN)							
	0:100	25:75	50:50	75:25	100:0			
			Vegetative					
DMD (%)	62.68	62.48	61.10	56.96	54.10	0.575	0.07	0.75
NH_3-N (mM)	34.76	33.45	31.83	31.30	27.96	0.996	0.29	0.14
CH^4 (mg g^{-1} DMD)	48.74	47.52	44.38	45.91	45.26	2.092	0.13	0.36
			Late Flower					
DMD (%)	54.13	51.70	52.40	50.03	48.78	1.238	0.11	0.26
NH_3-N (mM)	30.92	29.12	31.02	29.83	29.38	0.810	0.78	0.26
CH^4 (mg g^{-1} DMD)	49.47	51.61	53.48	50.21	50.16	1.565	0.24	0.12

NOTE: Means within the same row with different letters differ at the $P<0.05$. L, linear, Q, quadratic

Table 4.5. Effect of inclusion level of vegetative legume species with alfalfa on short chain fatty acid (SCFA) profile of sainfoin after 48 h of *in vitro* incubation

	Cicer milkvetch:alfalfa combination (DM) (CMV)						Contrasts	
	0:100	25:75	50:50	75:25	100:0	SEM	L	Q
Total SCFA (mmol)	94.94	91.63	93.88	93.67	88.40	5.461	0.82	0.47
Acetate[a]	59.49	58.34	59.37	59.12	57.94	0.929	0.79	0.32
Propionate	18.45	19.11	19.43	19.20	20.43	0.415	0.02	0.14
Butyrate	19.62	19.04	17.83	17.36	16.48	0.979	0.09	0.39
Isobutyrate	3.43	3.40	3.37	3.34	3.24	0.115	0.90	0.71
Valerate	5.35	5.39	5.33	5.28	5.30	0.113	0.64	0.60
Isovalerate	6.32	6.40	6.37	6.35	6.34	0.106	0.75	0.66
Caproate	0.53	0.56	0.57	0.56	0.63	0.016	0.75	0.11
A:P	3.23	3.05	3.06	2.96	2.84	0.104	0.16	0.17
	Sainfoin:alfalfa combination (DM) (SAIN)							
	0:100	25:75	50:50	75:25	100:0			
Total SCFA (mmol)	94.94	85.29	87.60	83.02	84.18	4.235	0.15	0.37
Acetate[a]	59.49	57.52	59.37	59.58	61.56	1.140	0.56	0.60
Propionate	18.45	19.10	18.79	18.78	18.15	0.470	0.23	0.56
Butyrate	19.62	18.49	17.71	16.6	15.62	0.521	<0.01	0.05
Isobutyrate	3.42	3.24	3.09	2.87	2.76	0.063	<0.01	0.20
Valerate	5.36	5.18	4.98	4.73	4.62	0.070	<0.01	0.34
Isovalerate	6.32	6.12	5.81	5.42	5.26	0.067	<0.01	0.53
Caproate	0.53	0.52	0.51	0.47	0.46	0.028	0.03	0.77
A:P	3.23	3.01	3.17	3.19	3.39	0.138	0.39	0.59

Note: Means in the same row with different letters differ at the $P < 0.05$. L, linear, Q, quadratic

[a]individual SCFA concentrations are reported as mol $100mol^{-1}$.

Table 4.6. Effect of inclusion level of late flowering legume species with alfalfa on short chain fatty acid (SCFA) profile after 48 h of *in vitro* incubation

	Cicer milkvetch:alfalfa combination (DM) (CMV)						Contrasts	
	0:100	25:75	50:50	75:25	100:0	SEM	L	Q
Total SCFA (mmol)	88.86	87.86	85.12	93.63	94.08	5.591	0.95	0.39
Acetate[a]	60.48	60.10	59.54	61.63	61.46	0.757	0.86	0.27
Propionate	17.87	18.19	18.64	18.79	19.08	0.364	0.04	0.22
Butyrate	18.16	18.05	17.55	17.37	17.18	1.119	0.28	0.86
Isobutyrate	3.02	2.93	2.87	2.88	2.89	0.139	0.07	0.31
Valerate	4.96	4.91	4.80	4.74	4.75	0.185	<0.01	0.70
Isovalerate	5.69	5.55	5.43	5.39	5.47	0.162	<0.01	0.32
Caproate	0.55	0.56	0.53	0.51	0.51	0.007	0.10	0.35
A:P	3.38	3.31	3.20	3.28	3.22	0.099	0.26	0.23
	Sainfoin:alfalfa combination (DM) (SAIN)							
	0:100	25:75	50:50	75:25	100:0			
Total SCFA (mmol)	88.86	82.27	83.66	85.39	82.70	3.428	0.38	0.07
Acetate[a]	60.48	60.48	59.74	61.45	60.18	0.708	0.53	0.31
Propionate	17.87	18.05	18.14	17.88	18.26	0.321	0.88	0.35
Butyrate	18.16	16.91	17.60	17.01	17.11	0.699	0.02	0.04
Isobutyrate	3.02	2.69	2.86	2.67	2.72	0.077	<0.01	0.03
Valerate	4.96	4.55	4.72	4.48	4.56	0.139	<0.01	0.05
Isovalerate	5.69	5.09	5.39	5.02	5.14	0.090	<0.01	0.04
Caproate	0.55	0.49	0.53	0.46	0.49	0.010	<0.01	0.52
A:P	3.38	3.35	3.30	3.44	3.30	0.096	0.68	0.32

Note: Means in the same row with different letters differ at the $P < 0.05$. L, linear, Q, quadratic

[a]individual SCFA concentrations are reported as mol 100mol^{-1}.

4.7.2 RUSITEC

Dry matter, ADF, and NDF disappearance increased quadratically ($P < 0.01$) with increasing vegetative CMV inclusion (Table 4.7). Total gas production (L d^{-1}), CH_4 concentration (%), CH_4 production (mg g^{-1} DMD), and total dissolved hydrogen (µmol L^{-1}) were unchanged by vegetative CMV inclusion; however, dissolved CH_4 was reduced linearly ($P = 0.03$) with CMV inclusion. Total microbial protein synthesis, LAB, FPB, and EMPS increased linearly ($P < 0.01$) with increasing vegetative CMV inclusion.

Media pH decreased linearly ($P < 0.01$) with increasing vegetative CMV inclusion (Table 4.8). Total ruminal SCFA production (mmol d^{-1}) was unchanged by increased vegetative CMV inclusion. Acetate production (mol 100mol^{-1}) decreased quadratically ($P < 0.01$) with increasing vegetative CMV inclusion, averaging 64.96 mol 100mol^{-1}. Propionate production (mol 100mol^{-1}) increased quadratically ($P < 0.01$) and butyrate production (mol 100mol^{-1}) decreased quadratically ($P < 0.01$) with increasing vegetative CMV inclusion. Isobuytrate production (mol 100mol^{-1}) decreased linearly ($P < 0.01$) with increasing inclusion of vegetative CMV. Isovalerate production (mol 100mol^{-1}) was not affected by vegetative CMV inclusion rate, but valerate and caproate (mol 100mol^{-1}) both increased quadratically ($P < 0.05$) with increasing vegetative CMV inclusion. Corresponding to the increase in propionate and decrease in acetate, the acetate to propionate ratio was reduced quadratically ($P < 0.01$) with increasing vegetative CMV inclusion. Ammonia nitrogen (NH_3-N) (mmol d^{-1}) decreased linearly ($P < 0.01$) with increasing vegetative CMV inclusion.

Table 4.7. Effect of cicer milkvetch inclusion on nutrient disappearance, gas production, and microbial protein synthesis in the RUSITEC

	Cicer milkvetch:alfalfa combination (DM) (CMV)					Contrasts	
	25:75	50:50	75:25	100:0	SEM	L	Q
Nutrient disappearance (%)							
Dry matter	74.44	77.78	80.65	82.54	0.391	<0.01	<0.01
Acid detergent fiber	53.85	59.24	62.13	66.86	1.452	<0.01	<0.01
Neutral detergent fiber	68.67	72.08	78.21	79.34	0.469	<0.01	<0.01
Gas production							
Total (L d^{-1})	0.97	1.10	1.10	1.13	0.066	0.14	0.16
CH_4, % of gas	3.89	4.15	3.59	3.71	0.332	0.45	0.58
CH_4, (mg g^{-1} DM digested)	6.89	7.74	6.02	6.71	0.861	0.56	0.38
Total dissolved hydrogen (µmol L^{-1})	0.35	0.10	0.23	0.15	0.079	0.19	0.28
Total dissolved CH_4 (mmol L^{-1})	0.32	0.30	0.30	0.27	0.015	0.03	0.62
Bacterial nitrogen							
Total (mg d^{-1})	35.89	42.24	48.63	52.48	3.463	<0.01	0.71
LAB (mg d^{-1})	26.54	32.88	39.21	41.86	3.493	<0.01	0.61
FPA (mg d^{-1})	5.07	4.62	4.52	4.79	0.434	0.63	0.41
FPB (mg d^{-1})	4.28	4.80	4.91	5.82	0.526	0.05	0.71
EMPS (g bacteria N kg^{-1} DM digested)	9.22	10.38	11.53	12.14	0.826	0.01	0.75

NOTE: Means within the same row with different letters differ at the $P<0.05$.L, linear, Q, quadratic; CH^4, methane; LAB, liquid associated bacteria; FPA, feed particle associated bacteria; FPB, feed particle bound bacteria; EMPS, efficiency of bacteria nitrogen synthesis.

Table 4.8. Effect of cicer milkvetch inclusion on ruminal pH, short chain fatty acid (SCFA) and ammonia (NH_3-N) production in the RUSITEC

	Cicer milkvetch:alfalfa combination (DM) (CMV)					Contrasts	
	25:75	50:50	75:25	100:0	SEM	L	Q
pH	7.03	7.02	7.01	6.99	0.007	<0.01	0.21
Total SCFA (mmol d^{-1})	41.19	43.37	44.94	42.23	1.917	0.59	0.21
Acetate (A)[a]	64.48	65.89	65.32	64.18	0.292	0.26	<0.01
Propionate (P)	20.54	20.26	21.44	22.54	0.212	<0.01	<0.01
Butyrate	8.33	7.40	6.72	6.39	0.112	<0.01	<0.01
Isobutyrate	1.51	1.43	1.39	1.37	0.021	<0.01	0.22
Valerate	2.11	2.06	2.19	2.48	0.069	<0.01	0.02
Isovalerate	2.68	2.57	2.56	2.54	0.055	0.11	0.24
Caproate	0.37	0.38	0.41	0.49	0.021	<0.01	0.11
A:P	3.14	3.27	3.05	2.86	0.042	<0.01	<0.01
NH_3-N (mmol d^{-1})	7.34a	6.81ab	6.40b	6.26b	0.217	<0.01	0.38

NOTE: Means within the same row with different letters differ at the $P<0.05$. L, linear, Q, quadratic.
[a]individual SCFA concentrations are reported as mol $100mol^{-1}$.

4.8 Discussion

Alfalfa is the most widely grown perennial legume in western Canada, owing to its high yield, nutritive value, and winter hardiness (McMahon et al. 1999). Due to concerns with frothy bloat for cattle grazing monoculture alfalfa, research evaluating bloat-free legumes, such as sainfoin and cicer milkvetch have increased (McMahon et al. 1999; Williams et al. 2011; Stewart et al. 2019). Cicer milkvetch is bloat safe due to its reticulate veined leaf pattern and thick epidermal layers which slows ruminal microbial digestion and has been reported to reduce ruminal NH_3-N concentration, reduce CH_4 production, and shift route of N excretion (Lees et al. 1982; Williams et al. 2011; Noviandi et al. 2014; Stewart et al. 2019). Conversely, legumes such as sainfoin and birdsfoot trefoil, are bloat safe and modulate rumen fermentation due to condensed tannins (CT) in plant tissues, which have been reported to increase host animal protein utilization and provide anti-parasitic properties (Min and Solaiman 2018; Mueller-Harvey et al. 2019). Furthermore, feeding CT forages may be environmentally advantageous, as they may reduce enteric CH_4 emission and urinary N excretion in ruminants, thus reducing greenhouse gas emissions (Mueller-Harvey et al. 2019; Stewart et al. 2019). In the present study, we evaluated how replacing alfalfa with CMV and SAIN and how inclusion rate affects in vitro digestion responses.

4.8.1 Dry Matter Disappearance, Short Chain Fatty Acid Production and Ammonia Nitrogen Production

In the current study, results were dependent on plant growth stage as increased inclusion of vegetative cicer milkvetch increased DMD in both batch culture and RUSITEC experiments and lowered CH_4 production in RUSITEC; however, increased inclusion of late flower cicer milkvetch had no effect on DMD or CH_4 production in batch culture incubations. Inclusion of vegetative sainfoin tended to decrease DMD, with no effect on CH_4 production, whereas inclusion of late flower sainfoin had no effect on DMD or CH_4 production. The negligible effect of late flower plant species on DMD was not unexpected as generally plant maturity increases lignification and decreases digestibility (NASEM 2016).

Increasing the inclusion rate of vegetative cicer milkvetch substrate when substituted for alfalfa substrate, linearly increased DMD in both experiments, and linearly increased ADF and NDF digestibility in the RUSITEC experiment. Stewart et al. (2019) found cattle fed a diet of

100% vegetative cicer milkvetch hay had greater nitrogen retention as compared to sainfoin and alfalfa, which the authors attributed to an increased supply of fermentable energy in the rumen because of greater NDF digestibility in cicer milkvetch than alfalfa. These results are similar to the study conducted by McGraw and Marten (1986) who reported greater IVDMD for vegetative cicer milkvetch as compared to vegetative sainfoin, birdsfoot trefoil and alfalfa. Increased DMD could potentially increase DMI (NASEM 2016); however, when compared to *in vivo* trials, the DMI potential of cicer milkvetch and sainfoin, have been reported to be similar to alfalfa regardless of plant maturity (Acharya, 2006; Stewart et al. 2019). In contrast, heifers fed early flower cicer milkvetch and sainfoin hay were reported to have lower DMI than those fed early flower alfalfa hay (Stewart et al. 2019). In the current study, vegetative sainfoin had the opposite impact of vegetative cicer milkvetch as DMD linearly decreased as it was substituted for alfalfa. Similarly, Stewart et al. (2019) reported heifers fed individual sainfoin and alfalfa hays experienced lower DMD than cows fed cicer milkvetch hay, which the authors attributed to greater NDF and ADF digestibility in cicer milkvetch hay compared to sainfoin or alfalfa (Stewart et al. 2019).

Effects of legume species on SCFA production were dependent on the level of inclusion. During batch culture experiment, although no difference was observed in the molar proportion of acetate or subsequent A:P ratio, the molar proportion of propionate was increased linearly as both vegetative and late flower cicer milkvetch increased in diet. This differed from vegetative and late flower sainfoin inclusions in which no differences in propionate, acetate, or A:P ratios were observed. The results of the vegetative cicer milkvetch incubations were echoed in the RUSITEC experiment, as the A:P ratio was linearly reduced, driven by greater propionate production and reduced acetate production as cicer milkvetch inclusion increased from 25% to 100% of diet, displacing alfalfa. These results are not unexpected as DMD was observed to increase with increased cicer milkvetch inclusion, and greater feed digestibility is associated with increases in proportion of propionate in fermentation end products (Janssen 2010). Other studies have reported similar results on propionate production with additive effect of cicer milkvetch. For example, Noviandi et al. (2014), reported propionate concentration increased from 10.5 m*M* and 12.8 m*M* when level of inclusion of cicer milkvetch replaced 25 to 75% of tall fescue (*Schedonorus arundinaceus*) in a mixed forage diet in continuous culture. Similarly, Williams et al. (2011) found that during continuous culture, fermenters fed a diet containing 70% cicer

milkvetch : 30% corn silage had higher propionate concentration (31.8 mmol) compared to those fed 70% alfalfa : 30% corn silage (27.3 mmol), which was reflected in a lower A:P ratio for those fermenters fed cicer milkvetch compared to alfalfa. Considering that propionate the main precursor for glucose synthesis, increased propionate production is beneficial as it supports host metabolism and energy production (NASEM 2016).

In the current study, butyrate concentrations were also reduced with additive inclusion of sainfoin and cicer milkvetch in both experiments. This is similar to Williams et al. (2011) who reported fermenters fed 70% cicer milkvetch had lower butyric concentration (5.13 mol 100mol^{-1}) compared to those fed 70% alfalfa or sainfoin (8.16 and 7.75 mol 100mol^{-1}). Although metabolic hydrogen [H] is produced in the first step of butyrate production (oxidative decarboxylation of pyruvate to acetyl-CoA), butyrate production also involves two [H] incorporating steps (conversion of acetoacetyl-CoA to β-hydroxybutyryl-CoA and crotonyl-CoA to butyryl-CoA) (Ungerfeld 2020). Therefore, although butyrate production results in a net release of [H], less [H] is produced when butyrate is formed in comparison to acetate (Janssen 2010). When H_2 is increased, carbon is diverted away from acetate and butyrate production in favour of propionate (Moss et al. 2000). Therefore, it is possible that butyrate production was decreased due to increased H_2 production in the cicer milkvetch incubations. Alternatively, Morgavi et al. (2008) reported that defaunated sheep experienced an increase in propionate production and a reduction in butyrate production which the authors attributed an excess of H_2 due to the reduction in CH_4 production (Morgavi et al. 2008). Since protozoa produce H_2 as a major fermentation product, they play a critical role in CH_4 production through interspecies H_2 transfer with methanogens and as mentioned previously protozoa are difficult to maintain in continuous cultures (Morgavi et al. 2008; Martinez et al. 2010).

As branched chain amino acids are deaminated, they are converted to branched chain SCFA, therefore both branched chain SCFA and NH_3-N are indicators of ruminal protein digestion (NASEM 2016). Concentration of branched chain SCFA (isovalerate and isobutyrate) were linearly reduced ($P < 0.01$) with increased inclusion of vegetative sainfoin and quadratically reduced ($P < 0.05$) with increased inclusion of late flower sainfoin within the batch culture experiment, reflective of a possible decrease in ruminal proteolysis (Dahlberg 1988). Similar results have been reported by other researchers, who observed a reduction in ruminal

fluid branched chain SCFA concentration from cattle fed sainfoin compared to cicer milkvetch (Dahlberg et al. 1988). McMahon et al. (1999) reported linear reductions in branched chain SCFA production as sainfoin was increased from 0 to 50 and 100 % of diet, displacing alfalfa. Both studies attributed the decrease in branch chain SCFA to decreased proteolytic activity due to protein-CT complexes, which would limit the supply of branched chain amino acids available for deamination to branch chain SCFA (Dahlberg et al 1988; McMahon et al. 1999).

Ammonia nitrogen production (mM, NH_3-N) remained unchanged by the inclusion of either cicer milkvetch or sainfoin in the batch culture experiment. McMahon et al. (1999), reported a decrease in NH_3-N, when vegetative sainfoin leaves were incubated at 100% inclusion in comparison to 0 and 25% inclusion displacing alfalfa, suggesting a decrease in ruminal proteolysis at higher legume inclusion levels. In the current study, during the RUSITEC experiment, NH_3-N (mMol d^{-1}) was linearly reduced ($P < 0.01$) with inclusion of vegetative cicer milkvetch. These results are similar to Williams et al. (2011) who reported NH_3-N flow (g d^{-1}) was lowest from continuous fermenters fed cicer milkvetch (0.18 g d^{-1}) compared to alfalfa (0.21 g d^{-1}). Similar additive effect results were observed by Noviandi et al. (2014) who reported no change to NH_3-N production when fermenters were fed cicer milkvetch at 25% of the diet along with alfalfa, but observed a reduction in NH_3-N if cicer milkvetch displaced more alfalfa in the diet (50% cicer milkvetch = 16.8 and 18.7 mg $100ml^{-1}$ for cicer milkvetch and alfalfa, respectively; 75% cicer milkvetch = 19.5 and 20.6 mg $100ml^{-1}$ for cicer milkvetch and alfalfa, respectively). Protein degradation of cicer milkvetch has been reported to be similar to alfalfa in continuous culture (Dalberg 1988), however, it is known that protozoal populations are difficult to maintain in continuous culture systems (Martinez et al. 2010). Since protozoal populations have high deaminase activity contributing greatly to ruminal NH_3-N production (Jouany and Ushida 1999), decreases in protozoal numbers are associated with lower deamination and increased uptake of NH_3-N by bacterial populations (Varzaneh et al. 2018). Therefore, it is possible that low protozoal populations contributed to the decrease of NH_3-N. However, conversely, it is possible that reductions in NH_3-N observed in the current study could indicate enhanced N utilization by ruminal microorganisms, as cattle fed cicer milkvetch hay have been reported experience increased N retention due to an increase in synchronous supply of fermentable energy and nitrogen (Stewart et al. 2019).

4.8.2 Microbial Protein Synthesis and Methane Production

During the RUSITEC experiment, DMD, fiber disappearance (ADF and NDF), microbial protein synthesis (mg d^{-1}) and EMPS (mg bacterial N g^{-1} DM digested) increased linearly with inclusion of cicer milkvetch; however, total gas production (L d^{-1}) and methane (CH_4, % gas; CH_4, mg g^{-1} DMD) remained unchanged. Dissolved H_2 (dH_2) remained unchanged with increased cicer milkvetch inclusion; however, dissolved CH_4 (dCH_4) was linearly reduced with increasing inclusion of vegetative cicer milkvetch. Wang et al. (2014) determined that dH_2 correlated with ruminal CH_4 production and led to fermentation pathways that produce less H_2 such as propionate and butyrate. As H_2 is easily lost, the measurement of dH_2 concentrations can be challenging and differences in results may be negligible, as observed in the current study (Wang et al. 2014). However, the reduction of dCH_4 observed in the current study, may be indicative of reduced CH_4 production by methanogens, which would be similar to the reduction in CH_4 gas observed in the batch culture experiment with vegetative cicer milkvetch inclusion. This could be explained by the decreased A:P ratio in cicer milkvetch incubations, which was driven by increased in propionate, as propionate acts as an electron accepting pathway lowering substrate availability for methanogenesis (NASEM 2016).

Gas production strongly correlates to digestibility of organic matter (Menke et al 1979), and microbial protein synthesis is related to the availability of carbohydrate and N in the rumen (Chumpawadee et al., 2006). Rumen degradable protein is the portion of intake protein degraded to ammonia, amino acids, or dipeptides in the rumen, and varies in its susceptibility to ruminal proteolysis depending on ruminal dilution rate, dietary forage:concentrate ratio, ruminal pH, nutrient interactions, and feed processing (NASEM 2016; Lardner et al. 2019). Rumen microbes require degradable protein or sources of non-protein nitrogen (NPN), such as NH_3-N and urea, for microbial protein synthesis; therefore, a portion of intake protein must be degraded in the rumen, with a requirement estimated to equal microbial protein synthesis (NASEM 2016). Ruminal pregastic fermentation transforms dietary protein and NPN into ammonia and microbial protein, the latter supplying the ruminant with all 10 essential amino acids. Excessive ruminal degradation of protein, as occurs with high protein diets, is energetically costly and metabolically burdensome as the NH_3 must be converted to urea in the liver and excreted in urine (Reynolds and Kristensen 2008; NASEM 2016).

In the current study, given the increased microbial protein synthesis, EMPS, ADF and NDF digestibility, combined with reduced NH_3-N production as dietary cicer milkvetch increased, it is possible that N and energy release in the rumen allowed for synchronous fermentation and greater microbial protein synthesis and energetic efficiency. The impact of sainfoin and other CT sources on microbial protein synthesis has been well reported (McMahon et al. 1999; Makkar et al. 1995a; Dahlberg et al. 1988), however research analyzing the impact of tannin free legumes, such as cicer milkvetch, on microbial protein synthesis is limited. Lees et al. (1982) observed a thick epidermal layer and reticulated secondary and tertiary veining pattern in cicer milkvetch leaves providing greater structural strength and resistance to mechanical damage, when compared to alfalfa and birdsfoot trefoil (Lees et al. 1982). Low digestibility of the thick epidermal layer, combined with epidermal layers that are firmly attached to mesophyll cells due to the leaf vein structure and pattern, results in reduced microbial digestion within the mesophyll tissue of the cicer milkvetch leaf (Lees et al. 1982). The leaf structure and epidermal layer did not appear to negatively impact digestibility during the current experiments, as DMD was increased with inclusion of vegetative cicer milkvetch, likely due to grinding of substrate in preparation for incubations. Dalberg et al (1988), reported decreased total bacterial N production (mg d^{-1}) when fermenters were fed cicer milkvetch compared to sainfoin. However, once substrate utilization was accounted for, cicer milkvetch had greater efficiency of microbial protein synthesis than both sainfoin and alfalfa (Dahlberg et al. 1988). With respect to the current study, it is possible that increased dry matter digestibility, propionate production, and microbial protein synthesis, could lead to greater energetic and production efficiency in cicer milkvetch than alfalfa fed livestock.

4.9 Conclusion

Past studies have recommended the inclusion of sainfoin in pasture blends at 20 or 35% (McMahon et al. 1999; Wang et al. 2006). The findings from the current study would support this recommendation as increased inclusion of vegetative sainfoin during batch culture incubations tended to linearly decrease dry matter disappearance, and branched SCFA production. In contrast, inclusion of vegetative cicer milkvetch in RUSITEC linearly increased DMD, microbial protein synthesis, efficiency of microbial protein synthesis, and increased propionate production all of which may increase energetic efficiency of ruminal fermentation. Results suggest dietary inclusion of cicer milkvetch may supply synchronous sources of energy

and nitrogen, which could enhance efficiency of production. Therefore, these results suggest cicer milkvetch could benefit cattle production when substituted for alfalfa in mixed pasture blends and grazed at the vegetative stage. However, although cicer milkvetch appears to have nutritional benefit to the ruminant, mixed pasture systems are dynamic and therefore more research is needed to determine agronomic best management practices for inclusion of cicer milkvetch in pastures, plant persistence of cicer milkvetch in mixed species pastures, and grazing management strategies which will allow for successful producer adoption of cicer milkvetch.

5 EFFECT OF SOD-SEEDED LEGUMES ON RUMINAL FERMENTATION, ENTERIC METHANE AND MICROBIAL POPULATIONS USING THE SULPHUR HEXAFLOURIDE (SF_6) TRACER

5.1 Abstract

A two-year (2017, 2018) replicated (n=6) *in vivo* experiment evaluated the effects of cicer milkvetch (CMV; *Astragalus cicer* L.) and sainfoin (SAIN; *Onobrychis viciifolia* Scop.) on grazing animal ruminal fermentation, enteric methane production, and ruminal microbial populations. Each yr, 15 ruminally cannulated cows (average 739 kg ± 40 kg) were randomly allocated to 1 of 15 replicated (n=6) treatment paddocks: grazing sod-seeded sainfoin (SAIN) paddocks arranged in a RCBD; grazing sod-seeded cicer milkvetch (CMV) paddocks; or grazing non-sod-seeded (CONT) paddocks. Total short chain fatty acid concentration (mmol), and the molar proportion of acetate ($P = 0.48$), propionate ($P = 0.22$), and butyrate ($P = 0.83$) did not differ among treatments. Acetate to propionate and acetate/butyrate to propionate ratio was lower ($P = 0.01$) for cows grazing CMV paddocks compared to cows grazing CONT and SAIN paddocks. Ruminal ammonia (NH_3-N) ($P = 0.03$) and plasma urea nitrogen (PUN) concentration ($P \leq 0.01$) were greater in cows grazing CMV treatment compared to SAIN and CONT paddocks which was attributed to greater forage crude protein. Enteric methane (g kg^{-1} DMI) ($P = 0.76$) and microbial populations were not changed by treatment.

5.2 Introduction

With the introduction of new legumes into an old established grass-legume pasture, it is important to understand how these new forages will impact ruminal fermentation and ruminal microbial populations. Ruminants require a symbiotic relationship with a vast and diverse microbial population, which can be modified by diet, to facilitate the anaerobic digestion of dietary feedstuffs to yield metabolizable energy and protein (Petri et al. 2012; Kocherginskaya et al. 2001).

Sainfoin contains condensed tannins (CT) in its tissues which have been observed to alter ruminal fermentation. Condensed tannins bind with protein in the rumen, which could be viewed as beneficial since this may reduce pre-gastric fermentation of protein, thereby increasing rumen undegradable protein fractions and increasing the metabolizable amino acid flow entering the small intestine (Barry and McNabb 1999). However, CT are not always beneficial for livestock,

as they can limit DM digestibility, or be disadvantageous when protein in the diet is limiting, as in poor quality pasture, or late fall grazing (Mueller-Harvey 2019). The effect of CT on livestock production is also dependent on plant species, as some may be more detrimental than others. For example, livestock fed birdsfoot trefoil have been observed to experience increased bodyweight, wool and milk production, whereas livestock fed other species such as sulla and lotus have been observed to experience decreases in bodyweight and wool production (Waghorn et al. 1998). Although cicer milkvetch does not contain CT, it can alter ruminal degradable protein fractions due to its thick epidermal layer and reticulate veining leaf structure, which can slow microbial activity in the rumen (Noviandi et al. 2014).

During the process of ruminal fermentation, enteric methane (CH_4) is produced due to the need to dispose of hydrogen (H_2) within the rumen (Newbold et al. 2005). Accounting for 63% of total emissions, enteric CH_4 is the largest source of greenhouse gas (GHG) emission from Canadian beef farms, and thus recent research has focused on strategies to mitigate CH_4 (Beauchemin et al. 2009; Beauchemin et al. 2010). Condensed tannins have been reported to linearly decrease enteric CH_4 emissions with increasing dose (Min and Solaiman 2018). The mechanism by which they impact CH_4 emissions is not well understood (Chapter 3), and reports are confounding as other studies report no differences in CH_4 emissions when ruminants are fed condensed tannin containing forages (Chung et al. 2013; Huang et al., 2018; Stewart et al. 2019). Condensed tannins may reduce methane and impact ruminal microbial populations in a direct manner by reducing methanogen species in relation to other species, or they may act in an indirect manner by modifying ruminal fermentation end products, thus decreasing available nutrients to other microorganisms that produce the reducing equivalents used by methanogens to reduce CO_2 to CH_4 (Patra and Saxena 2010; Carrasco et al. 2017). In contrast, cicer milkvetch has been reported to reduce methanogenesis due to changes in ruminal microbial activity and modification of fermentation end products due to its leaf structure as outlined above (Lees et al. 1982). However, the exact mechanism by which cicer milkvetch reduces CH_4 requires further research, as extracts of cicer milkvetch, which would have no structural barrier to microbial action, have been reported to reduce ruminal cellulose fermentation (Weimer et al. 1993; Williams et al. 2011).

Limited research is available evaluating the effects of incorporating sainfoin and cicer milk vetch as sod-seeded legumes into an established grass-dominated stand, particularly with

reference to ruminal fermentation, methane emissions, and ruminal microbial composition. Therefore, the objectives of this experiment were to determine *in vivo* ruminal short chain fatty acid and ammonia concentration, fecal and urinary N excretion, enteric methane production, and rumen microbial profile in ruminally cannulated cows grazing grass-legume pastures sod-seeded with sainfoin (*Onobrychis vicifolia*) and cicer milkvetch (*Astragalus cicer*) species. Mills (2018) published partial data from one year (2017) in an undergraduate thesis.

5.3 Materials and Methods

5.3.1 Study Site Description

A 2 yr (2017, 2018) replicated (n=6) study was conducted at Western Beef Development Center's (WBDC) Termuende Research Ranch located near Lanigan, Saskatchewan, Canada. The experimental site was located on Pasture 8, Section 27 (51°51 'N latitude; 105°02 'W longitude), as described in Chapter 6 of this dissertation.

5.3.2 Pasture and Sod-Seeding Management

A 30-ha meadow bromegrass (*Bromus riparius Rehm*)-alfalfa (*Medicago sativa* L.) pasture was subdivided into 15, two-ha (65 × 306 m) paddocks. Paddocks were then randomly assigned to 1 of 3 treatments including: a control treatment (CONT; n = 3), sod-seeded sainfoin (SAIN; n = 6), or sod-seeded cicer milkvetch (CMV; n = 6). Forage seeds were obtained from Brett Young Seeds (Winnipeg, MB, CA) (3 paddocks each were each seeded to common SAIN or Oxley II CMV) and Northstar Seeds (Neepawa, MB, CA) (3 paddocks each were seeded to AC Mountainview SAIN or 3 paddocks seeded to Veldt CMV). Varietal effects were not considered, and data were pooled, resulting in greater replication for CMV and SAIN relative to CONT paddocks. Prior to seeding, one application (May 28, 2015) of 1.24 L ha^{-1} glyphosate (N(phosphonomethyl) glycine), purchased from Blair's Fertilizer Ltd. (Lanigan, SK), was applied to the non-bloat legume treatment paddocks to control existing species competition. Sainfoin was sod-seeded at a rate of 26 kg ha^{-1} and CMV was seeded at a rate of 17 kg ha^{-1}as per the seed suppliers' recommendation. Cicer milkvetch seed was scarified prior to seeding and SAIN was seeded with inoculant. Sod seeding took place on June 9 and 10, 2015 at Lanigan using a 2.4 m zero till seed opener AGROPLOW AD130 drill (Molong, NSW, AU) with 15.2-cm row spacing and was seeded at a depth of 1.9 cm. The non-bloat legume paddocks were rolled after sod-seeding and plots were mowed during summer to aid in weed control. No field treatments were applied to the CONT paddocks.

Monthly temperature (°C) and precipitation (mm) data and the long-term average (LTA; 30 yr) were obtained using Environment and Climate Change Canada weather stations located near the research trial location (Leroy, SK stations). Grazing commenced in the spring of 2017 and grazing management is described in Chapter 6 of this dissertation.

5.3.3 Experimental Animals

All cattle were cared for in accordance with the Canadian Council on Animal Care (CCAC 2009) guidelines. Institutional approval for animal use was granted by the University of Saskatchewan Animal Research Ethics Board (Protocol No. 20090107). Each yr (2017 and 2018), 15 ruminally cannulated (9C model cannula; Bar Diamond, Parma ID, USA) cows (average 739 kg ± 40 kg) were randomly allocated to CONT, SAIN, and CMV paddocks (1 cow/ paddock) and remained in the paddock for the duration of the grazing period. Model 9C cannulas were used as they are a more suitable cannula for enteric CH_4 studies (Beauchemin et al. 2014) and cannulas were checked prior to and monitored throughout experiment to ensure a tight fit to minimize leakage. In addition to the cannulated cattle, 3 steers per paddock grazed concurrently; however, these steers were not included in the current study as they were part of a separate trial (Chapter 6). Each yr, the grazing period was 21 d and included 14 d of adaptation and 7 d for data and sample collection.

5.3.4 Botanical Composition, Forage Quality and Estimated Animal Intake

Botanical composition (% of stand) was assessed yearly to determine establishment and persistence of cultivars within the stand. Estimates of botanical composition were taken at start (available) and end (residual) of each grazing period using the Daubenmire (1959) technique. Within each replicate paddock, 20 random quadrats (0.25 m^2) with permanently located transects were visually assessed for composition by canopy cover and separated into as is percentage grass, alfalfa, non-bloat legume and other species. Other species included weeds, such as absinthe wormwood (*Artemisia absinthium*) and Canada thistle (*Cirsium arvense*).

To estimate forage nutritive value, in each paddock, 20 randomly distributed quadrats (0.25 m^2) were clipped to a 5-cm stubble height and composited in plastic bags. Two sub-samples from each composite at the start and end of each grazing period were placed in paper bags and dried in a forced-air oven at 55ºC for 72 h to determine DM and forage nutritive value. Samples were ground to pass through a 2-mm screen (Thomas Wiley Laboratory Mill Model 4;

Thomas Scientific, Swedesboro, NJ) (AOAC 2000), composited by paddock, and stored until analyzed. Duplicate samples were analyzed at Cumberland Valley Analytical Services (Waynesboro, PA, USA) were analyzed for DM (Goering and Van Soest, 1970), crude protein (AOAC; method 990.03), soluble protein (Krishnamoorthy et al. 1982), acid detergent fiber (AOAC, method # 973.18), ash corrected neutral detergent fibre (Van Soest et al. 1991), calcium (AOAC, method 985.01), and phosphorous (AOAC; method 985.01) (AOAC International, 2000).

Herbage NDF in conjunction with animal body weight was used as an estimate of forage dry matter intake (DMI) according to the following equation (NASEM 2016):

Estimated DMI (as percent of BW) = (120/NDF, % of herbage DM)……..…..(**5.1)**

5.3.5 Ruminal Fermentation and Nitrogen Excretion

Ruminal fluid was collected from each cannulated cow for determination of short chain fatty acid (SCFA) and ruminal NH_3 concentrations. On d 1 to 6 of the sampling period, fifty millilitres of ruminal fluid were collected daily at 0900 h each d from each cannulated animal (n=15), using a 500 mm stainless steel tube including a lure-lock fitting on one and a 50-mesh stainless steel screen filter on the other end (Bar Diamond, Inc., Parma, ID, USA). This sampling approach allowed for the collection of ruminal fluid without opening the ruminal cannula. A subsample of 10 mL of ruminal fluid was placed in 3 different test tubes (1 with 2-mL of chilled 25% (wt/vol) metaphosphoric acid (H_2PO_4) solution for SCFA analysis, 1 with 2-mL of chilled 1% (vol/vol) sulfuric acid (H_2SO_4) solution for NH_3 analysis, and 1 spare). Ruminal pH was measured d 1 to 6 using the Lethbridge Research Center continuous indwelling ruminal pH system (Dascor Inc. Escondido, CA, USA) as described in Penner et al. (2006).

All ruminal fluid samples were sealed and stored at -20°C until analysis. All thawed samples were mixed thoroughly and composited on an equal volume basis, for each replicate in each year (Li et al. 2009). All ruminal fluid samples were assayed in duplicate as described by Albornoz et al. (2013). In brief, prior analysis, the test tubes were thawed in a refrigerator overnight at 4°C. Samples were then inverted several times and then centrifuged at 12,000 × *g* for 10 min at 4°C. Samples were then sub-sampled into micro-centrifuge tubes and centrifuged

again at 16,000 × *g* for 10 min at 4°C. The final supernatant was added to Agilent GC vials (Agilent Technology Inc., Mississauga, ON) and isocaproic acid was added as an internal standard. The samples were analyzed using an Agilent 6890 Series Gas Chromatography auto sampling system equipped with an Agilent 7683 Series Injector. A mixed standard was also included in the analysis and consisted of known amounts of acetic, propionic, butyric, isobutyric, valeric, isovaleric, caproic and isocaproic acids to set up the calibration curve. Samples were run in duplicate for SCFA determination using an Agilent 6890 Series Gas Chromatography System with flame ionization detector and an Agilent 7683 Series Injector (Agilent Technology Inc., Mississauga, ON). Separation was achieved using a Zebron ZB-FFAP High Performance GC Capillary Colum (Phenomoenex, cat. # 7HM-G009-11 Zebron) with an injection split ratio of 10:1 and injection volume of 0.2µL. Initial and final oven temperatures were 90 and 170°C, respectively, with an increase rate of 10°C min^{-1} followed by a final 2 min hold. Helium was used as a carrier gas with a flow rate of 35mL min^{-1}. Ruminal NH_3–N was determined colorimetrically as described by Fawcett and Scott (1960).

5.3.6 Blood, Urine and Fecal

Blood, urine, and fecal samples were collected at 0900 h to minimize diurnal changes in sample composition (Hammond 1983). Blood samples were collected from each cow per treatment on d 2 and 3 of the sampling period. Blood samples were taken from the jugular vein into sterile evacuated tubes containing an anticoagulant (10 mL, lithium heparin, Vacutainer, Becton Dickinson, Oakville, ON). The blood was centrifuged at 3000 × *g* at 4°C for 20 min to obtain plasma and stored at -20°C until analysis of plasma urea N (PUN) concentration. Grab samples of feces and urine were collected on d 1 to 6 of the sampling period and stored at -20°C for analysis. Upon analysis, the urine sample was diluted 20 times with deionized water and analysed by Shimadzu TOC-VCPN analyzer (Shimadzu Corporation, Kyoto, Japan) unit for dissolved nitrogen and carbon. The fecal samples were air-dried at room temperature and then ground using the ball grinder (8000D Mixer/Mill, SPEX SamplePrep® LLC., Metuchen, NJ, USA). Dry matter content of feces was determined by measuring the weight loss after samples were dried at 65°C. Total fecal nitrogen and carbon was measured by dry combustion with a LECO C635CNS Analyzer (LECO Corporation, St. Joseph, MI).

5.3.7 Enteric Gas Production

Enteric methane (CH_4) and carbon dioxide (CO_2) production was determined on d 1 to 6 of the sampling period, using the sulphur hexafluoride (SF6) tracer gas method (Johnson et al. 1994) and as per methods outlined Grainger et al. (2009). Brass permeation tubes containing SF_6 with a predetermined release rate were placed in the rumen of each cannulated cow 14 d prior to the experiment start to allow for tracer gas to reach a steady state (Iwaasa et al. 2005). Gases expired were sampled using a collection apparatus fit around the neck and attached to a halter (Iwaasa et al. 2005). The apparatus was designed to be evacuated between -74 and -91 kPa and held a volume of 1 L. The cows were adapted to the apparatus for 3 d prior to collection and no visible effects on grazing behavior were observed.

The yoke was placed around the neck of each heifer just behind the ears and attached to a halter fitted with an air-tight connection to a 90-cm length of restriction tubing (0.127 mm i.d.) with an in-line 15-µm filter and flexible nose piece. Collection yokes were changed every 24-h as the collection system was developed to collect 45% of its volume over a 24-h period (Iwaasa, personal comm. 2016). Background air samples were collected during the sampling period by hanging 6 gas collection equipment (yoke, halter) in each corner of the study pasture.

At time of yoke exchange, the internal pressure was measured to ensure no capillary systems were blocked or leaking. The yokes were then pressurized to 103 kPa with pure N_2 and three samples (25 mL syringe) were obtained by inserting a 26-gauge needle through the septum on the yoke. Each 25 mL syringe sample was injected into an evacuated 12.5 mL excutainer (Labco Ltd., Wycombe, bucks, UK). Carbon dioxide (CO_2), CH_4 and SF_6 concentrations were analyzed via gas chromatography (GC; Varian 450-GC) as described in Grainger et al. (2007). The GC was equipped with three detectors; a thermal conductivity detector (TCD; for CO_2), a flame ionization detector (FID; for CH_4) and a electron capture detector (ECD; for SF6). A 5mL sample was injected and two ten-port valves controlled the collection (500uL loop) and delivery through a 2M X 1/8" Porapak QS column to the TCD and then FID. Then collection (4mL loop) and delivery through a 2M X 1/8" Haysep D column to the ECD. Helium was used as a carrier gas (1.0 mL min-1), and run time was 3.5 min with and oven temperature of 70°C.

Daily enteric CH_4 emission were estimated by multiplying the ratio of CH_4:SF_6 concentrations by the known permeation tube release rate and correcting for duration of sample

collection and the background CH_4 concentration using the following equation adapted from Grainger et al. (2009):

Daily enteric CH_4 emission……(**5.2)**

$$Q_{CH4} = \frac{C_{CH4} - C^b_{CH4}}{C_{SF6} - C^b_{SF6}} Q_{SF6} \frac{MW_{CH4}}{MW_{SF6}}.$$

Where Q_{CH4} is CH_4 emission (g d^{-1}), C_{SF6} and C_{CH4} is the mixing ratio (µmol mol^{-1}) sampled by the canister on the cows, C^b_{SF6} and C^b_{CH4} is the inlet air streams, and Q_{SF6} (g d^{-1}) is the predetermined SF_6 release rate from the permeation tube, and MW is the molecular weight of the gases.

5.3.8 Microbial Community Composition

Mixed ruminal digesta samples (5 g) were taken in duplicate from 4 distinct areas of the rumen on d 7 of the collection period and frozen in liquid nitrogen for further analysis as described by Gruninger et al. (2018). Metagenomic DNA from samples was extracted using the DNeasy PowerSoil Kit (Qiagen, Germany) according to the manufacturer's instructions. Absorbance values were collected from extracted samples using the SPECTROstar Nano microplate reader (BMG Labtech, Ortenberg, Germany) and DNA concentration was determined using and the MARS Data Analysis Software package (BMG Labtech). All extractions and quantifications were performed by Quantum Genetix (Saskatoon, Canada).

Sequencing was completed at Genome Quebec at McGill Sequencing Center (Montreal, QC, Canada) and bioinformatic analysis completed at the Canadian Center for Computational Genomics (Montreal, QC, Canada). In brief, the 16S rRNA gene sequence libraires were generated using a two-step protocol. The first PCT step amplified the V4 region of the 16S rRNA gene using the universal bacterial and archaeal primers 515-F (GTGCCAGCMGCCGCGGTAA) and 806-R (GGACTACHVGGGTWTCTAAT). The second PCR step was used to add a unique 10-bp barcode at the 5’ end of each amplicon as well as to add Illumina (Illumina, San Diego, CA, USA) adapter sequences. The 16S rRNA gene amplicons were quantified using a Quant-iT PicoGreen dsDNA assay kit (Invitrogen, Burlington, ON, Canada), pooled in equimolar ratios, and then purified with AMPure XP beads (Beckman Coulter, Mississauga, ON). Sequencing of 16S rRNA gene amplicons was carried out using an Illumina MiSeq (2 × 250; San Diego, CA) and the MiSeq Reagent Kit v2 (500 cycles; Illumina) according to manufacturer’s instructions.

The 16S rRNA gene sequences were processed at using the R-package DADA2 (Callahan et al. 2016) denoise method. The forward and reverse reads were each truncated at a length of 240 bp, quality control was done for the reads using the QIME2, with chimeric sequences identified and removed. The reads were merged, and taxonomy assigned to generate operational taxonomic units (OTUs) at 97% similarity. Data was analyzed at taxonomic class, however no difference was observed at this level, and therefore data was reported at taxonomic phyla level.

5.4 Statistical Analysis

Statistical analysis was conducted using a one-way analysis of variance (ANOVA) using the PROC Mixed Model procedure of SAS 9.2 (SAS, 2003) for a randomized complete block design. Each paddock was considered an experimental unit, for a total of 30 experimental units over a 2 yr period. Treatment was included as a fixed effect and year and paddock were included as random effects. Degrees of freedom were adjusted using the Kenward-Roger option and pooled SEM was reported. The PDIFF was adjusted using the Tukey method and were considered significant when $P \leq 0.05$ and included in the LSMEANS. Botanical composition data were analyzed using both logarithmic and arcsine transformations; however, untransformed data were determined to yield the best fit based on Akaike and Bayesian information criterion. The differential ruminal bacterial taxonomy based on phylum level was analyzed using MicrobiomeAnalyst (Dhariwal et al. 2020; Chong et al. 2020) to adjust the data, hierarchical cluster, clustering distance with Euclidean for comparison.

5.5 Results

Seasonal precipitation (Figure 5.1) was below the 30-yr average for Lanigan, SK, in the 2017, and above the 30-yr average in 2018. Cumulative precipitation from May to September was 85.0 and 343.3 mm in 2017 and 2018. The 30-yr average amount of rainfall for the same period at Lanigan is 295.3 mm. Average monthly temperature (Figure 5.2) was similar to the 30-yr average.

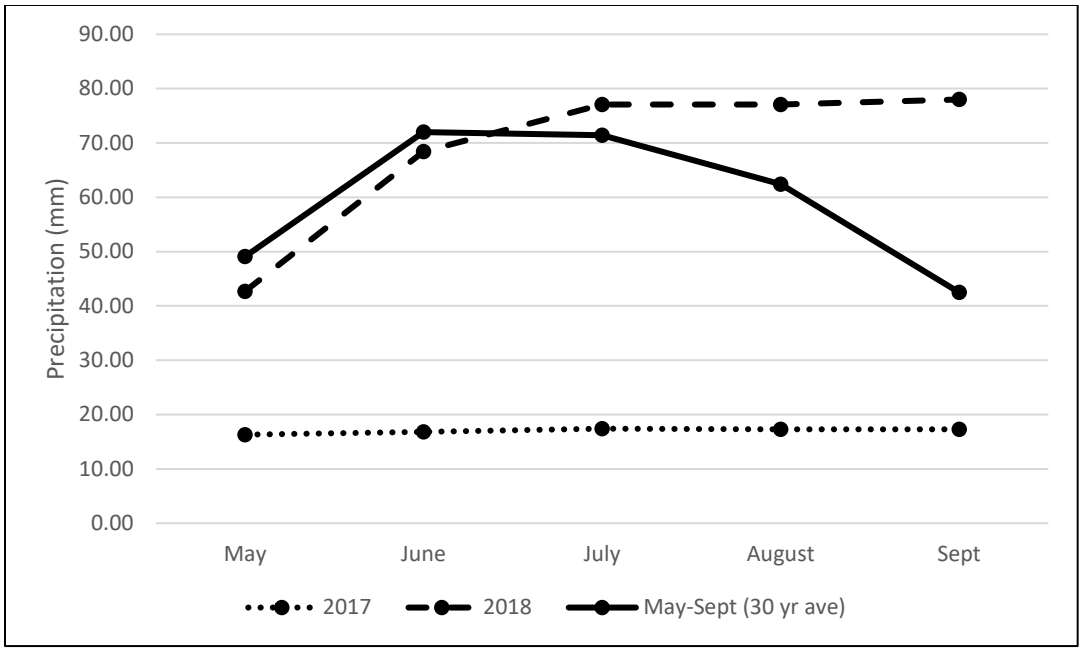

Figure 5.1 Average monthly precipitation during grazing season and monthly long-term (30yr) average at Lanigan, SK, Canada.
Note: Environmental data was collected using Environment and Climate Change Canada weather stations located near trial location (Leroy, SK stations) (www.weather.gc.ca)

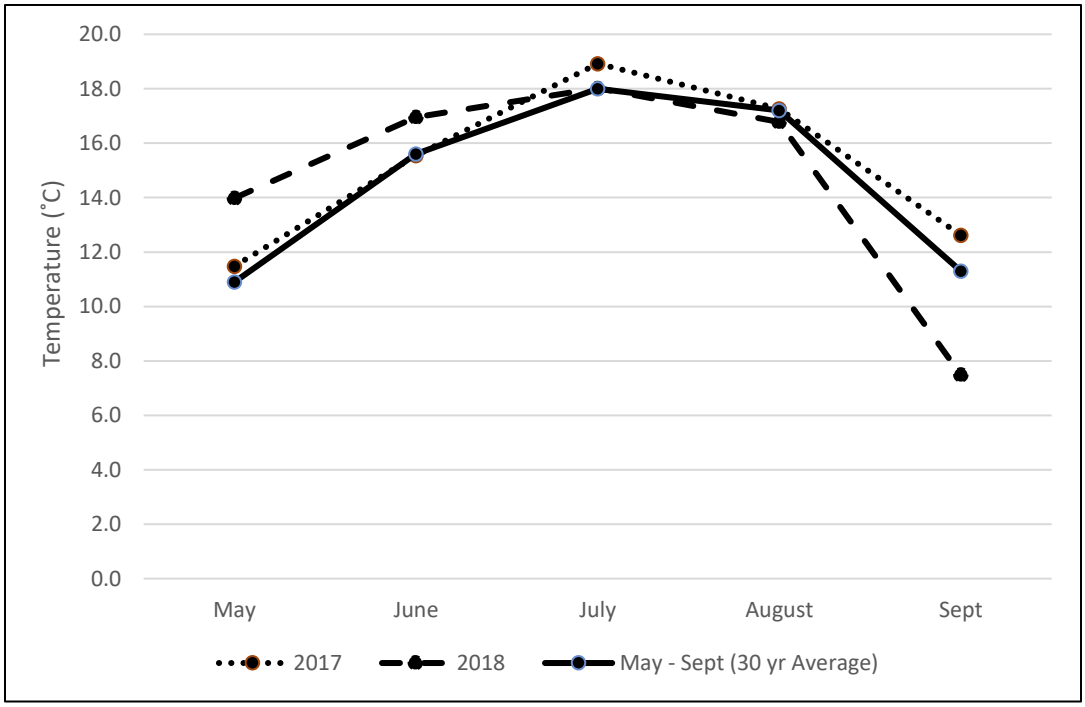

Figure 5.2. Average monthly temperature during grazing season and monthly long-term (30yr) average at Lanigan, SK, Canada.
Note: Environmental data was collected using Environment and Climate Change Canada weather stations located near trial location (Leroy, SK stations) (www.weather.gc.ca)

5.5.1 Botanical Composition and Forage Nutritive Value

The proportion of grass was greater in the CONT paddocks compared to the CMV and SAIN paddocks (Figure 5.3; treatment, $P < 0.01$). The proportion of alfalfa was greater in the SAIN and CMV paddocks compared to CONT (treatment, $P < 0.01$). The proportion of non-bloat legume was greater (treatment, $P < 0.01$) in the CMV paddock (19 % of stand) compared to the SAIN paddock (3 % of stand). There were no bloat-free legumes in the CONT paddock. The proportion of other species was lowest (treatment, $P < 0.01$) in the CONT paddocks, followed by the CMV paddocks and was highest in the SAIN paddocks. Proportion of total legume (alfalfa + non-bloat legume) was highest (treatment, $P < 0.01$) in the CMV paddock (28% of stand) compared to the SAIN (17% of stand) and CONT (0.5% of stand) paddocks.

Crude protein concentration was greater in CMV paddocks compared to CONT paddocks (Table 5.1; treatment, $P = 0.01$). The concentration of soluble protein (%, CP) was unchanged with introduction of CMV and SAIN into paddocks. Neutral detergent fiber concentration was lower (treatment, $P < 0.01$) in CMV and SAIN paddocks, compared to CONT paddocks. Acid detergent fiber concentration was lowest for CMV compared to CONT (treatment, $P < 0.01$). No difference in NEm (Mcal kg^{-1}), Ca or P concentration were observed between paddocks.

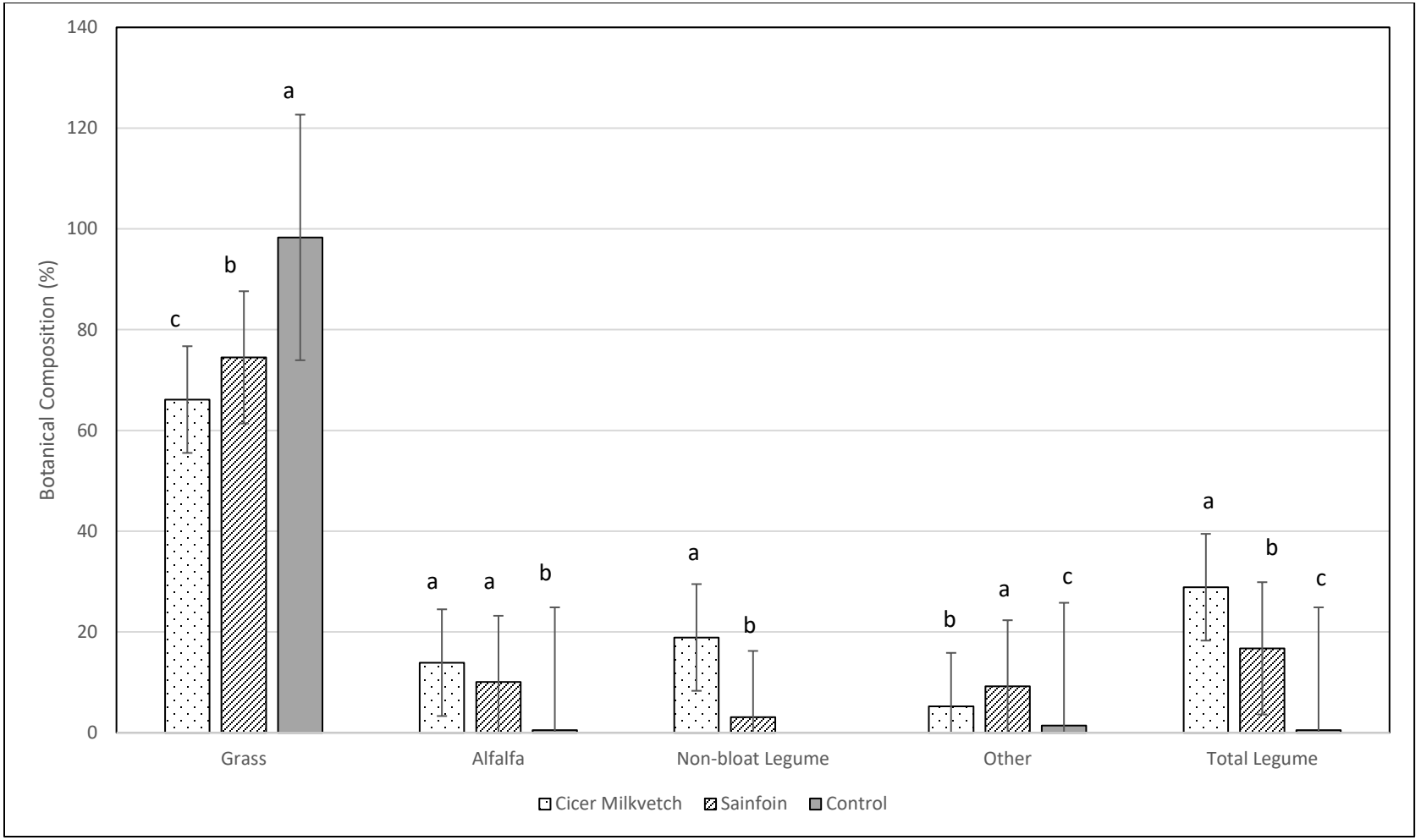

Figure 5.3. Botanical composition of treatment paddocks at Lanigan, SK, Canada over 2 yr
Note: Means within the same species with different letters differ at the $P < 0.05$.

Table 5.1. Chemical composition of forage grazed by fistulated cows over 2 yr (%, DM basis)

	Experimental Treatments				
Item	CMV	SAIN	CONT	SEM	*P* value
Crude Protein	14.73a	13.89ab	12.05b	1.347	0.01
Soluble Protein	34.03	33.68	33.95	1.284	0.92
Neutral detergent fiber	54.4b	55.67b	60.93a	2.438	<0.01
Acid detergent fiber	33.23b	34.12ab	35.95a	2.232	0.01
NEm	1.35	1.34	1.31	0.032	0.12
Calcium	0.67	0.67	0.46	0.102	0.12
Phosphorus	0.28	0.27	0.30	0.012	0.38

Note: Means in the same row with different letters differ at the $P < 0.05$. NEm, net energy maintenance (Mcal kg^{-1})

5.5.2 Rumen Fermentation and Nitrogen Excretion

Ruminal pH was not different between treatments (Table 5.2; treatment, $P = 0.91$). Total short chain fatty acid concentration (mmol), individual SCFA acetate, propionate, and butyrate concentrations (mol 100mol^{-1}) were unchanged by inclusion of non-bloat legumes into pasture (treatment, $P = 0.44$, 0.48, 0.22, 0.83, respectively). The acetate to propionate ratio was lower (treatment, $P = 0.01$) for cattle grazing CMV paddocks compared to cows grazing SAIN and CONT paddocks. Acetate/butyrate to propionate ratio was also lower (treatment, $P < 0.01$) in cows grazing the CMV paddocks, compared to cows grazing CONT and SAIN paddocks. Ruminal ammonia (NH_3-N) concentration was highest (treatment, $P = 0.03$) in cattle grazing CMV paddocks compared to SAIN and CONT paddocks. Plasma urea nitrogen was greater (treatment, $P < 0.01$) in cattle grazing CMV and SAIN paddocks compared to CONT paddocks. Blood glucose, non-esterified fatty acid, and beta-hydroxybutyrate concentrations were unchanged by treatment (treatment, $P = 0.32$, 0.60, 0.19, respectively).

Total urine N (g N L^{-1}) level was lower from cows grazing CMV paddocks, compared to cows grazing SAIN and CONT paddocks (Table 5.3; treatment, $P < 0.01$). Urine dissolved carbon (g C L^{-1}) was lower (treatment, $P < 0.01$) from cows grazing CMV and SAIN paddocks compared to cows grazing CONT paddocks. No treatment difference was reported in fecal DM (%) (treatment, $P = 0086$); however, total fecal N was higher (treatment, $P < 0.01$) from cows grazing CMV and SAIN paddocks compared to cows grazing CONT paddocks. Total fecal C remained unchanged by treatment (treatment, $P = 0.14$).

5.5.3 Enteric Gas Production and Microbial Community Composition

No treatment difference was observed in estimated DMI (Table 5.4; treatment, $P = 0.37$). No treatment difference was observed in CH_4 (g d^{-1}), CH_4 (g kg^{-1} DMI) or CO_2 (L d^{-1}) emission (treatment, $P = 0.64$, 0.76, 0.32, respectively).

A total of 30 samples (15 samples / year) were used to assess microbiomes and sequences were filtered for size, quality and for the presence of chimeras. A total of 1,983,284 reads (median = 66,109, minimum = 45,800, and maximum 85,792) were used to identify 10,378 unique bacterial operational taxonomic units (OTUs). Differential taxonomic comparison at the phyla level indicated no difference between treatments. Seven bacterial phyla were observed among samples (Figure 5.4), but *Fimicutes*, *Euyarchaeota*, and *Bacteroidetes* had the greatest relative abundance (Figure 5.5)

Table 5.2. Effect of pasture type on short chain fatty acid concentration, ammonia production, and blood metabolites

	Experimental Treatments				
Item	CMV	SAIN	CONT	SEM	*P* value
Mean ruminal pH	6.54	6.50	6.57	0.015	0.91
Ruminal fluid concentration					
Total SCFA (mmol)	117.19	109.18	107.55	12.063	0.44
Acetate (A)[a]	79.18	74.22	73.40	5.719	0.48
Propionate (P)	18.92	16.67	16.37	1.863	0.22
Butyrate (B)	10.95	10.59	10.36	1.400	0.83
A/B:P	4.78b	5.13a	5.12a	0.128	<0.01
A:P	4.21b	4.50a	4.50a	0.139	0.01
NH_3-N (mg dL^{-1})	11.3a	7.9b	7.3b	1.85	0.03
Blood concentration					
PUN (mg dL^{-1})	5.76*a*	4.22*b*	2.98*c*	0.460	<0.01
GLU (mg dL^{-1})	68.8	71.4	66.7	3.31	0.32
NEFA (µEq dL^{-1})	289.9	295.8	249.8	42.12	0.56
BHBA (mmol dL^{-1})	0.14	0.16	0.16	0.127	0.19

Note: Means in the same row with different letters differ at the $P < 0.05$. SCFA, short chain fatty acid; NH_3-N, ammonia; PUN, plasma urea nitrogen; GLU, plasma glucose; NEFA, non-esterified fatty acids; BHBA, beta-hydroxybutyrate.

[a]individual SCFA concentrations are reported as mol $100mol^{-1}$.

Table 5.3. Effect of pasture type on nitrogen and carbon content of feces and urine

	Treatment				
Item	CMV	SAIN	CONT	SEM	*P* value
Total urine N (g N L^{-1})	1.48c	2.14b	2.84a	0.075	<0.01
Urine dissolved carbon (g C L^{-1})	4.67b	6.22b	8.97a	0.430	<0.01
Fecal DM (%)	14.09	13.83	14.12	0.399	0.86
Total fecal N (g kg^{-1} DM)	2.37a	2.13b	1.95c	0.037	<0.01
Total fecal C (g kg^{-1} DM)	50.48	50.0	48.30	0.690	0.14

Note: Means in the same row with different letters differ at the $P < 0.05$. CMV, cicer milkvetch; SAIN, sainfoin; CONT, control; BW, body weight; DMI, dry matter intake.

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Table 5.4. Effect of pasture type on enteric gas production measured by SF^6 technique

	Experimental Treatments				
Item	CMV	SAIN	CONT	SEM	*P* value
Cow BW (kg)	723b	758ab	782a	14.9	<0.05
DMI (kg day^{-1})	16	16	15	0.5	0.37
Enteric gas production					
Methane (g day^{-1})	298.5	313.9	300.4	14.04	0.64
Methane (g kg^{-1} DMI)	18.9	19.6	18.7	0.94	0.76
Carbon dioxide (L d^{-1})	6685.8	5567.9	6706.7	682.75	0.32

Note: Means in the same row with different letters differ at the $P < 0.05$. CMV, cicer milkvetch; SAIN, sainfoin; CONT, control; BW, body weight; DMI, dry matter intake.

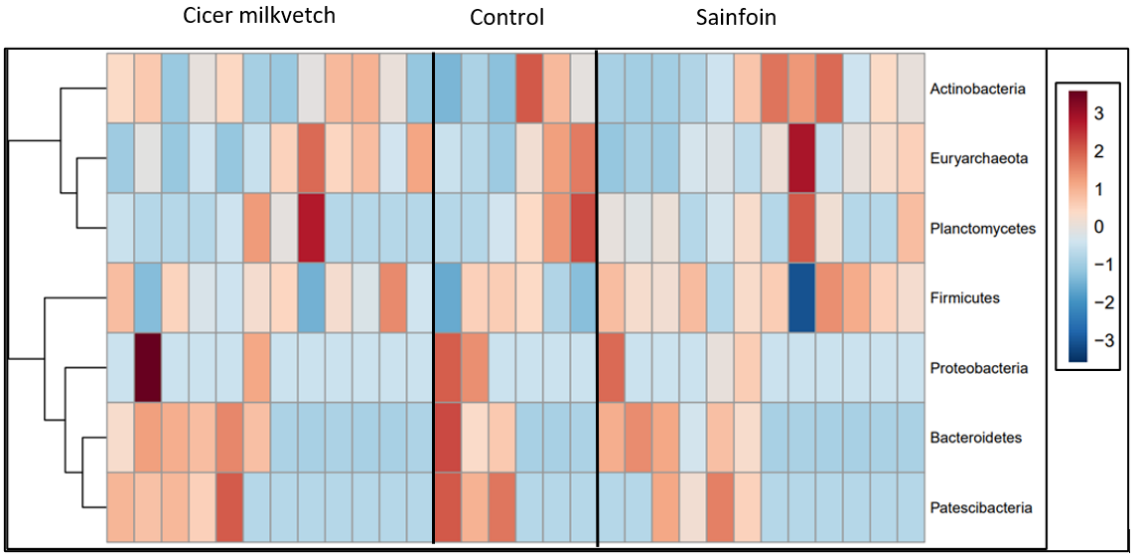

Figure 5.4. Heatmap of mean relative abundance of bacterial phyla identified in rumen digesta samples from cattle grazing cicer milkvetch, sainfoin and control pasture.

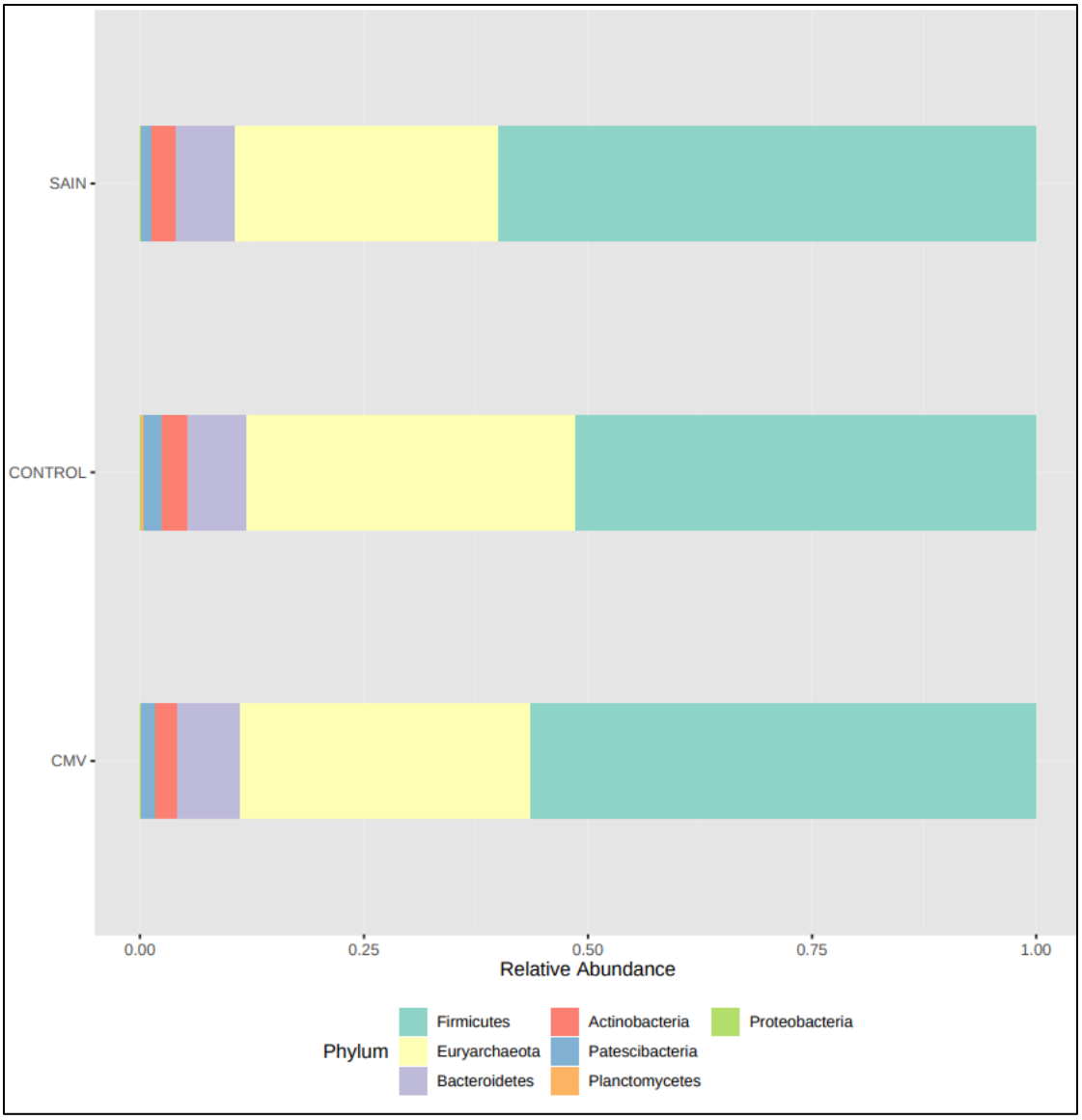

Figure 5.5 Relative abundance of bacterial 16S rRNA gene sequences at the phylum level observed in rumen digesta from cows grazing sainfoin, cicer milkvetch and control pastures.

5.6 Discussion

5.6.1 Botanical Composition and Forage Quality

As a percentage of stand, proportion of bloat-free legume was greater in cicer milkvetch paddocks compared to proportions of sainfoin. Although sainfoin is known to be extremely palatable, producer usage of sainfoin has been nominal in the past due to its low productivity, difficult, and sporadic establishment (Waghorn et al. 1998; Carbonero et al. 2011). As reflected in the low precipitation compared to 30-yr averages, especially in 2017, the growth of sainfoin in this experiment may have been impacted by environmental conditions (Bhattarai et al. 2016). Grazing on these paddocks was restricted until the year after seeding to allow for seed-set during the year seeded, however, as suggested by Chapter 6, pre-existing plant species may have negatively impacted the persistence of sainfoin at this site. It should be mentioned that the percentage of other undesirable species was increased after sod-seeding occurred, reflecting risk in sod-seeding by mechanically opening of the seedbank to allow for undesirable species to grow. Canada thistle, an undesirable species present in the current study, has been reported to prolifically produce seeds that can remain viable in the soil for as long as 20 years (Hodgson 1968). Therefore, the risk of increasing undesirable species must be considered when producers are contemplating rejuvenating pastures with sod-seeding. In addition, these undesirable species may have contributed to changes in forage quality observed between treatments. Therefore, a positive control consisting of glyphosate application and mechanical soil disturbance may be beneficial to include in future research projects. Crude protein concentrations were highest in cicer milkvetch paddocks compared to control, which is unsurprising since the percentage of total legume was higher in paddocks sod-seeded with bloat free legumes. Both cicer milkvetch and sainfoin paddocks had lower NDF concentrations compared to forage from control paddocks, which is to be expected, since sainfoin and cicer milkvetch have been reported to be highly digestible due to their low NDF concentrations (Acharya et al. 2006; Bhattari et al. 2016).

5.6.2 Rumen Fermentation

As with any diet, the proportion of ingredients will impact the degree to which modulation of rumen fermentation occurs. The percentage of cicer milkvetch in paddocks was 19%. In the current study, total short chain fatty acid production was not impacted by either sainfoin or cicer milkvetch treatments; however, acetate to propionate ratio (A:P) and acetate/butyrate to propionate was lower in cattle grazing cicer milkvetch compared to control

and sainfoin. These results echo the results observed in the previous batch culture and RUSITEC experiments, in which A:P ratio was linearly reduced with increasing levels of cicer milkvetch compared to sainfoin or alfalfa in continuous culture (Chapter 4). Experiments comparing inclusion rates of cicer milkvetch are lacking; however, Williams et al. (2011) reported greater propionate and lower A:P concentrations during continuous culture incubations when fermenters were fed 70% cicer milkvetch total mixed ration, compared to the same ratio of sainfoin or alfalfa total mixed ration. Noviandi et al. 2014, reported increased propionate concentration with increasing level of cicer milkvetch (25, 50, 75% inclusion) during continuous culture, compared to alfalfa and sainfoin, which was attributed to greater concentration of non-fiber carbohydrates in the cicer milkvetch. Sainfoin inclusion into paddocks resulted in no change in SCFA production, however the percentage of sainfoin in the stand was only 3% which is likely not high enough to elicit a response, as Wang et al. (2006) found no change in ruminal A:P ratio for cattle grazing pasture with less than 9% (DM basis) of sainfoin in stand. However, when sainfoin was included in pasture at greater than 9% (DM basis) of stand, ruminal A:P ratio was reduced when compared to cattle grazing pure alfalfa, leading improved energetic efficiency with sainfoin inclusion (Wang et al 2006).

Blood urea nitrogen (BUN) concentration is highly correlated with ruminal ammonia concentrations and can be used as a tool to indicate the protein to energy ratio in the diet. However, BUN concentration may be impacted by time of sampling, diet, or health and physiological stage of animal (Hammond 1983). Blood urea nitrogen samples were taken from animals at the same time of day to minimize diurnal variation. With respect to diet, BUN concentration can be influenced by dietary nitrogen content, nitrogen solubility, and energy content, or animal dry matter intake (Hammond 1983). In the current study, increased BUN and ruminal NH_3-N concentrations were observed in cattle grazing cicer milkvetch paddocks; however, this is likely due to the elevated CP in forage from cicer milkvetch paddocks. With respect to sainfoin, Stewart et al. (2019) reported cattle fed sainfoin hay (100% DMI) had lower BUN compared to cattle fed cicer milkvetch or alfalfa hay and attributed this difference to the impact of CT on ruminal protein digestion. In the current study, the lower BUN observed in the cows grazing sainfoin and control paddocks is likely attributed to reduced dietary CP intake, due to the reduced legume present in these paddocks (Hammond 1983).

Cows consuming cicer milkvetch paddocks had the lowest concentration of urine N concentration, followed by cows grazing sainfoin and then control, with the inverse observed for fecal N concentration. Since urine and fecal volumes were not measured this response can likely be attributed to differences in water intake and excreta output. With respect to urine, consumption of protein and minerals (Na and K) has a direct impact on the amount of urine produced daily by an animal (Dijkstra et al. 2013; Stewart et al. 2019). Animals fed high protein diets consume more water and excrete more urine (Van Vuuren and Smits 1997; Dijkstra et al. 2013), which would be viewed as environmentally unfavorable. Given the increased PUN and ruminal NH^3-N concentration in cows grazing cicer milkvetch forage and the higher CP concentration in cicer milkvetch forage, it is likely the lower urine N concentration in cows grazing these paddocks was due to dilution of urine N from increased water intake (Dijkstra et al. 2013).

5.6.3 Enteric Gas Production and Microbial Community Composition

Enteric methane (CH_4) is formed in the rumen primarily from hydrogen produced during ruminal fermentation, particularly high fiber diets, with diets that shift fermentation pathways to favor propionate over acetate and butyrate (such as high concentrate diets) generating less CH_4 (Noviandi et al. 2014). It is reported in literature that cattle grazing bloat-free legumes, such as birdsfoot trefoil, sainfoin and cicer milkvetch, can experience reductions in enteric CH_4 emissions; however, results from studies have been inconsistent (Williams et al. 2011; Bouchard et al. 2013; Noviandi et al. 2014; Stewart et al. 2019). In the present study, no difference was observed between treatment forages for enteric CH_4 emissions (g d^{-1} or g kg^{-1} DMI). icer milkvetch populations were below 25% of stand and sainfoin populations were below 5% of stand. Other studies have reported the effect of bloat-free legume on CH_4 emissions were dependent on the percentage of legume species in the diet. For example, McMahon et al. (1999) found that CH_4 (mL d^{-1}) production was linearly reduced with increasing level of sainfoin in the diet during continuous culture, whereas Noviandi et al. (2014) found no effect of increased inclusion of sainfoin or cicer milkvetch during continuous culture. Williams et al. (2011) found that diets containing a 70% cicer milkvetch or sainfoin in a total mixed ration (TMR) produced less CH_4 than diets containing 70% alfalfa in a total mixed ration in continuous culture. These differences in fermentation between species and legume inclusion are not surprising; however, it

highlights the complexity of microbial digestion of both CT forages and non-tannin containing forage.

Although the SF_6 tracer technique can be more variable than whole-animal chamber measurements, this technique is a valuable *in vivo* research technique allowing for measurement of CH_4 from large numbers of grazing animals in their natural environment (Ulyatt et al 1999; Pinares-Patino et al. 2011; Hammond et al. 2016). Measurement of enteric CH_4 emissions from ruminally-cannulated cattle using the SF_6 technique has been questioned due to the potential to increased variability in results (Beauchemin et al. 2012). When using cannulated cattle for SF_6 measurements it is recommended for the cannulas to be tight fitting to minimize leakage, such as the 9C cannula (Beauchemin et al. 2012), as was used in the current study. Concerns related to the cannulas impact on normal fermentation, with respect to air entering the rumen and losses of gas from the rumen headspace are valid. However, if properly managed using tight fitting cannulas and rumen fluid sampler syringes, as in the current study, differences in emission collection between cannulated and non-cannulated animals is reported to be minimal (Yonker et al. 2020).

The rumen microbial population is highly diverse and essential for the host animal, providing SCFA and microbial protein, which contribute the majority of metabolizable energy and metabolizable protein required by the animal (Petri et al. 2012; NASEM 2016). Although diet composition alters the diversity of the rumen microflora, rumen microbial populations and community structures are adaptive, with fermentation end products dependent on ruminal microbial population and substrate availability (Petri et al. 2012). As propionate is the primary glucogenic pathway and electron accepting pathway which lowers substrate availability for methanogenesis, research has attempted to develop strategies which shift rumen fermentation towards increase propionate production (NASEM 2016).

In the current study sod-seeding bloat-free legumes had no effect on the taxonomic comparison at the phyla level. Feed additives that alter ruminal microbial populations and fermentation end-products include ionophores, direct-fed microbials, essential oils, or plant secondary compounds (Beauchemin 2009; NASEM 2016). Attachment of microbiota to feed particles is critical for initiation of digestive processes, and therefore feed additives that prevent microbial attachment or promote detachment can inhibit cellulose digestion and alter

fermentation end products (McAllister 1994a). Most feeds contain a surface layer that is somewhat resistant to infiltration (McAllister 1994a); however, condensed tannins contained in forages, such as sainfoin, have been reported to inhibit growth and protease activity of *Butyrivibrio fribrisolvens* and *Streptococcus bovis* (Jones et al. 1994). In comparison, extracts of cicer milkvetch have been shown to inhibit *Fibrobacter succinogens,* and cellulose digestion (Weimer et al. 1993). However, in both studies, results were dependent on the dosage rate of CT and cicer milkvetch extract. As no differences were observed at deeper depth analysis (taxonomic class level), it is possible the level of sainfoin and cicer milkvetch legume in the diet did not modify rumen fermentation enough to impact on enteric methane emission or rumen bacterial populations.

5.7 Conclusions

In conclusion, incorporation of cicer milkvetch may increase energetic efficiency, as acetate to propionate and acetate/butyrate to propionate ratios decreased in cattle grazing cicer milkvetch paddocks. However, cattle grazing cicer milkvetch paddocks also experienced increased ruminal ammonia and blood urea nitrogen concentration, which indicates asynchronous ruminal protein and energy supply. In addition, sod-seeding appeared to increase the growth of undesirable species from the soil seed bank, especially in the sainfoin paddocks. This is an important risk factor that must be considered when producers are considering sod-seeding. More research is required to determine the effects of sod-seeding legumes into existing pasture and their impact on ruminal fermentation, enteric CH_4 emissions, and N excretion.

6 EFFECT OF SOD-SEEDING BLOAT-FREE LEGUMES ON PASTURE PRODUCTIVITY, STEER PERFORMANCE, AND PRODUCTION ECONOMICS[2]

6.1 Abstract

A five-year experiment evaluated the effects of sod-seeding sainfoin and cicer milkvetch into monoculture grass (Lanigan, SK) or legume (Lethbridge, AB) stands on pasture productivity, steer performance, and economics. At Lanigan, sainfoin decreased (treatment × year $P = 0.01$) from 13% in yr 1 to 2% in yr 2 (% plant population) and did not differ thereafter, while cicer milkvetch, maintained a proportion of 16% in the stand. Forage yield was greater (treatment × year; $P < 0.01$) in yr 1 in the sainfoin and cicer milkvetch treatments compared to control. DMI of steers was greater only in yr 5 and ADG was greater ($P < 0.01$) in sainfoin and cicer milkvetch treatments compared to control. At Lethbridge, sainfoin decreased (treatment × year; $P = 0.01$) from 46 to 17% (% DM yield), while cicer milkvetch maintained its proportion at 11%. Forage yield increased (treatment × year; $P < 0.01$) only in yr 2 and 3 of sainfoin, compared to cicer milkvetch or control. ADG gain was not affected by treatment. At Lanigan, sainfoin and cicer milkvetch generated greater gross returns compared to control; however, once establishment costs were applied there were no differences in present value of net returns.

Key Words: sainfoin, cicer milkvetch, legumes, rejuvenation, sod-seed

6.2 Introduction

Sainfoin (*Onobrychis viciifolia* Scop.) is a perennial legume belonging to the Fabaceae family, of the *Hedysareae* tribe (Bhattarai et al. 2016). This species is known to be palatable and drought and frost tolerant. However, producer usage of sainfoin has been minimal in the past due to its low persistence under grazing, difficult and sporadic establishment, and high seed cost (Waghorn et al. 1998; Carbonero et al. 2011). Sainfoin can be slow to establish and requires weed management practices to promote its establishment (Waghorn et al. 1998). Sainfoin can be seeded as a monoculture or in a mixture with other perennial legumes and grasses; however, it

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tends to compete better with caespitose than rhizomatous grasses if seeded in a blend (Bhattarai et al. 2016). Due to slow regrowth after cutting, and the requirement to time harvesting after flowering, it is recommended to limit harvesting to no more than twice yearly to allow for replenishment of root reserves and stand survivability (Waghorn et al. 1998; Carbonero et al. 2011). New cultivars of sainfoin that have greater persistence in grazed mixed stands have been developed and Sottie et al. (2014) reported that sainfoin varieties (LRC-3519) could persist in a grazed mixed stand at >20% of the above ground biomass when compared to older varieties which were not suited as they did not persist under grazing (Nova).

Cicer milkvetch (*Astragulus cicer* L.) is a long-lived perennial legume native to Europe that was introduced to North America from the USSR in 1931 (Acharya et al. 2006). Cicer milkvetch is adapted to the Black and Dark Brown soil zones in western Canada and has excellent winter hardiness (Acharya 2001) tolerating a wide range of soil types and environmental conditions including drought (Acharya et al. 2006). Although condensed tannins are responsible for the bloat safe properties of sainfoin, cicer milkvetch is bloat safe due to the mechanical strength, veining pattern, and structure of its leaves (Lees et al. 1982). The leaves of cicer milkvetch are pinnately-compound, have a thick epidermal layer, and a reticulate veining pattern which acts as a physical or mechanical barrier to microorganisms thereby slowing microbial digestion in the rumen (Lees et al. 1982; Acharya 2001). Cicer milkvetch maintains nutritional quality into late fall and has been shown to have higher total digestible nutrient (**TDN**) content than alfalfa (c.v. AC Grazeland) due to its ability to retain leaves after flowering (Acharya 2001; Lardner et al. 2018).

Hay yields in western Canada were reported to have declined over a 30-yr period, with variables such as fertilizer price negatively correlated to forage production (Jefferson and Selles 2007). Rejuvenation to increase pasture productivity may include fertilizer applications or break and reseed techniques; however, these techniques can be expensive and can expose the soil to wind and water erosion. Sod-seeding into existing pasture stands is a method of pasture rejuvenation that involves the introduction of a desirable species, such as a legume, into a low productive pasture stand. Due to the N_2 fixation by legumes, the need for costly N fertilizer can be minimized and the risk of soil erosion is reduced (Sequin 1998). Therefore, assessing the ability of bloat-free legumes to establish and persist under grazing management when sod-seeded

into existing monoculture grass and monoculture legume stands could provide producers with an alternate rejuvenation technique. The hypothesis of this experiment was that sod-seeding bloat-free legumes into existing pasture stands will improve forage yield, forage quality and grazing animal performance and prove to be an economically feasible pasture rejuvenation strategy for producers.

The objectives of this experiment were to: (i) determine botanical composition, forage yield, and forage quality of direct sod-seeded sainfoin and cicer milkvetch populations into either monoculture grass (Lanigan) or a monoculture legume stand (Lethbridge); (ii) determine grazing animal performance when grazing sod-seeded sainfoin or cicer milkvetch pastures relative to control (no sod-seed) pastures; and to (iii) conduct a capital investment analysis at Lanigan for pasture rejuvenation using sainfoin and cicer milkvetch.

6.3 Materials and methods

6.3.1 Experiment Site Description

A 5-yr (2016 through 2020 grazing seasons) replicated (n=6) experiment was conducted at Lanigan (SK, Canada) and a 3-yr (2016, 2017, 2018) replicated (n=4) experiment was conducted at Lethbridge, (AB, Canada). The Lanigan and Lethbridge locations were considered as separate studies due to difference in study duration, pre-existing pasture species, and data collection methodologies. The experimental site at Lanigan was located at the Western Beef Development Center's Termuende Research Ranch (51°51 'N latitude; 105°02 'W longitude). The site was located in the Thin Black soil zone and is a mixture of Oxbow Orthic Black and carbonated Oxbow with a loam texture (Saskatchewan Soil Survey, 1992). The experimental site at Lethbridge was located at Agriculture and Agri-Food Canada (AAFC) Lethbridge Research Center (49°42 'N latitude; 112°47 'W longitude) which was situated on a Dark Brown Chernozem soil that is a slightly alkaline clay loam (Larney and Janzen, 2012).

6.3.2 Experimental Site Management

At Lanigan, a 30-ha meadow bromegrass (*Bromus riparius Rehm*)-alfalfa (*Medicago sativa* L.) pasture was subdivided into fifteen, 2-ha (65 × 306 m) paddocks. Paddocks were then randomly assigned to 1 of 3 treatments: control (**CONT**; n = 3); sod-seeded sainfoin (**SAIN**; n = 6); or sod-seeded cicer milkvetch (**CMV**; n = 6). Three paddocks each were seeded to common sainfoin (Brett Young Seeds, Winnipeg, MB, CA), c.v. AC Oxley II cicer milkvetch

(Brett Young Seeds, Winnipeg, MB, CA), c.v. AC Mountainview sainfoin (Northstar Seeds, Neepawa, MB, CA) and c.v. AC Veldt cicer milkvetch (Northstar Seeds, Neepawa, MB, CA). Varietal effects were not considered. Prior to seeding, one application (May 28, 2015) of 1.24 L ha^{-1} glyphosate (N(phosphonomethyl) glycine), purchased from Blair's Fertilizer Ltd. (Lanigan, SK), was applied to CMV and SAIN paddocks to reduce existing species competition and allow for sainfoin and cicer milkvetch to establish. Sainfoin was sod-seeded at a rate of 26 kg ha^{-1} and cicer milkvetch was seeded at a rate of 17 kg ha^{-1}as per the seed suppliers' recommendations. Cicer milkvetch seed was scarified prior to seeding and sainfoin was seeded with an inoculant. Sod seeding took place on June 9 and 10, 2015 at Lanigan using a 2.4 m zero till seed opener AGROPLOW AD130 drill (Molong, NSW, AU) with 15.2-cm row spacing and was seeded at a depth of 1.9 cm. The SAIN and CMV paddocks were rolled after sod-seeding and paddocks were mowed once during summer to aid in weed control. No field treatments were applied to the control paddocks.

At Lethbridge, a 7.2-ha alfalfa pasture was subdivided into 18, 0.4 ha (15 × 270 m) paddocks. Each 0.4-ha paddock was randomly assigned to 1 of 3 replicated treatments: CONT (n = 2), SAIN (n =8), or CMV (n = 8). Forage seeds were obtained from Agriculture and Agri-Food Canada (Lethbridge, AB, Canada), and 4 paddocks each were seeded to c.v. AC Nova sainfoin, L3432 common sainfoin, c.v. AC Veldt cicer milkvetch, or c.v. AC Oxley II cicer milkvetch. All paddocks were mowed using a John Deere 972 flail forage harvester (Moline, Illinois, USA) on May 26 2015 and then received an application of 0.9 L ha^{-1} glyphosate on June 26 2015 to suppress the existing alfalfa stand that was at the early flower stage at the time of spraying. Stands were then mowed, a second time and the residue was removed using a John Deere 972 flail forage harvester in (Moline, Illinois, USA) on July 7 2015. The SAIN and CMV paddocks were sod-seeded in alternate rows (between existing rows of alfalfa) at 2.5 cm depth, and a 17 cm row spacing on July 9 2015 using a John Deere 5160 no-till pan drill (Moline, Illinois, USA). Sainfoin and cicer milkvetch were seeded at a rate of 33 kg ha^{-1} and 22 kg ha^{-1}, respectively. No field treatments were applied to the control paddocks. Lethbridge was irrigated and received 76 and 152 mm of irrigated water in June and September of 2017, and 50 mm of irrigated water in May, July, and August of 2018.

At both sites, germination and emergence were monitored in 2015. In the spring of 2016, all individual paddocks were fenced with permanent wire fencing and grazed to allow for similar

grazing each year. At both sites, grazing commenced each year when available forage was approximately 2000 kg ha^{-1} and steers remained on paddocks until forage was grazed to a uniform height of approximately 8 cm. Monthly temperature (°C) and precipitation (mm) data and the long-term average (LTA; 30 yr) were obtained using Environment and Climate Change Canada weather stations located near each research trial location (Lethbridge, AB and Leroy, SK).

6.3.3 Experimental Animals

All cattle were cared for in accordance with the Canadian Council on Animal Care (CCAC 2009) guidelines. Institutional approval for animal use was granted by the University of Saskatchewan Animal Research Ethics Board (Protocol No. 20090107) and the Lethbridge Research and Development Animal Care Committee (Protocol No. 1619).

The grazing periods at Lanigan occurred from June 10 to August 31, 2016 (yr 1, 82 d), June 28 to July 18, 2017 (yr 2, 20 d), June 25 to August 13, 2018 (yr 3, 48 d), July 10 to August 13, 2019 (yr 4, 34 d) and June 17 to August 12, 2020 (yr 5, 56 d). In yr 1, yr 4, and yr 5 60 spring-born fall-weaned yearling steers (4 steers/paddock) were used and in yr 2 and yr 3, 45 spring-born fall-weaned yearling steers (3 steers/paddock) were used. In each year, steers were stratified by BW (average BW = 326 ± 26 kg) and randomly allocated to CONT, SAIN, and CMV paddocks.

Grazing periods at Lethbridge were from July 12 to July 29, 2016 (yr 1, 18 d), July 6 to July 19, September 21 to October 3, and October 10 to October 19, 2017 (yr 2, 50 d), and June 27 to July 16 and September 19 to October 2, 2018 (yr 3, 32 d). Each yr, 32 spring-born, fall-weaned steers (average BW = 426 ± 43 kg) were stratified based on body weight (BW) and randomly allocated to CONT, SAIN, and CMV with 2 steers/paddock.

6.3.4 Botanical Composition

Botanical composition was assessed yearly to determine establishment and persistence of cultivars within the stand. At Lanigan, estimates of botanical composition were taken at the start (available) and the end (residual) of each grazing period. Using the Daubenmire (1959) technique, within each replicate paddock, 20 random quadrats (0.25 m^2) marked with a permanent transect (grid) were visually assessed for composition by canopy cover and separated into percentage grass, alfalfa, bloat-free legume and other species. Other species at Lanigan

included weeds, such as absinthe wormwood (*Artemisia absinthium*) and Canada thistle (*Cirsium arvense*).

At Lethbridge, estimates of botanical composition were taken at the start (available) and end (residual) of each grazing period. Within each replicate paddock three randomly distributed quadrats (0.36 m^2) were clipped, hand separated, dried in a forced air oven at 40°C for 72 h for DM determination and categorized by weight (% DM) into percent grass, alfalfa, bloat-free legume and other species. In the first year of the study at Lethbridge, other species in the CMV paddocks were SAIN regrowth from previous trials.

6.3.5 Estimated Forage Yield, Forage Quality and Estimated Dry Matter Intake

Available and residual forage was estimated at Lanigan at the start (available) and end (residual) of each grazing period, using the pre- and post-graze technique as described by Cook and Stubbendieck (1986). In each paddock, 20 randomly distributed quadrats (0.25 m^2) were clipped to a 5-cm stubble height, composited, and the fresh weight of all 20 samples was determined. To estimate changes in forage quality over each grazing season, two sub-samples from each composite at the start and end of each grazing period were placed in paper bags and dried in a forced-air oven at 55ºC for 72 h to determine DM and forage chemical composition (described below). Samples were ground to pass through a 2-mm screen (Thomas Wiley Laboratory Mill Model 4; Thomas Scientific, Swedesboro, NJ, USA) (AOAC 2000), composited by paddock, and stored until analyzed.

At Lethbridge, 5 randomly distributed quadrats (0.36 m^2) within each paddock were clipped to a 5-cm height and composited. To estimate changes in forage quality over each grazing season, one subsample was taken from each composite at the start and end of each grazing period, placed in paper bags and dried in a forced air oven at 55°C for 72 h for DM and forage quality determination. Samples were ground to pass a 1-mm screen (AOAC 2000) and stored until analyzed.

Duplicate samples from Lanigan were analyzed at Cumberland Valley Analytical Services (Waynesboro, PA, USA) for total DM (Goering and Van Soest, 1970), crude protein (AOAC; method 990.03), acid detergent fiber (AOAC, method # 973.18), ash corrected neutral detergent fibre (Van Soest et al. 1991), calcium (AOAC, method 985.01), and phosphorous (AOAC; method 985.01) (AOAC International, 2000).

Forage NEm (net energy maintenance) (Mcal kg^{-1}) = 1.37 × ME – 0.138 × ME^2 + 0.0105 × ME^3- 1.12……..……..**(6.1)**

At Lanigan animal body weight and dietary NEm concentrations were used as an estimate of forage dry matter intake (DMI) (consumption) according to the following equations (NASEM 2016):

NEm intake (Mcal d^{-1}) = $BW^{0.75}$ × (0.2435 ×NEm – 0.0466 × NEm^2 – 0.1128)……..**(6.2)**

DMI (kg d^{-1}) = NEm intake (Mcal d^{-1}) / dietary NEm concentration (Mcal kg^{-1} DM)……..…(**6.3)**

Where BW is the average BW for the feeding period and NEm is the dietary NEm concentration in Mcal kg^{-1} of dry matter.

Duplicate forage samples from Lethbridge were analyzed at Agriculture and Agri-food Canada laboratory for crude protein (AOAC; method 990.03), acid detergent fiber (AOAC, method # 973.18), ash corrected neutral detergent fibre (Van Soest et al. 1991), calcium (AOAC, method 985.01), and phosphorous (AOAC; method 985.01) (AOAC International, 2000). Since TDN was not calculated, due to the limited forage quality data collected at this site, DMI was not estimated at Lethbridge.

6.3.6 Steer Performance

A portable handling facility was located on site at Lanigan (Real Industries Ltd., Rathwell, MB, Canada) and a permanent handling facility was located at Lethbridge (Cattleac Cattle Equipment & ACC. Inc. Weatherford, OK, United States) for livestock handling and measurement of steer BW. Each yr, individual steers were weighed on 2 consecutive days at the start and end of the experiment. Average daily gain (ADG) was determined using start and end steer BW for each replicate paddock.

6.3.7 Soil Sampling and Analysis

Soil nutrients were measured at the start and end of trial to determine any changes in soil nutrient profile. In each paddock, soil samples were collected from 10 random locations at one depth (0 to 60 cm) using a hand auger and composited. All samples were stored at 4°C until they were air-dried and then ground to pass a 2-mm screen. Samples were analyzed for nitrate-N using the American Public Health Association, American Water Works Association and Water

Environment Federation (2005), standard method for the examination of water and wastewater (Houba et al. 2000), and plant available phosphorus and potassium using the modified Kelowna extraction method (Qian et al. 1994). Available P and K was only measured at the Lanigan.

6.3.8 Capital Investment Analysis

The costs and returns associated with each treatment were determined at the Lanigan experimental site only. Costs for rejuvenation were calculated from agronomic records and actual invoices incurred for the project. Costs to sod-seed the bloat-free legumes included spraying, seeding, land rolling, pre-seed herbicide and seed. Seed was the largest single cost for the rejuvenation. The cicer milkvetch seed was purchased for \$11.72 kg^{-1} and seeded at 16.84 kg ha^{-1} for a cost of \$197.29 ha^{-1}. The sainfoin seed was purchased for \$7.05 kg^{-1} and seeded at 25.82 kg ha^{-1} for a cost of \$182.16 ha^{-1}. Both forages were sod-seeded with a rented AgroPlow (\$38.29 ha^{-1}) and pulled by a rented 180 horsepower tractor (\$51.87 ha^{-1}) after one application of 1.24 L ha^{-1} glyphosate (\$7.90 ha^{-1}). The rate paid for custom application of glyphosate was \$29.64 ha^{-1}. After seeding, each paddock was rolled by a custom operator at a cost of \$14.10 ha^{-1}.

Annual returns for each treatment were based on annual DM yield multiplied by the price of standing hay reported in provincial annual price reports (Saskatchewan Forage Council 2016-2020). The published price for standing hay was \$0.033 kg^{-1} in 2016, \$0.046 kg^{-1} in 2017, and \$0.073 kg^{-1} for years 2018 to 2020 (Saskatchewan Forage Council 2016-2020).

Given sod-seeding required an up-front investment in yr 0 (2015) but generated returns (DM yield) over 5 yr capital investment analysis was conducted using the net present value method (Barry and Ellinger 2012).

Net present value (NPV)……..…….(**6.4)**

$$NPV = -INV + \frac{P_1}{(1+i)^1} + \frac{P_2}{(1+i)^2} + \cdots + \frac{P_N}{(1+i)^N} + \frac{V_N}{(1+i)^N}$$

Where,

INV is the initial investment
P_N is the net cash flows from the investment
i is the discount rate or required rate of return
V_N is the salvage value of the investment
N is the length of planning horizon

Annual returns (P_N) were discounted using a 5 percent required rate of return (i) to allow for comparison of treatments on a present value basis. The discounted returns were summed and the initial investment (INV) for the sod-seeding in yr 0 (2015) was subtracted to calculate the present value of net returns by treatment. The CONT treatment had no initial investment. The salvage value (V_N) for all treatments was assumed to be zero.

6.4 Statistical Analysis

Statistical analysis was conducted for each location independently using a one-way analysis of variance (ANOVA) with the PROC Mixed Model procedure of SAS 9.2 (SAS, 2003) for a completely randomized design. Paddock was considered as the experimental unit. Fixed effects were year, treatment, and their interaction with year included as a repeated measure. Degrees of freedom were adjusted using the Kenward-Roger option. The PDIFF was adjusted by the Tukey method and were considered significant when $P = 0.05$ and included in the LSMEANS. Botanical composition data (% of the stand in Lanigan and % of DM in Lethbridge) were analyzed using both logarithmic and arcsine transformations; however, untransformed data were determined to yield the best fit based on Akaike and Bayesian information criterion.

6.5 Results

Seasonal precipitation (May to September) was below the 30-yr average for Lanigan, SK in the first 2 yr of the study, above the 30-yr average in yr 3, and near average during yr 4 and 5, resulting in year-to-year variation in grazing days. Total precipitation at Lanigan from May to September was 176.6, 85.0, 343.3, 273.6, and 233.4 mm in 2016, 2017, 2018, 2019, and 2020 respectively (Table 3.1). The 30-yr average amount of rainfall for the same period at Lanigan is 295.3 mm, showing that yr 1 and yr 2 resulted in 40 and 70% lower levels of precipitation, compared to long-term average.

As Lethbridge was irrigated, total precipitation from May to September was 162.2, 87.5, and 125.1 mm in 2016, 2017, and 2018, respectively (Table 6.1) with an additional 228 mm in 2017 and 150 mm in 2018 added through irrigation.

Drought conditions in May each yr of the study compromised the duration of grazing length, resulting in an average trial length of 48 d (5 yr average) and an average stocking rate of 1.8 steers ha^{-1} at Lanigan, SK. Drought was not experienced at Lethbridge, as this site was

irrigated. The average grazing duration was 33 d (3 yr average) and the stocking rate was 5 steers per hectare.

6.5.1 Botanical Composition, Forage Yield, Forage Quality and Soil Nutrient Profile

At Lanigan, the proportion of grass increased from yr 2 to yr 3 in CMV and SAIN and remained stable thereafter, while the proportion of grass in CONT remained stable over 5 yr (Figure 6.1; treatment × year, $P < 0.01$). The proportion of alfalfa in CMV and SAIN decreased after yr 2 and remained stable thereafter (Figure 6.2; treatment × year, $P < 0.01$). There was no alfalfa present in the CONT paddocks. The proportion of bloat-free legumes in CMV was relatively stable despite some year-to-year variation, while for SAIN, the proportion of bloat-free legumes decreased from yr 1 to yr 2 and remained stable thereafter (Figure 6.3; treatment × year, $P < 0.01$). There were no bloat-free legumes recorded in the CONT paddocks. The proportion of other species in CMV and SAIN decreased from yr 1 to yr 2 and remained stable thereafter (Figure 6.4; treatment × year, $P = 0.05$). While the proportion of total legume was similar among CMV and SAIN in yr 1 and both decreased over the course of the study, the reduction in SAIN was greater than the reduction in CMV (Figure 6.5; treatment × year, $P < 0.01$); while the proportion of legume in CONT was nearly undetectable. At Lanigan, the yield of available forage prior to grazing was markedly greater for CMV and SAIN than for CONT (Figure 6.6; treatment × year, $P < 0.01$) in 2016, but no differences were observed in subsequent years. The average available forage yield over the 5 yr study was 4214, 4100 and 2878, kg DM ha^{-1}, for CMV, SAIN and CONT paddocks, respectively. While a treatment × year interaction was detected, there were no within year differences for the available forage yield after grazing (Figure 6.7).

Table 6.1. Average monthly temperature and precipitation during grazing season (May to September) and monthly long-term (30 yr) average (LTA) at Lanigan, Saskatchewan, and Lethbridge, Alberta, Canada

	Lanigan (2016-2020)				Lethbridge (2016-2018)			
	Temperature, °C		Precipitation, mm		Temperature, °C		Precipitation, mm	
Month	Mean	LTA	Mean	LTA	Mean	LTA	Mean	LTA
May	11.7	10.9	27.26	49.10	12.57	11.10	44.57	46.00
June	16.0	15.6	67.74	72.00	16.13	15.20	28.97	53.00
July	18.3	18.0	51.18	71.40	18.93	18.20	17.77	37.00
August	17.0	17.2	35.46	62.40	18.10	17.70	20.80	47.00
September	11.2	11.3	40.76	42.50	12.53	12.60	12.83	37.00
Mean	14.9	14.6	-	-	15.56	14.96	-	-
Total	-	-	222.40	297.40	-	-	124.93	220

Note: Environmental data was collected using Environment and Climate Change Canada weather stations located near each research trial location (Lethbridge, AB and Leroy, SK stations) (www.weather.gc.ca).

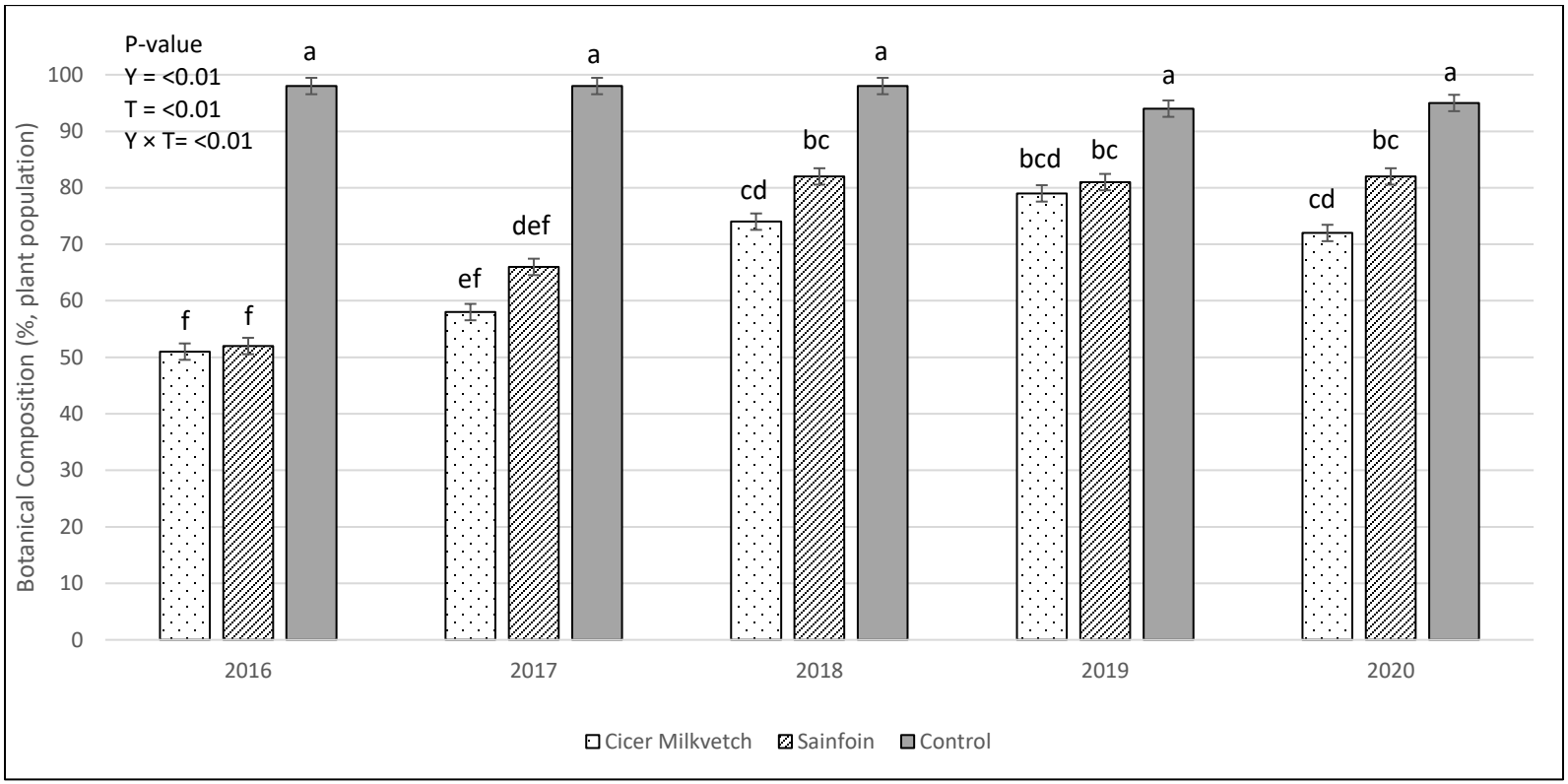

Figure 6.1. Effect of sod-seeding bloat-free legumes on grass composition in pasture at Lanigan, SK, Canada over 5 yr. Grass proportions associated with different letters are significantly different (treatment × year; $P < 0.01$). Y, year; T, treatment.

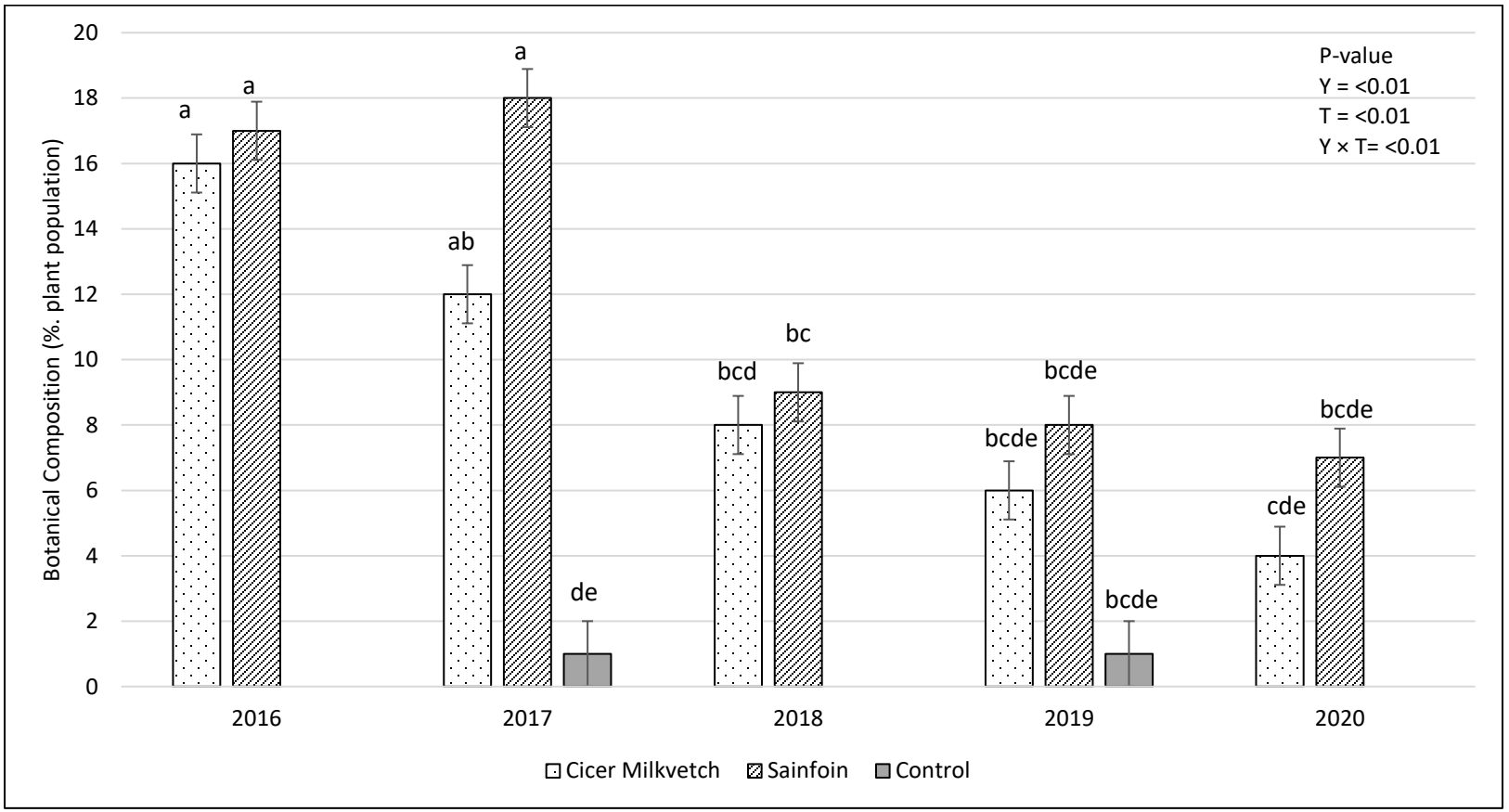

Figure 6.2. Effect of sod-seeding bloat-free legumes on alfalfa composition in pasture at Lanigan, SK, Canada over 5 yr. Alfalfa proportions associated with different letters are significantly different (treatment × year; $P < 0.01$). Y, year; T, treatment.

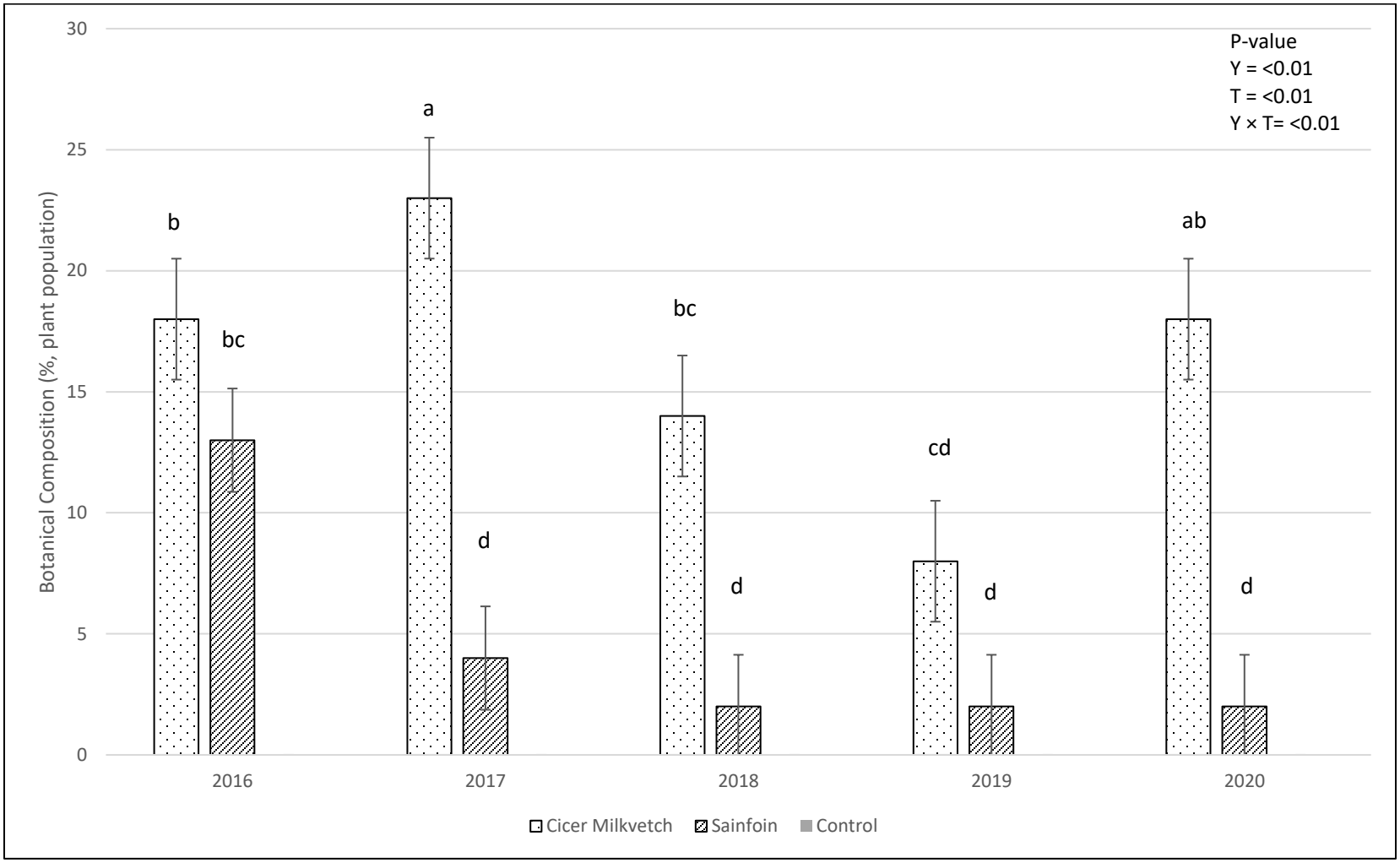

Figure 6.3. Effect of sod-seeding bloat-free legumes on bloat-free legume composition in pasture at Lanigan, SK, Canada over 5 yr.

Bloat-free legume proportions associated with different letters are significantly different (treatment × year; $P < 0.01$). Y, year; T, treatment.

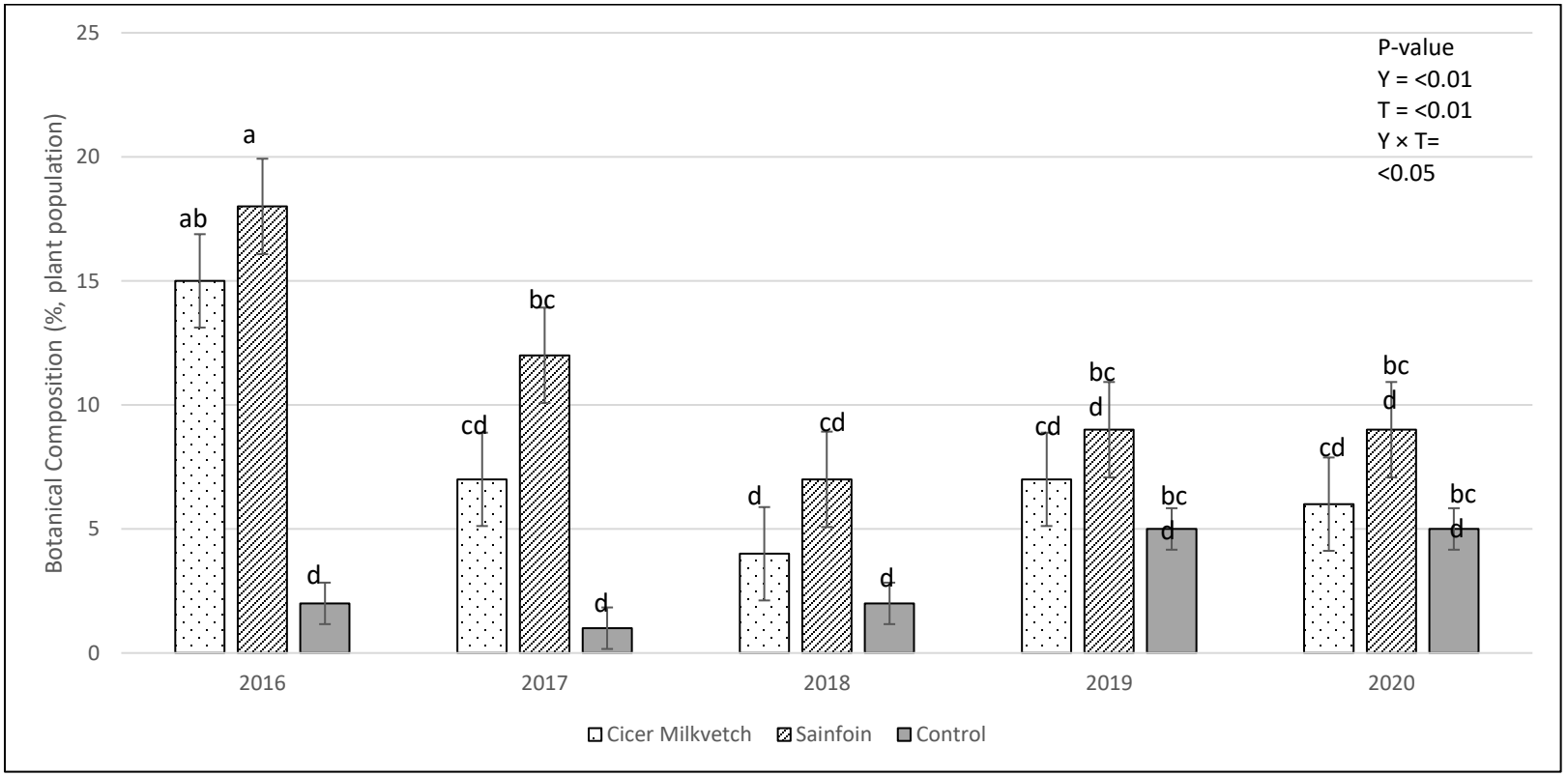

Figure 6.4. Effect of sod-seeding bloat-free legumes on other species composition in pasture at Lanigan, SK, Canada over 5 yr.

Other proportions associated with different letters are significantly different (treatment × year; $P < 0.05$). Y, year; T, treatment.

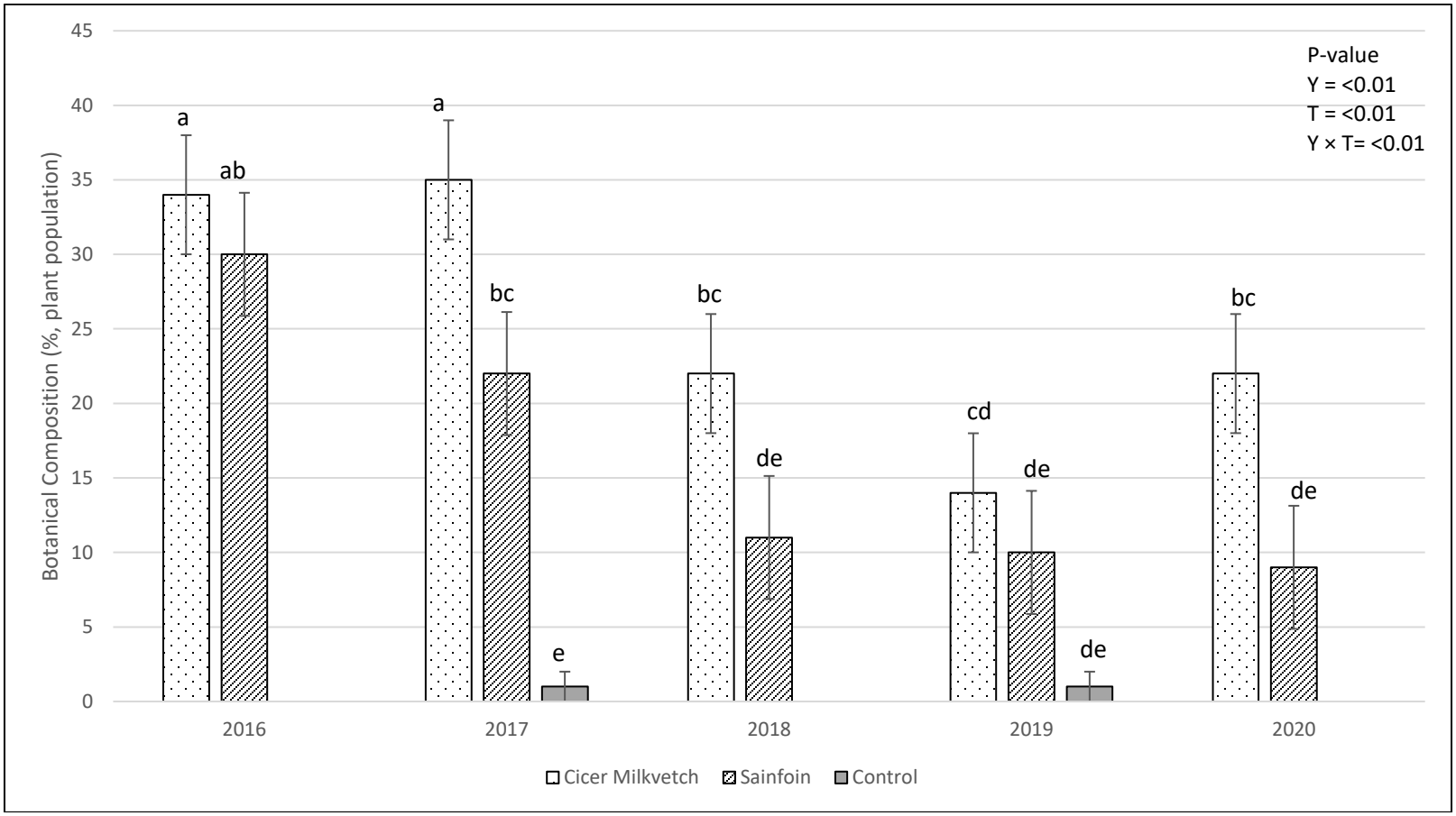

Figure 6.5. Effect of sod-seeding bloat-free legumes on total legume (alfalfa + bloat-free legume) composition in pasture at Lanigan, SK, Canada over 5 yr.

Total legume proportions associated with different letters are significantly different (treatment × year; $P < 0.01$). Y, year; T, treatment.

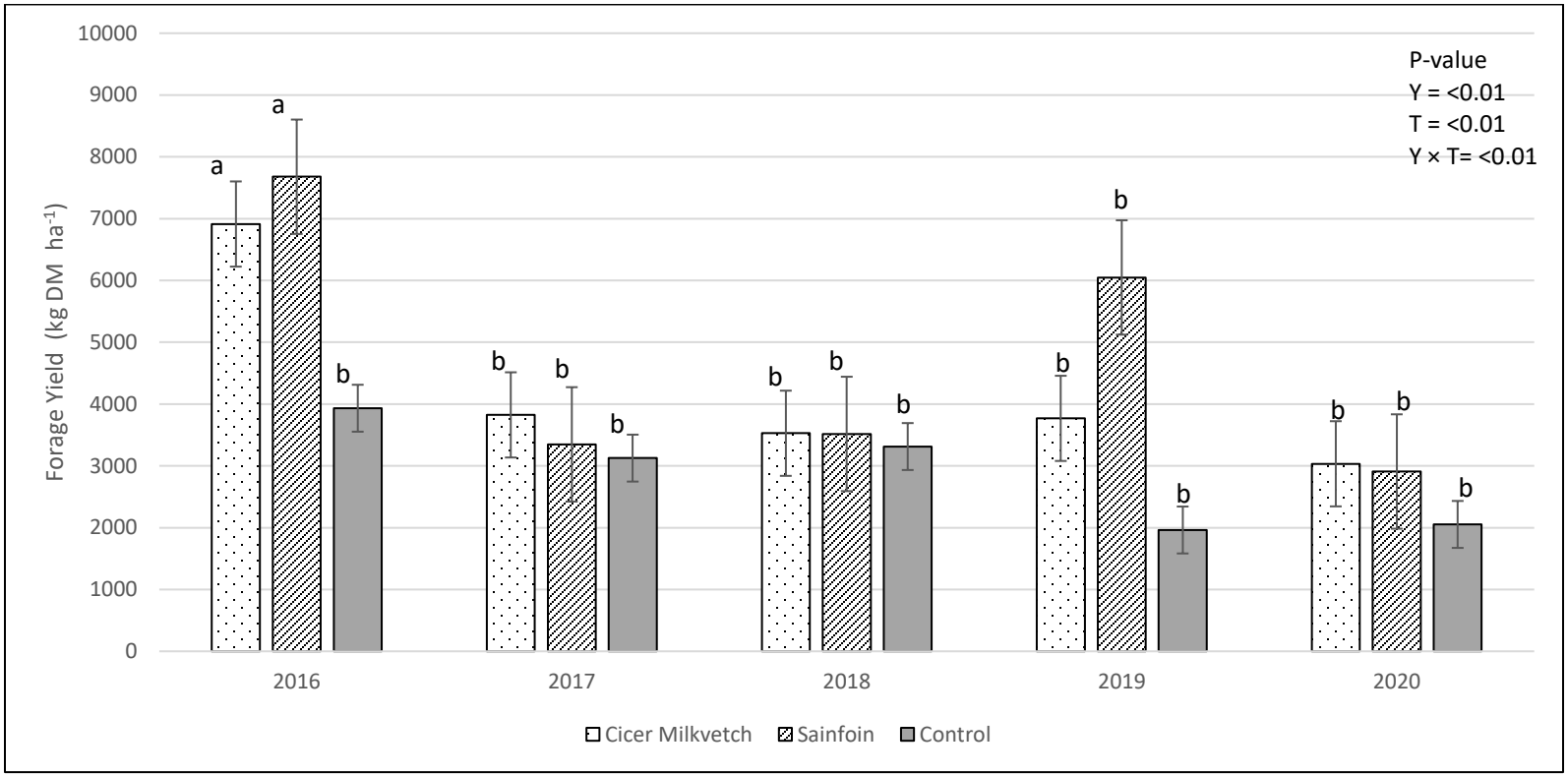

Figure 6.6. Effect of sod-seeding bloat-free legumes on start of trial forage yield in pasture at Lanigan, SK, Canada over 5 yr.

Forage yield associated with different letters are significantly different (treatment × year; $P < 0.05$). Y, year; T, treatment.

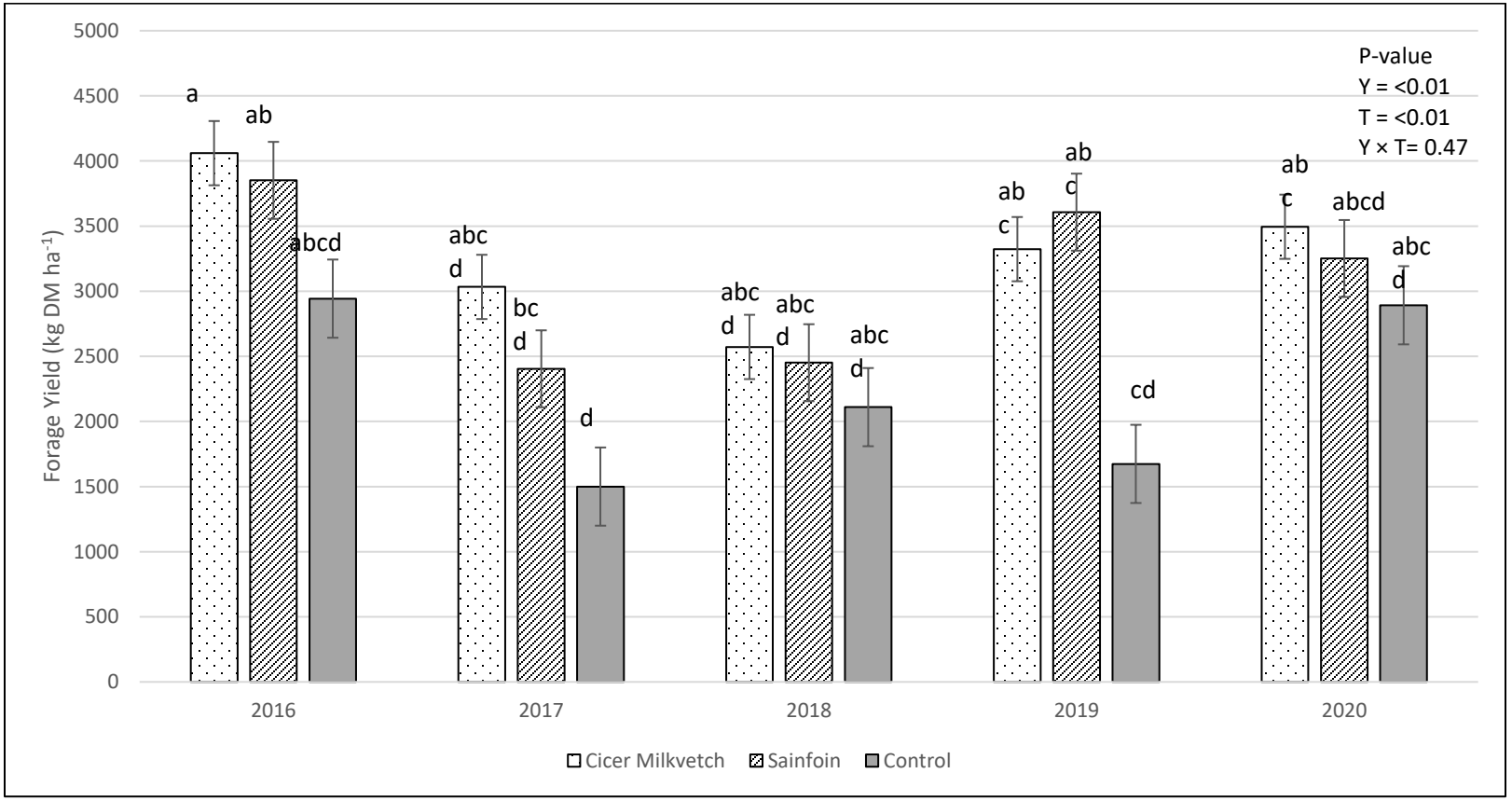

Figure 6.7. Effect of sod-seeding bloat-free legumes on end of trial forage yield in pasture at Lanigan, SK, Canada over 5 yr.

Forage yield associated with different letters are significantly different (treatment × year; $P < 0.05$). Y, year; T, treatment.

At Lethbridge, the proportion of grass (% DM) varied from year-to-year, as it increased from yr 1 to yr 2 and then decreased from yr 2 to yr 3 in the CONT treatment, with no grass present in CMV and SAIN treatments (Table 6.2; treatment × year, $P < 0.01$). The proportion of alfalfa increased from yr 1 to yr 3 in CMV and SAIN, and remained stable in CONT (treatment × year, $P < 0.05$). The proportion of bloat-free legume remained constant in CMV and declined in SAIN; however, notably SAIN had greater bloat-free plant population than CMV in 2016 and 2017 (treatment × year, $P < 0.01$). Other species were present in CMV; however, they declined from yr 1 to 2 and remained stable thereafter, and no other species were present in SAIN or CONT (treatment × year, $P < 0.01$). The proportion of total legume remained unchanged in SAIN and CONT treatments but increased in CMV over 3 yr (treatment × year, $P < 0.01$). At Lethbridge, total available forage at the start of experiment was increased in 2017 and 2018 (treatment × year, $P < 0.05$) in SAIN compared to CONT, and no difference was observed between CMV and CONT; however, average of available forage yield over 3 yr was 4043, 4263, and 2845 kg DM ha^{-1}, for CMV, SAIN and CONT treatments, respectively. End of trial available forage was decreased from yr 2 to yr 3 in both CMV and SAIN, but no decrease was observed in CONT (treatment × year, $P < 0.01$) At Lanigan, the CP concentration was greater (Table 6.3; treatment, $P < 0.01$) in CMV and SAIN, compared to CONT treatments, during yr 1 and yr 2; however, CP concentration decreased from yr 1 to yr 5 with no difference observed between treatments at the end of trial. In the CMV, SAIN and CONT treatments, NEm increased from yr 1 to yr 2 and then decreased year over year (treatment × year, $P < 0.01$). The concentration of NDF increased over 5 yr in CMV and SAIN treatments to concentrations that were similar to CONT, which remained unchanged (treatment × year, $P < 0.01$). The concentration of ADF increased in CMV and SAIN treatments over 5 yr, while CONT remained unchanged (treatment × year, $P < 0.01$). The concentration of Ca was greater in CMV and SAIN (treatment, $P <0.01$) compared to CONT. Concentration of P decreased in CMV treatments from yr 4 to yr 5 and remained unchanged in SAIN and CONT (treatment × year, $P < 0.01$).

At Lethbridge, CP concentration was unchanged in CMV, but decreased from yr 1 to yr 2 and remained stable thereafter in SAIN (Table 6.4; treatment × year $P < 0.01$). Concentration of CP was lowest in CONT compared to CMV and SAIN treatments; however, CP increased from yr 2 to yr 3 (treatment × year $P < 0.01$). Concentration of NDF was least in CMV and SAIN

compared to CONT (treatment $P < 0.01$). The concentration of ADF increased from yr 2 to yr 3 in all treatments, with CMV and SAIN having lower ADF than CONT (treatment × year $P < 0.01$). The concentration of Ca and P was greater in CMV and SAIN treatments, compared to CONT (treatment $P < 0.01$).

6.5.2 Estimated Dry Matter Intake and Steer Performance

At Lanigan, difference ($P < 0.01$) in DMI of steers was observed in year 5 when reported as kg d^{-1} and as a % BW with steers grazing CMV and SAIN treatments having greater DMI compared to CONT steers. (Table 6.5; treatment × year, $P < 0.01$). There was no difference (treatment, $P > 0.05$) among treatments for steer BW measured at the start of experiment; however, end of study BW increased over 5 yr (treatment × year $P < 0.01$). Steer average daily gain was increased when grazing CMV and SAIN treatments, compared to CONT (treatment $P < 0.01$). At Lethbridge (Table 6.6), there was no difference among treatments in steer BW at start or end of the experiment or average daily gain and individual DMI was not estimated.

Table 6.2. Effect of sod-seeding non-bloat legumes on botanical composition and forage yield at Lethbridge, AB, Canada over 3 yr

	Cicer Milkvetch			Sainfoin			Control				*P-value*		
	2016	*2017*	*2018*	*2016*	*2017*	*2018*	*2016*	*2017*	*2018*	SEM	*Y*	*T*	*Y x T*
	Botanical Composition (% DM)												
Grass	0c	0c	0c	0c	0c	0c	64b	80a	71b	1.2	<0.01	<0.01	<0.01
Alfalfa	47cde	69abc	80ab	54cd	66bc	83a	36de	20e	29de	4.9	<0.01	<0.01	0.02
Non-bloat legume	10c	17c	7c	46a	34b	17c	0c	0c	0c	4.1	<0.01	<0.01	<0.01
Other	46a	14b	13b	0b	0b	0b	0b	0b	0b	6.5	<0.01	<0.01	<0.01
Total legume	57b	86a	87a	100a	100a	100a	36bc	20c	29c	4.5	0.08	<0.01	<0.01
	Forage Yield[a] (kg DM ha^{-1})												
Trial Start	2811c	4418abc	4360abc	3073c	4603ab	4895a	3825abc	2586bc	2290c	286.1	0.12	0.03	0.02
Trial End	-	3345ab	2761c	-	3978a	3423bc	-	2719abc	1968bc	244.2	0.28	0.02	<0.01

Note: Means in the same row with different letters differ at the $P < 0.05$. Y, year; T, treatment.
[a]No forage yield measured in 2016 at end of trial.

Table 6.3. Effect of pasture type on start of trial forage quality grazed by steers at Lanigan, SK, Canada over 5 yr

	Cicer Milkvetch					Sainfoin					Control					*P-value*			
	2016	*2017*	*2018*	*2019*	*2020*	*2016*	*2017*	*2018*	*2019*	*2020*	*2016*	*2017*	*2018*	*2019*	*2020*	SEM	*Y*	*T*	*Y x T*
CP	15.0abc	16.4a	13.1cd	14.6abcd	12.9cd	16.2ab	14.9abc	12.8cd	13.9abcd	12.6cd	12.7cd	12.9cd	11.2d	11.9cd	12.6cd	0.64	<0.01	<0.01	0.17
NEm[a]	1.20cd	1.31a	1.26abc	1.23bcd	1.07f	1.18de	1.31ab	1.27abc	1.22cd	1.10ef	1.20cd	1.31ab	1.17def	1.18cde	1.16def	0.018	<0.01	0.63	<0.01
NDF	56.4abcd	51.9d	56.9abcd	57.9abcd	60.4a	53.5bcd	52.9cd	58.5abc	58.9ab	60.6a	63.6a	60.4ab	61.5a	60.0abc	56.3abcd	1.39	<0.01	<0.01	<0.01
ADF	35.4cd	30.8f	35.7c	34.5de	40.1a	33.8def	31.5ef	36.7bcd	35.1d	39.4ab	38.4abc	34.6de	37.3abcd	36.1abcd	36.4abcd	0.71	<0.01	0.09	<0.01
Ca	0.85a	0.75abcd	0.59abcd	0.53bcd	0.55abcd	0.83ab	0.77abc	0.57abcd	0.45d	0.52cd	0.49abcd	0.52abcd	0.42cd	0.40cd	0.47bcd	0.071	<0.01	<0.01	0.60
P	0.31ab	0.28abc	0.28abc	0.27abc	0.24c	0.32a	0.29abc	0.26bc	0.29abc	0.27abc	0.28abc	0.29abc	0.30abc	0.30abc	0.30abc	0.013	0.03	0.40	<0.01

Note: Means in the same row with different letters differ at the $P < 0.05$. Y, year; T, treatment; CP, crude protein (%, DM); NEm, forage NEm (Mcal kg^{-1}); NDF, neutral detergent fiber (%, DM); ADF, acid detergent fiber (%, DM); Ca, calcium (%, DM); P, phosphorus (%, DM).

[a]The NEm was calculated as (Mcal kg^{-1}) = $1.37 \times ME - 0.138 \times ME^2 + 0.0105 \times ME^3$-1.12.

Table 6.4. Effect of pasture type on start of trial forage quality grazed by steers at Lethbridge, AB, Canada over 3 yr

	Cicer Milkvetch			Sainfoin			Control			*P-value*			
	2016	*2017*	*2018*	*2016*	*2017*	*2018*	*2016*[a]	*2017*	*2018*	SEM	*Y*	*T*	*Y x T*
CP	19.8ba	22.3a	21.9ab	19.5c	22.7a	22.4a	-	13.9d	18.9c	0.46	<0.01	<0.01	<0.01
NDF	34.9bcd	38.3bc	35.1d	35.1bcd	38.1b	36.0cd	-	54.7a	51.8a	0.82	<0.01	<0.01	0.55
ADF	26.6cd	27.6c	29.2bd	26.8cd	27.5cd	30.7ab	-	29.2bc	33.2a	0.56	<0.01	0.02	<0.01
Ca	1.16cd	1.26c	2.39a	1.13cd	1.27c	2.35a	-	0.80d	1.81b	0.057	<0.01	<0.01	0.72
P	0.20cd	0.24c	0.30ab	0.20cd	0.24c	0.31a	-	0.18d	0.25bc	0.011	<0.01	<0.01	0.72

Note: Means in the same row with different letters differ at the $P < 0.05$. Y, year; T, treatment; CP, crude protein (%, DM); NDF, neutral detergent fiber (%, DM); ADF, acid detergent fiber (%, DM); Ca, calcium (%, DM); P, phosphorus (%, DM).
[a]forage quality was not analyzed in Control treatments in 2016

Table 6.5. Effect of pasture type on steer performance at Lanigan, SK, Canada over 5 yr (n=6)

	Cicer Milkvetch					Sainfoin					Control						*P-value*		
	2016	*2017*	*2018*	*2019*	*2020*	*2016*	*2017*	*2018*	*2019*	*2020*	*2016*	*2017*	*2018*	*2019*	*2020*	SEM	*Y*	*T*	*Y x T*
DMI[a]	7.1f	7.5ef	12.4b	10.2cd	16.0a	7.4ef	7.5ef	12.2bc	11.4bc	14.9a	6.2f	7.8def	11.9bc	9.8bcde	11.8bc	0.50	<0.01	<0.01	<0.01
DMIbw[b]	2.2g	2.6fg	4.1abc	3.3de	4.7a	2.3g	2.6fg	4.0bc	3.6cd	4.4ab	2.0g	2.7efg	3.9abcd	3.2def	3.5cde	0.15	<0.01	<0.01	<0.01
BWs	321ca	313e	300f	328b	339a	321cd	313e	301f	329b	338a	323bcd	315de	306f	327bc	338a	1.3	<0.01	0.38	0.25
BWe	383ca	327g	373de	363ef	414a	386c	326g	372def	370ef	410ab	363ef	330g	364ef	357f	394bc	3.0	<0.01	<0.01	<0.01
ADG	0.4h	0.7g	1.5a	1.0def	1.3abc	0.4h	0.7fg	1.4ab	1.1bcde	1.3abcd	0.2h	0.8efg	1.2abcde	0.8efg	1.0cdefg	0.07	<0.01	<0.01	0.12

Note: Means in the same row with different letters differ at the $P < 0.05$. Y, year; T, treatment; DMI = dry matter intake, (kg d^{-1}); BWs, start of test body weight (kg); BWe, end of test body weight (kg); ADG, average daily gain (kg d^{-1}).

[a]DMI is calculated as NEm intake (Mcal d^{-1}) / dietary NEm concentration (Mcal kg^{-1} DM).

[b]DMIbw = dry matter intake, % body $weight^{0.75}$.

Table 6.6. Effect of pasture type on steer performance over 3 yr at Lethbridge, AB (n=4)

	Cicer Milkvetch			Sainfoin			Control				*P-value*		
	2016	*2017*	*2018*	*2016*	*2017*	*2018*	*2016*	*2017*	*2018*	SEM	*Y*	*T*	*Y x T*
BWs	386bc	427a	442a	379c	433a	443a	378abc	410abc	449ab	14.1	<0.01	0.88	0.80
BWe	382c	419b	444a	376c	424ab	446a	384abc	404abc	456ab	11.9	<0.01	0.99	0.63
ADG	-0.2abc	-0.8bc	0.1ab	-0.2abc	-0.8c	0.1a	0.3abc	-0.7abc	0.3abc	0.4	<0.01	0.68	0.99

Note: Means in the same row with different letters differ at the $P < 0.05$. Y, year; T, treatment BWs, start of test body weight (kg); BWe, end of test body weight (kg); ADG, average daily gain (kg d^{-1}).

Table 6.7. Soil nutrients of sod-seeded pasture at Lanigan, SK, Canada over 5 yr (n=6)

	Cicer Milkvetch		Sainfoin		Control			*P*-value		
	2016	*2020*	*2016*	*2020*	*2016*	*2020*	SEM	*Y*	*T*	*Y x T*
NO_3-N	14.0	15.7	21.3	12.7	10.0	20.0	3.29	0.71	0.72	0.04
P	103.5a	34.7b	115.5a	45.7b	74.0ab	42.0b	11.12	<0.01	0.34	0.04
K	1309.7a	477.5b	1345.0a	521.8b	1345.0a	549.0b	26.32	<0.01	0.11	0.81

Note: Means in the same row with different letters differ at the $P < 0.05$. Y, year; T, treatment; NO_3-N, nitrate nitrogen; P, phosphate-P; K, potassium.

Table 6.8. Soil nutrients of sod-seeded pasture at Lethbridge, AB, Canada over 3 yr (n=4)

	Cicer Milkvetch			Sainfoin			Control				*P*-value		
	2016	*2017*	*2018*	*2016*	*2017*	*2018*	*2016*	*2017*	*2018*	SEM	*Y*	*T*	*Y x T*
NO_3-N	18.4	21.1	23.6	21.1	27.2	31.9	14.3	14.1	12.1	3.84	0.31	<0.01	0.62

Note: Means in the same row with different letters differ at the $P < 0.05$. Y, year; T, treatment; NO_3-N, nitrate nitrogen.

6.5.3 Soil Nutrients

At Lanigan, no difference in inorganic soil N, P, or K concentrations were observed among treatments; however, N and P levels were dependent upon year (Table 6.7). At Lethbridge, an increase (P <0.01) in inorganic soil N was observed in SAIN when compared to control (Table 6.8).

6.5.4 Economic Analysis

At Lanigan, total sod-seeding costs were \$339 ha^{-1} for CMV and \$324 ha^{-1} for SAIN (Table 6.9). Estimated forage value was increased (Table 6.10; P <0.05) by the sod-seed treatment in 3 out of 5 yr. After five years of grazing, the rejuvenated paddocks generated greater (P < 0.01) total gross returns (\$1154.87 ha^{-1} CMV, \$1095.98 ha^{-1} SAIN) compared to CONT (\$806.44 ha^{-1}). However, once annual gross returns were discounted (5% per year) to a present value basis and establishment costs were accounted for there was no difference in present value of net returns. The present value of net returns after 5 yr was \$625.83 ha^{-1} for SAIN, \$657.41 ha^{-1} for CMV and \$696.52 ha^{-1} for CONT.

Table 6.9. Comparison of establishment costs from pasture rejuvenation of a grass pasture with cicer milk vetch (CMV) and sainfoin (SAIN) at Lanigan (SK, Canada).

	CMV	SAIN
	$ ha^{-1}	
Pre-seeding glyphosate[a]	7.90	7.90
Spraying	29.64	29.64
Seeding equipment	90.16	90.16
Land rolling	14.10	14.10
Seed	197.29	182.16
Total establishment costs	339.09	323.96

[a]glyphosate applied at 1.24L ha^{-1}; SAIN seeded at 26 kg ha^{-1}; CMV seeded at 17 kg ha^{-1}; Agro-Plow ($38.29 ha^{-1}) and tractor ($51.87 ha^{-1}) rented for sod-seeding

Table 6.10. Estimated returns, costs and net returns of sod-seeded cicer milkvetch (CMV) and sainfoin (SAIN) pasture versus no sod-seeding (CONT) over 5-yr

	CMV	SAIN	CONT	SEM	*P*-value
Estimated forage value[a]	$ ha^{-1}				
2016	228.36*a*	253.36*a*	129.81*b*	18.096	<0.01
2017	176.71*a*	154.67*ab*	144.43*b*	5.410	0.05
2018	256.12	255.37	240.54	20.227	0.49
2019	273.63*a*	221.36*a*	142.55*b*	15.427	<0.01
2020	220.29*a*	211.22*a*	149.11*a*	30.734	0.31
5 yr total gross returns	1154.87*a*	1095.98*a*	806.44*b*	33.623	<0.01
5 yr present value of net returns[b]	657.41	625.83	696.52	35.226	0.43

Note: Means in the same row with different letters differ at the $P < 0.05$.
[a]estimated value was calculated as DM yield × annual published price of standing hay (SK Forage Council, https://www.saskforage.ca/resources).
[b]present value of net returns was calculated as the summation of discounted (5%) annual returns subtract sod-seeding costs.

6.6 Discussion

6.6.1 Botanical Composition, Forage Yield, Forage Quality and Soil Nutrient Profile

At both sites, the sainfoin proportion decreased in the stand. Others have stated that maintaining sainfoin is challenging due to its inability to compete with other plants (Sheppard et al. 2018). The results from Lethbridge are similar to Acharya et al. (2013), who found that varieties of sainfoin developed for grazing may only persist in the stand for 3 production years. However, Acharya et al. (2013) estimated that on the third production year, sainfoin could still be expected to contribute to 20% of the stand DM depending on the variety, which is evident at Lethbridge wherein sainfoin maintained close to 20% of stand after 3 yr.

Despite having a deep tap root, it is well known that sainfoin does not perform well in low moisture environments and populations will decline quickly during drought conditions (Bhattarai et al. 2016; Sheppard et al. 2018). Even though best management practices were followed, such as proper seeding rate, grazing removal, pre-existing plant species control, and allowing for proper seed set prior to grazing, given the limited drought tolerance, it is likely that unfavorable environmental conditions, combined with the sod-seeding technique and pre-existing plant competition in the current experiment did not allow for sainfoin to persist in the stand at Lanigan.

Research conducted with alfalfa and sainfoin, showed varietal differences affected persistence of sainfoin in the stand over 3 yr, with the Nova variety decreasing from 55 to 25% of the stand DM after three cuttings in the first production year, and providing less than 10% of the DM yield in the second production year (Sottie et al. 2014). In comparison, LRC-3519 only decreased from 55 to 45% of the stand DM yield after three cuttings in the first production year, and after three years of production maintained 28% of the stand DM yield (Sottie et al. 2014). Khatiwada et al. (2021) reported that pre-existing plant stand species play a role in pasture suitability for sod-seeding and that sainfoin may be better suited to sod-seeding into pre-existing alfalfa stands vs. pre-existing grass stands. Therefore, the pre-existing alfalfa species at Lethbridge may have allowed sainfoin to persist over time; whereas the pre-existing meadow bromegrass stands at Lanigan may not have been compatible for sainfoin populations, thus impacting the establishment and persistence ability of sainfoin over time under grazing management.

At Lanigan, cicer milkvetch remained at the same proportion (% of the plant population) in the stand over 5 yr. This is not unexpected as cicer milkvetch can be a long lived and more winter hardy species than alfalfa with some Canadian stands lasting greater than 30 yr (Acharya 2006). The CMV paddocks at Lethbridge decreased in cicer milkvetch population over the 3 yr grazing experiment. However, the level of establishment of cicer milkvetch in paddocks at Lethbridge was low. Cicer milkvetch can be hard to establish (Acharya et al. 2006), and Lethbridge had heavy weed pressure in these paddocks which may have affected the ability of the cicer milkvetch to establish, exhibited by the low population of cicer milkvetch at this site in yr 1 of the study. However, pre-existing plant species could impact cicer milkvetch establishment as Khatiwada et al. (2021), reported that sod-seeding of cicer milkvetch into pre-existing alfalfa resulted in poor establishment within 3 yr, and suggested that better performance of cicer milkvetch could result from varietal selection of cicer milkvetch suited to grow with alfalfa. Khatiwada et al. (2021) also reported differences in the ability of cicer milkvetch to establish in grass pasture, due to the competitive nature of the pre-existing grass species, with greater establishment success observed in pre-existing pasture containing a caespitose grass species compared to a rhizomatous grass species. Due to the short rhizomes of meadow bromegrass, this may have contributed to the success for establishment of cicer milkvetch at Lanigan. These results; however, are contradictory to Omokanye et al. (2018) who reported that when sod-seeding smooth bromegrass, alfalfa, and cicer milkvetch into pre-existing meadow bromegrass and alfalfa pasture, the cicer milkvetch failed to establish at multiple site locations. However, this result could have been confounded by the rhizomatous nature of species seeded, such as smooth bromegrass. Given the contradicting results between site locations in the current experiment and the mixed results in the literature, further research is required to determine the effect of different agronomic practices, soil zones, pre-existing plant species populations, and environmental conditions on establishment and persistence of sod-seeded bloat-free species.

At Lanigan, available forage (kg DM ha^{-1}) was dependent on year with an increase of 85% observed only for yr 1 in CMV and SAIN treatments when compared to CONT. This increase in forage yield was not sustained as there were no differences in forage yield among treatments thereafter. Lethbridge forage yield was also dependent on year, with SAIN treatment increasing in available forage by 60% over 3 yr and showing greatest yield in the third year of production, when compared to CONT and CMV treatments. Typically, sainfoin is known to have

lower yields than alfalfa; however, recent plant breeding efforts have shown new varieties may have increased yields over alfalfa (Acharya 2013). Although available forage at both sites was lower than levels measured in sainfoin/alfalfa pastures at Lethbridge by Sottie et al. (2014), yields at Lanigan were similar to mixed grass pastures measured at Lanigan by Anez (2015). It is possible that the mechanical aeration and soil disturbance caused by seeding could contribute to some increase in yield (Davies 1989); however, other studies have reported no increase in forage yield from mechanical aeration (Omokanye et al. 2019; Lardner 2000, Malhi et al. 2000)

Environment can cause significant year-to-year and seasonal variation in forage quality, due to alterations in aspects such as leaf/stem ratios, morphology, and chemical composition of the plant (Buxton and Fales 1994; Buxton 1996). Year to year variation may have been evident in the current study since start of trial forage quality was different between treatments and dependent on year. At both experimental sites, the inclusion of the bloat-free legumes (cicer milkvetch and sainfoin) increased the concentration of CP during the first 2 yr of trial in mixed forage when compared to CONT. At Lanigan, the CONT treatment consisted primarily of meadow bromegrass species, therefore it is not surprising that the inclusion of legumes increased the CP concentration. In addition, cicer milkvetch is known to have higher protein concentration than alfalfa, and sainfoin has been reported to have similar or lower protein level compared to alfalfa, at the same physiological stage (Parker and Moss 1980; Acharya 2006; Bhattarai 2016). These differences in CP concentrations are reflected in the higher CP concentrations in the CMV and SAIN treatments at Lethbridge, when compared to the CONT which contained 60 to 70% alfalfa and the remainder as meadow bromegrass.

Both cicer milkvetch and sainfoin are known to have lower NDF concentrations than alfalfa, making their forage highly digestible (Acharya et al. 2006; Bhattari et al. 2016). In addition, research has shown that cicer milkvetch forage maintains its nutritive value later in the growing season, which makes it superior to other legumes such as alfalfa for late season grazing (Acharya et al. 2006). The sustained reduction in NDF throughout the growing season is beneficial as other forages typically decline in production and digestibility during the fall season (Acharya 2006). At both experimental sites, the inclusion of bloat-free legumes into the pasture decreased the NDF concentration of the forage when compared to CONT; however, at Lanigan this result only lasted for the first 2 yr, likely due to change in botanical composition over 5 yr.

No change was observed among treatments for inorganic soil nutrient levels over the course of the 5 yr grazing experiment, however N and P levels were dependent upon year. There was an increase in soil N levels at Lethbridge in treatments seeded with sainfoin, compared to control and cicer milkvetch. Issah et al. (2020) compared the biological N_2 fixation rate of alfalfa, sainfoin and cicer milkvetch and found that percentage of nitrogen derived from atmosphere corresponded to 200, 128, and 65 kg ha^{-1} yr^{-1} for alfalfa, cicer milkvetch and sainfoin, respectively. Further research is required to determine the impact of environment, pre-existing plant species, and soil conditions on bloat-free legume seedling establishment, growth, nitrogen fixation, and persistence in mixed forage stands.

6.6.2 Estimated Dry Matter Intake and Steer Performance

Dry matter intake can be affected by forage availability, forage quality, plant species, and management (Minson 1990; NASEM 2016). The available forage (measured from a 5-cm height above ground) provided throughout the grazing experiment was above 2000 kg DM ha^{-1}, the minimum amount of forage available at which decreased bite size and DMI of grazing ruminants would be expected (Minson 1990). Therefore, forage availability was not thought to be a limiting factor for DMI in the current study. Dry matter intake was calculated at Lanigan with greater DMI only in year 5 for CMV and SAIN (both kg d^{-1} and % BW) than CONT. Given the method of DMI prediction, the response could possibly be attributed to the lower NEm level of forage in 2020. At Lanigan, differences in end of trial BW gain (kg d^{-1}) were dependent on year; with higher intakes attributed to increased ADG of steers.

At Lethbridge there was no effect of treatment on BW, but steers experienced negative BW gain. This could be attributed to the animal diet during non-grazing periods, due to the intermittent grazing that occurred at Lethbridge. At Lanigan, the steer ADG was similar to results reported by Sottie et al. (2017) and Popp et al. (2000) for steers grazing mixed legume pasture stands.

6.6.3 Economic Analysis

When an up-front investment is required, as is the case with sod-seeding, and varied returns accrue over multiple years a comparison on a present value basis is appropriate (Barry and Ellinger 2012). The present value of net returns for the SAIN and CMV means sod-seeding was profitable, but values did not differ from CONT.

The economic analysis of the current study suggests high risk associated with sod-seeding pastures with sainfoin and cicer milkvetch, as the resulting DM yields were not high enough to outperform the non-rejuvenated control treatment. These results are similar to Omokanye et al. (2018) who determined that spring sod-seeding a blend of smooth bromegrass, alfalfa, and cicer milkvetch species as a rejuvenation strategy was not economically feasible, with net losses per hectare, depending on site location with losses predicted at -$166 ha^{-1} and -$103 ha^{-1} over 3 yr. However, economic results are variable as a separate study by Omokanye et al. (2019) reported net profit of $380 ha^{-1} over 2 yr, when sod-seeding a meadow bromegrass, orchardgrass, timothy and alfalfa blend, whereby seed cost was $131 ha^{-1}, and total rejuvenation cost was $236 ha^{-1}, which is $100 ha^{-1} lower than the current study total sod-seeding cost. Although Omokanye et al. (2019) valued forage DM production (DM ha^{-1}), it measured costs and revenues for two years after rejuvenation. In the current study, SAIN populations were minimal after 5 yr, and thus re-seeding may be required after 5 yr for this species. The costs and revenues associated with sod-seeding pastures will depend on many factors including the cost of seed, valuation of forage, type of species, and environmental conditions. It is important to note that, present value of net returns could be increased if establishment costs could be reduced through use of a lower cost seed. Given the significant financial risk associated with rejuvenating pastures, more research is required to determine best management practices to increase the agronomic success, selection of species suited for sod-seeding, evaluation of pre-existing stands conducive to sod-seeding, and the economic benefit of sod-seeded bloat-free legumes.

6.7 Conclusions

The sod-seeded treatments had higher gross returns when compared to CONT; however, after accounting for establishment cost and adjusting to a present value basis, the present value of net returns for the sod-seeded treatments did not differ from CONT. These results suggest sod-seeding bloat-free legumes may increase productivity of unproductive pasture stands during the first year of production, but this rejuvenation strategy may not be economically feasible, as environment, sod-seed species selection, and pre-existing pasture species may create variable results over time. For successful producer adoption of a sod-seed rejuvenation strategy, longevity (>10 yr) of plant persistence and increased yield is critical to cover the initial cost of establishment. More research is required to determine varietal selection of cicer milkvetch and sainfoin species with persistence when sod-seeding, selection of pre-existing plant stands best

suited when sod-seeding bloat-free legumes, best management practices for pasture rejuvenation and grazing, and the economic feasibility of sod-seeding under different pasture conditions.

7 GENERAL DISCUSSION AND CONCLUSIONS

Pasture systems are dynamic and to understand the effect of management practices, it is critical to analyze the animal, plant, soil, and environment with systems-based research. The research provided in this thesis attempts to understand the impact of sod-seeded legumes on pasture productivity, animal performance, the environment, and system economics.

With respect to sainfoin, the CT contained in plant tissue, depending on their source, stoichiometry, and concentrations, are well known to impact ruminal protein and carbohydrate digestibility and microbial fermentation (McMahon 1999b; Stewart et al. 2019). During the batch culture incubations in Chapter 4 (Experiment 1), sainfoin inclusion linearly reduced DMD and NH_3-N and branched chain SCFA production, which could be attributed to reduced proteolysis due to protein-CT complexes which would limit the supply of branched chain amino acids available for deamination to branched chain short chain fatty acids (McMahon 1999b). A reduction in ruminal protein digestion could be viewed as beneficial as it could potentially increase ruminal by-pass protein, allowing for greater metabolizable protein and nitrogen retention, however this benefit is dependent on adequate supply of RDP (Waghorn 1998; NASEM 2016). An increase in rumen by-pass protein could also lead to environmental benefits, by shifting N excretion from the volatile urine N to the more stable fecal N, however increased ruminal by-pass protein could reduce ruminal N efficiency, and this must be taken into consideration (NASEM 2016; Mueller-Harvey 2019). For successful producer adoption of new species, increased forage production, forage quality, plant species persistence and livestock performance are critical. When sainfoin was analyzed in a large-scale *in vivo* trial as in Chapter 6 (Experiment 3), sainfoin populations decreased in the stand at both locations; however, it maintained a greater proportion at Lanigan compared to Lethbridge. Previous literature reports the role pre-existing plant populations play in the establishment and persistence of sainfoin (Khatiwada et al. 2021). Therefore, pre-existing species at Lethbridge (alfalfa) may have allowed sainfoin to persist over time, whereas the pre-existing stand at Lanigan (meadow bromegrass-alfalfa) may not have been compatible for sainfoin populations. Forage yield, forage quality, steer DMI and steer ADG were all variable throughout the 5 yr trial, with increased forage production only observed the first year after seeding. This suggests that at best, sod-seeding bloat-free legumes may only transiently increased forage productivity of unproductive pasture

stands, and this rejuvenation strategy may not give rise to long term (>10 yr) results. Enteric methane emissions were not impacted with inclusion of sainfoin during the batch culture experiment (Chapter 4) or the grazing experiment (Chapter 5). As sainfoin has been reported to impact end-products of rumen fermentation, it can reduce enteric methane emissions; however, results of sainfoin on methane emissions are variable (McMahon et al. 1999a; Stewart et al. 2019; Williams et al. 2011).

With respect to cicer milkvetch, during the batch culture incubations outlined in Chapter 4, increased inclusion of late flower cicer milkvetch relative to alfalfa did not impact DMD, NH_3-N or CH_4 production. However, vegetative cicer milkvetch inclusion resulted in greater DMD and lower CH_4 production compared to incubations of alfalfa. Similar results were observed in the subsequent RUSITEC experiment, in which, a linear increase in DMD, fiber (ADF and NDF) disappearance, and microbial protein synthesis and linear decrease in A:P ratio and NH_3-N production were observed with increasing cicer milkvetch inclusion. Chapter 5 results show that although total SCFA concentration was not impacted by treatment, A:P ratio was lowered by the inclusion of cicer milkvetch when dietary inclusion of cicer milkvetch was 19% as fed, displacing grass / alfalfa in the diet. No differences in treatments were reported with respect to CH_4 emission, when measured by the SF_6 technique. Although the SF_6 tracer technique can be more variable than whole-animal chamber measurements, this technique allows for collection of data from large numbers of grazing animals in their natural environment (Ulyatt et al 1999; Pinares-Patino et al. 2011). In chapter 5, cattle grazing cicer milkvetch paddocks experienced higher BUN levels and ruminal NH_3-N concentrations indicating asynchronous ruminal supply of nitrogen and energy which could increase urinary nitrogen excretion. Although cows grazing cicer milkvetch paddocks experienced reduced urine N, and increased fecal N concentration, these results were from spot urine and fecal samples which didn't account for volume or total production. Therefore, since CP concentration in cicer milkvetch forage was greater than control forage, and BUN and ruminal NH_3-N concentrations were elevated in cows grazing cicer milkvetch, it is likely that these cows experienced increased urinary nitrogen excretion (Hammond et al. 1983; Dijkstra et al. 2013). More research is required to fully understand the effect of non-tannin containing legumes, such as cicer milkvetch on ruminal protein digestion, nitrogen metabolism, and nitrogen excretion.

As stated previously, for successful producer adoption, rejuvenation strategies must be economically feasible, persistent, and long lasting. The sod-seeded treatments in Chapter 6 had higher gross returns when compared to the non-sod seeded paddocks. However, after accounting for establishment cost and adjusting to a present value basis, the present value of net returns for the sod-seeded treatments did not differ from control over the 5 yr experimental period. These results suggest sod-seeding bloat-free legumes may transiently increase productivity of unproductive pasture stands, but this rejuvenation strategy may be risky and may not result in medium to long term results. More research is required to determine varietal selection of cicer milkvetch and sainfoin species with persistence when introduced into existing pastures. In addition, research into the compatibility of species in relation to their ability to establish and persist together under grazing management will be crucial in understanding pasture rejuvenation.

The implications of these results benefit western Canadian beef and forage producers by providing research regarding sod-seeding of sainfoin and cicer milkvetch species into existing mixed pastures. The results of this research outline possible benefits of bloat-free legumes on animal performance, ruminal fermentation parameters, microbial protein synthesis, particularly with dietary inclusion of cicer milkvetch. However, this research also highlights potential risks to sod-seeding, such as increased undesirable species in sod-seeded pastures, variable persistence of sod-seeded species, transient forage and livestock performance in sod-seeded pastures and reduced economic feasibility of sod-seeding cicer milkvetch and sainfoin for pasture rejuvenation. In addition, this research provides valuable insight into the relationship between cicer milkvetch and sainfoin species and existing pasture stands over time (5 yr), which could play a critical role in the successful adoption of pasture rejuvenation strategies for western Canada. Finally, the results of this study show potential for new research, such as a need for better understanding the impact of non-tanniferous, bloat-free legumes (cicer milkvetch) on ruminal protein digestion and nitrogen metabolism, and the critical need for increased knowledge regarding the relationship between pre-existing plant species and newly introduced species in mixed pastures.

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