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Influence of Forage Diversity and Condensed Tannins on Livestock Foraging Behavior, Production and Environmental Impact

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INFLUENCE OF FORAGE DIVERSITY AND CONDENSED TANNINS ON

LIVESTOCK FORAGING BEHAVIOR, PRODUCTION AND

ENVIRONMENTAL IMPACTS

by

Sebastian P. Lagrange

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ABSTRACT

Influence of Forage Diversity and Condensed Tannins on Livestock Foraging Behavior,

Production and Environmental Impact

by

Sebastian P. Lagrange, Doctor of Philosophy

Utah State University, 2020

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I hypothesized that forage diversity, providing different types and concentrations

of nutrients and secondary compounds like condensed tannins (CT), benefit ruminant

production systems. Thus, I explored whether consuming increasingly diverse

combinations of tanniferous (*Lotus corniculatus*, birdsfoot trefoil; *Onobrychis viciifolia*,

sainfoin) and non-tanniferous legumes (*Medicago sativa,* alfalfa) improve animal

performance and reduce nitrogen (N) and methane (CH_4) emissions relative to forage

combinations of lower diversity. In Chapter 2, I found that offering choices among these

legumes to penned sheep enhanced intake and diet digestibility relative to feeding single

species. Sainfoin promoted lower blood urea nitrogen (BUN) concentrations and shifted

the site of N excretion from urine to feces. In Chapter 3, substrates from mixtures

selected by lambs in Chapter 2 (70:30 alfalfa:sainfoin or alfalfa:birdsfoot trefoil and

50:35:15 alfalfa:sainfoin:birdsfoot trefoil ratios, respectively) were incubated with

ruminal fluid and a buffer medium using the *in vitro* gas production technique and

exhibited greater gas production rates than equal parts mixtures (i.e., indifferent selection). In Chapter 4, I found that heifers grazing tanniferous legumes had lower concentrations of BUN, urinary N and greater fecal N concentrations than animals grazing alfalfa. In addition, 2-way choices between tanniferous legumes led to the greatest decline in urinary N concentration and heifers grazing the 3-way choice partitioned 20% less N into urine and retained 43% more N than the average of heifers grazing monocultures. This suggests that different types of tannins result in associative effects that enhance N economy in grazing ruminants and reduce N excretion. Enteric CH_4 emissions were not affected by treatment, but heifers in the 3-way choice showed the greatest body weight gains, which may imply reductions in the number of days to slaughter and reduced CH_4 emissions during the animal's lifetime. Finally, Chapter 5 showed that heifers grazing strips of legumes preferred sainfoin over birdsfoot trefoil or alfalfa, and birdsfoot trefoil over alfalfa. Heifers on choice treatments showed levels of hair cortisol, number of daily steps, and proportions of grazing events and standing time that were similar to heifers grazing monocultures. Collectively, my results suggest that offering choices of tanniferous legumes and alfalfa has the potential to increase animal productivity while reducing environmental impacts without affecting grazing efficiency or stress levels relative to legume monocultures, all benefits that lead to more sustainable pasture-based finishing systems.

(282 pages)

PUBLIC ABSTRACT

Influence of Forage Diversity and Condensed Tannins on Livestock Foraging Behavior,

Production and Environmental Impact

Sebastian P. Lagrange

Eating a combination of forages with different chemistries (i.e., nutrients, beneficial compounds such as tannins) may enhance ruminant nutrition and reduce environmental impacts relative to eating single forages. I explored the influence of offering sheep and cattle all possible combinations of tanniferous (i.e., plants with tannins; birdsfoot trefoil, sainfoin) and non-tanniferous legumes (i.e., plants without tannins; alfalfa) or their monocultures on animal performance, behavior, and methane and nitrogen (N) emissions. Offering choices among these legumes to penned sheep improved intake and diet digestibility relative to feeding monocultures. Mixtures selected by sheep were better digested than mixtures containing equal parts of the forages (indifferent selection), and similar to the legume of greatest digestion rate (alfalfa). In both sheep and cattle, tanniferous forages shifted the site of N excretion from urine to feces, which reduces environmental impacts, as fecal N is in the form of organic N and is metabolized at a slower rate than N in urine. Heifers grazing choices between tanniferous legumes showed the greatest decline in urinary N concentration, suggesting compounded effects that enhance N economy in grazing ruminants and reduce urinary N excretion to the environment. Enteric methane emissions were not affected by treatment, but heifers offered choices among all three legumes showed the greatest body weight gains, implying reductions in the number of days to slaughter, which reduces methane

emissions during the finishing process. Grazing behavior and stress levels in heifers offered choices among strips of the three legumes were similar to animals grazing monocultures. Thus, my results suggest that grazing forage combinations increased animal productivity and reduced environmental impacts without affecting behavior or stress levels relative to grazing single forages, all benefits that lead to more sustainable pasture-based finishing systems.

For my family

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Sebastian P. Lagrange

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CHAPTER 1

LITERATURE REVIEW

Conventional Finishing Systems in U.S:
Description and Environmental Impacts

Currently in the United States, conventional beef finishing systems are based on

high concentrate diets being fed to calves in feedlots during a period of three to six

months until calves are finished and slaughtered (USDA, 2019). Cattle in conventional

feedlots systems usually receive a balanced diet that include corn grain, grain byproducts,

oilseed meals, small fractions of roughages (alfalfa hay or corn silage) and

vitamin/mineral supplements (Drouillard, 2018). Conventional systems also include the

use of steroid implants, ionophores, and beta-adrenergic agonists which allow animals to

enhance growth and reduce time to slaughter (Capper, 2012); reaching live weight gains

that average 1.7-2.0 kg/d and feed conversion rates between 5.5-6.5 kg/kg (Xu et al.,

2014; MacAdam and Villalba, 2015; Ebert et al., 2017; Koenig et al., 2018). However,

grains might become a limited resource for feeding cattle in the near future as they might

compete as a food source for humans in a world with increasing population, or become

less profitable for the conventional beef industry if grains prices increase due to its close

dependence with oil energy sources (Holechek, 2009). In this context, grains might be

used for other meat industries as pork and poultry with higher conversions efficiencies

into meat than cattle. In addition, there are increasing consumer's concerns about the use

of hormones and sub-therapeutic doses of antibiotics fed to cattle in feedlots which might

generate bacterial resistance and affect human health (Provenza et al., 2019).

On the other hand, the presence of concentrated animal feeding operations might

represent a source of environmental pollution for the surrounding areas. According to Burkholder et al. (2007), the increasing number of feedlots in U.S presents a greater risk to water quality due to the increased volume of waste and manure management practices which do not protect adequately water resources from contamination. Feedlot's manure contains a variety of potential contaminants, such as high concentrations of nitrogen (N) and phosphorus (P), pathogens such as E. coli, growth hormones, antibiotics, chemicals used as additives, animal blood, copper sulfate, and pesticides (US EPA, 2013). Ground water and surface water can be affected by pollution from feedlots through the leaching of pollutants or runoff of nutrients, organics, and pathogens from fields and storage (Hribar and Schultz, 2010). The higher concentrations of N and P for example, can lead to the eutrophication of water bodies being harmful to wildlife and water quality in aquatic systems (Barth et al., 2004). Feedlots also contribute to the reduction of air quality through the emissions of particulate substances and gasses. The particulate substances and dust provoked for animal movements is carried out by wind and the odors of the manure promote frequent complaints from people who live near feedlots. Furthermore, the decomposition of animal manure releases several types of gas emissions such as ammonia (NH_3), hydrogen sulfide, and methane. Ammonia produced in feedlots mostly comes from urine spots where urea rapidly undergoes microbial breakdown through enzymatic hydrolysis, leading to ammonium (NH_4^+) formation and subsequent NH_3 volatilization (Todd et al., 2008). Generally, cattle production account for approximately 43% of the anthropogenic NH_3 emissions in the U.S (Battye et al., 1994). Ammonia is a respiratory irritant and can combine rapidly in the atmosphere with other air pollutants such as sulfuric and nitric acids to form fine particulate matter ($PM_{2.5}$)

(Hristov et al., 2011) which can cause respiratory disease, increasing asthma and chronic bronchitis in neighboring communities, especially in children and farmworkers (Hribar and Schultz, 2010).

Greenhouse Gas Emissions from Beef Production Systems

The greenhouse gas emissions (GHG) from beef cattle agriculture involve methane (CH_4), nitrous oxide (N_2O), and carbon dioxide (CO_2) (Rotz et al., 2019). In order to account for the effects of emissions of different gasses, and express them in a common scale, the Intergovernmental Panel on Climate Change developed the global warming potential (GWP), which standardizes the effect of different GHGs in "CO_2 equivalent units" (CO_2eq) (Myhre et al., 2013). According to this, the global warming potential of CH_4 and N_2O is 28 and 265 kg of CO_2eq /kg in a 100-year time horizon (IPCC, 2014), respectively, which means a 28 and 265 higher capacity than CO_2 for absorbing energy and warm the earth. In a recent life cycle assessment of the beef cattle national herd, Rotz et al. (2019) estimated that the GHG emissions from beef cattle industry in the U.S, considering direct emissions from soil (cultivated pastures, range and cropland) and the manufacturing of the operation's inputs (fertilizers, pesticides, electricity) is equivalent to 242.6 Tg CO_2eq, which represent 3.8% of the 6457 Tg CO_2eq of total anthropogenic GHG emissions in the U.S in recent years (US EPA, 2019). Approximately, 142 Tg CO_2eq proceed directly from cattle emissions (CH_4 and N_2O from enteric fermentation and manure management), which is near 60% of the total GHG emitted for beef cattle production (Rotz et al., 2019) or 2.1% of the total U.S anthropogenic GHG emissions (US EPA, 2019). When GHG emissions are expressed per

unit of product (GHG intensity), the U.S average for 2019 was approximately 21 kg CO_2eq/kg carcass weight, being the cow-calf system the biggest contributor with 70% of the total GHG emissions (Rotz et al., 2019). These GHG intensity values confirm previous values reported by Beauchemin et al. (2010) for Canadian beef cattle systems of 22 kg CO_2eq/ kg CW with the cow-calf system contributing 80% of total GHG emissions.

Methane Emissions of Beef Production Systems

The largest contributing source of GHG emissions from beef cattle production is enteric CH_4, accounting for 56% (Rotz et al., 2019) to 63% (Beauchemin et al., 2010) of all GHG from beef industry and 39% of all GHG emissions from the livestock sector (Gerber, 2013); thus, reducing emissions from this source would have the most impact. Methane is a byproduct of the microbial fermentation of feeds in the rumen and may also represent an energy loss to the animal that range between 2 to 12% of the gross energy consumed with the diet (Johnson and Johnson, 1995). The reduction of CO_2 to CH_4 by methanogenic archaea act as an H_2 sink, removing H_2 from the rumen and avoiding the negative effects of H_2 accumulation on microbial enzymatic activity and degradation of plant material (McAllister and Newbold, 2008). Methanogens use H_2 as their main energy source, producing CH_4 in the process through the following reaction:

$$CO_2 + 4\ H_2 = CH_4 + 2\ H_2O$$
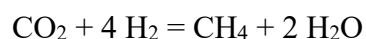
Methane is accumulated in the rumen and eructed by the ruminant to the atmosphere (Janssen, 2010), resulting in negative implications for environmental sustainability.

Several comprehensive reviews have described different strategies proposed by the scientific community to reduce enteric methane production and mitigate methane emissions (Broucek, 2018; Haque, 2018; Alemneh and Getabalew, 2019; Gerardo et al., 2019; Islam and Lee, 2019), but in order to be adopted for beef cattle producers they should be cost effective and socially accepted. Rumen defaunation for instance has been proved to reduce CH_4 emissions from ruminants by 50%, due to the fact that protozoal are large producers of H_2 and many methanogens are associated with protozoal (Hegarty, 1999); however, the lack of persistent response due to rapid adaptation and recovery of protozoal numbers along with impractical defaunation methods has limited its use (Martin et al., 2010). On the other hand, anti-methanogen vaccines have reduced CH_4 emissions up to 8% in sheep (Wright, 2004), however not always changes in methanogen populations lead to CH_4 reductions (Williams et al., 2009) and the development of a successful wide spectrum immunization is still in the far horizon for CH_4 abatement programs, limiting the application of such strategies as alternatives to reduce CH_4 emissions. Selection of "low CH_4" producing animals might be a promising strategy as a CH_4 mitigation options (Pickering et al., 2015), but it is still in an early stage of development. The use of ionophores that inhibit protozoal's growth (Guan et al., 2006), halogenated methane analogues, which inhibit growth and enzymatic activity of archaea in the rumen (Goel et al., 2009) or nitrate salts which have a greater affinity for H_2 than does CO_2 (Lee and Beauchemin, 2014) has been discarded due to consumer perception and potential negative effects on animal health, human health and the environment.

Finally, dietary manipulations like feeding highly digestible feed components like grains (Beauchemin and McGinn, 2005), or feeding organic acids like fumarate or malate

(Asanuma et al., 1999), which promote propionate production in the rumen and redirect H_2 to other reductive bacteria can reduce CH_4 emissions from the animal. The addition of lipids (Grainger and Beauchemin, 2011), condensed tannin extracts (Carulla et al., 2005; Grainger et al., 2009), essential oils (Benchaar and Greathead, 2011), exogenous enzymes and yeasts (McGinn et al., 2004) among others, which might be supplied along with concentrates in total mixed rations for confined livestock are still the most promising CH_4 mitigation options in terms of practical application and acceptance by farmers and consumers. Nevertheless, many ruminants consume forages as their sole diet in pasture-based livestock systems, and the need to supply feed additives in meals might difficult their practical implementation (Pacheco et al., 2014). In this case, CH_4 emissions may be reduced by using high digestible forage species with low content of fiber (McCaughey et al., 1999; Waghorn et al., 2002).

Diet quality affects the amount of CH_4 emitted by ruminants. Forages with high fiber concentration, may constrains passage rate and increase ruminal retention time (Allen, 1996; Meyer et al., 2010). Thus, if the retention time of a feed in the rumen increase, an increment in CH_4 production per unit of forage intake (CH_4 yield) is expected since the extent of rumen fermentation increase and there is more H_2 to be used as a substrate for methanogenic archaea (Moss et al., 2000). In addition to this, a more fibrous diet usually results in a more acetic type of fermentation, which increases CH_4 production (Johnson and Johnson, 1995; Ominski and Wittenberg, 2005). On the other hand, forages with lower fiber content increase passage rates and may favor propionate production which is considered a competitive pathway for H_2 use in the rumen (Moss et al., 2000), which in turn contributes to reduce CH_4 yield. In addition, forages with high

content of readily accessible carbohydrates as legume or brassica crops are digested more quickly and may also lead to greater propionate production relative to other forages like grasses (Sun et al., 2015), so cattle emit less CH_4 per unit of forage consumed. In support of this, Archimède et al. (2011) identified the structure of the fiber and ruminal retention time as the main factor influencing CH_4 production in a meta-analysis of data from ruminants fed C3 or C4 grasses and legumes, with 17% greater CH_4 yields from C4 than C3 grasses. In addition, animals fed warm legumes produced 20% less CH_4 than those fed C4 grasses.

Alternatively, forages with high concentration of non-fibrous carbohydrates (soluble carbohydrates plus pectin) that are rapidly fermented in the rumen, and low proportion of structural carbohydrates (cellulose and hemicellulose), might yield ruminal microorganism proportions similar to those contained in grain fed animals, increasing proportions of potentially propionate-forming bacteria and decreasing H_2 production, which might result in decreased CH_4 emissions relative to forages with lower content of non-fibrous carbohydrates (Sun et al., 2015).

Finally, grazing systems that rotate cattle across pastures, increasing plant density and diversity, could play a key role in reversing climate change through sequestering carbon from the atmosphere (Teague et al., 2016). Trampling helps work manure and other decaying organic matter into the soil, turning it into rich humus, promoting plant's root growth, water retention and microbe's development and contributing to keep CO_2 underground and out of the atmosphere (White, 2011). Pasturelands managed with regenerative grazing, with no-till farming practices and with active plant growth of perennial forages, help to increase CO_2 capture via photosynthesis and the carbon content

of the soil, acting as a net carbon sink and offsetting the enteric CH_4 emitted by cattle (Teague et al., 2016). Although forage fed cattle may produce more GHG than conventional grain-fed cattle per kilogram of beef produced (Capper, 2012) (i.e., since animals are slaughtered older and at lower finishing weight and consume forages with higher fiber content than grains), their net emissions might be reduced considerably when the soil organic carbon accrual is included in life-cycle assessments (Lupo et al., 2013). There is evidence that native grasslands and cultivated perennial pastures managed with regenerative grazing techniques that can sequester carbon in soils at a rate of 1400 to 1700 kg CO_2eq/ha/yr and result in net reductions of GHG (Liebig et al., 2010). Moreover, Teague et al. (2016) suggests that grass-fed beef produced through regenerative adaptive rotational grazing has a lower GHG impact than grain-fed beef once soil carbon sequestration and common soil carbon losses from croplands that grow grains for conventional feedlots are taken into consideration. This is due to the elimination of soil GHG emissions resulting from grain production and associated soil erosion.

Alternative Beef Production Systems

Grass-Fed Beef Production

Grass-finished beef is a niche market (represent 4% of the U.S beef market) that is growing rapidly in the United States due to consumer' concerns regarding human health, environmental impact and animal welfare (Cheung et al., 2017; Felix et al., 2018). This production system benefits from the fact that ruminants do not require concentrates such as grain, since they can derive energy from the cellulose of forages and other feeds which cannot be digested by swine or poultry (Van Soest, 2018). Rather than ship calves

to large animal feeding operations, mostly placed in the great plains region of the U.S (Drouillard, 2018), grass-finished beef can be produced locally, reducing the use of fossil fuels for transport of grains and cattle to feedlots, favoring the development of small local beef producers (Pollan, 2006; Holechek, 2009). In addition, perennial pastures can be grown on marginal lands and thus beef production systems do not compete for grain or croplands.

Grass-finished beef have lower total fat and lower concentrations of the saturated fatty acids (myristic and palmitic), considered to be detrimental to serum cholesterol levels, than grain-finished beef (Daley et al., 2010; Chail et al., 2016). Grass-finished beef also have two to six times higher levels of omega 3 (n-3) fatty acids, which makes a much lower ratio n-6/n-3 than grain-finished beef (1.53 vs 7.65; Daley et al., 2010), (2.78 vs 13.6; Leheska et al., 2008), (1.56 vs 4.84; Duckett et al., 2009), for grass and grain-fed beef respectively. Likewise, the n-6/n-3 ratio has increased even in pasture-raised beef supplemented with grains at only 1% BW (1.44 vs 3.17; Lagrange et al., 2006). The optimal ratio of n-6 to n-3 fatty acids in a healthy diet should not exceed 4:1(Gomez Candela, 2011) and many research studies have demonstrated that higher levels of omega 3 (antioxidant) fatty acids in the diet have benefits in the prevention or treatment of hearth diseases and stroke, different cancers and possible autoimmune problems such us lupus, eczema, and rheumatoid arthritis (Simopoulos, 2002; Wall et al., 2010; Gomez Candela, 2011). Finally, grass-finished beef also duplicate the concentration of conjugated linoleic acids (CLAs) (Leheska et al., 2008; Duckett et al., 2009; Daley et al., 2010) relative to grain-finished beef and contains higher levels of antioxidants as vitamin E (α-tocopherol), β-carotene (precursor of vitamin A) and enzymes that scavenge free

radicals and are beneficial for consumers (Duckett et al., 2009). CLAs are essential fatty acids that cannot be synthesized by humans and are only available from certain foods (Daley et al., 2010). Benefits of CLAs include reduced cancer risk and reduction of cardiovascular disease risk factors as cholesterol levels (Gebauer et al., 2011).

Despite the benefits mentioned regarding nutritional profiles of grass-finished beef in human health, most U.S consumers prefer the taste and tenderness associated with the white-fat marbling of the grain-finished beef (Maughan, 2011). However, in a research study conducted to evaluate grass-finished beef acceptance among U.S consumers based on taste panel rankings, Umberger et al. (2002) found that 23% of U.S consumers preferred argentine grass-finished beef over U.S corn-finished and were willing to pay a premium of $1.36 more per pound for the grass-finished beef.

Nevertheless, systems that use forages (typically grass) to finish cattle present several challenges concerning production and environmental impact, as they require longer finishing periods (10-12 months) and more animals and land to produce the same quantity of beef product (Mathews and Johnson, 2013), while producing a larger carbon and nitrogen footprint than conventional grain-based feedlot systems (Capper, 2012). In addition, nutritional value of grasses usually decline with the progress of the growing season, associated with plant reproductive development, increasing fiber content and decreasing N concentration as well as DM and fiber digestibilities (Fulkerson et al., 2007; Pelletier et al., 2010a), which leads to poor animal performances that average 0.5-0.6 kg of BW gain/d for the grass finishing system (Elizalde et al., 1998; Pelletier et al., 2010b; Capper, 2012; MacAdam and Villalba, 2015). In addition, grass-finishing beef production systems lead to low feed conversion efficiencies (10-12 kg of DM/kg of BW gain;

Lawrence et al., 2012) relative to grain-finishing systems. Grass-finished beef systems also might require irrigation in order to maintain biomass availability and grazing pressure during periods of drought or low precipitation and sustain high-quality forage year-round, which is required to increase weight gains in animals fed just forages (Mathews and Johnson, 2013). Finally, the use of big frame animals typically used in the U.S in conventional beef production systems might be a constraint for finishing cattle exclusively under forage diets, since large framed cattle reach physiological maturity and start fattening at a later age and at a heavier weight than do smaller-framed cattle, needing longer times to reach the same backfat thickness (Dolezal et al., 1993). In addition, it might be difficult for these animals to meet their higher nutrient requirements when the forage source is nutritionally unbalanced or present a low nutrient density.

Legume-Fed Beef Production

Forage legumes in beef feeding systems can offer economic and environmental advantages relative to grass-finishing systems. In contrast to grasses, forage legumes are lower in neutral detergent fiber (NDF), higher in N concentrations (Pelletier et al., 2010a; Phelan et al., 2015), present higher levels of non-structural carbohydrates (Fulkerson et al., 2007) and are digested more rapidly by ruminants at similar stage of forage maturity (Phelan et al., 2015). These characteristics lead to lower retention times in the rumen, so intake and production are higher than in grass-fed systems (Van Soest, 2018). This faster rate of digestion of forage legumes is primarily attributed to the faster rates of particle breakdown and faster fermentation rates in the rumen (Waghorn et al., 1989). Non-structural carbohydrates are also important in that they are a readily fermentable source

of energy for microorganisms in the rumen, providing energy in synchrony with the high

protein availability of forage legumes for the synthesis of microbial protein (Berthiaume

et al., 2010).

In addition, forage legumes do not decline in N concentration (Pelletier et al.,

2010a) and digestibility (Dewhurst et al., 2009) due to plant maturity to the same

magnitude as do grass forages. The higher nutritional composition of legumes usually

leads to greater DM intakes by ruminants than for grasses (Phelan et al., 2015), resulting

in greater liveweight gains (0.8 to 1.6 kg/d for beef steers) (Popp et al., 2000; MacAdam

et al., 2011; MacAdam and Villalba, 2015; Pitcher, 2015). This substantially decreases

days to slaughter and the amount of GHGs emitted (specially CH_4) per unit of intake or

beef product relative to cattle fed grasses (Phelan et al., 2015). In previous studies at Utah

State University, the enteric CH_4 emissions of beef cows grazing the forage legumes

birdsfoot trefoil (*Lotus corniculatus*) and cicer milkvetch (*Astragalus cicer*) were 167 and

159 g CH_4/d, respectively; which represent half of the emissions reported for the grass

meadow brome (*Bromus riparius*) with 355 g CH_4/d (Pitcher, 2015).

Cattle grazing legumes entail a realistic strategy to reduce enteric CH_4 emissions.

The higher fiber content in grass forages usually increases the proportion of acetate to

propionate in the rumen, increasing the production and release of CH_4 (Johnson and

Johnson, 1995). In support of this, the number of cattle required to produce 1 billion

pounds of beef when finished on pure birdsfoot trefoil pastures were approximately

500,000 less than when cattle were finished on grass (2.9 vs 3.4 million animals,

respectively; MacAdam and Villalba, 2015), approaching numbers required for

concentrate-based diets (2.7 million). Moreover, legume-finished beef results in greater

carcass weight, dressing percentage, backfat thickness and intramuscular fat percentage in the longissimus muscle than grass-finished beef (4.4% vs 2.9%, respectively), approaching values observed for grain-based finishing systems (5.8%; Chail et al., 2016). This outcome might be related to the high content of non-fibrous carbohydrates present in forage legumes. Likewise, tenderness, fattiness, juiciness and overall liking of legume-finished beef has no differences with grain-finished beef and both types of beef presented greater scores for these characteristics than grass-fed beef (Chail et al., 2016). In addition to these results, the n-6/n-3 ratio of fatty acids observed in legume-finished beef is much lower than the observed with concentrate diets and similar to grass-fed diets (2.41, 5.74 and 3.44, respectively), with greater n-3, as well as reduced n-6 in legume-finished beef (Chail et al., 2016), maintaining the benefits of the healthy fatty acids mentioned previously.

Unlike both cereal grains and pasture grasses, perennial legumes have the ability to form symbiotic associations with soil bacteria (Rhizobia spp.) and fix their own N, being productive for multiple years and replacing the need of N fertilization (Temperton et al., 2007; Pirhofer-Walzl et al., 2012). Finishing cattle on N-fixing forages promotes lower expenses and greater profits for producers and decrease GHG emissions related with production, transport (emission of CO_2) and use of N-based fertilizers (Phelan et al., 2015), as direct emissions of nitrous oxide (N_2O) are negligible from biological N fixation (Rochette and Janzen, 2005). Therefore, legume-finishing systems gives producers an alternative to follow a sustainable forage-finishing program while maintaining high animal performances and beef quality comparable with grain-finishing programs.

In this context, alfalfa (*Medicago sativa* L.) has been one of the most important crops grown in the western U.S, being the most high-yielding and nutritious forage available for feeding high-producing ruminants (Yost et al., 2020). Similarly, white clover (*Trifolium repens*) and red clover (*Trifolium pretense*) have been extensively used for grazing in Australia, New Zeeland and the United Kingdom. However, the direct use of these legumes as grazing forage has been limited due to the high risk of livestock losses caused by frothy bloat (Wang et al., 2012). Pasture bloat occurs in fresh, high-protein forages, with high rate of particle breakdown, that results in a rapid release of plant soluble proteins and disruption of chloroplasts, providing large quantities of gas and bacterial slime, which create a stable foam that prevents the animal eructation of fermentation gases (CO_2 and CH_4) (Majak et al., 2003). Ultimately, the rumen becomes distended, resulting in death from suffocation or cardiac arrest. Management techniques as grazing mature forage might reduce the risk, but at the expense of reducing the overall nutritional value of legume forages (Thompson et al., 2000). Grazing grass + legume mixtures still may impose a risk of bloat if animals are able to select and ingest the preferred legume species in high proportions.

Nitrogen Emissions in Legume-Finishing Systems

Only between 10% to 40% of ingested N is retained as animal product (meat or milk) by ruminants, with the majority of dietary N excreted in feces and urine (Calsamiglia et al., 2010). The high content of ruminal degradable protein in forage legumes usually exceeds the capacity of microorganism for uptake of NH_3 and synthesis of microbial protein due to a deficient energy supply for N capture (Julier et al., 2003).

The excess of ruminal NH_3 is absorbed across the rumen wall (Abdoun et al., 2006), transformed to urea in the liver, and excreted in the urine with an energetic cost for the animal (Lobley and Milano, 1997). However, when NH_3 detoxification capacity of the liver is overpassed, NH_3 accumulation in blood could be toxic for the ruminant and induce negative internal states which constraint DM intake (Provenza, 1995). In addition, high blood urea levels lead to high urinary N excretions (Kohn et al., 2005), which exacerbates the problem of low N retention with legume forages by increasing the proportion of N excreted as a highly labile form in the urine, which is a major environmental concern (Getachew et al., 2006). Once urine is excreted and deposited in the soil surface, urea is rapidly hydrolyzed by microbial urease to NH_4^+, which may be nitrified later to nitrite (NO_2) and nitrates (NO_3^-) (Dijkstra et al., 2013). Greater levels of urinary N excretions are associated with a greater and more rapid NH_3 volatilization and N losses as NO_3^- that may be leached into groundwater or run off to waterways (Dijkstra et al., 2013), contributing to eutrophication (Zonderland-Thomassen et al., 2014; Leip et al., 2015) and pollution of drinking water. In addition, N_2O is produced as an obligate intermediary during microbial nitrification and denitrification processes (Oenema et al., 2005; Huang et al., 2015), being one of the most important GHG, with a warming potential 265 times greater than CO_2 in a 100-year time horizon (IPCC, 2014). According to Bao et al. (2018), an increment in urinary N excretion of growing beef cattle from 29 to 50 g/d increases the estimated emission of N_2O by a 37% from 413 to 565 mg/d. However, the fraction of urine N released as N_2O also depends on the type, wetness and temperature of the soil. Regardless of these conditions, reductions in the proportion of N partitioned to urine in ruminants will be beneficial for the environment, since urinary N is

much more susceptible to gaseous losses than fecal N, which is in the form of organic

bound N and needs longer time to be mineralized to NH_4^+ before being susceptible to

volatilization or being available for nitrification process (Cai et al., 2017).

To counteract the fact that the use of legumes can result in high urinary N

excretion into the environment, a mitigation option is to use legume species that contain

bioactive secondary compounds known as condensed tannins (CT). The use of

tanniferous legumes with moderate concentrations of CT (i.e. 30-60 g/kg DM basis) in

monocultures or associated with other non-tanniferous legumes may reduce ruminal

protein degradability and alleviate malaise by inhibiting NH_3 production in the rumen,

thus increasing the pool of high-quality protein that reaches the small intestine (Barry and

McNabb, 1999), shifting N excretion from the urinary route to feces while improving N

utilization (Waghorn, 2008). Other benefits associated with the use of tanniferous

legumes are a decrease in the levels of enteric CH_4 emitted (13-16%) from forage diets

(Woodward et al., 2004), and a reduction of the risk of bloat (Wang et al., 2012),

allowing cattle to graze forage legumes at the greatest nutritional value and at the same

time contributing to solve some of the environmental problems mentioned previously.

Condensed Tannins in Beef Production Systems

Molecular Structure

Condensed tannins are plant secondary compounds (PSCs) also known as

proanthocyanidins, consisting of oligomers or polymers of flavan-3-ol monomers, which

differ due to the hydroxyl groups and the stereochemistry (spatial orientation) of the C-2

and C-3 in the C-ring (Aboagye and Beauchemin, 2019). Most of the CT occurring in

forage species are procyanidin (PC) (e.g., catechin and epicatechin) and prodelphinidin

(PD) subunits (e.g., gallocatechin and epigallocatechin) which possess an additional

hydroxyl group at C-5 of the B-ring (Zeller, 2019). Epicatechin and epigallocatechin have

a cis orientation of the C-2 and C-3 in the C-ring, while catechin and gallocatechin

possess a trans orientation (see Zeller, 2019).
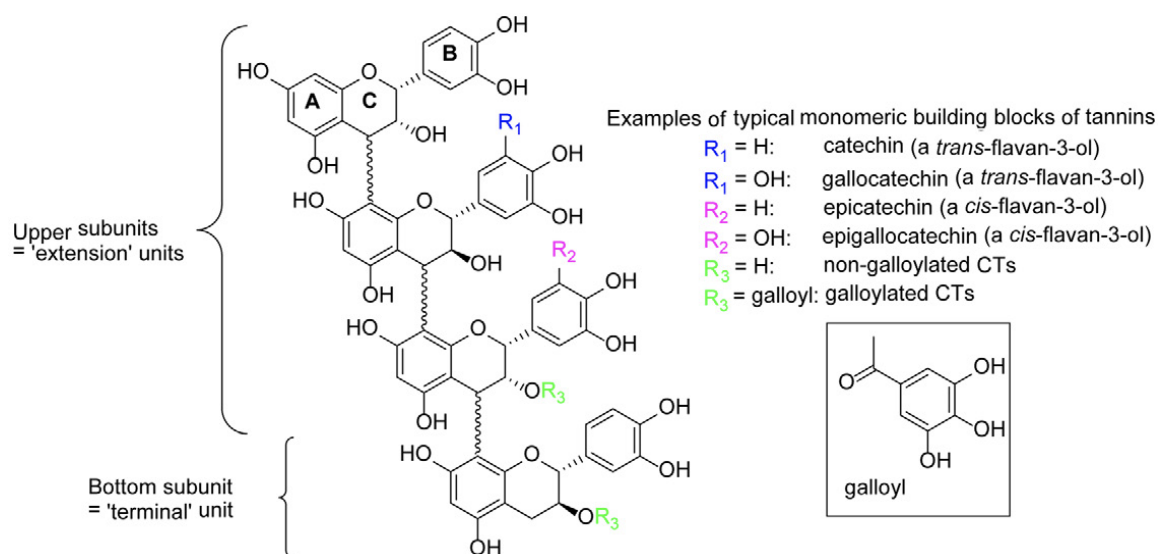
Figure 1-1. Condensed tannin molecule consisting of four flavan-3-ol monomers.
Adapted from Mueller-Harvey et al., 2019.

Monomers bind each other into oligomers and polymers through covalent

linkages of the C-4 in the C-ring of one flavan-3-ol to the C-8 or C-6 positions in the C-

ring of another monomer (Aboagye and Beauchemin, 2019) (Fig. 1-1). These oligomers

and polymers in common forage plants are typically present as mixtures of PC and PD

subunits which are randomly distributed throughout the CT molecule and linked through

different types of bindings, leading to many different chemical structures within the

group of CT (Zeller, 2019). Molecules of CT also differ in the number of flavan-3-ol

subunits they are built (degree of polymerization), resulting in structures that can vary in MW between 1900 to 28,000 Da (Aboagye and Beauchemin, 2019). Thus, plants contain structures of CT that vary in degrees of polymerization and composition of their subunits and they can differ between plant species, cultivars within the same species, and even parts (leaves, stems) within the same plant (Naumann et al., 2017). In addition, the contents of CT vary with phenological stage, reducing concentration as maturity progresses (Lees et al., 1995). For instance, leaves of sainfoin (*Onobrychis viciifolia Scop.*) have higher CT concentrations and a greater biological activity and PD proportion than stems (Theodoridou et al., 2010), thereby vegetative stages present a higher concentration of CT than mature plants (Berard et al., 2011) and therefore a greater CT-protein complexation potential (Aerts et al., 1999).

Condensed Tannins-Protein Complexes and Implications in Ruminants

Once plant tissues are chewed or degraded by microbial digestion, CT are released from vacuoles and bind to plant, salivary and microbial proteins, forming insoluble complexes in the rumen (Jonker and Yu, 2017). These complexes reduce protein solubilization and protect dietary proteins from microbial hydrolysis and deamination in the rumen, reducing the susceptibility of forage protein to microbial degradation (Min et al., 2000). In addition, CT can form complexes with extracellular and cell coat enzymes of proteolytic bacteria, inhibiting their activity and reducing protein degradation (Jones and McAllister, 1994). As a result, there is an increased outflow of undegraded plant protein to the intestines, and reductions in ruminal NH_3 concentrations (McNabb et al., 1996; Aufrère et al., 2013; Avila et al., 2015). The CT-protein complexes

are stable over the pH range 3.5 to 7.0, but can dissociate in the abomasum and anterior duodenum at a lower pH (Perez-Maldonado et al., 1995), releasing proteins for break down and increasing the proportion of plant amino acids available for post ruminal absorption (Bermingham et al., 2001), which increase the efficiency of N utilization by the ruminant.

The formation of the CT-protein complex is due to hydrogen bonding between the hydroxyl groups (–OH) of the CT molecule and the amino group (–NH) of peptides (Fig. 1-2), or by hydrophobic interactions between the phenol ring and the carboxyl group (–COOH) of proteins (Jonker and Yu, 2017). The formation of such complex depends on the structure of both the protein and the specific CT in the plant or plant part, the isoelectric point of the protein, the pH in the gastrointestinal tract, and the tannin-protein molar ratios (Naumann et al., 2017).
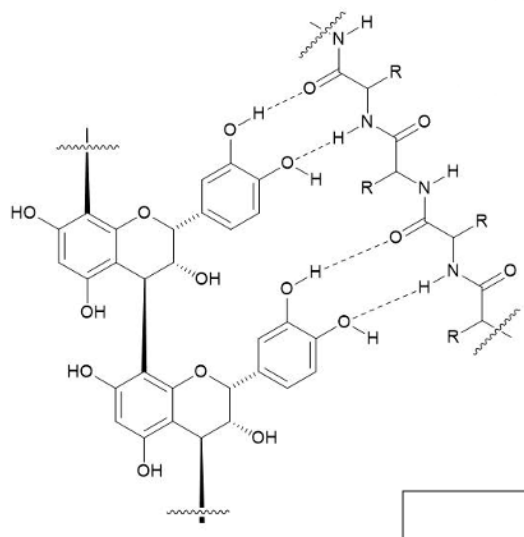
Figure 1-2. Hydrogen bonding involved in condensed tannin-protein complexation. Adapted from Zeller, 2019.

For instance, different studies have determined that as CT concentration (Naumann et al., 2014) or MW and mean degree of polymerization (Ropiak et al., 2017) increases, the protein precipitation capacity of CT also increases. AufrèRe et al. (2014) found a negative correlation between N solubility and CT concentration, PD/PC ratio, mean degree of polymerization and cis/trans ratio for three sainfoin varieties at several harvests.

Condensed tannins in birdsfoot trefoil have average molecular weights of 4400 Da (McAllister et al., 2005), with a degree of polymerization in the range of 6 to 14 of predominantly PC type subunits (Jonker and Yu, 2017), while sainfoin's CT are basically constituted by PD monomers of a mean MW of 5100 Da (McAllister et al., 2005), with polymer sizes that vary between 4-12 subunits (Jonker and Yu, 2017). Thus, differences between the molecular structure of CT between birdsfoot trefoil and sainfoin may result in different effects on protein degradability as it influences their binding capacities and their affinities for plant, microbial and mammalian proteins during herbivory. This may explain the higher protein precipitation capacity reported for sainfoin's CT relative to CT from birdsfoot trefoil (McAllister et al., 2005).

Several *in vitro* (Rufino-Moya et al., 2019) and *in vivo* studies (Scharenberg et al., 2007; Theodoridou et al., 2010; Theodoridou et al., 2012) have reported reductions in ruminal protein degradation, ruminal NH_3 concentrations and urinary N excretion with incubated sainfoin's substrates or when sainfoin was fed to sheep, relative to animals receiving polyethylene glycol (PEG), a polymer used to inactivate the effects of CT. In another *in vitro* study, Williams et al. (2011) found that NH_3 concentrations were lower when sainfoin was incubated in continuous cultures than when alfalfa (a non-tanniferous

legume) was used as the substrate. However, NH_3 was not different between birdsfoot trefoil and alfalfa in this study. Similar results were obtained later by Grosse Brinkhaus et al. (2016) who observed a 21% reduction in blood urea N and a 38% lower urinary urea N when dairy cows were fed sainfoin than when they were fed alfalfa pellets; however, no differences where observed for these parameters between the non-tanniferous alfalfa or birdsfoot trefoil.

When sainfoin is fed to ruminants, CT-protein complexes may not be completely dissociated in the abomasum and continue throughout the small intestine, preventing amino acid digestion and absorption (McNabb et al., 1998; Bermingham et al., 2001). The potential of these complexes for being reversible is dependent on the type of bonding (non-covalent or covalent) between CT and proteins (Le Bourvellec and Renard, 2012). Alternatively, CT may still be active under the pH level (5.0) of the proximal small intestine and interfere with endogenous and microbial proteolytic enzymes, increasing the proportion of protein ending in the feces (Aufrère et al., 2013). This may reduce N retention as observed for sainfoin diets (Azuhnwi et al., 2013). In contrast, the prevalence of PC type in birdsfoot trefoil may be associated with a greater protein digestion in the abomasum and small intestine and improved amino acid absorption and animal performance (Waghorn, 2008; Jonker and Yu, 2017).

Effect of Condensed Tannins on the Incidence of Frothy Bloat in Ruminants

Tanniferous legumes like birdsfoot trefoil and sainfoin are non-bloating and can therefore be grazed in pure stands. Complexes between CT and proteins prevent the plant protein from being solubilized into ruminal fluid and thus, formation of the

proteinaceous, gas-trapping foam is inhibited (McMahon et al., 2000). Condensed tannin

concentrations as little as 1 to 5 g/kg DM can prevent bloat (Li et al., 1996), so pastures

containing tanniferous legumes can be grazed without restriction. In addition, CT may

reduce the rate of gas production and proliferation of ruminal microbial populations in

the highly digestible alfalfa and the ruminal availability of soluble protein to form the

persistent foam (Wang et al., 2012). In support of this, the inclusion of sainfoin into

alfalfa pastures have reduced the incidence of bloat (Wang et al., 2006) and may

therefore be a practical and effective means of controlling this disorder. McMahon et al.

(1999) reported a marked reduction in pasture bloat when included as little as 10%

sainfoin in fresh alfalfa diets.

Effect of Condensed Tannins on Enteric Methane Emissions

Condensed tannins may inhibit CH_4 production in the rumen, which is beneficial

for improving nutrient utilization and reducing GHG emissions. Several studies have

reported reductions either in the gross emission of CH_4 (g/d) or in CH_4 yield (g/kg dry

matter intake), using forages with moderate concentration of CT (20 – 50 g/kg DM)

(Woodward et al., 2004; Moreira et al., 2013; Wang et al., 2018) or plant extracts

supplied with the feed (Piñeiro-Vázquez et al., 2018) or drenched directly to the animals

(Grainger et al., 2009). A meta-analysis from 15 *in vivo* experiments showed that

increasing tannin concentration in the diet decrease CH_4 production linearly when

expressed relative to dry matter intake (DMI) or digestible OM intake (Jayanegara et al.,

2015). Thus, low concentrations of CT (<20 g/kg DM) may not affect CH_4 production in

ruminants relative to control diets (Aboagye and Beauchemin, 2019).

Chemical structure of CT may also be an important factor affecting enteric CH_4 production, as was demonstrated *in vitro* by Hatew et al. (2016) who found differences in CH_4 emissions among CT extracts from four different sainfoin accessions. As mentioned previously for the protein precipitation capacity of CT, as degree of polymerization in CT increases, greater reductions in CH_4 production have been reported in *in vitro* studies (Tavendale et al., 2005). Likewise, higher molecular weight fractions of CT significantly decreased total methanogens numbers in vitro compared with lower molecular weight CT fractions (Saminathan et al., 2016).

The effect of CT on CH_4 emissions has been attributed to a direct effect on methanogenic archaea and/or their enzymatic activity (Tavendale et al., 2005; Tan et al., 2011; Saminathan et al., 2016) or more likely to an indirect effect on fiber digestion, adversely affecting cellulolytic bacteria and consequently reducing the amount of forage substrate fermented in the rumen (reduced digestion), and thus, H_2 producing acetate and the availability of H_2 for methanogenesis (Bodas et al., 2012; Jayanegara et al., 2015; Vasta et al., 2019). The mechanisms intervening on this effect are likely related to inactivation of extracellular microbial enzymes through the formation of CT-enzyme complexes and the subsequent reduction in their digestive activity (Bae et al., 1993) and/or direct inhibition of cellulolytic bacteria (McSweeney et al., 2001). In addition, formation of cell-associated protein-tannin complexes on the cell surface may interfere with microbial attachment to fiber and prevent microbial digestion (Bento et al., 2005). In support of this, Wang et al. (2015) and Barry and McNabb (1999) suggested that concentrations of CT in forages greater than 50 g/kg, might decrease DM digestibility in ruminants, and Chung et al. (2013) observed a lower NDF digestibility in sainfoin than in

alfalfa (45.3 vs 55.3%), even with CT concentration in sainfoin as low as 2.45%. A reduced fiber digestion due to an increased CT ingestion may also slow clearance of forage residues from the rumen, reducing voluntary DMI (Waghorn, 2008); thus, reductions in enteric CH_4 emissions due to a decreased fiber digestibility would not be a viable strategy.

Reductions in numbers of ciliate protozoa when CT are supplied with the ingestion of tropical legumes (Vaithiyanathan et al., 2007) could indirectly affect CH_4 emissions as mentioned previously with rumen defaunation, either by reducing methanogens symbiotically associated with protozoal populations or by reducing fiber digestion and H_2 supply to methanogenic archaea (Bhatta et al., 2009).

Tanniferous Legumes

<u>*Sainfoin*</u>

One of the forage species that grow well in the Mountain West USA and that naturally contain significant concentrations of CT in their leaves and stems is sainfoin, which contains 30 to 80 g CT/kg DM (Wang et al., 2015). The CT in Sainfoin are distributed throughout the aerial parts of the plant and restricted into the cell's vacuoles (Lees et al., 1993).

Condensed tannins in sainfoin enhance ruminant nutrition relative to other perennial legumes like alfalfa (Wang et al., 2015). Sainfoin is a legume species that have shown to decrease the urinary N losses without negatively impacting on the N retention by ruminants (Aufrère et al., 2008; Theodoridou et al., 2010), and reduce CH_4 production in *in vitro* studies (McMahon et al., 1999; Theodoridou et al., 2011; Niderkorn et al.,

2020), which is beneficial for improving nutrient utilization and reducing dietary energy loss and GHG emissions for eco-friendly animal production. Aufrère et al. (2005) showed in an *in vitro* study that mixing sainfoin with alfalfa could be an efficient way to reduce the N solubility of pure alfalfa. Sainfoin can serve as an alternative forage crop to alfalfa pastures in climate-adapted environments as it presents yields and nutritional value comparable to alfalfa (Sengul, 2003), leading to similar performances in sheep and cattle (Marten et al., 1987; Karnezos et al., 1994; Maughan et al., 2014). Huyen, (2016) found that replacing grass silage by sainfoin silage can improve milk yield and milk fatty acid profile of dairy cows.

As CT reduce the activity of specific rumen bacteria responsible for biohydrogenation of dietary fatty acids (Vasta et al., 2008), sainfoin diets may promote increments in conjugated linoleic acid and polyunsaturated fatty acids and reductions in saturated fatty acids in meat relative to animals consuming diets without CT (Vasta et al., 2009). In support of this, beef carcass from cattle fed sainfoin had greater marbling scores, quality grades and backfat thicknesses than alfalfa-fed cattle and steaks were redder in color than steaks from cattle finished on alfalfa and contained more unsaturated fatty acids (Maughan et al., 2014).

Birdsfoot Trefoil

Birdsfoot trefoil is a legume species that present a more prostrate growth habit relative to alfalfa or sainfoin (Grabber et al., 2014), with greater biomass per unit of area and higher bulk density (i.e., herbage weight per unit of canopy volume), which is correlated with a greater leaf area index (Gibb and Orr, 1997). It contains between 10 to

40 g CT/kg DM (Grabber et al., 2015) and yields approximately two-thirds as much as alfalfa in pure stands in the northern Mountain West (MacAdam and Griggs, 2013) with similar nutritional value to different alfalfa cultivars (Grabber et al., 2014). The unique CT produced by birdsfoot trefoil (Waghorn, 2008), as well as its high fiber digestibility (Christensen et al., 2015; Hunt et al., 2014a,b) enhance the efficiency of energy and protein use in ruminants relative to other perennial legumes like alfalfa.

A greater amino acid absorption has been linked to overall improvements in animal performance, including body weight gain, wool and milk production, reproductive performance and the ability to cope with gastrointestinal nematode burdens (Patra and Saxena, 2010). For instance, Min et al. (1999) reported increments of reproduction efficiency and wool production in sheep fed birdsfoot trefoil relative to animals receiving PEG, a polymer that binds and inactivates tannins. This response was produced without increments in voluntary intake, but authors reported a greater concentration of plasma essential amino acids, suggesting a higher intestinal absorption. Sheep grazing birdsfoot trefoil significantly increased performance compared with grazing alfalfa pastures (a non-tanniferous legume), resulting in increased ewe and lamb weight gains, carcass dressing-out percentage, and wool growth (Douglas et al., 1995). Harris et al. (1998) found that dairy cows grazing birdsfoot trefoil improved the efficiency of feed utilization and increased milk yield by 10% with increments in milk protein concentration relative to white clover (another non-tanniferous legume). Thus, one possible solution to the problems of low N utilization and high risk of bloat for cattle grazing non-tanniferous legume monocultures may entail the use of tanniferous legumes either as pure forages or

in association with free-CT legumes in order to increase efficiency of N use and improve the health of ruminants, humans and the environment.

Forage Diversity in Beef Cattle Production Systems

A diversity of forages and biochemicals available in pasturelands may enhance the benefits described above because complementary relationships among multiple food resources in nature improves the fitness of herbivores (Tilman, 1982), which in turn could reduce environmental impacts. Herbivores evolved grazing in diverse plant communities, consuming arrays of feeds of different chemical and physical characteristics (Provenza et al., 2007). Diverse diets offer ruminants a variety of nutrients and PSC which allow for a more balanced diet with more medicinal benefits than single forage species in monocultures (Westoby, 1978; Villalba et al., 2015). In addition, complementarities among nutrients and PSC may lead to a more efficient use of feeds, with improvements in animal welfare and productivity (Waghorn and McNabb, 2003) and reduced carbon and N emissions to the environment (Rochfort et al., 2008; Patra and Saxena, 2010).

The consumption of different legumes with contrasting chemical composition (different content of non-fiber carbohydrates, fiber and proteins) and presence of CT may lead to associative effects, like protein degradabilities lower than the average of the individual forages, as it has been demonstrated in *in vitro* conditions by Niderkorn et al., (2012) for a mixture of sainfoin and cocksfoot (*Dactylis glomerata*). Grazing tanniferous legumes in association with alfalfa may reduce enteric CH_4 emissions and N excretion relative to grazing forage monocultures. In support of this, Aufrère et al. (2007)

demonstrated that CT from sainfoin could bind and precipitate protein from alfalfa, and Naumann et al. (2015) found 65 and 25% reductions in CH_4 production when pure alfalfa was replaced in an *in vitro* study by the tanniferous legumes panicled-tick clover (*Desmodium paniculatum*) or sericea lespedeza (*Lespedeza cuneata*), respectively. In addition, McMahon et al. (1999) working with RUSITEC incubators observed a linear decline in CH_4 production as the proportion of sainfoin increased in binary mixtures with alfalfa. Sainfoin also diminished *in vitro* CH_4 production when the legume was associated with ryegrass (Niderkorn et al., 2011).

No reductions in DMI have been reported in the literature when high quality forages like alfalfa are partially replaced by tanniferous legumes in *in vivo* studies. For instance, Aufrère et al., (2013) did not observe any significant difference in DMI between sheep fed fresh alfalfa or different alfalfa and sainfoin mixtures (75% sainfoin-25% alfalfa or 25% sainfoin-75% alfalfa). Wang et al., (2006) observed similar feed intakes in beef steers grazing pure alfalfa or mixed alfalfa-sainfoin pastures containing up to 35% sainfoin, and Christensen, (2015) feeding a mixture of alfalfa-birdsfoot trefoil hays to dairy cows did not find differences in DMI relative to feeding pure alfalfa.

Some bioactive secondary metabolites in forage legumes can cause digestive interactions, so that the rumen fermentation pattern of a mixture of forages can differ from the average values of its components (Sinz et al., 2019), resulting in positive (synergistic) or negative (antagonistic) effects on ruminant nutrition. It may therefore be helpful to use more than one CT source and thus individual sources ingested at a lower dosage to avoid potential antinutritional effects of high concentrations of single CT (Sinz et al., 2019). As described previously, tannins produced by different forage species,

cultivars, plants, plant parts or during different seasons may have contrasting physical

and chemical properties which may impact herbivores in different ways (Waghorn and

McNabb, 2003). Thus, mixtures between legumes with different CT chemical structures

as sainfoin and birdsfoot trefoil may produce associative effects that enhance the effect of

single CT. Thus, interactions among CT may also influence the total amount of food an

herbivore can ingest (Villalba et al., 2004; Rogosic et al., 2007). It has been observed that

DMI by sheep increase as the number of tanniferous shrubs in the diet increases relative

to single shrub diets (Rogosic et al., 2007).

It has been suggested that food diversity may provide ruminants a positive

stimulus that increases their motivation to eat (Meuret and Bruchou, 1994). A diversity of

forages allows animals to incorporate different species to their diets which may delay the

onset of satiety (Chapman et al., 2007). Animals that are motivated to eat different

species (i.e., a choice of legumes) could also incur in increased locomotion activities in

order to gather different forages and achieve the challenge of building a balance diet

(Senft et al., 1987). In contrast, animals constrained to monocultures may reach satiety at

lower levels of feed intake due to the nutritional disbalances or too frequent of excessive

orosensorial exposure to limited stimuli. The sensory-specific satiety hypothesis

attributes changes in food preferences to transient food aversions caused by flavors,

nutrients, and toxins ingested too frequently or at high concentrations (Provenza, 1996).

This behavior has been observed in housed lambs which were fed the same mixed ration

offered in a diversity of flavors; unflavored, sweet, umami and bitter (diversity treatment)

vs. lambs receiving a monotonous ration with just one flavor (Villalba et al., 2011).

Lambs in the diversity treatment manifested partial preferences, consumed more total

feed with a more even distribution of their feeding patterns during the day, and performed

better than did lambs exposed to monotonous flavors.

Several studies have been observed synergistic effects when different forages

species have been consumed by ruminants either in choices or mixtures. In an experiment

using fresh forages fed to sheep, Niderkorn et al., (2014) observed positive associative

effects on DMI in the order of 9.5% with 50:50 mixtures of cocksfoot and red clover

silages or 5.6% for perennial ryegrass (*Lolium perenne*) and chicory (*Cichorium intybus*

L.) relative to the balanced median DMI values calculated from these forage when they

were fed separately. Similarly, in free-ranging conditions, Cortes et al., (2006) observed a

greater DMI by sheep grazing contiguous strips of perennial ryegrass and tall fescue

(*Festuca arundinacea*) than when grazing the same species as monocultures, which was

mediated by an increase in grazing time rather than an increase in intake rate. Finally,

Champion et al., (2004) found herbage intake increments by sheep grazing a free choice

of contiguous strips of white clover and perennial ryegrass vs. their respective

monocultures. In this case, the greater daily intake appeared to be due to a longer eating

time in the choice relative to pure white clover and to a greater intake rate relative to the

pure ryegrass.

The spatial aggregation of forage species in contiguous swards as opposed to an

intermingled mixture may reduce search time allowing animals being more efficient in

diet selection (Chapman et al., 2007). In a finely intermingled mix pasture, animals may

have to search for the preferred plant species, and this may reduce their intake rate

(Prache et al., 1998) and reduce daily voluntary intake relative to grazing monocultures.

Moreover, some less competitive species like sainfoin may be outcompeted in a mixture

with better adapted species like alfalfa. Alternatively, the most preferred herbage species could be overgrazed leading to resource degradation (Acharya et al., 2013; Sottie et al., 2014).

Finally, given choices to ruminants and allowing them to solve the problem of nutrient imbalances or excess of toxins may elicit positive emotional states and ultimately improve their welfare, relative to animals exposed to monocultures (Villalba and Manteca, 2019). Animals exposed to a diverse array of foods reduce some indicators of stress relative to animals ingesting single rations (Catanese et al., 2013) and they have the opportunity to learn about the postingestive consequences of foods and how to meet their needs through selecting a varied diet (Lyons and Parker, 2007). Diversity also allows animals to select a diet that is a function of their specific and dynamic needs. In contrast, single rations designed for the "average" individual may not satisfy all animals' needs given the inherent individual differences that exist among animals (Manteca et al., 2008).

Much of the research to date on the effects of forage diversity on animal behavior and performance has been conducted by contrasting monocultures with simple 2-species mixtures. Little is known about how higher order complementarities, like combinations of different tanniferous and non-tanniferous forage legumes presented in patches affect foraging behavior and performance of cattle through associative effects. In addition, there is a gap in knowledge regarding the potential complementary effects among different legumes, with different types and concentrations of CT and nutrients, on CH_4 and N emissions by ruminants. Thus, I hypothesized that ruminants grazing a diversity of legumes with different profiles and concentrations of nutrients and plant secondary

compounds (e.g., condensed tannins) promote associative effects that improve

productivity and reduce environmental impacts relative to grazing monocultures. I also

hypothesized that grazing tanniferous legumes would enhance animal performance and

reduce environmental impacts relative to grazing non-tanniferous legumes. With this

dissertation, I then tested the synergistic effect of increasingly diverse combinations of

tanniferous (sainfoin, birdsfoot trefoil) and non-tanniferous (alfalfa) forages on

digestibility, ruminant performance, foraging behavior and environmental impacts. In

Chapter 2, I explored single, binary and trinary choices among sainfoin, birdsfoot trefoil

and alfalfa on forage intake and preference, diet digestibility and N excretion by sheep

(Objective 1). I then determined (Chapter 3) the *in vitro* ruminal degradability and gas

production kinetics of the three legumes as single substrates, binary or trinary mixtures,

in order to better understand the significance of associations tanniferous legumes-alfalfa

relative to single-species. The proportion of legumes in the mixture was designed such

that the different species contributed in equal amounts to the mixture (i.e., indifferent

preference value) or in amounts that represented the selection displayed by lambs in

Chapter 2 (Objective 2). During Chapter 4, I evaluated the influence of grazing

monocultures of the tanniferous and non-tanniferous legumes described above, as well as

all possible 2- and 3-way choices among strips of the three legumes on performance,

enteric CH_4 emissions and N retention in beef cattle during the finishing phase of

production (Objective 3). With Chapter 5, I explored the foraging behavior, performance

and hair cortisol concentration (Objective 4) in beef cattle grazing the treatments

described for Chapter 4. Finally, Chapter 6 integrates results from Chapters 2 to 5,

providing implications for the future of forage-fed ruminants, as well as new avenues of

research aimed at enhancing the sustainability of forage-based beef production systems.

Expected Benefits

Grazing a chemically and morphologically higher diversity of forages containing complementary plant secondary compounds and nutrients (3-way and 2-way choices) leads to greater benefits - increased voluntary intake, livestock performance, welfare and reduced environmental impacts (improved efficiency of N use in ruminants, and further reduce CH_4 emissions) - relative to monocultures of the same pastures.

Due to positive associative effects, ruminants in choice treatments are expected to show a greater voluntary dry matter intake, gain at an even greater rate, show the lowest greenhouse gas (CH_4) emissions, and the lowest cortisol levels because animals offered choices have lower stress levels relative to animals constantly fed the same ration (monocultures). Furthermore, it is expected lower blood urea N and urinary N concentration on tanniferous legumes and N outputs to be higher in feces and lower in urine due to the positive effects of tannins on bypass protein. We expect beef cattle grazing monocultures to have fewer steps and standing time than cattle grazing in the 2-way or 3-way choices.

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CHAPTER 2

TANNIN-CONTAINING LEGUMES AND FORAGE DIVERSITY INFLUENCE

FORAGING BEHAVIOR, DIET DIGESTIBILITY AND NITROGEN

EXCRETION BY LAMBS[1]

ABSTRACT

Diverse combinations of forages with different nutrient profiles and plant secondary compounds may improve intake and nutrient utilization by ruminants. We tested the influence of diverse dietary combinations of tannin- (sainfoin-*Onobrichis viciifolia*; birdsfoot trefoil-*Lotus corniculatus*) and non-tannin- (alfalfa-*Medicago sativa* L.) containing legumes on intake and diet digestibility in lambs. Freshly-cut birdsfoot trefoil, alfalfa and sainfoin were offered in *ad libitum* amounts to 42 lambs in individual pens assigned to 7 treatments (6 animals/treatment): (i) single forage species [sainfoin (**SF**), birdsfoot trefoil (**BFT**) and alfalfa (**ALF**)], (ii) all possible 2-way choices of the three forage species [alfalfa-sainfoin (**ALF-SF**), alfalfa-birdsfoot trefoil (**ALF-BFT**) and sainfoin-birdsfoot trefoil (**SF-BFT**)], or (iii) a choice of all three forages [alfalfa-sainfoin-birdsfoot trefoil (**ALF-SF-BFT**)]. Dry matter intake (**DMI**) was greater in ALF than in BFT (P=0.002), and DMI in SF tended to be greater than in BFT (P=0.053). However, when alfalfa was offered in a choice with either of the tannin-containing legumes (ALF-

SF; ALF-BFT), DMI did not differ from ALF, whereas DMI in SF-BFT did not differ from SF (P>0.10). When lambs were allowed to choose between two or three legume species, DMI was greater (36.6 vs 33.2 g/kg BW; P=0.038) or tended to be greater (37.4 vs 33.2 g/kg BW; P=0.067) than when lambs were fed single species respectively. Intake did not differ between two- or three-way choice treatments (P=0.723). Lambs preferred alfalfa over the tannin-containing legumes in a 70:30 ratio for 2-way choices, and alfalfa>sainfoin>birdsfoot trefoil in a 53:33:14 ratio for the 3-way choice. *In vivo* digestibility (**DMD**) was SF > BFT (72.0 vs 67.7%; P=0.012) and DMD in BFT tended to be greater than in ALF (64.6%; P=0.061). Nevertheless, when alfalfa was offered in a choice with either sainfoin or birdsfoot trefoil (ALF-SF; ALF-BFT), DMD was greater than ALF (P<0.001 and P=0.007, respectively) suggesting positive associative effects. The SF treatment had lower blood urea nitrogen and greater fecal N/N Intake ratios than the ALF, BFT or ALF-BFT treatments (P<0.05), implying a shift in the site of N excretion from urine to feces. In conclusion, offering diverse combinations of legumes to sheep enhanced intake and diet digestibility relative to feeding single species, while allowing for the incorporation of beneficial bioactive compounds like condensed tannins into the diet.

INTRODUCTION

Alfalfa (*Medicago sativa* L.) is the most high-yielding and nutritious forage available for feeding high-producing ruminants in North America (NAAIC, 2017). Nevertheless, its use in pure stands has been associated with increased risk of bloat (Wang et al., 2012) and large urinary nitrogen losses caused by the rapid degradation of

alfalfa proteins in the rumen (Julier et al., 2003; Getachew et al., 2006; Dijkstra et al., 2013). In addition to ammonia volatilization to the atmosphere due to urinary N excretions (Whitehead, 2000), high levels of ammonia in urine "hot spots" are sources of nitrous oxide, a potent greenhouse gas (Forster et al., 2007) produced during microbial nitrification and denitrification processes (Oenema et al., 2005; Huang et al., 2014). Another problem with excesses of urinary N is the eutrophication of watersheds by nitrates, produced by ammonia oxidation and then leached into ground water, streams and lakes (Whitehead, 2000).

A strategy to reduce the aforementioned environmental impacts while maintaining high levels of animal productivity entails the provision of alfalfa in a diverse diet with bioactive-containing forages that increase N retention and/or reduce the proportion of urinary N losses. For instance, polyphenols like condensed tannins (**CT**) in legumes like sainfoin (*Onobrichis vicifolia*) or birdsfoot trefoil (*Lotus corniculatus*) bind to proteins and protect them from degradation in the rumen (Scharenberg et al., 2007b; Theodoridou et al., 2010; Theodoridou et al., 2012), altering the fate of the excreted N to greater fecal to urinary ratios (Mueller-Harvey, 2006). A shift in the route of N excretion from urine to feces means more stable N fractions in manure since N is mainly bound to organic compounds like neutral detergent and acid detergent insoluble N, which potentially lessens N losses to the environment as ammonia (Whitehead, 2000; Grosse Brinkhaus et al., 2016; Stewart, 2018).

In addition to the benefits of tannin-containing legumes, a diversity of forages and biochemicals available in pasturelands may enhance the benefits described above because complementary relationships among multiple food resources in nature improves the

fitness of herbivores (Tilman, 1982). Biodiversity in pasturelands may lead to positive associative effects among forages which improve the nutrition (i.e., N retention, diet digestibility) and welfare of livestock (i.e., reductions in stress caused by single forages with unbalanced nutrient profiles), while reducing environmental impacts. Sheep and goats eating mixed diets on rangeland display daily intakes two or more times greater than reference intake values obtained with animals fed single forages of similar nutritive value (Agreil and Meuret, 2004). On the other hand, differences in the chemical structures of CT in sainfoin and birdsfoot trefoil (McAllister et al., 2005) influence their capacities to bind proteins and microbial enzymes in the rumen (Mueller-Harvey et al., 2019), which may also lead to positive associative effects in diverse diets that influence protein degradability and the fate of nitrogen excretion.

Ruminants offered a diversity of forages (alfalfa, sainfoin, birdsfoot trefoil) may be able to build a diet that enhances nutrient retention and diminishes ammonia formation in the rumen, and consequently urinary N loses, relative to animals fed single forages. This response may occur because herbivores develop preferences based on the post-ingestive consequences of the foods experienced during the foraging process (Provenza, 1995; Provenza and Villalba, 2006).

We hypothesized that a diversity of tannin- and non-tannin containing legumes in ruminant feeding systems would lead to complementary relationships among nutrients and CT that: i) increase the ratio of fecal to urinary N excretions, ii) reduce blood urea N (**BUN**), and iii) maintain or increase food intake and digestibility relative to single forages. Thus, the aim of this study was to test the synergistic effects of increasingly diverse combinations of tannin-containing legumes (sainfoin or birdsfoot trefoil) and

alfalfa offered as single, binary or trinary choices.

MATERIAL AND METHODS

The study was conducted at the Green Canyon Ecology Center, located at Utah State University in Logan (41°45′59″ N, 111°47′14″ W), according to procedures approved by the Utah State University Institutional Animal Care and Use Committee (approval 2470). The experiment took place from May 20 to June 13, 2015.

Animals and Treatments

Forty-two commercial Columbia-Polypay-Suffolk crossbred lambs (4 month of age) with an average initial body weight (BW) of 24 ± 6 kg were housed outdoors under a protective roof in individual, adjacent pens measuring 1.5 m by 2.5 m (Fig. A-1). Lambs were fed *ad libitum* amounts of alfalfa pellets for 7 days to determine dry matter intake (**DMI**) for each lamb. After this 7-d period, a 7-d adaptation period was carried out to familiarize lambs to their respective legume diets, which were also fed during an ensuing 10-d experimental period. Throughout the study, lambs had free access to culinary water (Fig. A-2) and trace mineral salt blocks (mineral composition: minimum 96% NaCl, 320 mg/kg Zn, 380 mg/kg Cu, 2,400 mg/kg Mn, 2,400 mg/kg Fe, 70 mg/kg I, and 40 mg/kg Co).

Freshly-cut forage from two tannin-containing legume species – sainfoin (*Onobrichis vicifolia*), and birdsfoot trefoil (*Lotus corniculatus*) and from the non-tannin containing legume alfalfa (*Medicago sativa)* were offered in *ad libitum* amounts in seven diet treatments as (i) single forage species [sainfoin (**SF**), birdsfoot trefoil (**BFT**) and

alfalfa (**ALF**)], (ii) all possible 2-way choices of the three forage species [alfalfa-sainfoin (**ALF-SF**), alfalfa-birdsfoot trefoil (**ALF-BFT**) and sainfoin-birdsfoot trefoil (**SF-BFT**)], or (iii) a choice of all three forages [alfalfa-sainfoin-birdsfoot trefoil (**ALF-SF-BFT**)]. Lambs were sorted by their average intake of alfalfa pellets during the previous 7-d period and then randomly assigned to the seven treatment groups (6 lambs/group), such that treatments were balanced with regards to their intake capacity. Treatments were randomly distributed among pens.

Forages

Well-established and irrigated stands of sainfoin (cv. Shoshone), birdsfoot trefoil (cv. Langille) and alfalfa (cv. DK) seeded in August 2014 at the Utah State University Irrigated Pasture research facility in Lewiston, UT (41 56' N 111 52'W) provided the forages for this study. Pastures were irrigated using hand-line sprinkler sets running in 12 h cycles, which applied approximately 10.5 cm of water every 2 weeks.

Legumes were harvested from three monoculture plots of 0.17-ha each morning between 0700 – 0900 h in June 2015 at around 10-cm from ground level using a flail harvester (Rem Manufacturing Ltd., Swift Current, SK, Canada) with particle sizes varying between 2-4 cm, and immediately transported to the Green Canyon Ecology Center for daily feeding. Birdsfoot trefoil and alfalfa were cut at late bud stage and sainfoin in late flowering stage.

Adaptation Period (May 27 to June 2)

During this period, lambs were familiarized with the treatment diets and the experimental protocol. Each morning at 1100 h all lambs received freshly-cut forage of

each legume according to their assigned treatments, starting with 100 g (DM basis) on May 27. Different legume species in the 2- and 3-way choice treatments were offered in separate buckets that were simultaneously presented on a daily basis at random locations within each pen (Fig. A-3). Forage amounts were increased by 100 g daily until *ad libitum* amounts were fed to each lamb by the last day of the period (June 2). During adaptation, lambs offered SF and ALF were, in general, willing to consume greater amounts of forage than lambs offered BFT. Lambs eating ALF were monitored daily for symptoms of bloat (e.g., reduced intake, reluctance to move, distended rumen, and difficulty in breathing), which were not observed during the study.

Experimental Period (June 3 to June 13)

Each morning at 1100 h all lambs received legumes according to their assigned treatments and no other food was offered until the following day. Different legume species were presented as described for the adaptation period. The amounts of each legume offered per lamb during the experimental period ranged between 400 to 2200 g/d (DM basis) and they were adjusted on a daily basis depending on individual lamb intake such that refused amounts were always greater than 15% of the initial amounts of forage offered (DM basis). Refusals from each animal and for each legume were removed and weighed daily at 0900 h before fresh forage was offered to all animals according to their respective treatment.

Measurements

Intake and Preference

Dry matter intake of each legume was calculated on a daily basis for each lamb as the difference between the amount of forage offered and the amount of forage refused. Intake was expressed as g DM/kg BW. For multiple forage treatments, preference by lamb was estimated as the daily proportion of the DMI calculated for each legume species relative to the total amount of DMI.

Fecal DM Output and In Vivo Digestibility Calculations

Fecal DM output (**FO**) was determined using the concentration of an internal marker, acid detergent lignin (**ADL**), in the forage consumed and in feces (Van Soest, 2018). Fecal samples of at least 10 g (wet basis) were manually taken daily from the rectum of each lamb at 1300 h during the last 8 days of the experimental period (June 6 to June 13). Representative samples of forage offered and refused were collected daily during the same period. Forage and fecal samples were placed in plastic seal top bags, labelled and immediately stored in a freezer at -20°C until analyses. Samples were subsequently freeze dried (Free Zone 18 Liters, Labconco Corporation, Kansas City, MO) at -60°C until two consecutive weights did not differ in a 24-h period, and subsequently ground to pass the 1-mm screen of a Wiley mill (model 4; Thomas Scientific Swedesboro, NJ, USA). Fecal samples were then composited by lamb over the 8-d sampling period, combining approximately 2.5 g DM from each day. Samples of forages offered and refused were also composited over the 8-d period (0.75 g/d, DM basis) by species and analyzed in duplicates for ADL (see below). Fecal output was then

determined using the following formula: FO (g/d) = [DMI (g/d) × ADL in feed (g/g)] / ADL in feces (g/g) (Cochran and Galyean, 1994).

The ADL concentration in feed was calculated by the ratio of the difference between the amounts of ADL offered and refused for each legume and DMI as follows: [offered (ADL_{ALF} + ADL_{SF} + ADL_{BFT}) g – refused (ADL_{ALF} + ADL_{SF} + ADL_{BFT}) g] / DMI (g).

Once FO was determined, dry matter digestibility (**DMD**) was calculated for each lamb as: DMD (%) = {[DMI (g/d) – FO (g/d)] / DMI (g/d)} × 100 (Cochran and Galyean, 1994).

Neutral detergent fiber digestibility (**NDFD**) and acid detergent fiber digestibility (**ADFD**) were calculated by determining the concentration of neutral detergent fiber (**NDF**) or acid detergent fiber (**ADF**) in forages, refusals and feces (see below), and then applying the formula:

NDFD or ADFD (%) = {[NDF or ADF in feed (g/d) – NDF or ADF in feces (g/d)] / NDF or ADF in feed (g/d)} × 100 (Cochran and Galyean, 1994).

The NDF or ADF concentration in feed was calculated by the ratio of the difference between the amounts of NDF or ADF offered and refused for each legume and DMI as follows:

NDF concentration in feed (g/g) = [offered (NDF_{ALF} + NDF_{SF} + NDF_{BFT}) g – refused (NDF_{ALF} + NDF_{SF} + NDF_{BFT}) g] / DMI (g),

then: NDF in feed (g/d) = DMI (g/d) × NDF concentration in feed (g/g).

NDF in feces (g/d) = FO (g/d) × NDF concentration in feces (g/g).

ADF in feed (g/d) and ADF in feces (g/d) were calculated as described for NDF in

feed and feces.

Digestible dry matter intake (**DDMI**) was calculated as the product of DMI (g/d) and DMD.

The ratio of nitrogen excreted through the feces to consumed nitrogen (**Fecal N:Intake N**) was calculated by analyzing N concentration in the forage (offered and refusals) and fecal samples. The N excreted through the feces (g per lamb) was calculated by multiplying FO by the N concentration in feces. Intake of N was estimated for each lamb by difference between the total amount of the N offered with the legumes and the total amount refused every day as follows:

Intake N (g/d) = Offered (N_{ALF} + N_{SF} + N_{BFT}) − Refused (N_{ALF} + N_{SF} + N_{BFT}).

Blood Analyses

Blood samples (without EDTA added; Becton Dickinson Vacutainer System; Becton Dickinson and Company, Franklin Lakes, NJ; 10 mL serum vacutainer tubes) were collected via jugular venous puncture at 1000 h from each lamb prior to the beginning of the experimental period on May 29 and at the end of the experimental period on June 12. Samples were allowed to clot for 45 min before being centrifuged (1500 rpm for 15 min). The serum was extracted, placed in 1.5- mL microcentrifuge tubes and immediately submitted to the Utah Veterinary Diagnostic Laboratory (Logan, UT) for BUN analyses. The assay was performed with a Siemens Dimension Xpand Plus analyzer (Siemens Healthcare Diagnostics, Newar, DE) using Siemens urea N flex reagent, in an enzymatic method which uses urease enzyme in a bi-chromatic rate technique.

Chemical Analyses

One representative sample of each legume offered (alfalfa, sainfoin and birdsfoot trefoil) was taken daily before feeding, as well as one representative sample of refusal per legume. Legume and refusal samples were placed in paper bags and dried in a forced-air oven (VWR Scientific Inc., Radnor, PA) at 60°C for 48 h to determine moisture content and report voluntary intake on a DM basis.

One additional sample of each legume offered was collected at the same time, along with one additional sample of each legume refusal, and frozen in plastic seal top bags. Samples were subsequently freeze-dried at -60°C and ground to pass a 1-mm screen of a Wiley mill (model 4; Thomas Scientific Swedesboro, NJ, USA). Both legume and refusal samples were composited by species over the 10-d experimental period, taking approximately 2.0 g DM from each sample (samples from 06/03 to 06/13) and used for chemical analyses.

Composited forage, refusal and fecal samples were analyzed in duplicates for DM, N, ADF and aNDF concentrations. Dry matter was determined by drying the samples at 105°C for 3 h in a forced-air drying oven as recommended by the National Forage Testing Association (Shreve et al., 2006). Crude protein was calculated by analyzing the N concentration of the samples using a Leco FP-528 N combustion analyzer (AOAC, 2000; method 990.03) and applying the 6.25 conversion factor (Jones, 1931). aNDF (Mertens, 2002) and ADF (AOAC, 2000; method 973.18) determinations were modified by using Whatman 934-AH glass micro-fiber filters with 1.5 μm particle retention and a California Buchner funnel in place of fritted glass crucible. Determinations of ADL were modified from (Robertson et al., 1981) as follows: fiber

residue and filter from the ADF step was transferred to a capped tube and 45 mL of 72% sulfuric acid was added. Tubes were gently agitated for 2 h and filtered onto a second filter (same type as above) which was then rinsed, dried, weighed and finally ashed for 2 h in a furnace to remove lignin organic matter.

Analyses of total CT in legume samples were conducted in triplicate (assaying the samples three times in the same day), according to the butanol-HCl-acetone spectrophotometric assay of Grabber et al. (2013), using purified CT from sainfoin and birdsfoot trefoil as the reference standard.

Statistical Analyses

Dry matter intake, DDMI and FO were analyzed using a repeated measure design with day as the repeated measure. Diet (single forage species, 2-way and 3-way choices), day and the interaction diet x day were the fixed factors. Lambs (nested within diet) were included in the model as the random factor. The variance–covariance structure used was the one that yielded the lowest Akaike information criterion (compound symmetric). Nutritional composition of diets and feces, DMD, NDFD, ADFD, Fecal N excretion, Intake N, Fecal N:Intake N ratio and BUN, were analyzed as a completely randomized design, with diet as the fixed factor and lamb nested within diet as the residual component. BUN values were analyzed with initial BUN as a covariate. All analyses were computed using PROC GLIMMIX in SAS/STAT (SAS Inst., Inc. Cary, NC; Version 9.4 for Windows). Least squares means (LSMeans) were compared pairwise using the Least Significant Difference test (LSD) when F-ratios were significant (P<0.05) and reported along with their standard errors (SEM). A tendency was considered when

0.05<P<0.10.

In order to explore the potential associative effects in the 2- and 3-way choice treatments, the difference between the values observed for each response variable in a choice treatment and a linearly predicted value for the same variable was calculated as:

Associative effect (%) = 100 × [(Observed value – Estimated value) / Estimated value]. The estimated value was calculated as the weighted average of the values measured for each one of the legumes in the choice when they were fed as a single treatment (i.e., ALF, BFT, or SF). As an example, the estimated values for DMI in the ALF-SF choice was calculated as: (DMI_{ALF} × proportion of alfalfa selected in the choice) + (DMI_{SF} × proportion of sainfoin selected in the choice).

Preplanned contrasts were performed to compare observed vs estimated values using the LSMESTIMATE statement in PROC GLIMMIX. Contrasts were specified as the arithmetic difference between the observed value for the specific binary or trinary diet and the estimated value from the average of their components. Preplanned contrasts were also performed to compare the average of the three singles diets vs binary (2-way choices) or singles vs trinary treatments (3-species diets). A difference between the singles and binary or trinary diet groups or between observed and estimated values for a specific choice was considered significant when P values were < 0.05.

Proportion of each legume consumed within binary and trinary treatments (preference), was analyzed with day (fixed factor) as the repeated measure and lamb as the random factor. The confidence interval of the intercept was used to determine the range in which the true average proportion selected can vary. A legume species was considered "preferred" or "not preferred" in a specific two- or three-way choice

treatment, when the average proportion selected (intercept) for the legume was higher or lower than 0.50 or 0.33, respectively, and the confidence interval for the intercept did not include 0.50 or 0.33, respectively.

Assumptions of homoscedasticity of variance and normality were tested using studentized residuals and no apparent deviations from such assumptions were found. Normality of the random effect (lambs within diet) was tested using probability plots in PROC UNIVARIATE.

RESULTS

Chemical Composition of the Forages and Feces

The chemical composition of the legumes offered in the study, as well as the composition of refusals is reported in Table 2-1. On average across legumes, the refused forage was of lower nutritional quality than the forage on offer (i.e., lower CP, and greater ADF, aNDF, and ADL concentrations). Nevertheless, this difference was less evident for birdsfoot trefoil, which showed similar CP values between offered and refused forage.

Table 2-1. Nutritional composition (g/kg DM [mean (SEM)]) of legumes offered in the study and refusals

	Legumes	CP[1]	aNDF[2]	ADF[3]	ADL[4]	CT[5]
Offered	Alfalfa	177.0 (2.8)	376.0 (10.0)	317.0 (9.9)	65.0 (1.3)	1.8 (0.1)
	Birdsfoot Trefoil	191.0 (3.5)	374.0 (11.6)	333.0 (11.9)	70.8 (2.9)	13.0 (0.4)
	Sainfoin	138.0 (5.6)	430.0 (13.7)	383.0 (12.1)	86.2 (4.3)	27.1 (1.1)
Refusals	Alfalfa	134.0	514.0	427.0	95.9	0.8
	Birdsfoot Trefoil	191.0	461.0	394.0	88.9	9.9
	Sainfoin	112.0	581.0	508.0	115.1	14.1

[1]CP= crude protein.
[2]aNDF= amylase-treated neutral-detergent fiber.
[3]ADF= acid-detergent fiber.
[4]ADL= acid-detergent lignin.
[5]CT= Condensed tannins.

An estimation of the nutritional composition of the diets consumed by the lambs is reported in Table 2-2. The CP concentration was similar between BFT and ALF treatments (P=0.469), and both diets had greater CP concentration than SF (P<0.001). In contrast, the SF treatment presented the greatest concentrations of NDF, ADF and ADL, followed by BFT and then by ALF with the lowest values (P<0.05). Thus, when alfalfa was consumed with birdsfoot trefoil in 2-way choices (ALF-BFT), the CP concentration of the diet was greater (P<0.001) and the concentration of ADL tended to be lower (P=0.052) than in the ALF-SF treatment, due to the presence of sainfoin. The nutritional quality of the ALF-SF-BFT and ALF-SF treatments was similar.

Condensed tannin concentrations were greater (~ 2X) in SF than in BFT (P<0.001). Alfalfa is a non-tannin containing legume, confirmed by the low values of CT (Table 2-2).

Table 2-2. Nutrient concentration of diets and feces (lsmean; g/kg DM) when lambs were fed single forages, and 2- and 3-way choices of those forages: alfalfa (ALF), birdsfoot trefoil (BFT) and sainfoin (SF)

	Diet	CP[2]	aNDF[3]	ADF[4]	ADL[5]	CT[6]
Nutrient Concentration[1]	ALF	188.7[a]	338.5[cd]	287.1[c]	56.6[c]	2.1[e]
	BFT	191.0[a]	353.9[bc]	318.9[b]	66.6[b]	13.7[c]
	SF	147.7[d]	376.4[a]	338.3[a]	75.8[a]	31.2[a]
	ALF-SF	180.2[b]	325.1[de]	282.8[c]	57.8[c]	13.7[c]
	ALF-BFT	195.0[a]	314.1[e]	274.3[c]	53.2[c]	5.9[d]
	SF-BFT	160.7[c]	363.5[ab]	328.1[ab]	72.0[a]	26.9[b]
	ALF-SF-BFT	181.7[b]	313.7[e]	277.4[c]	56.3[c]	15.5[c]
	S.E.M	2.2	7.6	6.0	1.6	1.1
	Diet Effect	<0.001	<0.001	<0.001	<0.001	<0.001
Feces						
	ALF	142.2[d]	512.7[c]	411.2[e]	160.0[c]	
	BFT	157.0[bc]	574.7[b]	524.5[b]	206.8[b]	
	SF	166.0[ab]	614.8[a]	561.0[a]	270.9[a]	
	ALF-SF	159.2[abc]	540.2[c]	467.2[c]	201.6[b]	
	ALF-BFT	149.7[cd]	508.5[c]	436.7[de]	174.0[c]	
	SF-BFT	168.0[a]	598.7[ab]	549.3[ab]	258.9[a]	
	ALF-SF-BFT	160.0[abc]	534.2[c]	459.8[cd]	207.3[b]	
	S.E.M	3.7	11.7	9.2	5.5	
	Diet Effect	<0.001	<0.001	<0.001	<0.001	

[a-e]LSmeans in a column with different letters differ (P<0.05).

[1]Nutrient Concentration: Concentration of nutrients in lambs' diets calculated as: (Amount of forage offered × concentration of the nutrient in the forage – Amount of forage refused × concentration of the nutrient in the refusal) / DMI.

[2]CP= Crude protein.

[3]aNDF= amylase-treated neutral-detergent fiber.

[4]ADF= acid-detergent fiber.

[5]ADL= acid-detergent lignin.

[6]CT= Condensed tannins.

Fecal CP concentration was lower than the concentration observed in the ingested forages, with the exception of SF and SF-BFT treatments (Table 2-2), which presented greater values in the feces. SF also revealed greater protein concentration in feces than the ALF (P<0.001) and ALF-BFT (P=0.004) treatments, and this parameter also tended to be greater in SF than in BFT (P=0.096). Fecal CP concentration was also greater in

BFT than in ALF (P=0.008). Fecal NDF, ADF and ADL concentrations were on average ~1.5X, 1.5X and ~3.5X the concentration observed in the forages, respectively. Lambs fed SF showed the greatest fecal concentrations of NDF, ADF and ADL among the single diets (P<0.05; Table 2-2).

Intake and Preference

On average across diets, DMI differed throughout the experimental period (P<0.001; Fig. 2-1). Averaged across days, DMI in ALF was greater than intake displayed by lambs fed BFT (P=0.002; Table 2-3) and DMI in the SF treatment tended to be greater than in the BFT treatment (P=0.053). Nevertheless, when alfalfa was offered in a choice with either of the two tannin-containing legumes (ALF-SF or ALF-BFT), total DMI did not differ from ALF (P=0.503 and P=0.377, respectively). Similarly, DMI in the SF-BFT treatment did not differ from SF (P=0.584).

Comparisons between observed and estimated values did not reveal any positive or negative associative effects regarding DMI for lambs offered binary or trinary choices (P>0.10; Table 2-3). Nevertheless, DMI was on average 10% greater when lambs were allowed to choose between two legume species than when fed single species (36.6 vs 33.2 g/kg BW, respectively P =0.038), and overall DMI tended to be greater for 3-way choices than for single species (37.4 vs 33.2 g/kg BW, P=0.067; Table 2-3). In contrast, DMI did not differ between treatments when lambs were offered choices between two or three legume species (37.4 vs 36.6 g/kg BW, respectively; Table 2-3).
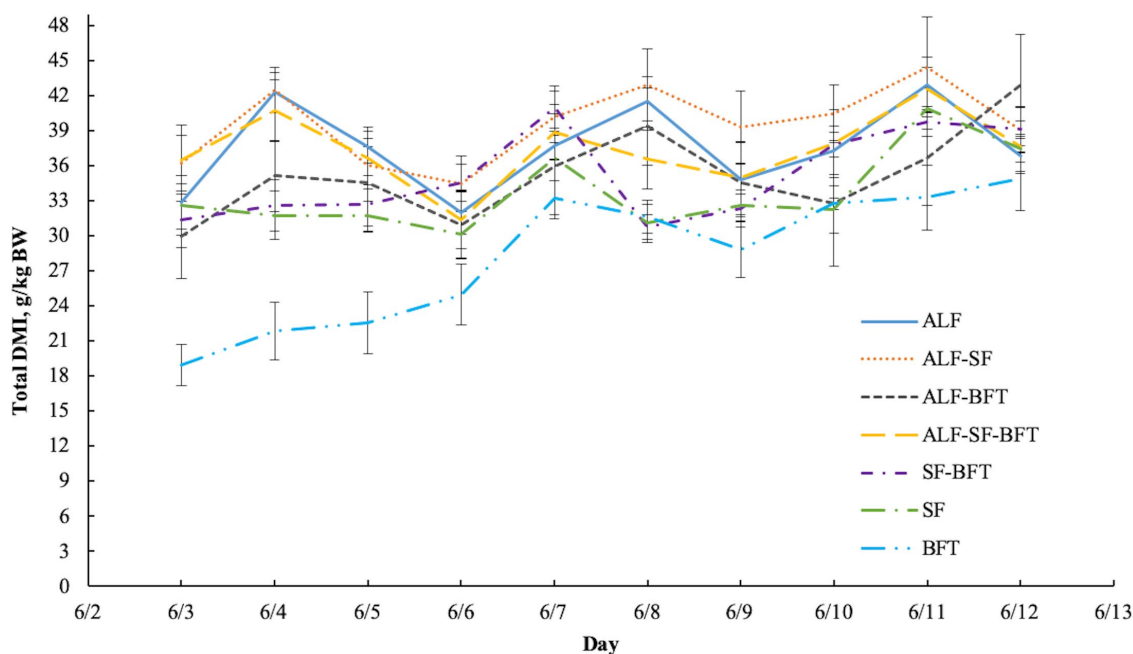
Figure 2-1. Daily total dry matter intake during the experimental period (g.kg BW^{-1}.d^{-1}; DM basis) of single forages and 2- and 3-way choices of those forages by lambs. Lambs were offered tannin-containing legumes (sainfoin; SF and birdsfoot trefoil; BFT) and the non-tannin containing legume alfalfa (ALF). Means are for 6 lambs per treatment. Bars represent SEM.

When offered the 2-way choice diets (ALF-SF, ALF-BFT or SF-BFT) alfalfa was preferred over sainfoin or birdsfoot trefoil (alfalfa>sainfoin and alfalfa>birdsfoot trefoil, Table 2-3), and sainfoin was preferred over birdsfoot trefoil (sainfoin>birdsfoot trefoil). Similarly, for the 3-way choice treatment, alfalfa was the most and birdsfoot trefoil the least preferred legume during the feeding period (alfalfa>sainfoin>birdsfoot trefoil, Table 2-3). Intake of each legume within each choice treatment expressed as g/kg BW is shown in Fig. 2-2. A day effect was detected for treatments containing birdsfoot trefoil (P<0.01; Table 2-3), driven by an increase in the proportion of birdsfoot trefoil selected by lambs towards the end of the experimental period and the concomitant decline in the proportions selected of the other components in the choice.

Table 2-3. Total dry matter intake (lsmeans) of legumes and proportions of these legumes selected by lambs when they were presented as a single forage or in 2- and 3-way choices: alfalfa (ALF), birdsfoot trefoil (BFT) and sainfoin (SF)

Diets	Total DMI, g·kg BW[-1]·d^{-1}	Proportions[3]		
		ALF	SF	BFT
ALF	37.6[ab]			
BFT	28.3[c]			
SF	33.7[bc]			
ALF-SF	39.4[a]	**0.67** (0.52-0.81)	**0.33** (0.19-0.48)	
ALF-BFT	35.1[ab]	**0.71** (0.60-0.81)[+++]		**0.29** (0.19-0.40)[+++]
SF-BFT	35.2[ab]		**0.71** (0.63-0.80)[+++]	**0.29** (0.20-0.37)[+++]
ALF-SF-BFT	37.4[ab]	**0.53** (0.32-0.74)[++]	**0.33** (0.11-0.55)[++]	**0.14** (0.08-0.20)[+++]
S.E.M	1.9			
P Values				
Diet effect	0.008			
Date effect	<0.001			
Diet x Date effect	<0.001			
2-species choice vs singles[1]	0.038			
3-species choice vs singles	0.067			
3-species vs 2 species choice	0.723			
Associative Effects[2]	% - (P-value)			
ALF-SF-BFT	6.8 (0.303)			
ALF-SF	8.6 (0.201)			
ALF-BFT	0.8 (0.907)			
SF-BFT	9.5 (0.216)			

[a-c] Total DMI LSmeans with different letters differ (P<0.05).

[1] Indicate that these are pre-planned contrasts between 2-way, 3-way choices and single diets.

[2] Associative effects (%): $100 \times$ [(observed value – Estimated value) / Estimated value]. Estimated value was the weighted average of the observed values for the single treatments.

[3] Proportions: numbers between parenthesis represent lower and upper values for 95% confidence interval of the mean; A legume species was considered "preferred" or "not preferred" when the average proportion selected was higher or lower than 0.50 (2-way choice) or 0.33 (3-way choice) and the confidence interval for the intercept did not include 0.50 or 0.33, respectively. + P<0.05; ++ P<0.01; +++ P<0.001, represents date effect for the proportion selected within each diet.
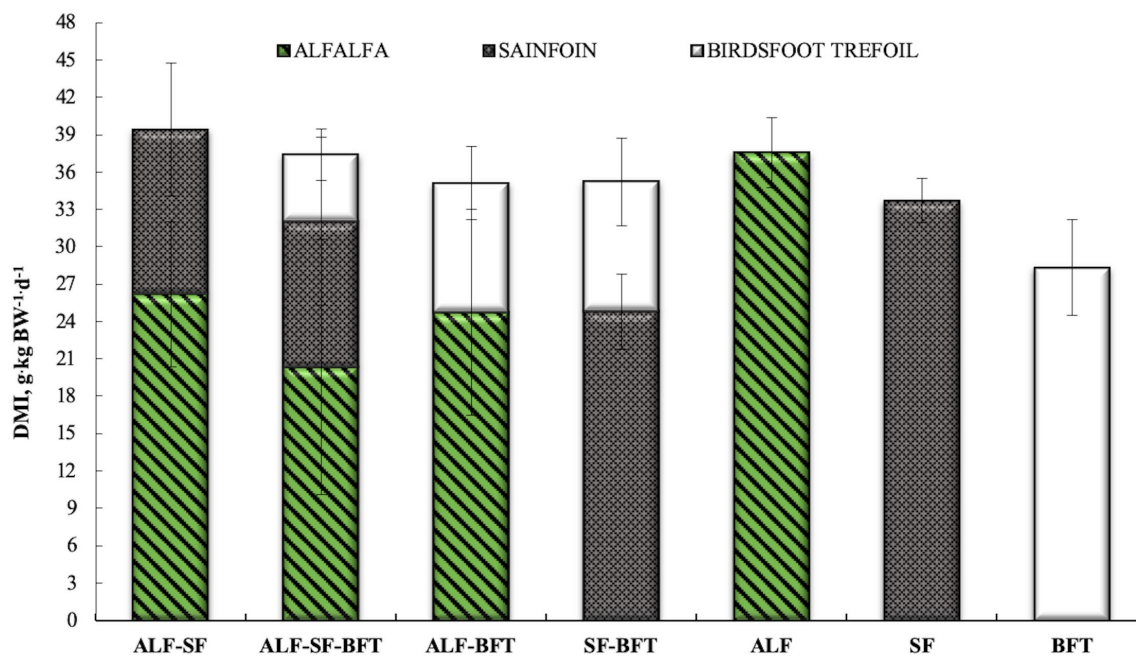
Figure 2-2. Dry matter intake (g·kg BW^{-1}·d^{-1}) of each legume consumed in the choice treatments. Bars represent 95% confidence intervals.

Digestibility and Fecal Output

Dry matter digestibility was SF > BFT (P=0.012) and digestibility in BFT tended to be greater than in the ALF treatment (P=0.061; Table 2-4). Nevertheless, when alfalfa was offered in a choice with sainfoin or birdsfoot trefoil, the inclusion of these tannin-containing legumes to the diet increased DMD relative to the single treatment ALF (ALF-SF and ALF-BFT > ALF; P<0.05). In fact, significant positive associative effects were observed for choices containing alfalfa and condensed tannin-containing legumes (Table 2-4). When both condensed tannin-containing legumes were consumed along with alfalfa (3-way choice), DMD was greater than for the BFT (P=0.005), ALF (P<0.001) or ALF-BFT (P=0.048) treatments (Table 2-4) and similar to the single and 2-way choice treatments containing sainfoin (e.g., SF, SF-ALF and SF-BFT; P>0.10). When lambs

were allowed to choose between two or three legume species, DMD was 2.4 and 4.3

percent units greater than treatments receiving single species (70.9 and 72.6 vs 68.1%,

respectively; P<0.01), but no significant differences in DMD were detected for lambs

receiving 2-way or 3-way choices of the legumes (Table 2-4) .

Table 2-4. Dry matter, NDF and ADF digestibility (lsmeans), digestible dry matter intake and fecal output (g·kg BW^{-1}·d^{-1}; DM basis) of legumes presented as single forages or in 2- and 3-way choices: alfalfa (ALF), birdsfoot trefoil (BFT) and sainfoin (SF)

Diets	DMD[1], %	NDFD[2], %	ADFD[3], %	DDMI[4], g·kg BW^{-1}·d^{-1}	FO[5], g·kg BW^{-1}·d^{-1}
ALF	64.6^d	46.5^c	49.3^{cd}	24.2^b	13.4^a
BFT	67.7^{cd}	47.6^c	46.9^d	19.1^c	9.1^b
SF	72.0^{ab}	54.3^a	53.6^{ab}	24.3^b	9.4^b
ALF-SF	71.2^{ab}	52.3^{ab}	52.6^{ab}	28.0^a	11.3^{ab}
ALF-BFT	69.3^{bc}	50.7^b	51.5^{bc}	24.0^b	11.1^{ab}
SF-BFT	72.2^{ab}	54.2^a	53.4^{ab}	25.4^{ab}	9.8^b
ALF-SF-BFT	72.6^a	53.5^a	54.8^a	27.0^{ab}	10.3^b
S.E.M	1.1	0.9	0.9	1.1	0.9
P values					
Diet effect	<0.001	<0.001	<0.001	0.001	0.033
Date effect				<0.001	<0.001
Diet x Date effect				<0.001	<0.001
2-species vs singles[6]	0.005	0.001	0.001	0.001	0.874
3-species vs singles	0.002	0.001	<0.001	0.002	0.765
3 vs 2 species choice	0.209	0.327	0.033	0.359	0.681
Associative Effects[7]	% - (P-value)				
ALF-SF-BFT	7.6 (0.001)	8.7 (0.001)	8.7 (0.001)	15.0 (0.014)	-9.9 (0.291)
ALF-SF	6.2 (0.006)	6.6 (0.009)	3.7 (0.095)	15.7 (0.011)	-5.9 (0.547)
ALF-BFT	5.8 (0.013)	8.3 (0.002)	5.9 (0.013)	5.6 (0.387)	-8.0 (0.390)
SF-BFT	2.0 (0.340)	3.4 (0.138)	3.4 (0.121)	11.6 (0.076)	4.8 (0.706)

$^{a-d}$ LSmeans in a column with different letters differ (P<0.05).
[1]DMD= *in vivo* Dry matter digestibility.
[2]NDFD= Neutral detergent fiber digestibility.
[3]ADFD= Acid detergent fiber digestibility.
[4]DDMI= Digestible dry matter intake.
[5]FO= Fecal Output.

[6]Indicate that these are pre-planned contrasts between 2-way, 3-way choices and single diets.
[7]Associative effects (%): 100 × [(observed value – Estimated value) / Estimated value]. Estimated value was the weighted average of the observed values for the single treatments.

NDFD and ADFD followed similar trends to those described for DMD, with values for SF being greater than for BFT (P<0.001) or ALF (P=0.001; Table 2-4). Similarly, when legumes were offered in 2 and 3-way choices, NDFD values were on average greater than values observed in single diets (52.4 and 53.5% vs 49.5, respectively; P=0.001; Table 2-4). In addition, some positive associative effects were detected for NDFD and ADFD, particularly when alfalfa was offered in a choice with condensed tannin-containing legumes in 2- and 3-way choices.

On average across diets, DDMI in ALF and SF was greater than DDMI in BFT (P=0.003; Table 2-4), particularly during the first three days of the experiment, which caused a treatment by day interaction (P<0.001). Overall, DDMI for the 3 and 2-way choices were 20 and 15% greater (P=0.002 and P=0.001) than for single diets (27.0 and 25.8 vs 22.5 g/kg BW, respectively). In contrast, no significant differences were detected between 2- and 3-way choices. The observed DDMI values for ALF-SF-BFT and ALF-SF were 15% greater than the calculated values from the weighted average of the individual legume components, indicating the presence of significant positive associative effects in these choices (Table 2-4).

BUN and Fecal Nitrogen Excretion

The proportion of Fecal N/Intake N was SF > BFT and ALF (P=0.008 and P=0.010, respectively) and no differences were observed between BFT and ALF treatments (P=0.932; Table 2-5). The treatment ALF-SF was not different from ALF

(P=0.471), but the proportion of Fecal N/Intake N for the ALF-BFT treatment tended to be lower than that observed for ALF (P=0.088) and significant negative associative effects were detected when these two species were combined (Table 2-5).

Table 2-5. Fecal nitrogen concentration (%) and excretion (g/d), proportion of the consumed nitrogen excreted through the feces (fecal N/intake N ratio) and BUN of legumes presented as single forage or in 2- and 3-way choices: alfalfa (ALF), birdsfoot trefoil (BFT) and sainfoin (SF)

Diets	Fecal N[1], %	Fecal N, g/d	Intake N[2], g/d	Fecal N/intake N, %	BUN[3], mg/dL
ALF	2.27[d]	7.6	27.9	26.7[bc]	19.2[b]
BFT	2.51[bc]	5.5	20.4	26.6[bc]	22.6[a]
SF	2.66[ab]	5.8	18.5	31.5[a]	16.1[c]
ALF-SF	2.55[abc]	7.4	28.9	25.5[c]	18.6[bc]
ALF-BFT	2.40[cd]	7.1	27.9	23.7[c]	22.2[a]
SF-BFT	2.69[a]	6.8	23.2	29.4[ab]	20.6[ab]
ALF-SF-BFT	2.56[abc]	7.0	28.5	24.2[c]	21.5[ab]
S.E.M	0.06	1.0	3.0	1.2	1.0
P values					
Diet effect	0.001	0.747	0.087	0.001	0.001
2-species choice vs singles[4]	0.209	0.346	0.084	0.044	0.140
3-species choice vs singles	0.257	0.559	0.084	0.006	0.065
3 vs 2 species choices	0.807	0.932	0.606	0.156	0.383
Associative Effects[5]			% - (P value)		
ALF-SF-BFT	5.2 (0.083)	4.4 (0.809)	19.8 (0.199)	-14.6 (0.007)	16.0 (0.022)
ALF-SF	6.0 (0.059)	6.1 (0.738)	16.4 (0.287)	-10.0 (0.071)	2.1 (0.673)
ALF-BFT	2.2 (0.505)	1.4 (0.941)	8.6 (0.563)	-11.1 (0.060)	10.7 (0.104)
SF-BFT	2.8 (0.336)	19.0 (0.402)	21.6 (0.287)	-2.3 (0.649)	15.1 (0.039)

[a-d] LSmeans in a column with different letters differ (P<0.05).
[1]Fecal N= Fecal nitrogen.
[2]Intake N= Intake nitrogen.
[3]BUN= Blood urea nitrogen.
[4]Indicate that these are pre-planned contrasts between 2-way, 3-way choices and single diets.
[5]Associative effects (%): 100 × [(observed value – Estimated value) / Estimated value]. Estimated value was the weighted average of the observed values for the single treatments.

The lowest and greatest values for BUN among single diets were observed for SF and BFT, respectively (P<0.05; Table 2-5). The addition of sainfoin to alfalfa in ALF-SF did not reduce the BUN values observed for ALF (P=0.703), but ALF-BFT increased BUN relative to pure ALF (P=0.033), even with proportions of birdsfoot trefoil in the diet as low as 30%. Thus, BUN from ALF-BFT was greater than in the ALF-SF (P=0.013) treatment. The observed values in SF-BFT and ALF-SF-BFT were significantly greater than the estimated values from their single components, indicating the presence of positive associative effects for BUN concentration in these treatments (Table 2-5).

DISCUSSION

Voluntary Intake and In Vivo Digestibility in Single Diets

Despite the presence of CT and the greater fiber concentration of the SF diet, lambs fed SF did not show any reduction in DMI relative to lambs fed ALF. It is likely that the 10% difference in NDF concentration observed between SF and ALF treatments was not high enough to induce a detrimental effect on DMI in SF diets. Similarly, the CT concentration observed in the SF diet (3.1% DM basis) was below the range of 6 to 12% mentioned by Aerts et al. (1999) or the threshold of 5.5% reported by Min et al. (2003) for causing feed intake reductions in ruminants fed tanniniferous forages. Consistent with our results, Aufrére et al. (2008) observed similar intakes in sheep fed fresh alfalfa or sainfoin when the concentration of CT in the tannin-containing legume was between 2.5 and 3.5% DM. In contrast, when CT content in sainfoin was around 6%, DMI in sheep was reduced by almost 20% relative to fresh alfalfa diets (Aufrére et al., 2013).

On the other hand, DMI in the BFT treatment was 25% lower than in ALF and tended to be lower than in the SF treatment (16% reduction). It is likely that the high concentration of CP in this forage (the highest out of the 3 legumes tested) accounted for the lower values of DMI observed in the BFT treatment. High intakes of readily degradable sources of N lead to increments in the concentration of ammonia in the peripheral circulation once the liver detoxification threshold is surpassed (Lobley and Milano, 1997), and may cause reductions in food intake as blood ammonia is one of the signals that control appetite (Provenza, 1995). This response is mediated through aversive post-ingestive feedback, which may occur very quickly within a meal (Villalba and Provenza, 1997). It is known than cattle are able to adjust their daily DMI to maintain blood ammonia nitrogen levels within a physiological limit of 2 mg/L (Nicholson et al., 1992). A restriction in DMI due to high concentration of CP in BFT is supported by the greater concentrations of BUN observed in the BFT than in the ALF or SF treatments.

The concentration of CT present in birdsfoot trefoil at the moment of being harvested for this study (13 g/kg) apparently was not high enough to reduce the degradation of CP in the rumen. In support of this, it has been suggested that the minimum concentrations of CT in birdsfoot trefoil to reduce the degradation of dietary protein and the production of ruminal ammonia through the formation of indigestible complexes is 20 g/kg DM (Aerts et al., 1999). In fact, previous studies using birdsfoot trefoil with less than 2% CT have shown that ruminal effective N degradability (Marichal et al., 2010) and ruminal concentrations of ammonia nitrogen (NH_3-N) (Williams et al., 2011; Christensen, 2015) were similar for birdsfoot trefoil and alfalfa diets with comparable concentrations of ruminal degradable protein. In contrast, sainfoin showed

greater amounts of undegradable crude protein after 8 and 24 h of *in vitro* incubations than birdsfoot trefoil (Scharenberg et al., 2007a), suggesting that the greater concentration of CT in sainfoin, as shown in this study (31.2 g/kg), was one of the reasons for preventing dietary protein from being degraded to ammonia in the rumen.

The lower DMD observed in this study for ALF and BFT may be due to the lower NDF and ADF digestibilities in these treatments than in SF. In a previous *in vitro* study, conducted with the same forages used in the present study (Lagrange et al., 2019), alfalfa and birdsfoot trefoil showed lower fiber concentrations and greater rates of fermentation and gas production (CH_4 and CO_2) at early incubation times than sainfoin. It is likely that ALF and BFT diets with a lower content of cell wall components, compounded with greater fermentation rates, increased passage rates of digesta through the rumen, which allowed for potentially digestible cell wall components and other forage constituents to escape ruminal digestion, explaining the observed reductions in fiber digestibility (Allen, 1996; Van Soest, 2018). Other studies (Aufrère et al., 2008; Chung et al., 2013) also observed a greater DMD for sainfoin than for alfalfa diets. The combination of high DM intakes and lower forage digestion in the ALF treatment resulted in lambs showing the greatest fecal outputs out of the three single species tested in the study, excreting 42.6% and 47.3% more feces than lambs eating SF or BFT, respectively.

Voluntary Intake, Preference and In Vivo Digestibility in Diverse Diets

It was clear that lambs were selective when they were presented with 2- and 3-way choices. In support of this, the nutritional composition of the ingested forages (Table 2-2) was greater than the composition of the forages on offer (Table 2-1). This pattern

appeared to increase with the increment in availability of alternatives, particularly for NDF, ADF and ADL (single legumes > 2-way > 3-way choices).

Herbivores manifest partial preferences, even when nutrients in single forages are adequate and toxins are not a concern (Provenza, 1996). A diverse diet allows herbivores to incorporate plants into their diets, that even when less nutritious, provide chemicals (i.e., flavors, antioxidants, compounds with medicinal properties) that enhance animal nutrition, health and welfare (Provenza et al., 2003; Villalba and Provenza, 2007). Consistent with this notion, lambs selected a diverse diet when offered choices among the three legumes used in the present study, and they preferred the species that showed greater DMI values when fed as single diets. For instance, lambs fed ALF-SF or ALF-BFT treatments preferred alfalfa to the alternative legume in a 70:30 ratio, but this combination did not constrain overall DMI as lambs offered those choices showed DMI values comparable to lambs receiving just alfalfa. Similarly, when lambs had to choose between all three species (ALF-SF-BFT), they selected a diet with proportions of the species: ALF > SF > BFT (53:33:14) that did not constrain DMI relative to the ALF treatment. Finally, when lambs had to choose between sainfoin or birdsfoot trefoil (SF-BFT treatment), they preferred sainfoin to birdsfoot trefoil in a 70:30 ratio, and DMI of the combination did not differ from intake values observed for the SF treatment. The lower preference manifested for birdsfoot trefoil could be a consequence of the high concentration of CP present in this species, as described above. In support of this, by selecting 30% of birdsfoot trefoil in SF-BFT, lambs increased their BUN concentration relative to lambs consuming the SF treatment, suggesting that an excess of N prevented further incorporation of birdsfoot trefoil into the SF-BFT diet.

An *in vitro* study (Lagrange et al., 2019) using the same forages used in this study shows that fermentation rates and total gas production were similar between alfalfa and substrates representing the 70:30 ratio of alfalfa:sainfoin or alfala:birdsfoot trefoil selected by lambs in the present study. In contrast, fermentation rates and gas production declined when substrates were composed of equal proportions (50:50 ratio) of the same binary choices (i.e., indifferent preference). Similarly, substrates representing the 3-way choice selected by lambs in this study (50:35:15 ALF:SF:BFT ratio) showed greater *in vitro* fermentation parameters than a mixture composed of equal proportions of the three legumes (33:33:33 ALF:SF:BFT). Thus, when lambs had *ad libitum* access to more than one legume, they selected a diverse diet in proportions that yielded fermentation rates (and DMI) similar to those observed for ALF. Thus, instead of just selecting the forage that offered the greatest fermentation rates and one of the greatest intake values (alfalfa), lambs incorporated tannin-containing legumes into their diet in proportions that did not reduce those parameters. This behavior provided the benefit of incorporating bioactive compounds like CT into the diet, which contributed to reduce the incidence of bloat (Howarth et al., 1978; McMahon et al., 1999) and improved the efficiency of N utilization (Barry and McNabb, 1999; Min et al., 2003; Chung et al., 2013). In addition, a diverse diet prevents reductions in DMI caused by the continuous and frequent exposure to the same orosensorial characteristics of a single diet (i.e., sensory-specific satiety, Provenza, 1996; Scott and Provenza, 1998; Atwood et al., 2001). Finally, interactions among chemicals in a diverse diet may lead to positive associative effects that enhance DMI and improve the nutrition of lambs (Görgülü et al., 1996; Keskin et al., 2004). In support of this idea, the mean DMI value of the 2-species choice was greater and the 3-

species choice tended to be greater than the mean value for single diets. Another example of positive associative effects is that 2- and 3-way choices resulted in improvements of DMD, NDFD and ADFD relative to the ALF treatment, with the 3-way choice yielding the highest synergic effect on digestibility. Likewise, lambs in the ALF-SF treatment had greater (14 %) DDMI and lambs in ALF-SF-BFT tended to consume more digestible DM (10%) than lambs in the ALF treatment. Such improved forage digestion with the addition of sainfoin and birdsfoot trefoil to alfalfa reduced FO in the 3-way choice relative to the ALF treatment.

Fecal and Blood Urea Nitrogen

No differences were observed in the ratio of Fecal N/Intake N between BFT and ALF treatments, but SF showed the greatest ratio. This response is likely mediated by the presence of CT, which form insoluble complexes with protein under the mild acidic-neutral conditions of the rumen (Perez-Maldonado et al., 1995; Le Bourvellec and Renard, 2012), and inhibit the proteolytic activity of ruminal bacteria (Jones and McAllister, 1994). Some tannin-bound proteins are released in the abomasum and anterior duodenum at lower pH values and then digested, but the process may be incomplete and some proportion of those proteins bound to tannins may end up in the feces (Waghorn et al., 1987), a process that has been reported for sainfoin (McNabb et al., 1998). Thus, the lower concentrations of CT observed in birdsfoot trefoil compounded with their lower precipitation capacity (McAllister et al., 2005) explain the reduced proportion of N into feces in the BFT relative to the SF treatment.

Greater ruminal protein degradation in lambs fed BFT, in addition to the high CP

values observed in the birdsfoot trefoil forage, explain the greatest BUN values observed among the single diets for lambs fed the BFT treatment, since high BUN values result from the absorption of excess ammonia from the rumen (Huntington and Archibeque, 2000). Protein degradation and ruminal ammonia-N concentration have been reported to be greater (Dahlberg et al., 1988) or similar (Christensen, 2015) in birdsfoot trefoil than in non-tannin containing legumes like alfalfa. In contrast, lambs fed SF showed the lowest concentrations of BUN, which suggest lower urinary excretions as there is a positive correlation between BUN and urinary N (Kohn et al., 2005). Thus, it is likely that there was a shift in the partition of N from urine to feces in the SF treatment, a pattern that may contribute to reduce environmental N pollution, as fecal N outputs are considered to be less harmful to the environment than urinary N (de Klein and Eckard, 2008). Urinary N is rapidly converted to ammonia and then oxidized to nitrite, nitrates and to volatile nitrous oxide (Oenema et al., 2005) which is a potent greenhouse gas (Forster et al., 2007). In addition, the runoff and leaching of nitrates into ground water contribute to eutrophication of streams and lakes (Whitehead., 2000; Huang et al., 2014). In contrast, fecal N is converted to ammonium at a much slower rate, retained to the soil and contributing to accumulation of soil organic matter (de Klein and Eckard, 2008).

Ingestion of sainfoin and birdsfoot trefoil in this study had different effects on fecal N concentration and BUN when they were ingested in a choice with alfalfa. Lambs consuming 30% of sainfoin in the ALF-SF treatment showed greater concentrations of N in feces than lambs fed ALF, and this parameter tended to be greater in ALF-SF than in ALF-BFT, although the proportion of Fecal N/Intake N or BUN values were similar to lambs in the ALF treatment. In contrast, lambs ingesting a 30% proportion of birdsfoot

trefoil in the ALF-BFT treatment had greater BUN values and showed a trend for lower Fecal N/intake N ratios than lambs in ALF. These results suggest that CT in birdsfoot trefoil did not affect the fate of N excretion or that the high concentrations of CP in birdsfoot trefoil just added more highly degradable protein to the rumen.

CONCLUSIONS

Tannin containing legumes like sainfoin and birdsfoot trefoil have the potential to reduce environmental impacts and enhance the nutrition of ruminants when presented in a diverse diet with other legumes such as alfalfa. Alfalfa fed as a single diet led to one of the highest DMI values for the study, but FO and BUN values were also proportional to such intake values, suggesting potential for increased environmental impacts. Sainfoin fed as a single forage led to greater concentrations of fecal N and reduced concentrations of BUN, whereas BFT increased BUN likely due to the high CP concentration of this forage. When offered choices among all legumes in 2-way choices, lambs mixed alfalfa with 30% sainfoin or birdsfoot trefoil, and when offered 3-way choices they mixed alfalfa with 33% sainfoin and 14% birdsfoot trefoil. Such selection was proportional to the intake and digestion rates of single forages, without reducing overall DMI relative to the pure alfalfa diet. Mixing legumes also led to positive associative effects that increased forage digestibility relative to ALF. Our results suggest that diverse combinations of legumes have the potential to enhance DMI and DMD relative to feeding single species, while allowing for the incorporation of beneficial bioactive compounds like CT into the diet. Some of the benefits of these compounds entail reductions in ruminal ammonia concentration and increases in the proportions of fecal N, an environmentally less

harmful form of N than urinary N. In addition, selecting from an array of legumes also

provides benefits related to dietary diversity in generalist herbivores, like improvements

in animal welfare and reductions in sensory-specific satiety.

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CHAPTER 3

GAS PRODUCTION KINETICS AND IN VITRO DEGRADABILITY OF TANNIN-

CONTAINING LEGUMES, ALFALFA AND THEIR MIXTURES[2]

ABSTRACT

The aim of this study was to determine *in vitro* ruminal degradability and gas

production kinetics of sainfoin (*Onobrichis viciifolia;* SF), birdsfoot trefoil (*Lotus

corniculatus;* BFT), alfalfa (*Medicago sativa* L.; ALF) and their binary or trinary

mixtures using the gas production technique. The proportions in the mixtures represented:

(1) those selected by lambs in a free-choice experiment (70:30 and 50:35:15 ratios for

binary and trinary combinations, respectively), or (2) equal proportions (50:50 or

33:33:33 ratios for binary or trinary mixtures, respectively). Organic matter digestibility

was greater in ALF and BFT than in SF (0.791 and 0.796 vs 0.751; $P<0.05$) and this

variable decreased as the proportion of SF in the binary mixtures increased. ALF showed

greater ($P<0.05$) gas production rates (R_{Max} =17.7 ml h^{-1}) than BFT (16.5 ml h^{-1}) or SF

(12.9 ml h^{-1}), reaching half of the asymptote of gas production (Parameter *B*= 7.3, 7.0 and

9.5 h, respectively) and maximum gas production rates at earlier times (2.4, 2.6 and 3.0 h,

respectively; $P<0.05$). The potential gas production (Parameter *A*) was ALF (210.6 ml) >

SF (198.3 ml) > BFT (187.6 ml) ($P<0.05$), and gas production rates decreased relative to

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pure ALF as the proportions of SF or BFT increased in the mixtures (P<0.05). The presence of two or three species in the substrate did not lead to positive associative effects. Nevertheless, lambs' preferred mixtures exhibited greater gas production rates and lower times to reach half potential gas production than mixtures formed with equal parts of each of the species (P<0.05). Thus, mixing alfalfa with sainfoin and/or birdsfoot trefoil in a diet at a 70:30 ratio may allow sheep to maintain fermentability values as high as pure alfalfa while ingesting a diverse diet with some bioactives (e.g., condensed tannins) that provide benefits to the internal environment such as reduced bloat and ammonia formation in the rumen, as well as advantages related to dietary diversity in generalist herbivores like improvements in food intake due to reductions in sensory-specific satiety.

INTRODUCTION

Alfalfa (*Medicago sativa* L.) is one of the most high-yielding and nutritious forages available, used widely for beef and dairy cattle production around the world. Nevertheless, its use in pure stands has been limited by the associated risk of bloat (Berg et al., 2000). In addition, the inefficient protein use observed in ruminants consuming pure alfalfa may lead to nitrogen (N) losses via urinary excretion, being detrimental to the environment (Julier et al., 2003; Getachew et al., 2006).

In contrast to alfalfa, legume species containing moderate levels of condensed tannins (CT) such as sainfoin (*Onobrichis vicifolia*) or birdsfoot trefoil (*Lotus corniculatus*) are non-bloating (Howarth et al., 1978) and show an increased efficiency of N utilization by ruminants (Barry and McNabb, 1999). Condensed tannins are

polyphenolic compounds that limit plant protein degradation in the rumen and increase the pool of high-quality protein that reaches the small intestine (Koenig and Beauchemin, 2018). Thus, the use of tannin-containing legumes in association with alfalfa may represent an effective alternative to reduce N pollution by shifting the site of N excretion from urine to more stable forms of N in feces (Wang et al., 2006; Aufrère et al., 2013). In addition, the presence of CT in legumes has been shown to reduce methanogenesis in both *in vitro* (Niderkorn et al., 2012) and *in vivo* (Ramírez-Restrepo and Barry, 2005) studies. Nevertheless, associations between alfalfa and tannin-containing legumes need to be achieved in a context where dry matter degradability and ruminal fermentation rates are not constrained. Otherwise, intake and productivity could be negatively compromised when animals ingest such mixtures. Alternatively, combinations of legumes may lead to associative effects that enhance productivity and reduce environmental impacts.

Previous studies report that high concentrations of CT may depress fiber digestion (McAllister et al., 2005), although the effect of condensed tannins on ruminal digestion may vary depending on their concentration in the diet and on their chemical structure (Wang et al., 2015; Mueller-Harvey et al., 2017). Differences between content and molecular structure of CT in birdsfoot trefoil and sainfoin may have differential effects on rumen fermentation, with potential synergies or antagonisms when these legumes are consumed together. Alternatively, the differential effects of CT may vary when tannin-containing legumes are ingested as the sole forage source or diluted with non-tannin containing legumes such as alfalfa.

Therefore, the aim of this study was to determine *in vitro* ruminal degradability and gas production kinetics of birdsfoot trefoil, sainfoin and alfalfa as single species,

binary or trinary mixtures in order to better understand the significance of associations CT-containing legumes-alfalfa relative to single-species. The proportion of legumes in the mixture was designed such that the different species contributed in equal amounts to the mixture or in amounts that represented the selection displayed by lambs during 2- or 3-way choices in cafeteria tests. Thus, our second objective was to compare the gas production kinetics of preferred proportions to equal proportions (i.e., indifferent preference value) of legume mixtures.

MATERIAL AND METHODS

Gas production kinetics and extent of degradation was determined using the gas production technique described by Theodorou et al. (1994) and modified by Mauricio et al. (1999).

Substrates and experimental design

Samples of CT-containing legumes (birdsfoot trefoil; **BTF**, cv. Langille and sainfoin; **SF**, cv. Shoshone), and CT-free alfalfa (**ALF***, cv. DK), were collected on June 07, 2015 on three monoculture plots of 0.17 ha each (spatial replications) seeded in August 2014 at the Utah State University Intermountain Irrigated Pasture Project research facility in Lewiston, northern Utah (41 56' N 111 52'W). Birdsfoot trefoil and alfalfa were cut at late bud stage and sainfoin in late flowering stage using a flail harvester (Rem Manufacturing Ltd., Swift Current, SK, Canada) at around 10 cm from ground level. Immediately after harvesting, samples of 250 g (particle size 2-4 cm) from each species were frozen at -20 °C and then freeze dried at -60 °C (Labconco Corporation Kansas

City, MO, USA) until constant weight and ground to pass a 1-mm screen with a Wiley mill (Thomas Scientific, Swedesboro, NJ, USA). Ground material of these legumes were combined in eleven different ratios (treatments). Treatments were: 1) ALF, 2) BFT, 3) SF (single forages), binary and trinary mixtures with proportions of species selected by lambs during a free-choice experiment (Lagrange and Villalba, 2016): 4) 70:30 ratios of: ALF/BFT (A70-B30), 5) ALF/SF (A70-S30), 6) SF/BFT (S70-B30), and 7) 50:35:15 ratio of ALF/SF/BFT (A50-S35-B15). Finally, treatments involved the equal part combinations (i.e., "no preference") of the legumes: 50:50 ratios for binary (8) A50-B50; 9) A50-S50; 10) S50-B50) and 11) 33:33:33 ratio for trinary mixtures (A33-S33-B33).

Five hundred milligrams of each one of these mixtures were weighted in small aluminum cups and placed in 125 ml serum flasks (Wheaton, Boston, USA) by triplicate. A total of 36 flasks (11 treatments x 3 replicates) plus 3 blanks were incubated in each batch.

Inoculum

Rumen fluid was taken 2 h post-feeding from a rumen-cannulated Angus cow on an *ad libitum* diet of tall fescue hay (Utah State University Institutional Animal Care and Use Committee, Approval # 2470). Rumen fluid pH was measured with a potentiometer (HI 991002, Hanna Instruments, Woonsocket, RI, USA) and averaged 6.9 ± 0.3.

In vitro fermentation procedure and gas production measurements

Forty ml of buffer medium prepared according to Menke (1988), were slowly added to each 125 ml serum flasks while flushing simultaneously with CO_2 for five seconds. Flasks were subsequently sealed with 20 mm butyl rubber stoppers and aluminum crimp caps (Wheaton Cia, Boston, USA), and stored overnight at 4°C. On the

next day, 20 ml of rumen fluid were injected into the flasks directly through the rubber

stopper, using a 25 ml syringe with a 18 gauge needle, 1:2 (v:v) rumen fluid : buffer

medium ratio. This time was considered time zero where the incubation process started.

pH of the buffer and ruminal fluid mixture at this time averaged 7.0 ± 0.1. The rumen

fluid was kept at 39°C untill all flasks were filled and shaked frequently in order to keep

adequate environmental conditions for the microorganisms. The portion of gas displaced

by the added liquid into the flask was allowed to escape prior to removing the needle

from the stopper. Then, flasks were shaked and placed in a preheated incubator (Percival,

Boone, IA, USA) at 39°C.

Head-space gas pressure in the flasks was read with an USB output pressure

transducer, (type PX409-015GUSBH, Omega Engineering Inc., Stamford, CT, USA)

connected to a PC that enabled to chart, log, display, and output data coming from the

transducer (Mauricio et al., 1999; Fig. A-4). Readings were taken at regular intervals of

2, 4, 6, 8, 10, 12, 18, 24, 36 and 48 h during the incubation period, inserting through the

flasks stoppers a 23-gauge needle which was attached to the pressure transducer through

a luer fitting-type connector (Fig. A-4). After the last reading, flasks were opened and the

pH of the solution measured. Flasks were placed into a fridge at 4ºC to slow down the

incubation and their contents immediately filtered.

Gas production kinetics

Gas volume estimates were generated for each incubation time from the gas

pressure values previously registered by the pressure transducer using the equation

reported by Frutos et al. (2002; Eq. (1)). Gas volumes were corrected for the amount of

substrate organic matter (OM) incubated and gas released from blanks (ruminal fluid plus buffer medium without substrate). Organic matter in the substrate was determined by ashing substrates at 550°C for 6 h (Thiex and Novotny, 2012). Corrected gas production estimates for each incubation time were then added in order to construct the gas production profiles of each treatment and gas production parameters were obtained using the Groot et al. (1996)'s single phasic model (Eq. (2)),

(1)   Head-space gas volume (ml) = 5.3407*gas pressure (psi)

(2) $G = A/(1 + (B^c/t^c))$

where G represents the amount of gas produced per unit of organic matter incubated at time t after the beginning of the incubation, A is the asymptotic gas production (ml g^{-1} OM); B (h) is the time after starting incubation at which half of the asymptotic amount of gas has been formed, representing the speed of gas production, and C is a constant determining the sharpness of the switching characteristics of the curve; as the value of C increases, the curve becomes sigmoidal with an increasing slope. The maximum rate of gas production (R_{Max}) and the time at which it occurs (T_{Max}) were calculated according to the fallowing equations (Bauer et al., 2001).

(3) R_{Max} (mL h^{-1}) = $(A*B^C*C*T_{Max}^{(-C-1)})/((1 + B^C*T_{Max}^{-C})^2)$

(4) T_{Max} (h) = $B*(((C-1)/(C+1))^{1/C})$

R_{Max} is obtained when the microbial population is big enough such that it no longer limits the fermentation process of the substrate and digestion is not reduced by chemical or structural barriers of the potentially digestible material at this point (Groot et al., 1996).

Substrate disappearance

Organic matter disappearance at 48 h incubation (organic matter degradability; dOM), was determined by filtering the flasks contents with 50 µm porosity (10 x 5 cm) ankon filter bags (Ankon Technology, Macedon, NY), previously oven dried and weighted. Bags were then washed with deionized water and dried in an air-forced oven at 60ºC to constant weight. Residual dry matter values were obtained by weighting bags with the digestion residues and extracting the empty dry bag weights. Dry matter degradation was calculated then by difference between the substrate and residue dry weights and corrected by the residual material measured in the blanks. Organic matter degradation (dOM) was determined by ashing the fermentation residues (see below). Finally, substrate disappearance allows for the calculation of a partitioning factor (*PF*) (Blümmel et al., 1997) which relates the amount of organic matter degraded *in vitro* to the gas volume produced by such amount, providing an estimate of fermentation efficiency.

Chemical analyses

Forages were analyzed for dry matter (DM), crude protein (CP), neutral (aNDF) and acid (ADF) detergent fiber, ADL (acid detergent lignin), condensed tannin (CT) content and ash. DM was determined using a two-step process. First, a partial drying using a forced-air drying oven at 60°C for 48 h, and secondly drying the samples at 105°C for 3 h in a forced-air drying oven as recommended by the National Forage Testing Association (Shreve et al., 2006). Crude protein was calculated by measuring the N content of the samples using a Leco FP-528 nitrogen combustion analyzer (AOAC,

2000; method 990.03) and applying the 6.25 conversion factor. aNDF (Mertens, 2002), ADF (AOAC, 2000; method 973.18) and ADL (Robertson et al., 1981) determinations were modified by using Whatman 934-AH glass micro-fiber filters with 1.5 μm particle retention and a California Buchner funnel in place of fritted glass crucible. Ash was determined burning samples at 550ºC for 6 h (Thiex and Novotny, 2012). Organic matter (OM) was calculated by difference between dry matter and ash. Analyses of total condensed tannins in the legumes were conducted in triplicate, according to the butanol-HCl-acetone spectrophotometric assay of Grabber et al. (2013), using purified CT from sainfoin and birdsfoot trefoil as the reference standard.

Statistical analyses

The experimental design was a completely randomized block design with three plots (spatial replications) and eleven treatments (different forage mixtures). Substrates and blanks were run twice per plot (experimental units), each run was conducted on a different week with three serum flasks (measurement units) per treatment, totalizing six runs in six consecutive weeks with 36 flasks/run.

Gas production parameters were estimated using PROC NLIN in SAS/STAT (SAS Inst., Inc. Cary, NC; Version 9.4 for Windows) with $A=200$, $B=20$ and $C=1$, as initial values. The estimated gas production parameters, maximum rate of digestion (R_{Max} Eq. (3)), time at which maximum rate occurs (T_{Max}; Eq. (4)), organic matter degradation (dOM) and partitioning factor (PF) were compared using a mixed model in which treatment was the main factor, plot and run as random factors. Analyses were performed using PROC GLIMMIX in SAS. Plot variation was found non-significant and therefore

dropped from the mixed model. Least square means (LSMeans) were compared pairwise using Tukey's multiple comparison test when F-ratios were significant ($P<0.05$) and reported along with their standard errors (SEM). Differences among LSmeans with $P<0.05$ were considered statistically significant. A tendency was considered when $0.10> P> 0.05$.

In order to explore the potential associative effects in the legume mixtures, observed to estimated values for gas production parameters and organic matter degradability were calculated as: 100 x [(Observed value – Estimated value)/Estimated value]. The estimated value was the weighted average of the observed values for the single substrates. Preplanned contrasts were performed to compare observed vs estimated values using the LSMESTIMATE statement in PROC GLIMMIX. Contrasts were specified as the arithmetic difference between the observed value for the specific binary or trinary diet and the estimated value from the average of their components (e.g. A70-S30 observed value vs ALF*0.7 + SF*0.3 observed values).

In addition, preplanned contrasts were performed to compare the average of gas production parameters for single species vs binary mixtures, single species vs trinary mixtures or trinary vs binary mixtures. A difference between the average of singles, binary or trinary mixtures groups or between observed and estimated values (associative effects) was considered significant when P values were < 0.05. Inspections of studentized residuals revealed no deviations from homoscedasticity of variance or normality.

RESULTS

Chemical composition of substrates

Chemical composition of the substrates assayed in the study is shown in Table 3-1. The greatest content of CP was observed in BFT, followed by ALF and then SF. In contrast, fiber concentration (NDF and ADF) was greater in SF than in BFT and ALF. Condensed tannin contents were on average 2.5 X greater in SF than in BFT. Alfalfa is a non-tannin containing legume, confirmed by the very low values of CT revealed in the assay (Table 3-1).

Table 3-1. Chemical composition (g/kg dry matter [SEM]) of the forages used in the study.

Species	CP	NDF	ADF	ADL	Ash	OM	CT
Alfalfa	168.0 (4.0)	364.0 (4.7)	306.7 (5.8)	65.0 (1.3)	89.8 (1.8)	910.2 (1.8)	1.9 (0.1)
Birdsfoot Trefoil	189.3 (2.0)	385.0 (2.0)	334.0 (4.0)	70.8 (2.9)	146.8 (3.1)	853.2 (3.1)	12.9 (0.7)
Sainfoin	131.7 (3.4)	438.0 (10.2)	391.0 (5.3)	86.2 (4.3)	73.9 (4.8)	926.1 (4.8)	31.0 (1.4)

CP= crude protein; NDF= neutral-detergent fiber; ADF= acid-detergent fiber; ADL= acid detergent lignin; OM= organic matter and CT= Condensed tannin content.

Organic matter disappearance

Regarding single substrates, ALF and BFT degradabilities were similar (P=0.999) and greater than SF (P<0.001) for both species, respectively (Table 3-2). The substitution of sainfoin for alfalfa significantly reduced the extend of degradation in the A50-S50 mixture, however A70-S30 did not differ from ALF (P>0.05). Similarly, the replacement of sainfoin for birdsfoot trefoil only reduced BFT degradability significantly at the higher proportion of sainfoin in the mixture (S70-B30). On average across all the mixtures, either 2- or 3-way combinations did not differ significantly from single forages and no

associative effect were observed in any of the mixtures (P>0.05).

Gas production kinetics

Cumulative gas production profiles, rate of gas production curves and parameters describing the cumulative gas production for each substrate are presented in Fig. 3-1 and Table 3-2, respectively. The mono-phasic model of Groot et al. (1996), fitted the gas production data obtained during the fermentation process (R^2 mean value = 0.999). All parameters were found different among substrates (P<0.01). The asymptotic gas production (A) was ALF > SF (P<0.001) > BFT (P<0.001).

The inclusion of the tannin-containing legumes (SF or BFT) in the 2- or 3-way mixtures, reduced (P<0.05) the asymptotic gas production compared with single ALF (Table 3-2). However, mixtures with BFT (A70-B30 and A50-B50) had significant lower *A* than mixtures with SF (A70-S30 and A50-S50). In fact, significant negative associative effects were observed for mixtures containing ALF and BFT (Table 3-2), to the point that no differences were observed in the asymptotic gas production between the A50-B50 mix and the BFT treatment (190.4 vs 187.6 ml/g OM, respectively).

Table 3-2. Organic matter degradation and gas production kinetics (LSmeans) of Alfalfa, Sainfoin and B. Trefoil mixtures incubated as single forages or in 2- and 3-way combinations.

Substrates	dOM	A (ml/g OM)	B (h)	C	R_{Max} (ml/h)	T_{Max} (h)	PF (mg/ml)
ALF	0.791 [abc]	210.6 [a]	7.3 [de]	1.48 [ab]	17.7 [a]	2.41 [c]	4.19 [d]
BFT	0.797 [ab]	187.6 [f]	7.0 [e]	1.57 [a]	16.5 [bc]	2.66 [abc]	4.75 [a]
SF	0.751 [e]	198.3 [cd]	9.5 [a]	1.45 [b]	12.9 [h]	3.00 [ab]	4.58 [abc]
A70-S30	0.775 [bcde]	203.3 [bc]	7.7 [cd]	1.48 [ab]	16.1 [cd]	2.56 [abc]	4.37 [cd]
A50–S50	0.766 [de]	206.0 [ab]	8.7 [b]	1.43 [b]	14.7 [ef]	2.62 [abc]	4.42 [bcd]
A70–B30	0.785 [abcd]	197.1 [cde]	7.0 [e]	1.53 [ab]	17.3 [ab]	2.51 [bc]	4.46 [bcd]
A50–B50	0.801 [a]	190.4 [ef]	7.7 [cd]	1.57 [a]	15.2 [de]	2.90 [abc]	4.77 [a]
S70–B30	0.768 [cde]	194.4 [def]	8.8 [b]	1.45 [b]	13.7 [fgh]	2.73 [abc]	4.68 [ab]
S50–B50	0.780 [abcd]	187.8 [f]	8.0 [c]	1.52 [ab]	14.3 [efg]	2.83 [abc]	4.77 [a]
A50–S35–B15	0.781 [abcd]	197.1 [cde]	8.2 [c]	1.50 [ab]	14.8 [e]	2.78 [abc]	4.59 [abc]
A33–S33–B33	0.787 [abcd]	193.4 [def]	8.9 [b]	1.50 [ab]	13.3 [gh]	3.06 [a]	4.80 [a]
S.E.M	0.007	2.5	0.2	0.03	0.6	0.18	0.13
P-value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.002	< 0.001
2-way vs singles	0.848	0.030	0.802	0.876	0.006	0.988	0.112
3-way vs singles	0.423	0.010	< 0.001	0.989	< 0.001	0.027	0.001
3- vs 2-way choices	0.283	0.294	< 0.001	0.880	< 0.001	0.015	0.017
Associative Effects:							
A70-S30 (%)	-0.5 ns	-1.7 ns	-2.9 ns	1.0 ns	-0.3 ns	0.9 ns	1.4 ns
A50-S50 (%)	-0.7 ns	0.8 ns	3.2 ns	-2.1 ns	-3.9 *	-2.4 ns	0.5 ns
A70-B30 (%)	-1.0 ns	-3.2 ***	-3.7 ns	1.8 ns	0.3 ns	1.5 ns	2.4 ns
A50-B50 (%)	0.7 ns	-4.4 ***	7.5 ***	3.1 ns	-10.7 ***	16.2 **	6.5 ***
S70-B30 (%)	0.3 ns	-0.3 ns	0.1 ns	-2.5 ns	-1.9 ns	-5.0 ns	1.0 ns
S50-B50 (%)	0.6 ns	-2.6 **	-2.7 ns	0.6 ns	-2.1 ns	1.4 ns	2.2 ns
A50-S35-B15 (%)	0.1 ns	-2.9 **	2.3 ns	1.1 ns	-6.8 ***	6.5 ns	4.1 *
A33-S33-B33 (%)	0.8 ns	-2.7 **	12.7 ***	0.2 ns	-15.2 ***	15.0 **	6.3 ***

dOM: Coefficient of organic matter digestibility; *A:* Asymptotic gas production (ml/g OM); *B*: time to half of the asymptote (h); *C*: Constant determining the sharpness of the curve; R_{Max}: maximum gas production rate (ml h^{-1}); T_{Max}: time at which R_{Max} occurs (h); *PF*: Partitioning Factor (mg OM disappeared/ml gas produced); *Associative effects (%):* 100 x [(observed value – Estimated value)/Estimated value]. Estimated value was the weighted average of the observed values for the pure substrates. Means in a column with different letters differ significantly (P<0.05); ns: non-significant; * P<0.05; ** P<0.01; *** P<0.001
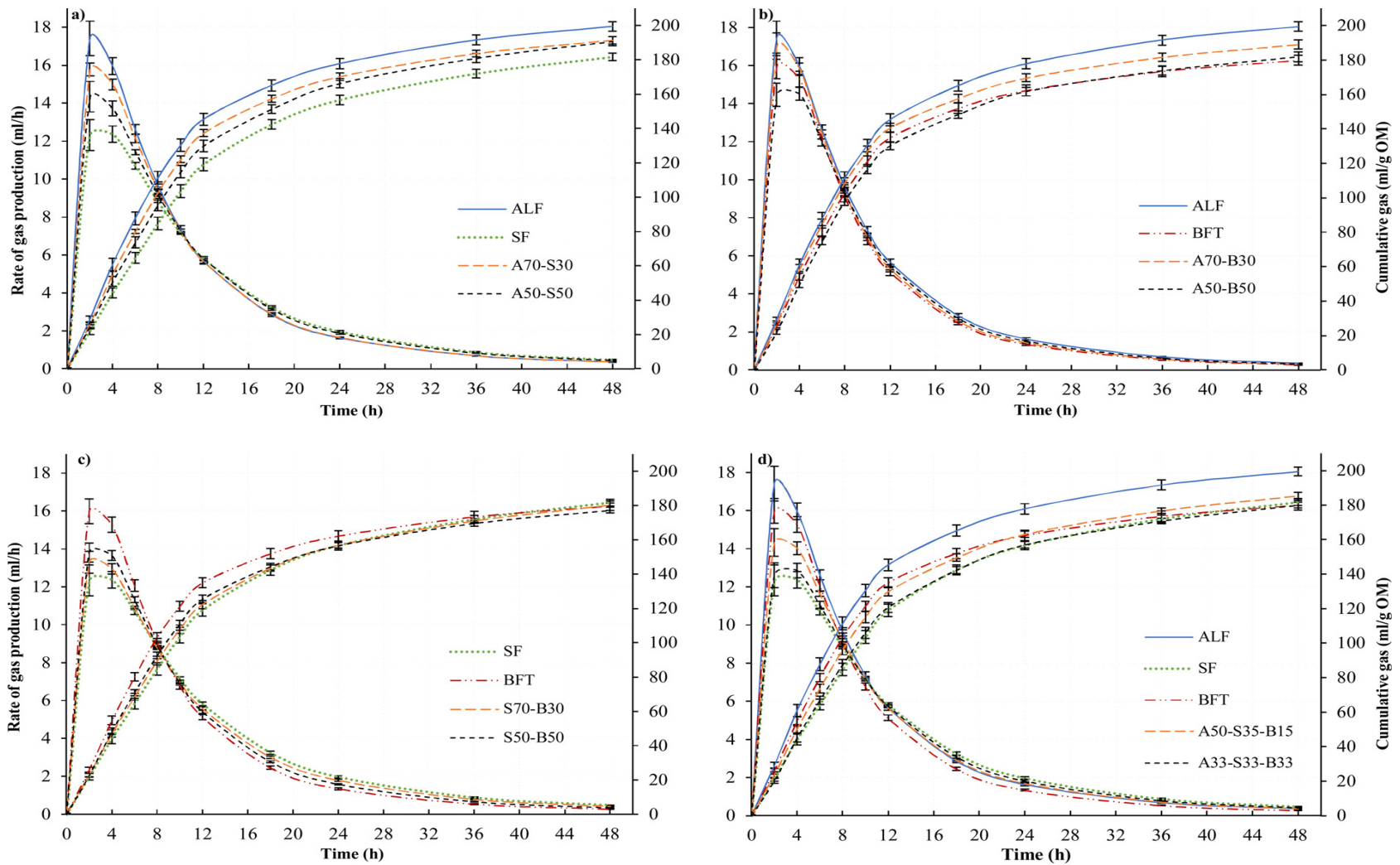
Figure 3-1. Cumulative gas production and rate of gas production profiles from different mixtures of a) Alfalfa and Sainfoin; b) Alfalfa and B. trefoil; c) Sainfoin and B. Trefoil; d) Alfalfa, Sainfoin and B. Trefoil. Bars represent standard errors of the mean (SEM).

Similarly, maximum gas production rates were reached faster (*Tmax*: 2.4 vs 3.0 h; P=0.016) and they were greater (R_{Max}: 17.7 vs 12.9 ml/h; P<0.001) for ALF than for SF, respectively, with A70-S30 and A50-S50 presenting intermediate values between ALF and SF treatments (Table 3-2). However, after *Tmax* was reached, ALF gas production rates deaccelerated faster than in SF such that after 8 h of incubation, gas production rate profiles looked very similar among all ALF-SF mixtures (Fig. 3-1a), and by 18 h the SF rate was greater than that of ALF maintaining this trend towards the end of the incubation period. Consistent with this trend, the ALF treatment required less time than SF to reach half of the potential gas production (parameter *B*: 7.3 vs 9.5 h; P<0.001, respectively) and that time was extended as the proportion of SF in the mixture increased.

Maximum gas production rate was also greater for ALF than for BFT (R_{Max}: 17.7 vs 16.5 ml/h, respectively; P=0.023), but in contrast with SF, gas production rates in BFT began to deaccelerate rapidly after 12 h of incubation, and gas production almost reached its asymptotic value after 18 h (Fig. 3-1b). Similar to ALF, the rates of gas production in BFT were greater than in SF only at early incubation times (e.g., between 2 – 8 h; Fig. 3-1c), decreasing R_{Max} (P<0.05) as the proportion of SF increased in the BFT-SF mix (Table 3-2).

In general, no significant differences in gas production parameters were observed between the average of binary mixtures and the average of single substrates, except for the potential gas production (*A*: 198.8 vs 196.5 ml/g OM) and maximum rate of gas production (R_{Max}: 15.7 vs 15.2 ml/h) for single forages vs 2-way mixtures, respectively (Table 3-2), likely driven for the negative associative effects observed for these parameters in the ALF-BFT mixtures.

When both tannin-containing legumes were incubated with alfalfa, regardless of their proportions in the 3-way mixtures, reduced the rates of gas production at the beginning of the incubation process relative to ALF (R_{Max}: 17.7, 14.8 and 13.3 ml/h for ALF, A50-S35-B15 and A33-S33-B33, respectively), and extended the time to reach half of the potential gas production (P<0.05). The delays in gas production increased with increments in the proportion of tannin-containing legumes in the mixture (T_{max}: 2.4, 2.8 and 3.1 h; Parameter B: 7.3, 8.2 and 8.9 h; for ALF, A50-S35-B15 and A33-S33-B33, respectively; P<0.05). In fact, the gas profile for the A33-S33-B33 mixture was very similar to the profile observed for pure sainfoin (SF), while A50-S35-B15 showed intermediate values between the singles substrates (Fig. 3-1d).

Some negative associative effects were present specially for R_{Max} (P<0.001) when equal proportions of alfalfa and birdsfoot trefoil were combined (A33-S33-B33) as in the binary mixture (50A-50B) and in T_{Max} and parameter B where the time was longer for observed than for estimated values (P<0.001). In fact, the average of both trinary mixtures (A50-S35-B15 and A33-S33-B33) showed a lower gas production rate (R_{Max}: 14.1 vs 15.7 ml/h; P<0.001) and potential gas production (A: 195.3 vs 198.8 ml/g OM; P<0.05) than the average of the three single substrates respectively, and a greater T_{Max} (2.9 vs 2.7 h; P<0.05) and parameter B (8.6 vs 7.9 h; P<0.001).

DISCUSSION

Organic matter disappearance

The greater OM degradability in ALF and BFT than in SF is likely attributable to the lower concentrations of ADF in the first two forages, in line with the more advanced

stage of maturity of sainfoin at the time of being sampled. BFT and ALF were cut at the early flowering stage, while SF was harvested in the late flowering stage of the first growth cycle. Our results are consistent with prior research (Niderkorn et al., 2011), showing greater values of *in vitro* degradability in ALF than in SF.

Previous studies found negative correlations between *in vitro* OM disappearance and CT contents (Frutos et al., 2002) with concentrations of CT generally greater than 50 g/kg. It is likely that the lower content of CT in the legumes of this study compounded with their chemistries (Mueller Harvey et al., 2017) did not influence forage degradability. In fact, we did not find any differences for this parameter between a non-tannin (alfalfa) containing legume and birdsfoot trefoil. Consistent with this notion, previous *in vitro* (Wang et al., 2007) and *in vivo* (Theodoridou et al., 2010, 2012) studies did not find any increments in sainfoin OM digestibility when polyethylene glycol (PEG), a binding agent that suppresses tannin activity, was included in the incubation or dosed directly to the rumen, discarding any influence of CT on sainfoin degradability.

Degradability values in mixtures were in general a linear combination of the values found in the pure substrates, but there were some exceptions. For instance, when the proportion of sainfoin in the mix with alfalfa was 0.30, dOM values did not differ from those observed for ALF. However, when proportion of SF in the mix grew to 0.50, there was a significant reduction in dOM relative to ALF. These results suggest that alfalfa might be mixed with sainfoin up to a proportion of 0.30 without negative impacts on organic matter degradability, which help explain the proportions of alfalfa and sainfoin selected by lambs in a cafeteria test (Lagrange and Villalba, 2016). Ruminants select diets based on the association between the taste of a food and its post-ingestive

consequences (Provenza, 1995), so it is likely lambs selected a 70:30 ratio of alfalfa/sainfoin to maintain degradability values of the mix as high as pure alfalfa with the benefit of including a tannin-containing legume like sainfoin in the diet.

The addition of SF to BFT (SF-BFT mixtures) also reduced dOM relative to BFT, but only with the highest proportion of sainfoin in the mixture (S70-B30). In this case, lambs offered a free choice of the same two legumes used in this study preferred sainfoin over birdsfoot trefoil in a 70:30 ratio, suggesting that factors other than digestibility might have driven this selection. Considering the high CP content observed in BFT, the proportion selected may represent the need to attenuate the accumulation of NH_3 in the rumen through the ingestion of CT from sainfoin (Chung et al., 2013; Copani et al., 2015), particularly given that excesses of NH_3 in the rumen fluid are aversive, promoting reductions in food intake (Provenza, 1995; Villalba and Provenza, 1997).

Finally, another important result of this study is that the presence of sainfoin along with alfalfa and birdsfoot trefoil in trinary mixtures resulted in digestibilities values comparable to those observed for pure ALF or BFT. Thus, the selection that lambs performed during preference tests (A50-S35-B15) with the same forages used in this study allowed for an increased dietary diversity with digestibility values comparable to those observed in single legumes that exhibited the greatest values for this parameter.

Gas production kinetics

The slower rate of gas production for SF at early incubation times could be due in part to its advanced stage of maturity, with greater concentration of cell walls and lower crude protein content (Guglielmelli et al., 2011). In support of this, Niderkorn et al.

(2011) found that *in vitro* fermentation of sainfoin led to lower VFA concentration and gas production than alfalfa during the first hours of incubation. We also observed that ALF and BFT - with lower contents of fiber and greater concentration of protein –had greater gas production rates and greater levels of gas produced at shorter periods of time than in the SF treatment.

According to Groot et al., (1996), the initial time of the incubation period is related to the fermentation of the soluble, fast fermentable fraction of the substrate (i.e., soluble carbohydrates) and microbial protein synthesis, whereas the last portion of the incubation period is related to the fermentation of the insoluble but potentially degradable components like the NDF fraction. This is in line with the proportionally greater amounts of gas production observed during the latter incubation times of the SF treatment, which contrasts with the deaccelerating gas production process observed for ALF and especially for BFT. In addition, sainfoin is characterized for a very low fiber digestion at early incubation times (Niderkorn et al., 2011), and previous studies reported a negative impact of CT on gas production for sainfoin substrates (Theodoridou et al., 2011). Thus, it can be concluded that CT in SF may be contributing along with the greater contents of fiber and lower concentration of protein to the reductions in the rate of gas production and potential gas production observed in this study. The influence of CT in BFT might be different from that described for SF since gas production rates at early incubation times were greater for BFT than for SF. The different CT concentrations measured in SF and BFT along with differences in chemical structures (McAllister et al., 2005; Mueller-Harvey, 2006) may be driving the distinct effect of CT on the digestion process.

The asymptotic gas production in BFT was lower than in ALF despite similar

organic matter degradabilities and nutritional composition. It is possible that the organic

matter degraded in BFT led to lower production of VFA and gasses, shifting more

substrate to microbial synthesis (Blümmel et al., 1997), supported in this study by the

greater partitioning factor observed for BFT.

The amount of gas produced by the different forages at the beginning of

incubation could be used to predict a ranking of DMI intake across species when

presented as single forages or preference when presented in cafeteria tests since gas

production is positively correlated with greater digestibility, greater energy content of the

forage and potentially reduced fill effect (Blummel et al., 2005). According to our *in vitro*

results, we might expect the greatest dry matter intake for ALF, because of its greater gas

production rate at the beginning of incubation, potential gas production and degradability

values, followed by BFT and then by SF. When lambs were offered the legumes used in

this study (Lagrange and Villalba, 2016), intake values were ALF > SF > BFT. It is likely

that other variables like the high concentration of CP in BFT limited the ingestion of this

forage since it is known that ruminants reduce intake of forages high in CP content in

order to maintain blood ammonia concentration below toxic levels (Provenza, 1995).

Mixing sainfoin with alfalfa in a ratio that represented lambs' preference (A70-

S30), reduced the rate of gas production (R_{Max}) compared to pure ALF. However, the

time to reach maximum rate (T_{Max}) and half of potential gas production (B) was not

modified by the inclusion of SF in the mix. These results suggest that ruminants might

take advantage of the extra benefits of incorporating sainfoin to their diets while

maintaining high rates of ruminal fermentation. On the other hand, when the proportion

of sainfoin in the mix increased to 0.50, both parameter B and R_{Max} were lower than for

pure ALF.

Our results suggest that BFT may be mixed with ALF in a proportion of 0.30 without producing any changes in the rate of gas production relative to pure ALF, although the potential gas production may be affected when BFT is at that level in the mix. When the amount of BFT increased up to 0.50 in the mix, potential gas production and gas production rates declined, resulting in a gas production profile more similar to pure BFT than to the average of values observed by the two singles substrates. This slightly antagonistic effect observed on the gas production rates for the A50-B50 mixture is then translated into lower amounts of total gas production at the end of the incubation period. Additionally, these results also contribute to explain lambs' preference for ALF over BFT (70:30) when they had *ad libitum* access to both forages. At this ratio, the rate of gas production was not different from pure ALF and lambs incorporated a tannin-containing legume to their diet with the benefits of reduced incidence of bloat described above.

The lambs' preferred trinary mixture (A50:S35:B15) exhibited better gas production rates which occurred at earlier incubation times than for the equal parts mixture (i.e., indifferent preference value; A33:S33:B33). As in the binary mixtures, the presence of the three species together did not trigger any synergic effects with regards to gas production kinetics, and the combination of these three species in general slowed down the fermentation process relative to the responses observed when the forages were incubated as single species. Moreover, some antagonistic effects on gas production rate were observed when these species were combined at equal proportions (A33:S33:B33). Therefore, the proportion at which these three legume species are combined affects the

fermentation process, and combinations do not appear to enhance gas production kinetics in terms of rate or potential gas production. Nevertheless, certain proportions –like those selected by lambs when fed the same forages assayed in the present study- improve fermentation relative to others (i.e., equal proportions) – allowing the animal to take advantage of other benefits of diet mixing, i.e., reduced bloat and NH_3 formation in the rumen or reduced sensory specific satiety (triggered by ingesting the same food too frequently or in excess), which reduces food intake (Provenza, 1996).

CONCLUSIONS

This study shows a greater OM degradability and rate of gas production in alfalfa and birdsfoot trefoil than in sainfoin, attributable to the greater contents of cell walls and lower concentration of protein in sainfoin. The 70:30 alfalfa/sainfoin or alfalfa/birdsfoot trefoil ratio showed greater rate of gas production than mixtures formed with equal proportions of the legumes (i.e., indifferent preference). The presence of the two tannin-containing legumes along with alfalfa in the trinary mixtures did not trigger any positive associative effects on degradability or gas production kinetics. In addition, trinary mixtures were not as fermentable as binary mixtures, which contained a greater proportion of alfalfa. In conclusion, mixing tannin-containing legumes with alfalfa could give ruminants the advantage of maintaining high rates of ruminal fermentation while ingesting beneficial bioactive compounds, as well as benefits related to dietary diversity in generalist herbivores like improvements in food intake due to reductions in sensory-specific satiety.

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CHAPTER 4

GRAZING DIVERSE COMBINATIONS OF TANNIFEROUS AND NON-

TANNIFEROUS LEGUMES: IMPLICATIONS FOR BEEF CATTLE

PERFORMANCE AND ENVIRONMENTAL IMPACT

ABSTRACT

Combinations of "non-traditional" legumes that have lower concentrations of fiber and greater concentrations of nonstructural carbohydrates than grasses, coupled with different types and concentrations of secondary compounds such as condensed tannins (*Lotus corniculatus*, birdsfoot trefoil; *Onobrychis viciifolia*, sainfoin) can create more sustainable beef production systems than monocultures of grasses or non-tanniferous legumes such as alfalfa (*Medicago sativa*). We tested the effect of increasingly diverse combinations of these legumes on cattle performance, methane (CH_4) emissions and nitrogen (N) balance. Forty-two heifers (401 ± 49.6 kg, 2 per treatment replication) grazed three spatial replications of 7 treatments: monocultures of birdsfoot trefoil (BFT), sainfoin (SF), or alfalfa (ALF), and 2- and 3-way choices among strips of sainfoin and birdsfoot trefoil (SF-BFT), alfalfa and birdsfoot trefoil (ALF-BFT), alfalfa and sainfoin (ALF-SF), and alfalfa, sainfoin and birdsfoot trefoil (ALF-SF-BFT) in a completely randomized block design in two 15-d periods during two consecutive years. Average daily gains (ADG) in heifers grazing the tanniferous legumes (1.05 kg/d) were 40% greater (P<0.10) than in heifers grazing ALF (0.74 kg/d) during the first year, but not the second year, of the study. Heifers grazing 3-way choices had greater intakes of dry matter (DMI) (10.4 vs 7.8 kg/d; P=0.064) and greater ADG than animals grazing monocultures

(1.21 vs. 0.95 kg/d, P=0.054 and 1.43 vs. 0.95 kg/d; P=0.085 for the first and second year of the study, respectively), suggesting a synergism among legumes. In the first year of the study, CH_4 emissions for tanniferous vs. non tanniferous legumes were 249.9 and 335.6 g/kg body weight (BW) gain (P=0.216). During the second year, the average CH_4 emissions for tanniferous and non-tanniferous legume monocultures was 220.2 and 224.7 g/kg BW gain (P=0.922), and for monocultures vs. 2- and 3-way choices, 221.7 vs. 202.0 and 161.8 g/kg BW gain (P>0.10), respectively. Heifers grazing SF and BFT had lower blood urea N (14.3 and 16.8 vs 20.8 mg/dL; P<0.05) and urinary N concentrations (3.7 and 3.5 vs 6.0 g/L; P<0.05) and greater fecal N concentrations (34.5 and 35.5 vs 30.5 g/kg; P<0.05) than those grazing ALF. Combining both tanniferous legumes (SF-BFT) led to the greatest declines in urinary N (2.24 g/L) and urea-N (1.71 g/L) concentration, suggesting that different types of tannins in different legume species result in associative effects that enhance N economy in grazing ruminants. In addition, heifers grazing 3-way choices partitioned less N into urine (40.7 vs 50.6%; P=0.037) and retained more N (36.1 vs 25.2%, P=0.046) than the average for heifers grazing monocultures. Collectively, these responses would contribute to reductions in NH_3 volatilization and N_2O emissions to the environment. In summary, combinations of tanniferous legumes with alfalfa improved animal performance and reduced environmental impacts relative to pasture monocultures, resulting in a more sustainable approach to beef production in pasture-based finishing systems.

INTRODUCTION

Forage-finished beef is a niche market that is growing rapidly in the United States

(Cheung et al., 2017). This production system benefits from the fact that ruminants do not require concentrates such as grain, as they can derive energy from the cellulose of forages and other feeds that cannot be digested by swine or poultry (Van Soest, 2018). Nevertheless, grass-based forage finishing systems require more animals and land than grain-based feedlot systems for equal annual red meat production, while producing larger carbon and nitrogen (N) footprints (Capper, 2012).

In contrast to both cereal grains and pasture grasses, perennial legumes fix their own N, and unlike annual grain crops, perennial legumes are productive for multiple years after establishment without additional cultivation or planting (Temperton et al., 2007; Pirhofer-Walzl et al., 2012). Legume forages are digested more rapidly than grasses by ruminants (Phelan et al., 2015), so intake and production are higher than for forage grasses (Van Soest, 2018). Alfalfa (*Medicago sativa* L.) is the most high-yielding and nutritious forage available for feeding high-producing ruminants in North America (Yost et al., 2020), although pure stands of this legume present a high risk of bloat to grazing ruminants (Wang et al., 2012). In addition, the high concentration and ruminal degradability of alfalfa protein along with insufficient energy concentration, results in poor protein utilization by rumen microorganisms, which leads to buildup of NH_3 in the rumen and high urinary urea excretion (Getachew et al., 2006). Nitrogen excretion contributes to environmental pollution via NH_3 or nitrous oxide (N_2O) volatilization from the soil surface or nitrate (NO_3) in soil that may be leached into ground water (Leip et al., 2015).

Tanniferous legumes like birdsfoot trefoil (*Lotus corniculatus*) and sainfoin (*Onobrychis viciifolia* Scop) are non-bloating and can therefore be grazed in pure stands.

The unique condensed tannins (CT) produced by birdsfoot trefoil (Waghorn, 2008) as well as its elevated fiber digestibility (Hunt et al., 2014a, 2014b; Christensen et al., 2015) and non-fibrous carbohydrate concentration (Chail et al., 2016) enhance the efficiency of protein use in ruminants relative to other perennial legumes. Likewise, CT in sainfoin enhance ruminant nutrition relative to other perennial legumes like alfalfa (Wang et al., 2015). In addition to these benefits, CT may also suppress ruminal methanogenic microbes (Saminathan et al., 2016) and inhibit fiber digestion (Vasta et al., 2019), reducing enteric methane (CH_4) production. Methane is a greenhouse gas (GHG) 28 times more potent than CO_2 (IPCC, 2014) and enteric fermentation from ruminants is a major source of GHG emissions, accounting for 39% of all GHG emissions from the livestock sector (Gerber, 2013), and between 11-13% of global CH_4 emissions (Beauchemin, 2009).

Greater available diversity of forages and resulting enhanced chemoscapes in pasturelands (Villalba et al., 2019) may enhance the benefits described above because complementary relationships among multiple food resources in nature improves the fitness of herbivores (Tilman, 1982), which in turn could reduce environmental impacts. There is a gap in knowledge regarding the potential complementary effects of different legumes on N use efficiency and on the GHG footprint of beef cattle.

We hypothesized that forage diversity and tanniferous legumes would improve productivity and reduce environmental impacts relative to non-tanniferous legumes or forage monocultures. Thus, the aim of this study was to evaluate the synergistic effect of increasingly diverse combinations of tanniferous (birdsfoot trefoil; sainfoin) and non-tanniferous (alfalfa) legumes on performance, enteric CH_4 emissions and N retention in

beef cattle during the finishing phase of production.

MATERIAL AND METHODS

The study was conducted at the Utah State University pasture research facility in Lewiston, UT (41 56' N 111 52'W, 1382 m altitude), according to procedures approved by the Utah State University Animal Care and Use Committee (approval 2566). The experiment took place from June 21 to September 2 in 2016 and from June 5 to August 23 in 2017.

Pastures and experimental design

Seven pastures treatments (three single forage species, three 2-way and one 3-way combinations) were established in a randomized complete block design with 3 blocks (replications). Pastures were grazed during two periods in two consecutive years, 2016 and 2017 (Table 4-1). Treatments included monocultures of two tanniferous legume species: sainfoin (*Onobrychis viciifolia* cv. Shoshone; SF) and birdsfoot trefoil (*Lotus Corniculatus* cv. Langille; BFT), and one non-tanniferous legume, alfalfa (*Medicago sativa* cv. Vernal; ALF), and all 2- and 3-way choices among these legumes, sainfoin and birdsfoot trefoil (SF-BFT), alfalfa and birdsfoot trefoil (ALF-BFT), alfalfa and sainfoin (ALF-SF), and alfalfa, sainfoin and birdsfoot trefoil (ALF-SF-BFT). All treatment plots had an area of 0.5 ha each and were randomly distributed within each block. Within each plot of the legume combinations, there were two 0.25-ha strips (2-way choices), or three 0.165-ha strips (3-way choice), seeded in random order with alfalfa, sainfoin and/or birdsfoot trefoil, depending on the treatment (Fig. A-5). Thus, in each 2- and 3-way choice

plot, cattle could freely graze on any of the two or three species on offer (Fig. A-6 and A-7). The perimeters of the experimental plots were defined by electric fencing (Fig. A-8).

Pastures of sainfoin, birdsfoot trefoil and alfalfa were seeded on irrigated land at the research facility at rates of 36.8, 11.0, and 19.7 kg of pure live seed/ha, respectively, on September 2, 2015. Seeds of sainfoin and alfalfa were previously inoculated with Bradyrhizobium and Sinorhizobium meliloti (N-Dure; INTX Microbials LLC, Kentland, IN), respectively. Seeds of birdsfoot trefoil were inoculated with Rhizobium loti (Exceed; Visjon Biologics, Wichita Falls, TX) before planting. During the first year of establishment, all plots were sprayed with 2.5 L/ha of Butyrac® 200 (2,4-DB; Albaugh Inc., Ankeny, IA) for broadleaf weed control on April 15, 2016. On May 19, 2016, the first growth cycle of the legumes was mowed, cured and baled. On June 10, 2016 900 ml/ha of Plateau® (imazapic; BASF Corp., Durham, NC) was sprayed in all pastures for control of grass weeds. In year 2 (2017), all plots were sprayed with 440 ml/ha of Thunder® (imazethapyr; Albaugh Inc., Ankeny, IA) for broadleaf weed control and 730 ml/ha of Volunteer® (clethodim; Tenkōz Inc., Alpharreta, GA) for grass weeds on May 3, 2017. Grazing was delayed according to the manufacturer's recommendations.

Table 4-1. Dates and duration of grazing periods, phenological stages and measurements in both years of the study.

DATE	Year 2016		Year 2017	
5-Jun			Adaptation period 1	
6-Jun				
7-Jun				
8-Jun				
9-Jun				Initial Weight
10-Jun				
11-Jun				
12-Jun				
13-Jun				
14-Jun				
15-Jun			Experimental Period 1	
16-Jun				
17-Jun			Phenological Stage	Forage quality sampling
18-Jun			(First growth cycle)	Pre-grazing herbage availability
19-Jun				CH4 Sampling
20-Jun				CH4, Fecal and Urine sampling
21-Jun	Adaptation period 1		ALF: Full bloom	CH4, Fecal and Urine sampling
22-Jun			BFT: Full bloom	CH4, Fecal and Urine sampling
23-Jun			SF: Early seed pod	CH4, Fecal, Urine and Blood sampling
24-Jun				Fecal sampling
25-Jun				
26-Jun				
27-Jun				
28-Jun				Final weight, Post herbage availability
29-Jun				
30-Jun	Experimental Period 1	Initial Weight		
1-Jul				
2-Jul		Forage quality sampling		
3-Jul		Pre-grazing herbage availability		
4-Jul		CH4 Sampling		
5-Jul		CH4, Fecal and Urine sampling		
6-Jul		CH4, Fecal and Urine sampling		
7-Jul	Phenological Stage	CH4, Fecal and Urine sampling		
8-Jul	(Second growth cycle)	CH4, Fecal, Urine and Blood sampling		
9-Jul	ALF: Early bloom	Fecal sampling		
10-Jul	BFT: Early bloom			
11-Jul	SF: Full bloom	Post-grazing herbage availability		
12-Jul				
13-Jul				
14-Jul				
15-Jul			Overflow pasture of endophyte-free Tall Fescue	
16-Jul				
17-Jul				
18-Jul		Final Weight		
19-Jul				
20-Jul				
21-Jul				
22-Jul				
23-Jul				
24-Jul				
25-Jul				
26-Jul				
27-Jul				
28-Jul	Overflow pasture of endophyte-free Tall Fescue			
29-Jul				
30-Jul				
31-Jul				
1-Aug			Adaptation period 2	
2-Aug				
3-Aug				
4-Aug				Initial Weight
5-Aug				
6-Aug				
7-Aug				
8-Aug				
9-Aug				
10-Aug			Experimental Period 2	
11-Aug				
12-Aug	Adaptation period 2	Initial Weight		Forage quality sampling
13-Aug				Pre-grazing herbage availability
14-Aug			Phenological Stage	CH4 Sampling
15-Aug			(Regrowth)	CH4, Fecal and Urine sampling
16-Aug			ALF:Full bloom	CH4, Fecal and Urine sampling
17-Aug			BFT: Full bloom	CH4, Fecal and Urine sampling
18-Aug	Experimental Period 2		SF: Late bud	CH4, Fecal, Urine and Blood sampling
19-Aug				Fecal sampling
20-Aug		Forage quality sampling		
21-Aug		Pre-grazing herbage availability		
22-Aug		CH4 Sampling		Post-grazing herbage availability
23-Aug		CH4, Fecal and Urine sampling		Final Weight
24-Aug	Phenological Stage	CH4, Fecal and Urine sampling		
25-Aug	(Regrowth)	CH4, Fecal and Urine sampling		
26-Aug	ALF: Late bud	CH4, Fecal, Urine and Blood sampling		
27-Aug	BFT: Late bud	Fecal sampling		
28-Aug	SF: Late vegetative	Post-grazing herbage availability		
29-Aug				
30-Aug				
31-Aug				
1-Sep				
2-Sep		Final Weight		

Animals and grazing protocol

In each year, a different set of 42 Angus heifers were sorted by body weight (BW) and distributed among 7 groups of 6 animals with similar total weight per group. Groups were randomly assigned to the 7 treatments. Heifers within treatments were grouped in pairs with similar individual weight and each pair was randomly assigned to one of the 3 treatment replications (blocks) (n=3). The heifers' initial and final mean BW was 394 ± 54 kg and 436 ± 55 kg, respectively, for 2016, and 352 ± 40 kg and 421 ± 42 kg, respectively, for 2017.

Each experimental period was preceded by a 10-day adaptation phase to familiarize all animals with their respective dietary treatment (Table 4-1). Period 1 of 2016 occurred from June 30 to July 18, and Period 2 from August 18 to September 2. During 2017, Period 1 occurred from June 15 to June 28, and Period 2 from August 10 to August 23. Measurements were taken during 5 consecutive days in each experimental period (collection period; Table 4-1). Between experimental periods, animals grazed an overflow pasture of endophyte-free tall fescue, until legumes grew sufficiently to be grazed again. All pastures were irrigated using hand-line sprinklers in 12-h sets that applied approximately 10.5 cm of water.

Heifers strip-grazed their respective pastures behind electric fences that were moved approximately every three days to give access to fresh forage, and back-fenced to prevent access to previously grazed forage and allow legumes to re-grow. In Period 2, heifers grazed legumes that had regrown for approximately 45 days. Heifers were moved to a new section once they had grazed 20 to 30% of the available biomass in monocultures or when any legume strip was grazed to that extent in 2- or 3-way choice

treatments. This procedure ensured *ad libitum* forage availability for all of the species present in each treatment.

Throughout the adaptation and sample collection phases, animals had free access to water and trace-mineral salt blocks (mineral composition: minimum 960 g/kg NaCl, 320 mg/kg Zn, 380 mg/kg Cu, 2,400 mg/kg Mn, 2,400 mg/kg Fe, 70 mg/kg I, and 40 mg/kg Co). Animals on all treatments had access to bloat protectant blocks with Poloxalene 6.6% (Sweetlix® Pressed Bloat Guard®, Ridley USA Inc., Mankato, MN) for 2 days before entering the adaptation phase in order to reduce the likelihood of frothy bloat in animals that were assigned to ALF. All animals were given ear tags to control flies before beginning the first experimental period in each year.

Data collection

Herbage availability

Herbage dry matter (DM) availability per unit area in each plot was assessed before animals entered new paddocks (pre-grazing herbage mass) on July 3 and August 21, 2016 and on June 18 and August 13, 2017 for the first and second experimental periods, respectively (Table 4-1). Herbage availability was also evaluated after animals grazed these paddocks (post-grazing herbage mass). Measurements were made by taking 60 readings in each paddock (monocultures) or in each monoculture strip of 2- and 3-way choices using a rising plate pasture meter (Electronic Plate Meter Jenquip EC-10, Agriworks Ltd, NZ). Each paddock was sampled in a "lazy W" pattern and every four steps the plate meter was lowered vertically onto the herbage. Calibration curves for each legume were developed from individual rising plate meter readings of pre- and post-

grazing herbage at a range of heights. All forage under the plate meter was cut to the ground using a 0.10-m^2 quadrant frame, the same area as the plate meter, and dried at 60°C to constant weight. Linear relationships for each experimental period and each legume were estimated from calibration curves of DM herbage biomass on plate meter readings.

Forage quality sampling

Representative samples of the herbage ingested by heifers were collected on day 3 of each experimental period from each replication of each forage treatment. Herbage samples were collected between 1000 and 1300 h by walking a transect across a pasture section and hand-plucking the top 15-20 cm of the sward every few steps, mimicking the plant parts grazed by heifers. Samples were placed in plastic bags, covered with dry ice, and frozen at –20°C until they were freeze-dried (Free Zone 18 liters, Labconco Corporation, Kansas City, MO), and ground to pass the 1-mm screen of a Wiley mill (model 4; Thomas Scientific Swedesboro, NJ, USA) for chemical analyses.

Nutritional composition of diets, fecal composition
and in vivo digestibility calculations

Fecal grab samples were taken from the rectum of the animal in each pair that was also used for CH_4 emissions and N utilization calculations, during days 6 to 10 of the sample collection period. Samples were collected between 0800 and 1000 h in 2016 and between 0730 and 0930 h in 2017. Samples were immediately frozen under dry ice, then frozen at -20 °C until they were freeze dried and ground. Daily samples were composited proportionally for each heifer for the 5-d collection phase and analyzed for total N, acid

detergent fiber (ADF) and acid detergent lignin (ADL).

Diet DM digestibility (DMD) was determined using the concentration of ADL in the forage consumed and in feces as an internal marker (Van Soest, 2018) as follows:

%DMD = 100 – 100 × [% ADL in forage consumed / % ADL in feces] (Cochran and Galyean, 1994).

In the 2- and 3-way choice treatments, the concentration of ADL in the forage consumed was calculated as the weighted average of the proportion of forage that disappeared (calculated as pre- minus post-grazing herbage mass) from each allocated forage (Alfalfa$_{Dissap}$, Sainfoin$_{Dissap}$, Birdsfoot trefoil$_{Dissap}$) as follows:

% ADL in forage consumed = [ADL]$_{Alfalfa}$ × (% Alfalfa$_{Dissap}$/100) + [ADL]$_{Sainfoin}$ × (% Sainfoin$_{Dissap}$/100) + [ADL]$_{Birdsfoot\ trefoil}$ × (% Birdsfoot trefoil$_{Dissap}$/100).

The same approach was used to estimate the concentration of ADF, CP and CT in the forage consumed.

The digestion coefficient for different nutrients in the feed can be measured as follows

Digestibility (%) = 100 -100 (% marker in the feed / % marker in the feces) × (% nutrient in the feces / % nutrient in the feed) (Cochran and Galyean, 1994).

Therefore, acid detergent fiber digestibility (ADFD) was calculated using the concentration of ADL in the forage consumed and in feces as an internal marker (Van Soest, 2018), and the concentration of ADF in the forage consumed and in feces, applying the formula:

%ADFD = 100 – 100 × [(% ADL in forage consumed / % ADL in feces) × (% ADF in feces / % ADF in forage consumed)].

Urine and blood sampling

Urine samples were collected from days 6 to 9 of the sample collection period in conjunction with fecal sampling and from the same animals used for fecal collection by inducing urination through vulvar stimulation. Twenty-five ml of urine were collected and transferred to a prelabeled 120 ml specimen container with 3.125 ml of 0.2N HCl to acidify the sample and avoid N losses through volatilization. Acidified urine pH was measured with a pH meter (HI 991002, Hanna Instruments, Woonsocket, RI, USA) and averaged 3.0. Urine samples were immediately placed in a cooler with dry ice until all the samples were collected and then stored in freezer at -20 °C. Prior to assay, samples were thawed, composited by heifer over the 4-d collection phase and frozen again at -20 °C until analyses.

Blood samples were collected from the same animals used for fecal and urine collection on day 9 of each sample collection period. Samples were collected between 0800 and 1000 h in 2016 and between 0730 and 0930 h in 2017 from the coccygeal vein, using prelabeled 10 mL serum vacutainer tubes (without EDTA added; Becton Dickinson Vacutainer System; Becton Dickinson and Company, Franklin Lakes, NJ) and 18-gauge needles. Samples were allowed to clot for at least 30 min before being centrifuged at room temperature (1500 rpm for 15 min) using a benchtop centrifuge (ELMI CM-7S, CA, USA). Serum samples were frozen at -20 °C until analyses.

Methane emissions

Enteric CH_4 was measured using the sulfur hexafluoride (SF_6) tracer gas technique (Johnson et al., 2007) in two of the treatments in 2016 (ALF and SF; Fig. A10

and A-11, respectively), and in all treatment diets for 2017. A brass permeation tube with a known release rate of SF_6 (average 4.13 ± 0.475 mg/d) was placed in the reticulorumen of each heifer using a balling gun to serve as an internal tracer 20 days before the beginning of the collection period. The release rate of SF_6 from each permeation tube was the change in mass of tubes per week during 9 weeks of incubation at 39°C.

Exhaled gas from each heifer was collected in an evacuated 10 cm diameter, 28 cm long, 220 psi PVC canister, with a volume of 2.38 L fitted with Swagelok ball valves. Exhaled gas was collected using capillary tubing attached to a halter placed on the animal's head with a filtered inlet near the nostrils and mouth (Johnson et al., 2007) (Fig. A-9). Background SF_6 values were collected from the pasture study site prior to study initiation using canisters and capillary tubing staked at grazing height in an ungrazed location. Control canisters and capillary tubing were placed in each of the three spatial replications (blocks) and managed daily as described above for heifers. These canisters were placed on top of fence posts (1.5 m aboveground) to measure background atmospheric concentrations of CH_4 and SF_6 and were used to correct values obtained from exhaled air collected from the animals (Williams et al., 2011).

The CH_4 emission rate was calculated from the ratio of CH_4 and SF_6 and the known release rate of SF_6 as follows:

CH_4 emission rate (g/d): SF_6 release rate (g/d) $\times$ ([g CH_4]$_A$ – [g CH_4]$_B$) / ([g SF_6]$_A$ – [g SF_6]$_B$) (Johnson et al., 2007),

where A and B are CH_4 and SF_6 emitted from the animal or present in the background, respectively.

Enteric CH_4 emissions were also expressed as grams of CH_4 per kg of dry matter

intake (DMI), and grams of CH_4 per unit of BW gain.

Methane sampling

Daily CH_4 emissions were sampled from the same animal in each pair used for fecal and urine collection during days 5 to 9 (5 days/animal) of each collection period. Every morning between 0800 and 1000 h in 2016 and between 0730 and 0930 h in 2017, heifers were fitted with a halter and evacuated canister (Fig. A-9). All canisters were evacuated in the lab to less than 0.250 psi and initial pressures were recorded. Canister valves connected to air collecting capillary tubes were opened, the time recorded, and animals were returned to their respective pastures. Approximately 24 h later, canister valves were closed, the time was recorded, and heifers were fitted with fresh canisters and halters for the next 24-h period. Canisters were transported to the lab and filled to 1.1 atm with N_2 gas (positive pressure), allowed to equilibrate, and a gas subsample was transferred to evacuated 12 ml glass vials (Model 838 W, Labco Limited, Lampeter, UK) for CH_4 and SF_6 determination by gas chromatography.

Feed intake and average daily gain calculations

Heifers were weighed individually using a load cell scale (Rice Lake Weighing Systems, Rice Lake, WI) located under a squeeze chute, at the middle of each adaptation period and at the end of each experimental period (~20 days) to estimate average daily gain (ADG). Feed and water were restricted from 1800 h until the next morning when animals were weighed at 0900 h, before returning to pastures. Methane emissions were expressed per unit of BW gain.

Forage intake by individual animals in each treatment was estimated with the

Beef Cattle Nutrient Requirements Model (NRC, 2016 software version 1.0.37), using the individual ADG of each animal and the total digestible nutrient (TDN) concentration of each diet. The software estimated the DMI required by each animal (DMIR) to achieve the observed ADG. The DMIR uses the goal-seek tool of Microsoft® Excel® to change a previously entered DMI value until the first-limiting metabolizable energy (ME) or metabolizable protein (MP)-allowable gain matches animal ADG.

Fecal outputs (FO) were determined by relating individual animal DMIR and the *in vivo* digestibility of the diets estimated using ADL as an internal marker as described previously and applying the following formula: FO (g/d) = [DMIR (kg/d) × ADL in feed (g/kg)] / ADL in feces (g/kg) (Cochran and Galyean, 1994).

Nitrogen balance calculations

Daily intake of N (g/d) was estimated for each heifer by multiplying their individual DMIR and the N concentration of the forage consumed. The N excreted in the feces (g/d) was calculated for each sampled heifer by multiplying their FO by the corresponding N concentration of their feces. The total daily N excreted through urine (g/d) was determined as the product of the urinary N concentration in the sample (g/L) and the total daily urine output volume (L/d) of each heifer. Urine output (L/d) was estimated using urinary creatinine (a waste product of muscle metabolism) concentration as daily urinary creatinine excretion (UCE, mg/d) divided by urinary creatinine concentration (mg/L) (Valadares et al., 1999). The urinary creatinine concentration was determined in urine samples, and UCE was estimated according to Valadares Filho et al. (2016) using the allometric equation: UCE (mg/d) =37.88 × $BW^{0.9316}$.

The proportion of N that was excreted as urea was calculated by dividing the concentration of urinary urea N (UUN) by the concentration of total urinary N.

The proportion of N intake that was excreted in urine and feces, and the proportion of N partitioned to urine or feces were calculated as follows:

Nitrogen excretion (%) = [N excreted in urine (g/d) + N excreted in the feces (g/d)]/N intake (g/d) × 100

Nitrogen excreted in urine (%) = [N excreted in urine (g/d)/N intake (g/d)] × 100

Nitrogen excreted in feces (%) = [N excreted in feces (g/d)/N intake (g/d)] × 100

Finally, N retention was calculated as the difference between N intake and excretion (% N retention), expressed as (N intake – N excretion)/N intake × 100.

Chemical analyses

Forage and fecal samples were analyzed for DM, total N concentration, ADF, ADL, and CT. Dry matter was determined by drying the samples at 105°C for 3 h in a forced-air drying oven (AOAC, 1990; method 930.04). Total N concentration was analyzed using a Leco FP-528 N combustion analyzer (AOAC, 2000; method 990.03) with crude protein (CP) concentration calculated as N concentration × 6.25. Concentration of ADF was determined according to AOAC (2000; method 973.18), modified using Whatman 934-AH glass micro-fiber filters with 1.5 μm particle retention and a Buchner funnel in place of a fritted glass crucible. Determinations of ADL were modified from Robertson et al (1981) as follows: fiber residue and filter from the ADF step was transferred to a capped tube and approximately 45 ml of 72% sulfuric acid was added. Tubes were gently agitated for 2 h and filtered onto a second Whatman 934-AH

glass micro-fiber filter which was then rinsed, dried, weighed and finally ashed for 2 h in a furnace to remove lignin organic matter. Analyses of total CT in legume samples were conducted in triplicate according to the butanol-HCl-acetone spectrophotometric assay of Grabber et al. (2013), using CT isolated from sainfoin and birdsfoot trefoil as the standards.

Urine and serum samples were analyzed for UUN and blood urea N (BUN) with a Siemens Dimension Xpand Plus analyzer (Siemens Healthcare Diagnostics, Newark, DE) using Siemens urea-N flex as the reagent. Urine samples were also analyzed for total N concentration using a Leco FP-528 (Leco Corporation, Saint Joseph, MI) N combustion analyzer (AOAC, 2000; method 990.03), and for creatinine using a Siemens Dimension Xpand Plus analyzer (Siemens Healthcare Diagnostics, Inc., Deerfield, IL) and Siemens Dimension CRE2 as the reagent. Aliquots of each urine sample were first centrifuged to remove particulate matter.

Breath samples were analyzed for CH_4 and SF_6 concentrations at the Lethbridge Research and Development Centre (Agriculture and Agri-Food Canada, Lethbridge, Alberta) using a Varian 450 gas chromatograph with a flame ionization detector (FID) for CH_4 and an electron capture detector (ECD) for SF_6. Helium was used as the carrier gas for the FID detector and argon for the ECD detector. Prepared standards were used to standardize both gas chromatographs for SF_6 (Praxair Inc., Danbury, CT), and for CH_4 (Messer Canada Inc., Mississauga, ON). The concentration of each gas (ppm or ppt) for CH_4 and SF_6 respectively, was calculated using the area of each gas in their chromatograms and the slope and intercept of the standard curves. These values were then expressed in grams by multiplying by their molecular weights.

Statistical analyses

Nutritional composition of diets and feces*, in vivo* DMD and ADFD, BUN and UUN, urinary N concentration, urinary and fecal N excretions (g/d), partition of N into urine and feces and proportion of retained N were analyzed using a 2-way factorial treatment structure (year × period) in a randomized complete block design using a generalized linear mixed model. Seven treatments (three single forage species, three 2-way and one 3-way combinations), period (2), year (2) and all interactions were the fixed factors. Block, block × treatment and block × treatment × year were included in the model as random factors. Methane emissions, DMIR and ADG were analyzed separately within each year of the study, and therefore, the model only included treatment, period and their interaction as fixed factors.

Dry matter availability was analyzed using the same model, but with "species" rather than treatment as a fixed factor (alfalfa, sainfoin or birdsfoot trefoil). Block, block × species and block × species × year were included in the model as random factors.

All analyses were computed using PROC GLIMMIX in SAS/STAT 14.2 (SAS Inst., Inc. Cary, NC; Version 9.4 for Windows). Least square means (LSmean) were compared pairwise using the least significant difference test when the overall test for treatment effect was significant ($P \leq 0.10$). Means were reported along with their standard errors (SEM). Treatment differences were considered a tendency when $0.10 < P \leq 0.15$.

Preplanned contrasts were performed to compare the 3-way choice LSmean with the average LSmean for the three monoculture legume treatments or with the average LSmean for all 2 way-choices, using the LSMESTIMATE statement in PROC

GLIMMIX. Contrasts were specified as the arithmetic difference between ALF-SF-BFT and (0.33ALF + 0.33SF + 0.33BFT) or (0.33ALF-SF + 0.33ALF-BFT + 0.33SF-BFT) respectively. Contrasts between the average of 2-way choices and the average of monoculture treatments were also performed. A difference was considered significant when P values were ≤ 0.10. Treatment differences were considered a tendency when 0.10 < P ≤ 0.15. Assumptions of homoscedasticity of variance and normality were tested using studentized residuals. Diet CT concentration values were transformed to their cube roots in order to meet these assumptions and back-transformed LSmeans and SE values are reported.

<div align="center">RESULTS</div>

Herbage availability

Herbage availability for both years of the study was generally high, ranging from 4 to 8 Mg/ha (Table 4-4), with greater biomass observed for the first than for the second sampling period (Table 4-3) during 2017 (period × year interaction, P<0.001). Averaged across treatments and periods, availability of alfalfa, sainfoin and birdsfoot trefoil was 60, 17 and 22% greater in 2017 than in 2016 (P<0.001; P=0.021; P=0.001, respectively) (Table 4-4). Comparing the first sample collection periods during both years, herbage availability increased from 2016 to 2017 by 74, 37 and 35% for alfalfa, sainfoin and birdsfoot trefoil, respectively (P<0.001; Table 4-4).

During 2016, pre-grazing biomass averaged across periods and treatments was greater for birdsfoot trefoil than for alfalfa or sainfoin (5.5 vs 4.4 and 4.2 Mg/ha, respectively; P=0.002; Table 4-4). In contrast, no differences were observed for this

variable between birdsfoot trefoil and alfalfa during 2017, but biomass of both species was greater than biomass of sainfoin, which presented the lowest pre-grazing biomass (6.7 and 7.0 vs 4.9 Mg/ha; $P < 0.001$, respectively). When averaged across treatments, periods and years, the proportion of herbage biomass that disappeared was 0.27, 0.20 and 0.18 of pre-grazing measurements for sainfoin, alfalfa and birdsfoot trefoil plots, respectively.

For 2-way choices, the proportion of biomass of each species that disappeared ranged between 0.36 and 0.70 (sainfoin), 0.30 and 0.64 (alfalfa), and 0.31 to 0.56 (birdsfoot trefoil) of the total biomass that disappeared in the choice treatments, depending on the year and period of study (Table 4-2 and Table 4-3). For the 3-way choice, the proportions of biomass that disappeared ranged between 0.18 and 0.40 (sainfoin), 0.28 and 0.45 (alfalfa), and 0.26 to 0.51 (birdsfoot trefoil) of the total biomass that disappeared in the choice treatment.

Table 4-2. Means and (SEM) of pre and post-grazing DM herbage availability and biomass disappearance (Mg/ha) of the legume monocultures and 2- and 3-way choice treatments during the first year of the study (2016).

2016		Alfalfa		Sainfoin		Birdsfoot Trefoil	
Treatment		P 1 (early bloom)	P 2 (Late Bud)	P 1 (Full bloom)	P 2 (Late vegetative)	P 1 (early bloom)	P 2 (Late Bud)
ALF	Pre	4.3 (0.2)	4.1 (0.2)				
	Post	3.3 (0.2)	3.0 (0.04)				
	Disappearance	1.0	1.1				
SF	Pre			4.2 (0.7)	4.3 (0.4)		
	Post			3.0 (0.3)	3.7 (0.2)		
	Disappearance			1.2	0.7		
BFT	Pre					5.0 (0.3)	5.6 (0.3)
	Post					4.3 (0.2)	4.6 (0.1)
	Disappearance					0.7	1.0
ALF-SF Choice	Pre	4.5 (0.1)	4.4 (0.2)	4.7 (0.5)	4.1 (0.2)		
	Post	3.8 (0.1)	3.2 (0.1)	2.9 (0.2)	3.4 (0.1)		
	Disappearance	0.8	1.2	1.8	0.7		
	Proportion[a]	0.30	0.64	0.70	0.36		
ALF-BFT Choice	Pre	4.7 (0.3)	4.0 (0.2)			5.6 (0.2)	5.4 (0.2)
	Post	3.8 (0.2)	3.0 (0.1)			4.6 (0.1)	4.5 (0.1)
	Disappearance	0.9	1.0			1.0	0.9
	Proportion[a]	0.48	0.52			0.52	0.48
SF-BFT Choice	Pre			4.6 (0.5)	4.1 (0.1)	5.6 (0.2)	5.4 (0.1)
	Post			2.9 (0.2)	3.5 (0.05)	4.9 (0.1)	4.7 (0.1)
	Disappearance			1.6	0.6	0.7	0.7
	Proportion[a]			0.69	0.44	0.31	0.56
ALF-SF-BFT Choice	Pre	4.7 (0.1)	4.2 (0.1)	3.8 (0.6)	3.9 (0.1)	5.5 (0.2)	5.4 (0.2)
	Post	3.6 (0.2)	3.1 (0.05)	2.5 (0.2)	3.4 (0.02)	4.7 (0.1)	4.6 (0.1)
	Disappearance	1.1	1.1	1.3	0.5	0.8	0.8
	Proportion[a]	0.34	0.45	0.40	0.20	0.26	0.34
Average[b]	Pre	4.6	4.2	4.3	4.1	5.4	5.5
	SEM	0.2	0.2	0.2	0.2	0.2	0.2
	Period effect P-value	0.110		0.432		0.903	

[a] Proportion = Biomass disappeared of each species (Mg) / Total biomass disappeared in the choice (Mg).
[b] Average values are means for 3 spatial replications (blocks), and 4 treatments within each species (n=12).
[a-b] LSmeans in a row with different letters within the same species differ (P<0.10).

Table 4-3. Means and (SEM) of pre and post-grazing DM herbage availability and biomass disappearance (Mg/ha) of the legume monocultures and 2- and 3-way choice treatments during the second year of the study (2017).

2017		Alfalfa		Sainfoin		Birdsfoot Trefoil	
		P 1 (Full Bloom)	P2 (Full Bloom)	P 1 (Early Pod)	P 2 (Late Bud)	P 1 (Full Bloom)	P 2 (Full Bloom)
ALF	Pre	7.8 (0.5)	6.1 (0.3)				
	Post	6.2 (0.4)	4.8 (0.1)				
	Disappearance	1.6	1.3				
SF	Pre			5.6 (0.8)	4.7 (0.1)		
	Post			4.0 (0.1)	2.5 (0.04)		
	Disappearance			1.6	2.2		
BFT	Pre					6.8 (0.2)	6.3 (0.3)
	Post					6.0 (0.03)	4.8 (0.1)
	Disappearance					0.8	1.5
ALF-SF Choice	Pre	8.1 (0.1)	6.1 (0.3)	6.6 (0.1)	3.6 (0.2)		
	Post	6.8 (0.1)	4.9 (0.3)	4.8 (0.1)	2.7 (0.1)		
	Disappearance	1.4	1.2	1.8	0.9		
	Proportion[1]	0.44	0.58	0.56	0.42		
ALF-BFT Choice	Pre	8.2 (0.4)	6.2 (0.3)			7.9 (0.3)	6.1 (0.4)
	Post	6.7 (0.3)	5.1 (0.1)			6.3 (0.3)	4.6 (0.02)
	Disappearance	1.5	1.1			1.6	1.4
	Proportion[1]	0.48	0.44			0.52	0.56
SF-BFT Choice	Pre			6.6 (0.1)	3.9 (0.2)	7.3 (0.1)	5.9 (0.2)
	Post			4.6 (0.2)	2.6 (0.04)	6.2 (0.2)	4.6 (0.03)
	Disappearance			2.1	1.4	1.1	1.3
	Proportion[1]			0.65	0.50	0.35	0.50
ALF-SF-BFT Choice	Pre	7.7 (0.4)	5.7 (0.1)	4.3 (0.2)	2.8 (0.2)	7.3 (0.7)	6.0 (0.2)
	Post	7.0 (0.5)	5.0 (0.2)	4.0 (0.1)	2.3 (0.1)	6.3 (0.2)	4.7 (0.1)
	Disappearance	0.7	0.7	0.4	0.5	1.0	1.2
	Proportion[1]	0.33	0.28	0.18	0.22	0.49	0.51
Average[2]	Pre	8.0[a]	6.1[b]	5.9[a]	3.8[b]	7.3[a]	6.1[b]
	SEM	0.2	0.2	0.2	0.2	0.2	0.2
	Period effect *P-value*	<0.001		<0.001		<0.001	

[a] Proportion = Biomass disappeared of each species (Mg) / Total biomass disappeared in the choice (Mg).
[b] Average values are means for 3 spatial replications (blocks), and 4 treatments within each species (n=12).
[a-b] LSmeans in a row with different letters within the same species differ (P<0.10).

Table 4-4. Average of pre and post-grazing DM herbage availability (Mg/ha) (LSmeans) for alfalfa, sainfoin and birdsfoot trefoil across treatments during two periods (P1 and P2) and years (2016 and 2017), and the overall mean across treatments, periods and years.

	Alfalfa			Sainfoin			Birdsfoot Trefoil			Treatment effect
	P 1	P2	Average	P 1	P 2	Average	P 1	P 2	Average	P-Value
2016 Pre-grazing, Mg/ha	4.6[b]	4.2[b]	4.4[b B]	4.3[b]	4.1	4.2[b B]	5.4[b]	5.5[b]	5.5[b A]	0.002
2017 Pre-grazing, Mg/ha	8.0[a]	6.1[a]	7.0[a A]	5.9[a]	3.8	4.9[a B]	7.3[a]	6.1[a]	6.7[a A]	<.0001
SEM, Mg/ha	0.2	0.2	0.1	0.2	0.2	0.1	0.2	0.2	0.1	
Year effect, *P-value*	<.0001	<.0001	<.0001	<.0001	0.324	0.021	<.0001	0.036	0.001	

	Overall		
	Alfalfa	Sainfoin	Birdsfoot trefoil
Pre-grazing, Mg/ha	5.6	4.5	6.1
Post-grazing, Mg/ha	4.5	3.3	5.0

[a-b]LSmeans in a column with different lower-case superscripts differ (P<0.10).
[A-B] Average LSmeans in a row with different upper-case superscripts differ (P<0.10).
Values are means for 3 spatial replications (blocks), and 4 treatments within each species (n=12). Values at the bottom half of the table are means for 3 blocks, 4 treatments within each species, 2 years and 2 periods within each year of the study (n=48).

Diet composition and fecal chemistry

Crude protein concentration averaged across years and periods was lower in SF than in BFT or ALF (P<0.10; Table 4-5), and concentration of CP in the treatments with forage diversity was in general lower when sainfoin was present than when it was absent from the choice. No differences between ALF and BFT treatments were observed for CP concentration, either during the first or second experimental periods. Averaged across years, treatment diets consumed by heifers had greater CP concentration in the first than in the second period of the study (P<0.001). However, a treatment by period interaction was observed (P=0.005), mainly driven by the SF treatment, as in contrast to ALF and BFT, heifers in SF maintained the concentration of CP across periods (P=0.235).

Table 4-5. Average diet nutritional composition and *in vivo* digestibility (LS means) for cattle grazing monocultures, 2- and 3-way choices of: alfalfa (ALF), birdsfoot trefoil (BFT) and sainfoin (SF) during two periods (P1 and P2) during 2016 and 2017.

	CP[a], g/kg	ADF[b], g/kg	ADL[c], g/kg	CT[d], g/kg	DMD[e], %	ADFD[f], %
ALF	263.9[a]	188.6[cd]	38.6[d]	1.3[f] (0.1)	72.4	53.5[ab]
BFT	257.5[a]	160.8[f]	44.6[bc]	16.9[d] (1.4)	74.8	48.2[c]
SF	212.7[c]	232.2[a]	52.8[a]	58.9[a] (4.7)	71.3	49.0[c]
ALF-SF	239.1[b]	211.8[b]	48.0[b]	24.2[c] (1.9)	73.4	53.8[a]
ALF-BFT	257.0[a]	170.8[ef]	42.8[c]	8.9[e] (0.7)	72.5	50.1[bc]
SF-BFT	229.6[b]	204.0[bc]	51.7[a]	38.6[b] (3.1)	71.2	44.2[d]
ALF-SF-BFT	253.5[a]	182.9[de]	45.3[bc]	21.6[cd] (1.9)	73.8	50.3[abc]
S.E.M	4.8	6.3	1.4		1.1	1.5
Period 1	256.5[a]	196.2	44.3[b]	17.0[b]	73.4	51.8[a]
Period 2	233.0[b]	189.8	48.5[a]	20.5[a]	72.1	47.9[b]
Year 2016	240.5[b]	181.5[b]	44.9[b]	18.6	73.5[a]	50.5
Year 2017	249.0[a]	204.5[a]	47.9[a]	18.8	72.0[b]	49.2
P values						
Treatment effect	<0.001	<0.001	<0.001	<0.001	0.260	0.008
Period effect	<0.001	0.198	<0.001	0.039	0.108	<0.001
Year effect	0.033	<0.001	0.015	0.907	0.099	0.255
Treatment × period	0.005	0.078	0.712	0.069	0.276	<0.001
Treatment × year	0.275	0.049	0.533	0.699	0.009	0.398

	CP[a], g/kg		ADF[b], g/kg		ADL[c], g/kg		CT[d], g/kg		DMD[e], %		ADFD[f], %	
	P1	P2	P1	P2	P1	P2	P1	P2	P1	P2	P1	P2
ALF	279.3[a A]	248.5[a B]	184.5[c]	192.7[abc]	34.8[e B]	42.4[c A]	1.5[d] (0.2)	1.2[f] (0.1)	75.2	69.6	58.5[a A]	48.5[ab B]
BFT	274.0[a A]	241.0[a B]	162.2[d]	159.5[d]	42.5[cd]	46.6[bc]	16.7[b] (1.9)	17.2[d] (2.0)	75.6	73.9	47.5[d]	48.9[ab]
SF	207.2[c]	218.3[b]	250.7[a A]	213.7[a B]	53.3[a]	54.3[a]	44.6[a B] (5.1)	76.1[a A] (8.7)	70.1	72.5	53.0[bc A]	45.0[b B]
ALF-SF	245.4[b]	232.9[ab]	216.8[b]	206.8[ab]	45.8[bc B]	50.3[ab A]	19.7[b B] (2.2)	29.3[c A] (3.3)	73.7	73.1	56.7[ab A]	50.9[a B]
ALF-BFT	280.2[a A]	233.7[ab B]	163.1[cd]	178.6[cd]	40.1[d B]	45.6[c A]	10.0[c] (1.1)	7.9[e] (0.9)	73.5	71.5	48.4[d]	51.7[a]
SF-BFT	240.2[b A]	219.0[b B]	216.2[b A]	191.8[bc B]	49.8[ab]	53.6[a]	33.6[a] (3.8)	44.1[b] (5.0)	72.2	70.3	48.9[cd A]	39.6[c B]
ALF-SF-BFT	269.3[a A]	237.7[a B]	180.2[cd]	185.5[bc]	43.8[cd]	46.8[bc]	21.1[b] (2.7)	22.1[cd] (2.8)	73.8	73.7	49.8[cd]	50.8[a]
SEM	6.6	6.6	9.0	9.0	1.9	1.9			1.5	1.5	1.9	1.9
	2016	2017	2016	2017	2016	2017	2016	2017	2016	2017	2016	2017
ALF	263.5[a]	264.3[a]	180.2[ab]	197.0[c]	36.3[d]	41.0[d]	1.3[f] (0.1)	1.4[e] (0.2)	71.8[c]	73.0[b]	53.0[ab]	54.1
BFT	245.7[b B]	269.3[a A]	157.3[c]	164.3[d]	45.0[c]	44.2[cd]	15.2[d] (1.7)	18.7[c] (2.1)	72.4[bc B]	77.1[a A]	46.7[cd]	49.7
SF	213.3[c]	212.2[c]	198.0[a B]	266.3[a A]	50.8[ab B]	56.8[a A]	64.9[a] (7.4)	53.3[a] (6.1)	74.7[abc A]	67.8[c B]	49.3[bcd]	48.8
ALF-SF	242.8[b]	235.5[b]	194.3[a B]	229.2[b A]	45.8[bc]	50.2[b]	22.9[c] (2.6)	25.5[c] (2.9)	76.8[a A]	70.0[bc B]	56.9[a]	50.6
ALF-BFT	250.1[ab]	263.8[a]	167.3[bc]	174.3[d]	41.6[c]	44.1[cd]	7.8[e] (0.9)	10.1[d] (1.2)	71.4[c]	73.6[ab]	51.1[bc]	49.0
SF-BFT	222.4[c]	236.8[b]	196.7[a]	211.3[bc]	51.8[a]	51.6[b]	40.1[b] (4.6)	37.1[b] (4.2)	71.4[c]	71.0[bc]	44.5[d]	44.0
ALF-SF-BFT	246.0[b]	261.0[a]	176.9[abc]	188.9[cd]	43.0[c]	47.7[bc]	22.7[c] (2.6)	20.5[c] (2.9)	75.8[ab]	71.7[bc]	52.2[ab]	48.4
SEM	6.6	6.6	9.0	9.0	2.0	2.0			1.5	1.5	2.1	2.1

[a-f] LSmeans in a column with different lower-case superscripts differ (P<0.10). [A-C] LSmeans in a row with different upper-case superscripts within the same parameter differ (P<0.10). Values at the top half of the table are means for 3 spatial replications (blocks), two years, and two periods within each year of the study (n=12). Values at the bottom half of the table are means for 3 blocks and two years within each period (n=6) or 3 blocks and two periods within each year of the study (n=6). [a]CP= crude protein; [b]ADF= acid-detergent fiber; [c]ADL= acid-detergent lignin; [d]CT= condensed tannins; [e]DMD= dry matter digestibility; [f]ADFD= acid detergent fiber digestibility.

On average across periods and years, the concentration of ADF in the treatment diets was SF > ALF (23% less) > BFT (44% less) (P<0.01; Table 4-5). However, treatment by period and treatment by year interactions were observed, mainly driven by the SF treatment, with greater concentration of ADF in the first than in the second period of the study (P=0.007), and in 2017 than in 2016 (P<0.001).

The concentration of ADL across years and periods was the greatest for SF, intermediate for BFT and the lowest for ALF (P<0.10, Table 4-5). Averaged across treatments, ADL concentration was greater in 2017 than in 2016 (P=0.015), and it was greater during the second than during the first experimental period (P<0.001).

When averaged across years and periods, concentration of CT was ~3.5 fold greater in SF than in BFT (P<0.001; Table 4-5). Concentration of CT in the SF treatment was also greater in the second than in the first experimental period (P=0.002). In contrast, concentration of CT in BFT did not differ between periods, which caused a period by treatment interaction (P=0.069). Alfalfa is a non-tanniferous legume, confirmed by the very low contents of CT detected.

On average across periods and years, concentration of ADF, ADL and CT were lower (P<0.10) in the ALF-BFT treatment than in the ALF-SF or SF-BFT treatments due to the presence of sainfoin, which presented the greatest concentrations of these variables (Table 4-5).

Concentration of fecal N was greater for heifers grazing the tanniferous legumes than for animals grazing the non-tanniferous ALF treatment (i.e., SF = BFT > ALF), and this parameter was also greater for ALF-BFT than for ALF-SF when averaged across periods and years of the study (P<0.10; Table 4-6).

Table 4-6. Average dry matter percentage, and concentration of N, ADF and ADL in feces (LS means) of heifers grazing monocultures, 2- and 3-way choices of: alfalfa (ALF), birdsfoot trefoil (BFT) and sainfoin (SF) during 2016 and 2017.

Feces:	DM, %	N, g/kg	ADF[a], g/kg	ADL[b], g/kg
ALF	13.1	30.5[c]	320.3[de]	142.1[d]
BFT	12.6	35.5[a]	336.6[de]	180.3[ab]
SF	13.4	35.0[ab]	406.9[a]	190.0[a]
ALF-SF	12.2	32.8[bc]	370.2[bc]	184.2[ab]
ALF-BFT	12.8	34.1[ab]	311.6[e]	156.7[c]
SF-BFT	13.1	33.8[ab]	393.9[ab]	181.5[ab]
ALF-SF-BFT	11.8	35.0[ab]	346.7[cd]	174.0[b]
S.E.M	0.6	1.0	12.0	5.7
Period 1	12.9[a]	33.9	354.2	169.0
Period 2	12.5[b]	33.7	356.1	176.4
Year 2016	12.4	35.1[a]	338.9[b]	172.3
Year 2017	13.0	32.5[b]	371.4[a]	173.1
			P Values	
Treatment effect	0.561	0.052	<0.001	<0.001
Period effect	0.097	0.708	0.776	0.102
Year effect	0.179	0.002	0.001	0.867
Treatment × period	0.243	0.068	0.221	0.844
Treatment × year	0.508	0.578	0.494	0.012

[a-e] LSmeans in a column with different letters differ (P<0.10). Values are means for 3 spatial replications (blocks), two years, and two periods within each year of study (n=12).
[a]ADF= acid-detergent fiber,
[b]ADL= acid-detergent lignin.

In vivo digestibility

A treatment by year interaction (P=0.009) was observed for DMD, mainly driven by the lower DMD in SF during the second year of study (P<0.001; Table 4-5). No differences in DMD were observed among ALF, SF or BFT treatments during 2016, (P>0.10), but during 2017, it was BFT > ALF (P=0.073) > SF (P=0.028). There were no differences in DMD between the 3-way choice (73.8%) and the averages of 2-way choices (72.4%; P=0.317) or monocultures (72.8%; P=0.485).

On average between years, ADFD was ALF > SF > BFT during Period 1 (P<0.10; Table 4-5). However, no differences in ADFD were detected among monoculture treatments during Period 2 (P>0.10). A treatment by period interaction was detected (P<0.001), which was mainly caused by a reduction in ADFD in ALF (P<0.001) and SF (P=0.002) treatments from the first to the second experimental period. Finally, ADFD for the 3-way choice (50.8%) during Period 2 was greater than for the average value observed in all monocultures (47.5%; P=0.072), and ADFD was the lowest for the SF-BFT treatment during both periods of the study (P<0.10).

Average daily gains and estimated intakes

During 2016, heifers on the tanniferous treatments (SF and BFT) gained more weight than heifers on the non-tanniferous legume ALF (P=0.050 and P=0.084, respectively; Table 4-7). When tanniferous legumes were offered along with alfalfa in 2-way choices (ALF-SF or ALF-BFT), heifers showed similar ADG to animals grazing ALF (P>0.10). Nevertheless, when all three legumes were offered in a choice, the heifers' growth rate was greater than for animals grazing ALF (P=0.008; Year 2016).

Moreover, ADG in the 3-way choice during 2016 was 32% greater than the average growth rate of the 2-way choice treatments (1.21 vs. 0.91 kg/d; P=0.031) and 28% greater than the average of the three monoculture treatments (1.21 vs. 0.95 kg/d; P=0.054). No differences in ADG were detected among treatments (P=0.429) during 2017 (Table 4-8). However, heifers grazing the 3-way choice gained 50.5% more weight than in monoculture treatments (1.43 vs. 0.95 kg/d; P=0.085 respectively). Likewise, no differences were detected for DMIR among treatments (2016: P=0.466; 2017: P=0.357), although animals in the ALF-SF-BFT treatment (2017) averaged 33% greater intakes than animals grazing monocultures (10.4 vs. 7.8 kg/d; P=0.064).

Table 4-7. Methane emissions, ADG and DMI required (LSmeans), of cattle grazing alfalfa (ALF), birdsfoot trefoil (BFT) or sainfoin (SF), and 2- or 3-way choices of those forages during the first year of the study (2016).

Treatments	Methane[a], g/d	ADG[b], kg/d	Methane per unit BW gain[c], g/kg	DMIR[d], kg/d	Methane[e], g/kg DMIR
	2016				
ALF	236.2	0.74[c]	335.6	7.8	30.3
BFT		1.04[ab]		9.1	
SF	237.1	1.06[ab]	249.9	8.8	27.9
ALF-SF		0.87[bc]		7.6	
ALF-BFT		0.82[bc]		8.5	
SF-BFT		1.04[ab]		9.7	
ALF-SF-BFT		1.21[a]		9.3	
S.E.M	15.0	0.10	33.9	0.8	2.0
Period 1	206.7[b]	1.20[a]	213.3[b]	9.3[a]	24.4[b]
Period 2	266.6[a]	0.74[b]	372.1[a]	8.1[b]	33.8[a]
	P Values				
Treatment effect:	0.971	0.093	0.216	0.466	0.486
Period effect	0.046	<0.001	0.030	0.009	0.031
Treatment × period	0.872	0.259	0.944	0.347	0.559
2-way choices vs monocultures[6]		0.706		0.922	
3-way choice vs monocultures[f]		0.054		0.413	
3-way vs 2-way choices[g]		0.031		0.449	

[a-c] LSmeans in a column with different letters differ ($P<0.10$). Values are LSmeans of 3 spatial replications (blocks) and two periods within each year of study (n=6).
[a]Daily gross CH_4 emissions (g/d), [b]ADG: average daily gain (kg/d).
[c]Methane per unit production: CH_4 (g/d)/ADG (g/d), [d]DMIR: dry matter intake required (kg/d).
[e]Methane per unit of intake (g/kg).
[f]Indicate that these are pre-planned contrasts between 2-way, 3-way choices and monoculture treatments.

Table 4-8. Methane emissions, ADG and DMI required (LSmeans), of cattle grazing alfalfa (ALF), birdsfoot trefoil (BFT) or sainfoin (SF), and 2- or 3-way choices of those forages during the second year of the study (2017).

Treatments	Methane, g/d	ADG, kg/d	Methane per unit BW gain, g/kg	DMIR, kg/d	Methane, g/kg DMIR
	2017				
ALF	199.5	0.96	224.7	8.2	24.8
BFT	202.5	1.15	187.6	7.9	26.1
SF	180.2	0.74	252.8	7.2	23.8
ALF-SF	222.1	1.19	200.1	9.7	23.5
ALF-BFT	210.8	0.93	232.4	8.0	26.3
SF-BFT	177.4	1.04	173.6	8.4	21.6
ALF-SF-BFT	214.6	1.43	161.8	10.4	21.3
S.E.M	17.6	0.18	35.0	0.9	2.1
Period 1	179.4[b]	1.03	185.3[b]	8.1[b]	22.9
Period 2	222.6[a]	1.09	224.1[a]	9.1[a]	24.9
	P Values				
Treatment effect:	0.575	0.429	0.648	0.428	0.582
Period effect	0.001	0.450	0.060	0.058	0.227
Treatment × period	0.290	0.153	0.209	0.215	0.229
2-way choices vs monocultures	0.541	0.521	0.533	0.269	0.561
3-way choice vs monocultures	0.416	0.085	0.258	0.064	0.253
3-way vs 2-way choices	0.658	0.161	0.438	0.198	0.413
Tanniferous vs Non-tanniferous[a]	0.712	0.955	0.922	0.579	0.952

[a-c] LSmeans in a column with different letters differ (P<0.10). Values are LSmeans of 3 spatial replications (blocks) and two periods (n=6). [a]Indicate that this is a pre-planned contrast between the average LS means of tanniferous monocultures (SF and BFT) and non-tanniferous monoculture ALF. Other acronyms as in Table 4-7.

Methane emissions

Daily emissions of CH_4 did not differ between ALF or SF treatments in 2016 (Table 4-7), or among all treatments in 2017 (Table 4-8), either when emissions were expressed as g/d, g/kg BW gain, or g/kg DMIR (CH_4 yield). Average emissions for the 3-way choice, 2-way choices and monocultures during 2017 were 161.8, 202.0 and 221.7 g/kg BW gain (P=0.438 and P=0.258 for 3-way vs. 2-way choices or monocultures,

respectively), or 21.3, 23.8 and 24.9 g CH_4/kg DMIR (P=0.413 and P=0.253; for 3-way vs. 2-way choices or monocultures, respectively Table 4-8). A period effect was observed for CH_4 emissions for both years, as a result of greater levels of emissions during the second than during the first period of evaluation. This pattern was observed when emissions were expressed either in absolute amounts (g/d), per unit of production (g/kg BW gain) or as CH_4 yield (g/kg DMIR).

Excretion of nitrogen in urine and feces and blood urea nitrogen

Averaged across periods and years, concentrations of BUN, urinary N and UUN were greater (P<0.05) in heifers grazing ALF than in animals grazing tanniferous legumes (SF and BFT; Fig. 4-1). In addition, the SF treatment showed a tendency towards lower BUN concentrations than the BFT treatment (P=0.144). Grazing alfalfa along with sainfoin or birdsfoot trefoil in 2- or 3-way choices (e.g., ALF-SF, ALF-BFT or ALF-SF-BFT) led to lower concentrations of urinary N, UUN and BUN than in animals grazing ALF (P<0.10). Moreover, heifers grazing a choice of tanniferous legumes (SF-BFT) showed lower levels of total urinary N and UUN concentration than heifers grazing the same legumes in monoculture (e.g., SF-BFT < SF or BFT; P<0.10). In addition, proportions of urinary N as urea-N was less for heifers in the SF-BFT or SF treatments (75.1 and 76.8%, respectively) than for heifers grazing ALF, BFT, ALF-BFT or ALF-SF-BFT (85.3, 81.9, 83.9 and 85.2% respectively; P<0.10).

Averaged across treatments and years, total urinary N and UUN concentrations were lower during the second than during the first period of assessment (urinary N: 4.7 vs. 3.4 g/L and UUN: 3.8 vs. 2.9 g/L, for periods 1 and 2, respectively; P<0.001) (data not shown).
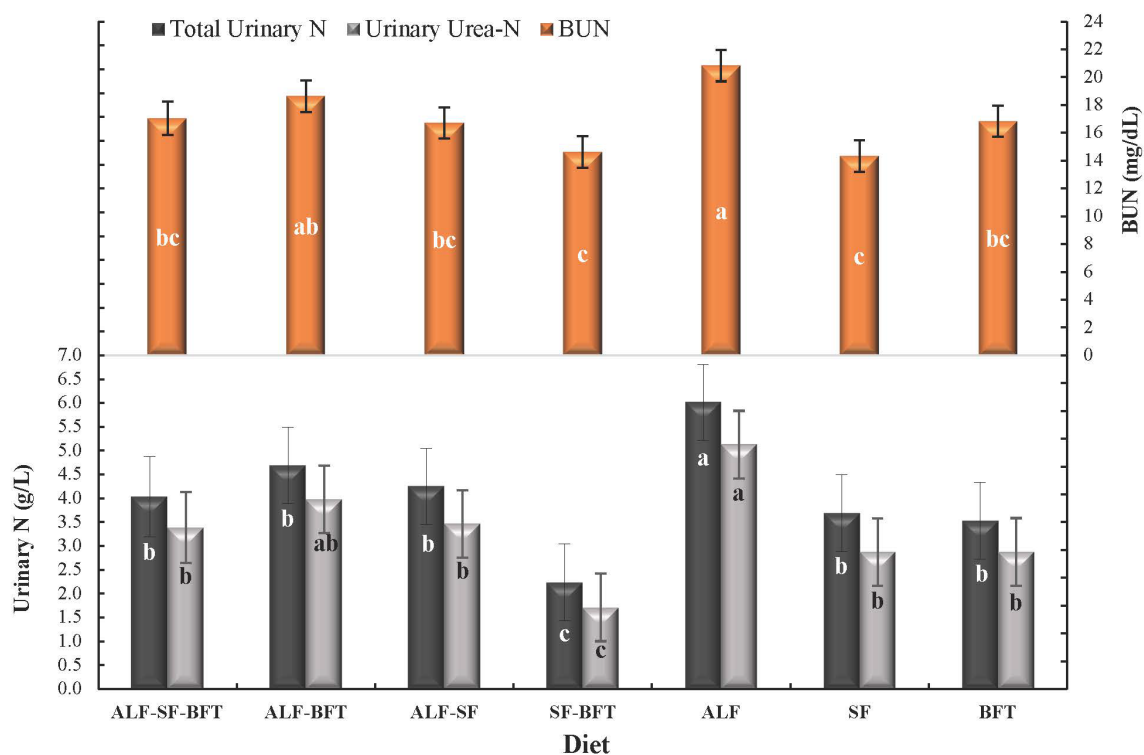
Figure 4-1. Total urinary N, urinary urea N (UUN) and blood urea N (BUN) in heifers that grazed alfalfa (ALF), sainfoin (SF), birdsfoot trefoil (BFT), and 2- or 3-way choices of these forages. Values are LSmeans with SEMs for 3 spatial replications (blocks), two years, and two periods within each year of study (n=12). Bars of the same parameter with different letters differ (P<0.10).

Despite the greater urinary N concentration observed for cattle grazing ALF (Fig. 4-1), no differences were found in daily urinary N excretion among treatments (P=0.176; Table 4-9). Similarly, there were no differences in daily fecal N excretion among treatments (P=0.428; Table 4-9), although fecal N concentrations (g/kg) were greater in both tanniferous legume treatments (SF and BFT) than in ALF (P < 0.10; Table 4-6). Thus, total N excretion (urinary and fecal N), did not differ among treatments (P=0.635; Table 4-9). Similarly, no differences among treatments were observed for the amount of N ingested daily (N intake), when averaged across periods and years (P=0.520).

When animals grazed the 3-way choice (ALF-SF-BFT), the partitioning of N to

urine was 22 and 20% less than for 2-way choices or the average of the three

monocultures (40.7 vs. 52.0%, P=0.022 and 50.6%, P=0.037, respectively; Table 4-9). In

addition, the proportion of retained N increased by 43% (P=0.046) relative to the average

of monocultures (36.1 vs. 25.2%, respectively) and by 52% (P=0.029) relative to the

average of 2-way choices (36.1 vs. 23.7%, respectively), suggesting positive associative

effects for the 3-way choice. Averaged between both years of the study, all treatments

showed a reduction in the proportion of retained N during the second relative to the first

period of evaluation (P=0.041).

There was a significant effect of treatment (P=0.007) on the proportion of N

partitioned to feces (Table 4-9). Averaged across periods and years, the SF treatment

partitioned more N to feces than BFT (P=0.003) or ALF (P<0.001), although during

2016, SF did not differ from BFT (27.7 vs 26.3%; P=0.656), causing a significant

treatment by year interaction (P=0.068). When both tanniferous legumes were consumed

together in the choice (SF-BFT), heifers partitioned more N to feces than with the ALF-

SF (P=0.074) or ALF-BFT (P=0.049) treatments. Averaged between years, heifers

partitioned more N to feces in the second than in the first period of the study (P=0.003).

Table 4-9. Excretion of nitrogen in urine and feces, N balance (LS means) in heifers grazing alfalfa (ALF), birdsfoot trefoil (BFT) and sainfoin (SF), or choices (2- and 3-way) among these forages.

Treatments	[a]Urinary N excretion, g N/d	[b]Fecal N excretion, g N/d	[c]Total N excretion, g N/d	[d]N intake g N/d	[e]Total N excretion, % of N intake	[f]Urinary N, % of N intake	[g]Fecal N, % of N Intake	[h]N Retention, % of N Intake
ALF	181.3	69.3	250.6	346.6	73.7	53.8	19.9[c]	26.3
BFT	168.2	73.1	241.0	336.0	73.4	50.6	22.7[c]	26.6
SF	135.0	84.1	219.6	293.1	77.3	47.4	30.1[a]	22.7
ALF-SF	146.3	73.6	219.2	314.9	71.7	48.3	23.2[c]	28.3
ALF-BFT	191.1	75.7	267.4	345.6	78.7	56.5	22.3[c]	21.3
SF-BFT	170.0	91.7	262.2	336.0	78.4	51.3	27.1[ab]	21.6
ALF-SF-BFT	152.2	89.2	241.1	387.3	63.9	40.7	23.3[bc]	36.1
SEM	16.1	9.0	22.9	29.6	4.4	3.9	1.5	4.4
Period 1	165.8	77.0	243.3	351.4[a]	71.2[b]	48.8	22.4[b]	28.8[a]
Period 2	161.1	82.0	242.7	322.6[b]	76.6[a]	50.8	25.8[a]	23.4[b]
P Values								
Treatment effect	0.176	0.428	0.635	0.520	0.274	0.180	0.007	0.274
Period Effect	0.518	0.212	0.941	0.026	0.041	0.389	0.003	0.041
Year Effect	0.866	0.144	0.527	0.825	0.897	0.665	0.172	0.897
Treatment × period	0.747	0.550	0.571	0.184	0.264	0.505	0.249	0.264
Treatment × year	0.103	0.029	0.520	0.624	0.448	0.491	0.068	0.448
2-way vs monocultures[i]	0.532	0.499	0.497	0.777	0.669	0.639	0.959	0.669
3-way vs monocultures	0.596	0.193	0.877	0.108	0.046	0.037	0.617	0.046
3-way vs 2-way choices	0.345	0.394	0.747	0.153	0.029	0.022	0.649	0.029

a-d LSmeans in a column with different letters differ (P<0.10). Values are means for 3 spatial replications (blocks), two consecutive years (2016 and 2017), and two periods within each year of study (n=12).
[a]Calculated as the product of total urinary N concentration (g/L) and total urine output (L/d) estimated from urinary creatinine concentration as: 37.88 x $BW^{0.9316}$(mg/d)/creatinine concentration (mg/L) (Valadares Filho et al., 2016).
[b]Calculated from individual fecal outputs (kg/d) and fecal N concentrations (g/kg; Table 4-6).
[c]Total N excretion= [Urinary N excretion (g/d) + Fecal N excretion (g/d)].
[d]Calculated from estimated DM intakes (kg/d) and N concentration of the consumed diet (g/kg; Table 4-5).
[e]Total N excretion, % = [(Urinary N excretion (g/d) + Fecal N excretion (g/d))/ N intake (g/d)] × 100
[f]N partitioned to urine, % = [Urinary N excretion (g/d)/N intake (g/d)] × 100.
[g]N partitioned to feces, % = [Fecal N excretion (g/d)/N intake (g/d)] × 100.
[h]N Retention, %= [N retention (g/d)/N intake (g/d)] × 100.
[i]Indicate that these are pre-planned contrasts between 2-way, 3-way choices and monocultures.

DISCUSSION

Forages are nutrition centers and pharmacies with vast arrays of primary (nutrients) and secondary compounds (e.g., CT) that can provide multiple services vital for agroecosystems (Crozier et al., 2006; Villalba et al., 2019). Diversity in natural systems may improve productivity (Tilman et al., 1997; Picasso et al., 2011), resilience to environmental stress (Sanderson et al., 2007), and efficiencies of nutrient capture and nutrient cycling (Tilman et al., 2002; Isbell et al., 2017). From the standpoint of ruminant nutrition, complementary relationships among multiple food resources in nature improve animal fitness (Tilman, 1982). Within this context, we hypothesized that plant secondary compounds and pasture diversity offer ruminants a wider array of beneficial chemicals with potential for synergism to improve ruminant nutrition while reducing C and N footprints. We explored the value of co-grazing three legumes, all with exceptional nutritive value but varying in concentration of secondary metabolites to determine their effects on cattle production, enteric CH_4 emissions, and N losses to the environment.

Forage availability and disappearance

Forage availability greatly exceeded demand during all periods and in both years of the study and therefore allowed animals to be maximally selective. For the 2- and 3-way choice treatments, sainfoin was the legume depleted to the greatest extent during the study, evidenced by the greater proportion of sainfoin biomass disappearance in choice treatments, particularly during Period 1. Thus, as in previous studies using cattle and sheep, heifers preferred sainfoin over alfalfa (Maughan et al., 2014; Villalba et al., 2015) or birdsfoot trefoil (Lagrange and Villalba, 2019). This preference may be explained by the presence of CT in sainfoin, which may have reduced NH_3 formation in the rumen through reductions in proteolysis (i.e., excess of rumen NH_3 is one of the signals that control appetite and may causes negative post-ingestive effects) (Costes-Thiré et al., 2018), or high concentrations of non-fibrous carbohydrates (Marais et al., 2000) that provided energy in synchrony with protein availability (Richardson et al., 2003).

Chemical and taxonomic diversity in pasturelands:
Impacts on digestibility, intake and animal performance

Concentration of CT in SF averaged 59 g/kg DM in the present study (39 to 82 g/kg DM range) with the greatest values observed during the second period in both years of the study, probably due to the greater proportion of leaves in SF regrowth, where the majority of CT in sainfoin accumulates (Theodoridou et al., 2010). Condensed tannin concentrations are consistent with values reported for this species in previous studies: ranging from 5 to 140 g/kg DM, depending on variety, phenological stage and growing conditions (Wang et al., 2015). Concentrations of CT in BFT were lower than in SF, fluctuating between 14.6 and 19.0 g/kg (average: 17 g/kg), and in the range (14 to 32

g/kg) of those reported for North American and European birdsfoot trefoil cultivars (Grabber et al., 2015).

A higher proportion of leaves in SF regrowth (late vegetative and late bud stage for 2016 and 2017, respectively) may explain the lower concentration of ADF observed for SF during Period 2 of the study, as well as the sustained concentration of CP observed across periods for this treatment (AufrèRe et al., 2014). In contrast, ALF and BFT evidenced lower concentrations of CP and greater contents of ADL during the second than during the first grazing period. These legumes regrew at faster rates than SF, thus reaching a more advanced stage of maturity in the second than in the first period. The nutritional value of forages typically decreases with maturity and reproductive development, as NDF concentration increases and N concentration and forage digestibility decline (Fulkerson et al., 2007; Pelletier et al., 2010a).

Although no differences among treatments were observed for DMD in 2016, this parameter was less for SF than for ALF or BFT treatments during 2017. Similar results were found by Stewart et al. (2019) who reported lower DMD for heifers fed sainfoin hay than for heifers fed birdsfoot trefoil hay. In contrast to 2016, pastures were not mowed before the first experimental period during 2017, and were therefore grazed at a more advanced stage of maturity. This was evidenced in SF by greater ADF and ADL concentrations than in 2016, which may contribute to explain the lower values of DMD observed for this treatment (Van Soest, 2018).

Heifers in the 3-way choice showed greater DMD values than heifers grazing ALF in the first year of the study, suggesting associative effects among tanniferous legumes and alfalfa. However, the inclusion of tanniferous legumes in 2-way choices

with alfalfa did not modify DMD relative to monocultures. Likewise, Aufrère et al. (2013) reported no reductions in alfalfa total tract digestibility when this legume was mixed with sainfoin at different proportions, although Wang et al. (2007) showed that the apparent digestibility of alfalfa-sainfoin mixtures either fed to sheep as hay or silage was improved relative to feeding pure alfalfa.

The increase in the concentration of ADL in ALF with progression of the growing season could explain the reductions in ADFD (10 percent units) across periods of the study in both years (Jung et al., 1997). In fact, ADFD was greater for ALF than for the rest of the monocultures during Period 1, likely due to lower ADL concentration in ALF, but this difference disappeared during Period 2, as ADL concentration in ALF increased.

Mixing the two tanniferous legumes (SF-BFT) led to the lowest values of ADFD recorded (Period 2; both years; Table 4-5), likely due to negative interactions among CT or other chemical constituents in the legumes that produced negative associative effects. The increase in CT concentration from 44.6 to 76.1 g/kg in SF regrowth may explain reductions in ADFD (8 percentage units) from the first to the second period of the study. This is supported by results from Scharenberg et al. (2007) and Azuhnwi et al. (2013), who used polyethylene glycol to inactivate CT in sainfoin and reported a concomitant increase in ADF digestibility. This CT effect may be due to inactivation of extracellular microbial enzymes through the formation of CT-enzyme complexes and consequent reduction in their digestive activity (Bae et al., 1993) and/or direct inhibition of cellulolytic bacteria (McSweeney et al., 2001). In addition, formation of cell-associated protein-tannin complexes on the cell surface may interfere with microbial attachment to fiber and prevent microbial digestion (Bento et al., 2005).

Despite the reduced ADFD in tanniferous legumes relative to ALF, heifers in the BFT or SF treatments showed greater (40%) BW gains than animals in ALF during 2016. Reductions in intake by the ALF treatment, likely due to the lower nutritional value of the regrowth as described earlier, and NH_3 buildup in the rumen (see below) may explain this pattern. Reductions in the nutritional quality of the regrowth may also explain the lower BW gains observed in Period 2 of the study.

Heifers grazing the 3-way choice showed intake levels 33% greater (2017) and BW gains 28% (2016) and 50% (2017) above the average observed for monoculture diets, which supports our hypothesis regarding the benefits of forage diversity on pasture systems. Associative effects may enhance intake and livestock performance, as observed in previous studies with increments in the diversity of rations (Görgülü et al., 1996; Villalba et al., 2004) or forages (Cortes et al., 2006; Rogosic et al., 2008; Lagrange and Villalba, 2019). In fact, ADG by heifers grazing legumes in the present study (0.95 kg/d) was much greater than reported for grass-finishing diets (0.6 kg/d; Elizalde et al., 1998; Pelletier et al., 2010b; Capper, 2012), although lower than those reported in conventional feedlots (1.7-2.0 kg/d; Xu et al., 2014; Ebert et al., 2017; Koenig et al., 2018). Greater BW gains in legume vs. grass-finishing systems imply a reduction in the number of days to slaughter, which would result in reduced environmental impacts, and less land and water use for forage-based beef production systems (Capper, 2012; Hristov et al., 2013). The improved ADG observed in the 3-way choice treatment would further enhance these benefits.

Greater BW gains in the 3-way choice than the average observed for monocultures could be explained through greater intakes as predicted by the NRC model

(NRC 2016). Ruminants satiate on forages with the same orosensorial and postingestive characteristics (Provenza, 1996), and they display greater intakes when exposed to a diversity of forages of different nutritional composition (Villalba and Provenza, 2005; Agreil et al., 2006; Villalba et al., 2011). Alternatively, some CT like those in sainfoin and birdsfoot trefoil may enhance the efficiency of CP use in ruminants (Waghorn, 2008; Wang et al., 2015). Thus, the synergistic effect of ingesting a diversity of types and concentration of nutrients and CT with sainfoin and birdsfoot trefoil, in conjunction with the ingestion of protein-dense legumes like alfalfa and diverse orosensorial experiences likely contributed to enhance BW gains in heifers grazing 3-way choices of legumes (Douglas et al., 1995; Aufrère et al., 2013; Sottie et al., 2017).

Enteric methane emissions in monocultures

Enteric CH_4 emissions are the most important emission source (~60%) contributing to the carbon footprint of beef cattle production systems (U.S average GHG intensity: ~23 kg CO_2eq/kg carcass weight), with the cow-calf system showing the greatest sensitivity to mitigation practices in life cycle assessments (Beauchemin et al., 2010; Rotz et al., 2019). Daily emissions of CH_4 (g/d) observed in our study were slightly greater than those values reported by the International Panel on Climate Change Tier 1 approach for beef cattle in North America (173 g/d; (IPCC, 2019). However, the IPCC values include fast-growing beef steers/heifers finished in feedlots on grain-based rations, which emit at low rates (13-15 g/kg DMI; Beauchemin and McGinn, 2005; Vyas et al., 2014; Cottle and Eckard, 2018). Nevertheless, CH_4 emissions by grazing heifers in this study were much lower than those typically reported for grass-finishing diets (36–37 g/kg

DMI; Ominski et al., 2006; Fitzsimons et al., 2013; Stewart et al., 2019), and comparable to emissions observed in the backgrounding phase with silage-grain based diets (24.6 g/kg DMI, Beauchemin and McGinn, 2005; 22.0 g/kg DMI, Vyas et al., 2016).

Despite the lower ADFD observed for tanniferous legumes, which may reduce acetate production and the availability of H_2 for methanogenesis (Bodas et al., 2012; Jayanegara et al., 2015; Vasta et al., 2019), no differences among treatments were observed for the amount of CH_4 emitted daily (g/d), per unit of intake (i.e., yield), or per unit of gain (i.e., CH_4 emission intensity). Nevertheless, heifers grazing the SF treatment in 2016 and BFT in 2017 emitted 25 and 17% less CH_4/kg BW gain, respectively, than heifers grazing the ALF treatment, driven by the similar amounts of CH_4 emitted daily and the greater ADG observed for the tanniferous legume treatments.

Condensed tannins may increase the efficiency of energy use in ruminants through reductions in the production of CH_4 (Carulla et al., 2005; Animut et al., 2008; Junior et al., 2017), since CH_4 represents an energy loss between 2 to 12% of the gross energy consumed with the diet (Johnson and Johnson, 1995). Prior research showed *in vitro* reductions in CH_4 production when the concentration of CT in sainfoin was 28 (80 g/kg DM; Hatew et al. 2016) to 49% (113 g/kg DM; McMahon et al. 1999) greater than the concentration observed for the SF treatment in this study. Likewise, declines in CH_4 production occurred when the concentration of CT in birdsfoot trefoil was 50% greater (26 g/Kg DM; Woodward et al. 2004) than concentrations found for BFT in this study, which may contribute to the lack of differences in CH_4 production observed between tanniferous and non-tanniferous legumes. Additionally, the lower contents of ADF in ALF than in SF could have reduced CH_4 emissions in animals grazing ALF (Johnson and

Johnson, 1995), counter-balancing the positive effects of CT in SF in reducing CH_4 emissions. Finally, several *in vitro* (Rufino-Moya et al., 2019) and *in vivo* (Beauchemin et al., 2007; Chung et al., 2013; Ebert et al., 2017) studies show no differences in CH_4 production between tanniferous and non-tanniferous substrates. Regardless of the effects of CT, the CH_4 emission values observed for tanniferous monocultures in this study indicate their high nutritional value, comparable to ALF.

Enteric methane emissions in diverse diets

Consistent with results from monoculture diets, mixing alfalfa with tanniferous legumes did not reduce emissions of gross CH_4 (g/d) production, or yield (g/kg DMIR) relative to monocultures, although heifers in the ALF-SF-BFT treatment showed a non-significant 14% reduction in CH_4 yield relative to the average of monoculture treatments, likely driven by the greater levels of intake in the former treatment. It is known that DMI is one of the most important factors influencing CH_4 emissions in ruminants (Jiao et al., 2014), as CH_4 yield declines as intake increases because greater intakes are the result of lower retention times of digesta in the rumen, which reduces fiber fermentation and thus CH_4 production (Moss et al., 2000; Pinares-Patiño et al., 2009; Lima et al., 2016). Heifers offered 3-way choices also showed non-significant reductions (27%) in CH_4 emission intensity relative to monoculture treatments, explained by greater BW gains for 3-way choices.

Nitrogen excretion in monocultures

The increase in efficiency of protein use by animals consuming tanniferous forages has been attributed in part to the enhancement in the absorption of essential

amino acids from the small intestine, shifting N excretion from urine to feces (Waghorn, 2008). Consistent with this concept, urinary N concentration in heifers grazing tanniferous legumes in the present study relative to animals grazing ALF was reduced by 40% (Fig. 4-1). Concomitantly, BUN concentrations in heifers grazing BFT or SF were 19 and 31% lower, respectively, than in heifers grazing ALF, which showed the greatest concentration of UUN. This was likely a consequence of the high concentration of CP in ALF leading to a greater production of NH_3 in the rumen (Getachew et al., 2006). In contrast, CT reduce the extent of proteolysis in the rumen (Waghorn, 2008) and thus the rate of formation of urea by the liver (Huntington and Archibeque, 2000), explaining the lower BUN and UUN concentration in SF and BFT treatments. Similarly, feeding fresh sainfoin to sheep (Aufrère et al., 2008) and beef heifers (Chung et al., 2013) reduced total tract N digestibility, effective ruminal N degradability, and urinary N excretion relative to feeding fresh alfalfa. In addition, strong negative correlations were found between concentration of CT in sainfoin and ruminal N degradation (AufrèRe et al., 2014). Alternatively, a deficient energy supply to ruminal microorganisms may contribute to greater BUN and UUN concentrations in ALF, as tanniferous legumes may provide greater levels of readily available sources of energy to the rumen (via non-structural carbohydrates) (Christensen et al., 2015; Chail et al., 2016; Stewart et al., 2019).

Reduced concentration of urinary N by heifers in SF was accompanied by a high fecal N concentration in this treatment and greater partitioning of the N consumed to feces (30%). Similar results were observed for sheep (Aufrère et al., 2008) and cattle (Stewart et al., 2019) consuming sainfoin relative to animals consuming alfalfa. Previous studies have also reported greater concentrations of N in feces of ruminants fed sainfoin

compared with those fed birdsfoot trefoil (Grosse Brinkhaus et al., 2016; Lagrange and Villalba, 2019). Differences in the chemical structure of CT between sainfoin and birdsfoot trefoil might be responsible for differential affinities of these secondary compounds for dietary CP and microbial or endogenous proteolytic enzymes (Mueller-Harvey et al., 2019). Condensed tannins in sainfoin precipitate proteins more effectively than CT in birdsfoot trefoil (McAllister et al., 2005). Thus, greater concentrations of CT in SF compounded with greater precipitation capacity (McAllister et al., 2005) may explain the increased partition of N into the feces of animals grazing SF.

No differences were observed in the percentage of total N partitioned into urine among monocultures, although lower BUN, UUN and total urinary N concentrations in heifers grazing SF or BFT than in heifers grazing ALF, suggests a lower proportion of N partitioned into urine. It is likely that the methodology used (urinary creatinine concentration) overestimated daily urine outputs (and thus urinary N excretion), given that estimates of daily urinary creatinine concentration may be reduced during spot urine sampling (Chen et al., 1992; Rennó et al., 2008). Nevertheless, total N retention values and similar N partitioning values to urine in sheep fed alfalfa diets in total urine collection studies (e.g., Aufrère et al., 2008), suggest that estimation of urine output in the present study is accurate.

The N retention values observed in this and other studies for growing beef cattle grazing forage legumes were comparable to those typically reported for beef heifers and steers fed finishing diets with more than 90% concentrates (25-35%; Koenig and Beauchemin, 2013; Ebert et al., 2017; Koenig and Beauchemin, 2018), despite the fact that concentration of CP in the legumes was much greater (213 to 264 g/kg DM) than

concentrations usually present in feedlot finishing diets. In addition, animals in this study

showed a greater proportion of N retention/N intake than that reported for growing beef

animals fed monoculture grass diets (11% for *Lolium perenne* silage; Kirkpatrick et al.,

1997), 11-14% for a grass-prairie hay (Coffey et al., 2000) or 18.5% for meadow brome

(*Bromus riparius*) hay (Stewart et al., 2019). This pattern may be attributed to the

positive effects of CT and greater concentrations of soluble carbohydrates in legumes

(Chail et al., 2016), as described before.

Nitrogen excretion in diverse diets

The reductions in BUN and UUN observed for heifers grazing the 2- and 3-way

choices relative to heifers grazing ALF (Fig. 4-1), can also be attributed to the beneficial

effects of CT described above. Nevertheless, these parameters were similar between the

ALF and ALF-BFT treatments. Previous studies in dairy cows have shown that adding

birdsfoot trefoil to alfalfa diets did not reduce urinary N excretion relative to grazing pure

alfalfa stands (Christensen et al., 2015), or ruminal protein degradability in batch cultures

(Grosse Brinkhaus et al., 2017), likely due to the low concentration of CT in birdsfoot

trefoil. In contrast, mixes of sainfoin, which has a greater concentration of CT than

birdsfoot trefoil and different types of CT, with alfalfa led to decreases in proteolysis and

ruminal NH_3 concentration (McMahon et al., 1999; Wang et al., 2006), with increments

in the proportion of undigested protein escaping the rumen (Aufrère et al., 2013) relative

to diets of alfalfa alone.

A combination of tanniferous legumes (SF-BFT) led to declines in urinary N and

UUN concentrations that were even greater than the reductions observed for the single

tanniferous species individually. This novel finding suggests a positive associative effect on the reduction of ruminal protein degradation, possibly due to the different chemical structure of their CT, as discussed above. Combining different nutrient profiles from tanniferous legumes may also have promoted synergism. In addition, heifers consuming a choice of tanniferous legumes showed much lower proportions of UUN (75%) than those treatments where alfalfa was present in the choice (80-85%). The positive associative effect of consuming different tanniferous legumes led to reduction of urinary N concentrations that was also observed in the 3-way choice, where there was a 10% unit reduction in the ratio of urinary N to intake N relative to the average observed for animals grazing monocultures.

Reductions in N partitioning to urine for the 3-way choice treatment were not accompanied by a proportional increment in N partitioning to feces, resulting in a partition value (23.3%) that was similar to the average observed for monocultures (24.3%). Thus, the 3-way choice resulted in greater N retention values (52% greater than in 2-way choices and 43% greater than the average observed for monocultures, Table 4-9). This outcome is also indicative of a positive associative effect among legumes, contributing to the greater ADG in animals grazing the 3-way choice.

Reductions in the proportion of N partitioned to urine are beneficial for the environment. In addition to the negative effects of NH_3 volatilization (Campbell, 2016) and NO_3^- leached to groundwater and waterways (Zonderland-Thomassen et al., 2014; Leip et al., 2015), urinary N is a source of the potent GHG N_2O. For instance, increments in urinary N excretion from 29 to 50 g/d in growing beef cattle led to a 37% increase in estimated N_2O emissions, from 413 to 565 mg/d (Bao et al., 2018). In addition, a shift in

the route of N excretion from urine to feces contributes to reducing the detrimental effect of N excretion, as fecal N is mainly in the organic form and has to be mineralized to ammonium (NH_4^+) before being susceptible to volatilization (Cai et al., 2017). Finally, CT–protein complexation inhibits the mineralization process, slowing the breakdown of protein from feces to NH_4^+ and then to leachable NO_3^- (Eckard et al., 2010).

Collectively, our results suggest that offering cattle the greatest species diversity and including tanniferous legumes in pastures with alfalfa reduces urinary N excretion with minimal changes in fecal N excretion relative to monocultures, with positive effects on N retention, soil organic matter and animal growth.

CONCLUSIONS

Heifers grazing legume monocultures had performance intermediate between grass-fed and grain-based finishing systems. Animals grazing tanniferous legumes (SF, BFT) showed improved gains and a shift in the site of N excretion from urine to feces, relative to a non-tanniferous legume (ALF). A diversity of legumes in 3-way choices enhanced animal performance and N economy of animals compared with animals grazing monocultures. These results have important implications for the abatement of NH_3 and N_2O volatilization, and NO_3^- leaching to groundwater and waterways. Enteric CH_4 emissions did not differ between heifers grazing monocultures of tanniferous legumes or ALF, or between animals grazing a diversity of legumes or legume monocultures. However, greater BW gains than for grass-finishing systems would reduce the number of days to slaughter and thus reduce CH_4 production over the animal's lifetime. Collectively, our results suggest that these productive and environmental benefits would

lead to a more sustainable beef production system, with lower environmental impacts at greater levels of productivity, shorter finishing times and reduced land area per animal.

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CHAPTER 5

GRAZING DIVERSE COMBINATIONS OF TANNIFEROUS AND NON-

TANNIFEROUS LEGUMES: IMPLICATIONS FOR FORAGING

BEHAVIOR, PERFORMANCE AND HAIR CORTISOL IN

BEEF CATTLE

ABSTRACT

A diversity of forages with different types and concentrations of nutrients and plant secondary compounds may lead to complementary relationships that enhance cattle performance and welfare. We determined whether grazing combinations of "non-traditional" tanniferous legumes (*Lotus corniculatus*, birdsfoot trefoil, *Onobrychis viciifolia*, sainfoin) and alfalfa (*Medicago sativa*) influence foraging behavior, performance and hair cortisol concentration in beef cattle compared with grazing the same legumes as monocultures. Twenty-one pairs of heifers grazed three spatial replications of seven treatments: monocultures of birdsfoot trefoil (BFT), sainfoin (SF), or alfalfa (ALF), and 2- and 3-way choices among strips of sainfoin and birdsfoot trefoil (SF-BFT), alfalfa and birdsfoot trefoil (ALF-BFT), alfalfa and sainfoin (ALF-SF), and alfalfa, sainfoin and birdsfoot trefoil (ALF-SF-BFT) in a completely randomized block design in 2 periods of 25 d each during two consecutive years. The lowest incidence of grazing events occurred in the BFT treatment (42.0% of the total scans recorded; P<0.10), with the rest of the treatments ranging between 47.8 (SF-BFT) and 52.6% (ALF-SF) of the total scans recorded. Heifers selected a varied diet, preferring sainfoin over birdsfoot trefoil or alfalfa in a 46:27:27 ratio for the 3-way choice, and in a 70:30

ratio for both 2-way choices. Heifers preferred birdsfoot trefoil over alfalfa (62:38 ratio) in a 2-way choice. All treatments followed similar daily grazing patterns (P>0.10), with two major grazing events (1 hour after sunrise and 3 hours before dark). No differences among treatments were observed for the number of steps taken by heifers on a daily basis, motion index, or the percentage of time heifers spent standing (1,600, 5,356, and 45.3%, respectively; P>0.10), suggesting that heifers on choice treatments did not invest extra time in walking, searching or patch switching activities relative to heifers grazing monocultures. Heifers grazing the 3-way choice gained more BW (1.27 Kg/d) than the average gains observed for animals in all legume monocultures (1.00 kg/d; P=0.014) or 2-way choices (0.97 kg/d; P=0.007), suggesting a synergism among pasture species for the treatment with the highest diversity. No differences in hair cortisol concentration were observed among treatments, with values ranging between 1.4 (BFT) and 2.12 ng/g (3-way choice) (P>0.10). Thus, forage diversity has the potential to enhance animal performance, likely driven by interactions among condensed tannins and dietary protein, without affecting hair cortisol levels or grazing efficiency, likely explained by the spatial arrangement of the forage species presented in the study.

INTRODUCTION

Legume-based finishing systems take advantage of the unique ability of ruminants to utilize significant amounts of plant fiber for energy (Van Soest, 2018), and the high nutritional quality and fermentation rates of legumes relative to grasses (Villalba et al., 2019). Nevertheless, monocultures of legumes like alfalfa (*Medicago sativa* L.) impose limitations to production in part caused by the risk of bloat (Wang et al., 2012), and by

the inefficient use of nitrogen due to imbalances in the ratio of nitrogen to energy commonly observed in these species (Getachew et al., 2006). One solution to this problem involves offering a diversity of forages with different types and concentrations of biochemicals (e.g., protein, non-fibrous carbohydrates, and plant secondary compounds like condensed tannins; CT), thus promoting complementary or associative relationships among multiple feed resources that improve animal fitness (Tilman, 1982) while reducing carbon and nitrogen (N) footprints (Rochfort et al., 2008; Patra and Saxena, 2010). For instance, the use of alfalfa in association with tanniferous legumes like birdsfoot trefoil (*Lotus corniculatus*), or sainfoin (*Onobrychis viciifolia*) overcomes the problem of excessive ruminal protein degradability (Aufrère et al., 2013; Grosse Brinkhaus et al., 2016), which reduces urinary N excretions and improves N retention in sheep and cattle (Lagrange and Villalba, 2019; Lagrange et al., 2020).

In addition to the aforementioned benefits, forage diversity provides animals with varied sensorial and post-ingestive stimuli that increase the motivation to eat (Meuret and Bruchou, 1994; Villalba et al., 2011). Herbivores grazing monocultures of single species satiate on the orosensorial characteristics of single feeds due to transient food aversions caused by flavors, nutrients, and toxins ingested too frequently or in excess, and satiety can be stressful (Provenza, 1996). However, if diverse options are available, animals may continue responding to other orosensorial or post-ingestive stimuli, achieving an adequate state of nutrition based on their individual and changing needs (Provenza et al., 2003; Villalba et al., 2015b). Thus, forage diversity contributes to enhanced animal welfare because generalist herbivores exposed to diverse arrays of feeds have less likelihood of experiencing stressful situations, like frustration due to lack of food alternatives available

to build a balanced diet, or satiety due to repeated or excessive exposure to the same single feeds (Villalba et al., 2010; Catanese et al., 2013).

Accumulation of cortisol in hair during a specific period of hair growth, provide information of retrospective cortisol levels during an established period of time, which is not provided by other more common matrixes like serum or saliva. Thus, hair cortisol concentrations have been validated as a useful biomarker of long-term stress in cattle and a feasible methodology to objectively assess cattle welfare (Heimbürge et al., 2019).

Finally, the level of spatial aggregation of forage species in diverse systems, ranging from uniform mixes to separated swards may influence ingestive behavior and performance in ruminants (Chapman et al., 2007). In a finely intermingled mix pasture, animals may have to search for and handle the preferred plant species, and these time-consuming activities may reduce intake rate relative to grazing monocultures (Prache et al., 1998). On the other hand, spatial segregation of plant species into patches may reduce the time animals need to select and handle desired amounts of specific forages, while at the same time overcoming many agronomic difficulties inherent in establishing and maintaining mixed pastures (Chapman et al., 2007). Previous studies have found that offering different forage species as ryegrass and white clover to grazing sheep and goats in contiguous strips rather than in mixtures increases voluntary intake and performance (Champion et al., 2004). Nevertheless, there is a gap in knowledge regarding the potential complementarity among patches of legumes of different chemistries and their potential associative effects on beef production systems. Therefore, the aim of this study was to evaluate the synergistic effect of offering increasingly diverse combinations of tanniferous (birdsfoot trefoil; sainfoin) and non-tanniferous (alfalfa) legumes as

monocultures with no choice, or in 2 or 3-way combinations of species, on foraging behavior, animal performance and a welfare parameter (hair cortisol) in grazing cattle during the finishing process. Our hypothesis was that monocultures with no choice were more likely to engender stress than 2- or 3-way choices among species with or without CT.

MATERIAL AND METHODS

The study was conducted at the Utah State University irrigated pasture research facility in Lewiston, UT (41 56' N 111 52'W, 1382 m altitude), according to procedures approved by the Utah State University Institutional Animal Care and Use Committee (approval 2566). The experiment took place from June 21 to September 2 in 2016 and from June 5 to August 23 in 2017.

Pasture and experimental design

Pastures and experimental design utilized in this study were the same as presented in the previous chapter (Chapter 4). Briefly, three blocks (replications) of seven pasture treatments were established on irrigated land at the research facility in September of 2015. Treatments included monocultures of two tanniferous legume species: 1) sainfoin (*Onobrychis viciifolia* cv. Shoshone; SF) and 2) birdsfoot trefoil (*Lotus Corniculatus* cv. Langille; BFT), 3) the non-tanniferous legume alfalfa (*Medicago sativa* cv. Vernal; ALF), and all 2- and 3-way choices among these legumes presented in strips: 4) alfalfa and sainfoin (ALF-SF), 5) alfalfa and birdsfoot trefoil (ALF-BFT), 6) sainfoin and birdsfoot trefoil (SF-BFT) and 7) alfalfa, sainfoin and birdsfoot trefoil (ALF-SF-BFT).

All treatment plots had an area of 0.5 ha each and were randomly distributed within each block. For monocultures, the entire 0.5 ha was planted to a single species; for choice treatments, there were either two 0.25-ha strips approximately 30 m wide x 82 m long, or three 0.165-ha strips of 20 m wide x 82 m long; strips within each block were randomly assigned to alfalfa, sainfoin or birdsfoot trefoil, depending on treatment (Fig. A-5). Thus, in each 2- and 3-way choice plot, cattle could freely graze on any of the two or three species on offer (Fig. A-6 and A-7). The perimeters of the experimental plots were defined by electric fence (Fig. A-8).

Pastures of sainfoin, birdsfoot trefoil and alfalfa were seeded at rates of 36.8, 11.0, and 19.7 kg of pure live seed/ha respectively on September 2, 2015. Seeds were previously inoculated with the appropriate rhizobium inoculant (N-Dure; INTX Microbials, LLC, Kentland, IN) before planting. During the first year of establishment, all plots were sprayed with 2.5 L/ha of Butyrac® 200 (2,4-DB; Albaugh Inc., Ankeny, IA) for broadleaf weed control on April 15, 2016. On May 19, 2016, the initial growth of the legumes was mowed, cured and baled. On June 10, 2016, 900 ml/ha of Plateau® (imazapic; BASF Corp., Durham, NC) was applied to all pastures for control of grass weeds. In year 2 (2017), all plots were sprayed with 440 ml/ha of Thunder® (imazethapyr; Albaugh Inc., Ankeny, IA) for broadleaf weed control and 730 ml/ha of Volunteer® (clethodim; Tenkōz Inc., Alpharreta, GA) for grass weeds on May 3, 2017. Grazing was delayed according to the manufacturer's recommendations.

Animals and grazing protocol

Animals and grazing protocol utilized in this study were the same as presented in

the previous chapter (Chapter 4). Briefly, pastures were grazed during two periods (P1 and P2) in two consecutive years, 2016 and 2017. During each year, a different set of 42 Angus heifers were sorted by body weight (BW), and distributed among seven groups of 6 animals with similar total weight per group. Groups were randomly assigned to the 7 treatments. Heifers within treatments were grouped in pairs (n=3) with similar individual weight and each pair was randomly assigned to one of 3 treatment replications (blocks). The heifers' initial and final mean BW was 394 ± 54 kg and 436 ± 55 kg, respectively, for 2016, and 352 ± 40 kg and 421 ± 42 kg, respectively, for 2017.

Each experimental period included a 10-day adaptation phase to adjust animals to their respective diet treatment. Period 1 of 2016 occurred from June 30 to July 18, and P2 from August 18 to September 2. During 2017, P1 occurred from June 15 to June 28, and P2 from August 10 to August 23. Samples were collected during 5 consecutive days at the end of each experimental period (collection period).

During 2016, at the beginning of P1 (June 30), birdsfoot trefoil and alfalfa swards were in the early bloom stage of their second growth cycle, whereas sainfoin swards were in the full bloom stage. On August 18 (P2), alfalfa and birdsfoot trefoil's third growth cycle was in late bud – early bloom stage, whereas sainfoin (with a slower regrowth) was in the late vegetative to early bud stage. In contrast to 2016, pastures were not mowed in the spring of 2017, and accumulated birdsfoot trefoil and alfalfa was grazed at full bloom stage and sainfoin at the early seed pod stage beginning June 15. On August 10 (P2), alfalfa and birdsfoot trefoil regrowth was in full bloom but sainfoin was at the late bud and early flowering stage.

Between experimental periods, animals grazed on an overflow pasture of

endophyte-free tall fescue, until legumes regrew sufficiently to be grazed again. All pastures were irrigated using hand-line sprinklers in 12-h sets that applied approximately 10.5 cm of water.

Heifers strip-grazed their respective pastures behind electric fences that were moved approximately every three days to give access to fresh forage, and back-fenced to prevent access to previously grazed forage and allow legumes to re-grow. In P2, heifers grazed legumes that had regrown for approximately 45 days. Heifers were moved to a new section of the same treatment once they grazed 20-30 % of the initial available biomass for monocultures, or when any of the legume strips was grazed to that extent in 2- or 3-way choice treatments. This procedure ensured *ad libitum* forage availability for all the species present in each treatment.

Throughout the adaptation and sample collection phases, animals had free access to water and trace-mineral salt blocks (mineral composition: minimum 960 g/kg NaCl, 320 mg/kg Zn, 380 mg/kg Cu, 2,400 mg/kg Mn, 2,400 mg/kg Fe, 70 mg/kg I, and 40 mg/kg Co). Animals on all treatments had access to bloat protectant blocks with Poloxalene 6.6% (Sweetlix® Pressed Bloat Guard®, Ridley USA Inc., Mankato, MN) for 2 days before entering the adaptation phase in order to reduce the likelihood of frothy bloat in animals that were assigned to ALF. All animals were tagged with ear fly tags before beginning the first experimental period (P1) in each year for external parasite prevention.

Measurements

<u>*Herbage availability*</u>

Herbage dry matter (DM) availability per unit area in each plot was assessed before animals entered new paddocks (pre-grazing herbage mass) on July 3 and August 21 (2016) and on June 18 and August 13 (2017) for P1 and P2, respectively. Herbage availability was also evaluated after animals grazed these paddocks (post-grazing herbage mass). Measurements were made by taking 60 readings in each paddock (monocultures) or in each monoculture strip of 2- and 3-way choices using a rising plate pasture meter (Electronic Plate Meter Jenquip EC-10, Agriworks Ltd, NZ). Each paddock was sampled in a "lazy" W pattern and every four steps the plate meter was lowered vertically onto the herbage. Calibration curves for each legume were built from individual raising plate meter readings of pre and post-grazing herbage at different heights. All forage under the plate meter was cut to the ground using a 0.10-m^2 quadrant frame, the same area as the plate meter, and dried at 60°C to constant weight. Linear relationships for each experimental period and each legume were estimated from calibration curves of DM herbage biomass on plate meter readings.

<u>*Forage quality sampling*</u>

Representative samples of the herbage ingested by heifers were collected on day 3 of each experimental period from each forage and replication of each treatment. Herbage samples were collected between 1000 and 1300 h by walking a transect across a pasture section and hand-plucking the top 15-20 cm of the sward every few steps, mimicking the plant parts grazed by heifers. Samples were placed in plastic bags, covered with dry ice,

and frozen at –20°C until they were freeze-dried (Free Zone 18 Liters, Labconco

Corporation, Kansas City, MO), and ground to pass the 1-mm screen of a Wiley mill

(model 4; Thomas Scientific Swedesboro, NJ, USA) for chemical analyses.

Scan sampling

The foraging behavior of the pair of heifers in each treatment plot was recorded

using game cameras (PC800 HyperFire Professional IR, Reconyx Inc, Holmen, WI) and

the incidence of feeding on each of the forage species in the choice treatments was then

determined. During the experimental period, seven cameras were distributed among the

seven treatment plots in a spatial replication (block), allocating a camera at one side of

each paddock. Cameras were placed immediately after heifers had access to fresh strips

of pasture and they were kept in the same plot for 48 h in order to capture images of the

heifer's locations and behaviors (see below) with a time-lapse of 5 min intervals.

Cameras were active from 0500 (dawn) until 2200 (last light), a period of 17 h. This

procedure allowed scanning for daily grazing patterns during the first and second day

after animals accessed fresh pasture, when all forage species present in the paddock were

available in ad *libitum* amounts. Subsequently, cameras were moved to a different block

for the first 48 h on fresh pasture breaks, and then to the third replication for the same

amount of time. These rotations continued until the three replicates for each treatment

were recorded twice in each one of the experimental periods in 2016 and 2017. Pictures

were then visualized individually using Preview version 10.1 (Apple Inc.).

Scan samples were used (Altmann, 1974), to assess the incidence of feeding on

each forage species within each paddock (grazing) and bouts of inactivity such as not

eating, resting, searching and drinking water (non-grazing). The incidence of feeding in

each treatment was evaluated as the percentage of the total number of scans in which

heifers were feeding during each 48-h recording period relative to the total number of

scans recorded (grazing and non-grazing events). In 2- or 3-way choices, frequency of

feeding on each legume (preference) was calculated as a percentage of the number of

grazing scans recorded on each of the legume species within each treatment relative to

the total number of grazing scans recorded for each 48-h recording period. Pairs normally

grazed together (>90% of the time) on the same plant species. If individuals were

performing different behaviors, each behavior was recorded for each individual. A total

of 61,640 pictures were analyzed from two experimental periods and two years of study.

Behavioral levels of activity

One animal from each of the 21 pairs of heifers used in the study were used for

activity measurements and fitted with a pedometer (Icetag3D^{TM}, IceRobotics, Roslin,

UK) on their left rear leg from the beginning of each experimental period and removed

during the last day of the period. Activity levels and posture (number of steps taken,

motion index, lying and standing bouts) were measured with the use of these pedometers

which took second-to-second readings throughout the period. The motion index provides

a broader measure of the animal's activity level and complements the step count,

considering the magnitude of the 3-D acceleration, and as such it is related to the total

amount of energy used by the animal over a given period. The calculation is performed

per second and then summed to provide the total activity per minute in G's/10 (Ice

Robotics, 2020). Data were downloaded with the provided IceRobotics software (version

2012) in a format of 1 summary record per day. Standing and lying times were calculated by summing the time in seconds during the day animals spent standing and lying, respectively.

Average daily gain calculations

Heifers were weighed individually using a load cell scale (Rice Lake weighing systems, Rice Lake, WI) located under the squeeze chute at the beginning and end of each experimental period to estimate average daily gains (ADG). Feed and water were withheld from 1800 h until the following morning, when animals were weighed at 0900 h before transfer to pastures.

Hair sampling and cortisol extraction

Assessing cortisol in hair samples is thought to reflect long term adrenocortical activity over many weeks or months, which is a more precise indicator of chronic stress than other matrices like blood, saliva or fecal samples (Meyer and Novak, 2012). Hair samples were taken from one animal of each pair of heifers. Heifers were shaved the first day of the adaptation phase and hair samples were collected on the last day of the experimental period, a hair growth period of 18 and 25 days in 2016 and 21 and 19 days in 2017, for P1 and P2, respectively. The hair samples contained only new black hair grown during each period, and was taken from a 100 cm^2 square area on the forehead. Samples were collected using an electric hair clipper (Model AGR+ ANDIS; Sturtevant, WI) to acquire the longest possible hair sample (approximately 1 cm), while at the same time avoiding scratching the skin. Each hair sample was placed into pre-labeled zip-lock plastic bags and stored in the freezer until cortisol extraction.

Extraction of cortisol from the hair sample was performed according to Tallo-Parra et al., (2015). Briefly, 250 mg of hair from each sample was weighed and placed into a 15-ml conical tube (Falcon®, BD Biosciences, Franklin Lakes, NJ). Each sample was washed by adding 2.5 ml of isopropanol (2-propanol 99.5%, J.T. Baker® Avantor, Phillipsburg, NJ) and vortexed (Vortex Genie 2, 3030A, Daigger Scientific, Vernon Hills, IL) for 2.5 min in order to remove external steroid sources. The isopropanol was drained and the process was repeated twice (three washes in total). The hair samples were left to dry completely for approximately 5 days in darkness at room temperature. Then hair was ground to a fine powder using a ball mill (MM 301 Mixer mill, Retsch GmbH; Haan, Germany) with 10-ml stainless steel grinding jars and one single 12-mm stainless steel grinding ball for 5 min at 30 Hz. Then, 50 mg of ground hair from each sample was weighed and placed into a pre-labeled 2-ml microcentrifuge tube (Fisher Scientific®, Waltham, MA) and stored in darkness at room temperature.

For cortisol extraction, 1.5 ml of pure methanol (Fisher Scientific®, Waltham, MA) was added directly to the 2-ml tubes and then all tubes were placed in an orbital shaking water bath at 30ºC (Model 3545, Lab-Line Instruments, Melrose Park, IL) and shaked at 100 rpm for 18 hours. Following extraction, samples were centrifuged at 7000 × g for 2 min (Centrifuge 5415 C, Eppendorf GmbH, Hamburg, Germany) and subsequently 0.75 ml of supernatant from each tube was transferred into new pre-labeled 2-ml microcentrifuge tubes and placed in a digital dry heat block (ISOTEMP 125D, Fisher Scientific, Waltham, MA) at 38 ºC with the lids open. Once the methanol was completely evaporated (approximately after 24 hours), the dried extracts were reconstituted with 0.2 ml of EIA buffer (Cortisol ELISA KIT; Neogen Corporation,

Lexington, KY) and shaken for 30 seconds and immediately stored at -20 ºC until analysis.

Chemical analyses

Forage samples were analyzed for DM, total N concentration, acid detergent fiber (ADF), acid detergent lignin (ADL), and CT. Dry matter was determined by drying the samples at 105°C for 3 h in a forced-air drying oven (AOAC, 1990; method 930.04). Total N concentration was analyzed using a Leco FP-528 N combustion analyzer (AOAC, 2000; method 990.03) with crude protein (CP) concentration calculated as N concentration × 6.25. Concentration of ADF was determined according to (AOAC 2000; method 973.18), modified by using Whatman 934-AH glass micro-fiber filters with 1.5 µm particle retention and a Buchner funnel in place of a fritted glass crucible. Determinations of ADL were modified from Robertson et al (1981) as follows: fiber residue and filter from the ADF step was transferred to a capped tube and approximatelly 45 ml of 72% sulfuric acid was added. Tubes were gently agitated for 2 h and filtered onto a second Whatman 934-AH glass micro-fiber filter which was then rinsed, dried, weighed and finally ashed for 2 h in a furnace to remove lignin organic matter. Analyses of total CT in legume samples were conducted in triplicate, according to the butanol-HCl-acetone spectrophotometric assay of Grabber et al. (2013), using CT isolated from sainfoin and birdsfoot trefoil as the standards.

Cortisol concentrations from hair extracts were determined on duplicate samples using a cortisol ELISA (Enzyme-Linked Immuno Sorbent Assay) detection kit (Neogen Corporation, Lexington, KY). After samples were thawed, 50 µL of each sample

(hormonal extracts) and 50 μL of each of the standards (provided by the kit) were transferred to a well of a 96 well microplate in duplicate. Next, 50 μL of diluted enzyme conjugate (110 μL of enzyme conjugate provided by the kit mixed with 5.5 mL of EIA buffer per plate) was added to each well and the plates were gently on a rotary shaker while incubating at room temperature for one hour. After incubation, contents of the plate are dumped and tapped out thoroughly on clean lint-free wipes. The plate is then washed three times using 300 μL of diluted wash buffer per well (20 mL of wash buffer provided by the kit plus 180 mL of deionized water) to remove all unbound material. Finally, 150 μL of substrate (kit provided) were added to each well, which detects bound enzyme conjugate by generating a color reaction after 30 min of incubation at room temperature. Cortisol concentrations were obtained by measuring and comparing the absorbance of sample wells against the standards with a microplate reader (SpectraMax M5, Molecular devices LLC, San Jose, CA) at 650 nm.

Cortisol Calculations:

[Cortisol] (ng/g hair) = [(Cortisol ng/ml × 0.2 ml) × (1.5 ml / 0.75 ml methanol)] / 0.05 g ground hair

Statistical analyses

Average daily gain and concentration of hair cortisol was analyzed using a 2-way factorial treatment structure (year × period) in a randomized complete block design using a generalized linear mixed model. Treatment (7; single forage species, 2-way and 3-way combinations), Period (2), Year (2) and all interactions were the fixed factors. Block, Block × Treatment and Block × Treatment × Year were included in the model as random

factors.

Percentage of total grazing scans, number of steps per day, daily motion index and percentage of standing time per day were analyzed using a similar design but with Period nested within Year, because experimental periods were not performed at the same time in both years and there was a photoperiod shift that may affect the response. Thus, the fixed factors were Treatment, Year and Period (Year) and random factors were Block, Block × Treatment and Block x Treatment × Period (Year). In addition, percentage of total grazing scans was analyzed using a binomial distribution (event/trial syntax). In this case, the binomial model used the number of grazing scans (y) and the total number of scans (n) as the response variable.

Percentage of grazing scans and standing time at each hour of the day (grazing patterns) were analyzed separately for each experimental period of each year of the study, due to differences in daylight hours at each experimental period affecting these variables. Thus, the generalized lineal mixed model included Treatment and time of the day and their interaction as fixed factors and Block and Block × Treatment were included in the model as random factors. Percentage of grazing scans per time interval also included Block × Time as random factors and used a binomial distribution which better fitted the nature of the scans data. In order to address overdispersion of the data for the binomial distribution, the residual variance Block × Treatment × Time was also included in this model as a random factor.

Finally, CP, ADF, ADL and CT concentrations in legume species as well as DM availability was analyzed using the same model, but with Species rather than Treatment as a fixed factor (alfalfa, sainfoin or birdsfoot trefoil). Block, Block × Species and Block

× Species × Year were included in the model as random factors.

All analyses were computed using PROC GLIMMIX in SAS/STAT 14.2 (SAS Inst., Inc. Cary, NC; Version 9.4 for Windows). Least squares means (LSmeans) were compared pairwise using the Least Significant Difference test when the overall test for Treatment effect was significant ($P \leq 0.10$). Means were reported along with their standard errors (SEM). Treatment differences were considered a tendency when $0.10 < P \leq 0.15$. Additionally, preplanned contrasts were performed to compare the 3-way choice LSmean vs. the average LSmean for the three monoculture treatments or the average LSmean for all 2 way-choices, using the LSMESTIMATE statement in PROC GLIMMIX. Contrasts were specified as the arithmetic difference between ALF-SF-BFT and (0.33ALF + 0.33SF + 0.33BFT) or (0.33ALF-SF + 0.33ALF-BFT + 0.33SF-BFT) respectively. Contrasts between the average of 2-way choices and the average of monoculture treatments were also performed. A difference was considered significant when P values were < 0.10. Treatment differences were considered a tendency when $0.10 < P \leq 0.15$.

Assumptions of homoscedasticity of variance and normality were tested using studentized residuals when analysis used a normal distribution. Hair cortisol and CT concentrations were transformed to natural logarithm, and percentage of standing time per time interval were transformed to the Logit scale in order to meet homogeneity of variance assumptions, and back transformed to report LSmeans and SE.

Forage preference (percentage of grazing scans recorded for any single species relative to the total number of grazing scans recorded in a choice treatment) was assessed separately for each of the 2-way and 3-way choice treatments. Data were analyzed using a generalized linear mixed model for a 2-way factorial treatment structure (Year and

Period) in a RCBD with a binomial distribution (y/n: number of grazing scans of any

species / number of total grazing scans in the choice). The residual Block × Period × Year

was included as random factor in order to address overdispersion. Due to lack of

independence of scans data within each treatment, the overall mean percentage of each

species in a specific choice treatment was estimated as the average over the 4 year ×

period combinations, and reported along with their 90% confidence intervals. A legume

species was considered "preferred" or "not preferred" in a specific 2- or 3-way choice

treatment, when the overall mean percentage selected (intercept) for the legume was

higher or lower than 50% or 33%, respectively, and the confidence interval for the

intercept did not include 50% or 33%.

RESULTS

Nutritional composition of the forages

The average nutritional composition of the legumes used in the study for both

years 2016 and 2017 is reported in Table 5-1. All forage legumes contained high

concentrations of CP (20-30%; DM basis), low levels of ADF (<25%) and intermediate

levels of ADL (3.5-5.5%). The nutritional composition of birdsfoot trefoil and alfalfa was

similar in both years of the study, and both legumes showed declines in their

concentrations of CP and incremental increases in their concentrations of ADF and ADL

from P1 (e.g., late June – early July) to P2 (e.g., middle to late August). In contrast,

sainfoin contained the lowest concentrations of CP and the greatest concentrations of

ADF and ADL during P1 in both years, although the concentration of ADF was less in P2

regrowth, and contents of CP and ADL became similar to the rest of the legumes assayed.

Condensed tannin concentration in sainfoin was 3- to 6-fold (2016), and 2- to 4-fold (2017) the concentration observed in birdsfoot trefoil for P1 and P2, respectively. Alfalfa is a non-tanniferous legume, confirmed by the low levels of CT revealed in the assay (Table 5-1).

Table 5-1. Nutritional composition (g/kg DM) of legumes during both periods and years of study.

2016	CP		ADF		ADL		CT	
Species	P1	P2	P1	P2	P1	P2	P1	P2
Alfalfa	289.8[a A]	246.7[a B]	176.7[b]	175.3	35.6[b B]	39.9[c A]	1.5[c A] (0.1)	1.0[c B] (0.1)
B. Trefoil	264.8[b A]	215.2[b B]	169.2[b]	157.1	45.0[a]	46.2[b]	15.0[b] (1.1)	14.3[b] (1.1)
Sainfoin	217.8[c]	219.9[b]	224.5[a A]	172.3 [B]	50.0[a]	52.2[a]	47.2[a B] (3.6)	90.5[a A] (6.7)
SEM	7.1	7.1	7.7	7.7	2.2	2.2		
P value	<0.001	0.003	<0.001	0.201	<0.001	<0.001	<0.001	<0.001
2017	CP		ADF		ADL		CT	
Species	P1	P2	P1	P2	P1	P2	P1	P2
Alfalfa	275.7[a A]	235.8[ab B]	175.4[b B]	227.4[a A]	37.7[b B]	51.3 [A]	1.5[c B] (0.1)	1.8[c A] (0.1)
B. Trefoil	287.0[a A]	248.8[a B]	146.9[c B]	174.9[b A]	42.3[b B]	50.0 [A]	20.3[b A] (1.6)	17.4[b B] (1.3)
Sainfoin	217.9[b]	225.0[b]	256.7[a A]	237.7[a B]	53.6[a]	55.0	40.8[a B] (3.1)	61.4[a A] (4.7)
SEM	7.4	7.4	7.9	7.9	2.3	2.3		
P value	<0.001	0.066	<0.001	<0.001	<0.001	0.272	<0.001	<0.001

a-c LSmeans in a column with different lower-case superscripts differ (P<0.10).
A-B LSmeans in a row with different upper-case superscripts within the same parameter differ (P<0.10).
CP= crude protein; ADF= acid-detergent fiber; ADL= acid-detergent lignin and CT= Condensed tannin concentration. Values are means for 3 spatial replications (blocks).

Herbage availability

Herbage availability for both years of the study was in general high, ranging from 4 to 8 Mg/ha (Table 5-2), with greater biomass observed for P1 than for P2 in 2017 (period × year interaction; P<0.001). Averaged across treatments and periods, availability of alfalfa, sainfoin and birdsfoot trefoil was 60, 17 and 22% greater in 2017 than in 2016, (P<0.001; P=0.021; P=0.001, respectively). Considering only Period 1, herbage

availability increased from 2016 to 2017 by 74, 37 and 35% for alfalfa, sainfoin and

birdsfoot trefoil, respectively (P<0.001).

Table 5-2. Average of pre and post-grazing DM herbage availability (Mg/ha) (LSmeans) for alfalfa, sainfoin and birdsfoot trefoil across treatments during two periods (P1 and P2) and years (2016 and 2017), and the overall mean across treatments, periods and years.

Species	Alfalfa, (Mg DM/ha)			Sainfoin (Mg DM/ha)			Birdsfoot Trefoil (Mg DM/ha)			Treatment effect
	P 1	P2	Average	P 1	P 2	Average	P 1	P 2	Average	P-Value
2016 Pre-grazing	4.6[b]	4.2[b]	4.4[b B]	4.3[b]	4.1	4.2[b B]	5.4[b]	5.5[b]	5.5[b A]	0.002
2017 Pre-grazing	8.0[a A]	6.1[a B]	7.0[a A]	5.9[a A]	3.8[B]	4.9[a B]	7.3[a A]	6.1[a B]	6.7[a A]	<.0001
SEM	0.2	0.2	0.1	0.2	0.2	0.1	0.2	0.2	0.1	
Year effect, *P-value*	<.0001	<.0001	<.0001	<.0001	0.324	0.021	<.0001	0.036	0.001	

	Overall		
Species	Alfalfa (Mg DM/ha)	Sainfoin (Mg DM/ha)	Birdsfoot trefoil (Mg DM/ha)
Pre-grazing	5.6	4.5	6.1
Post-grazing	4.5	3.3	5.0

[a-b]LSmeans in a column with different lower-case superscripts differ (P<0.10). [A-B] Average LSmeans in a row with different upper-case superscripts differ (P<0.10). Values are means for 3 spatial replications (blocks), and 4 treatments within each species (n=12). Values at the bottom half of the table are means for 3 blocks, 4 treatments within each species, 2 years and 2 periods within each year of the study (n=48).

During 2016, pre-grazing biomass averaged across periods and treatments was

greater for birdsfoot trefoil than for alfalfa or sainfoin (5.5 vs. 4.4 and 4.2 Mg/ha,

respectively; P=0.002). In contrast, no differences were observed for this variable

between birdsfoot trefoil and alfalfa during 2017, but biomass of both species was greater

than biomass for sainfoin, which showed the lowest pre-grazing biomass (6.7 and 7.0 vs.

4.9 Mg/ha; P <0.001, respectively). When averaged across periods and years, the

proportion of herbage biomass that disappeared was 0.27, 0.20 and 0.18 of pre-grazing

measurements for sainfoin, alfalfa and birdsfoot trefoil plots, respectively (Table 5-2).

Scan sampling

<u>Preference</u>

Figure 5-1 shows the percentage of grazing scans in each species relative to the total number of grazing events recorded for animals grazing a choice of legumes for each year and period. Heifers offered 3-way choices were observed more times grazing sainfoin (46% of the total grazing scans recorded) than birdsfoot trefoil or alfalfa (27% each) (Fig. 5-1a). During P1 of both years of the study, heifers spent approximately half of their grazing activity during daily 17-h sessions grazing sainfoin (47 and 49% of the grazing events recorded for 2016 and 2017, respectively, Fig. 5-1a). However, preference for this legume declined during P2 to 43 and 44% of the total grazing events recorded as a consequence of an increment in grazing activity on birdsfoot trefoil. However, the confidence intervals of these means included 33%, indicating indifference or no selection preference.

When animals were offered 2-way choices containing sainfoin, they preferred this legume over alfalfa or birdsfoot trefoil, particularly during P1 of 2016, with 80% of the total grazing events recorded on sainfoin strips (Fig. 5-1b and Fig 5-1c, respectively). Percentage of grazing scans recorded on birdsfoot trefoil and alfalfa slightly increased throughout periods and years, but they were always the least preferred species in a choice with sainfoin. On average across years and periods, heifers preferred sainfoin over alfalfa or birdsfoot trefoil in a 69:31 and 71:29 ratio, respectively (Fig. 5-1b and Fig 5-1c).

When heifers were exposed to 2-way choices between ALF and BFT, they preferred birdsfoot trefoil over alfalfa in three out of the four grazing periods of the study

and only in P1 of 2017, heifers showed no preference among species (Fig. 5-1d). Averaged across years and periods, heifers preferred birdsfoot trefoil over alfalfa in a 62:38 ratio.

Total grazing scans

The average percentage of total grazing events recorded across years and periods was the lowest for the BFT treatment ($P<0.10$; Table 5-3), and no treatment × period ($P=0.679$) or treatment × year ($P=0.255$) interactions were detected. Consistent with the overall pattern, the BFT treatment showed the lowest percentages of grazing scans in P1 of both years ($P<0.10$; data not shown). No differences among treatments were observed during P2 in both years of the study (2016; $P=0.332$ and 2017; $P=0.496$).

Pre-planned contrasts showed that the average of the grazing events recorded for 2-way choices was greater than the average value for all single species (49.9 vs. 47.0%, SEM=2.0%, respectively; $P=0.080$; Table 5-3), with no additional differences observed for the rest of the contrasts performed (Table 5-3). No differences in grazing scans were observed between P1 and P2 during 2016 (50.4 vs. 48.1%, SEM=1.1%), but the percentage of grazing scans was greater in P1 than in P2 during 2017 (49.9 vs. 45.6%, SEM=1.1%, respectively).
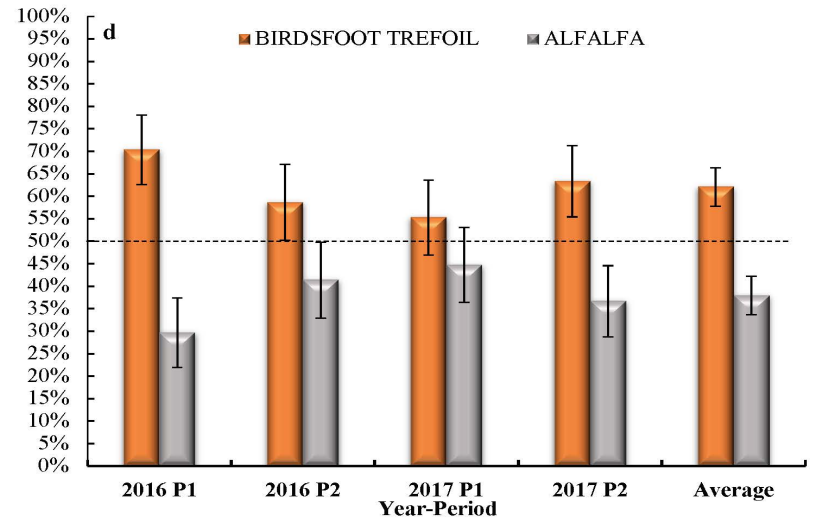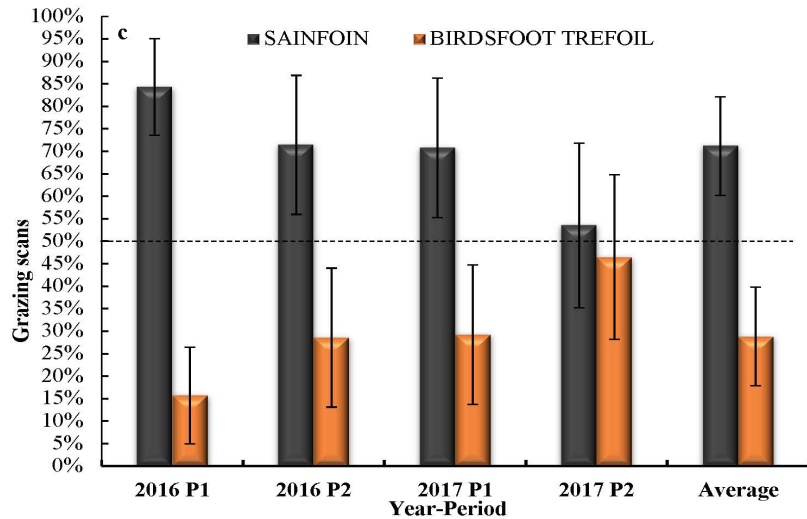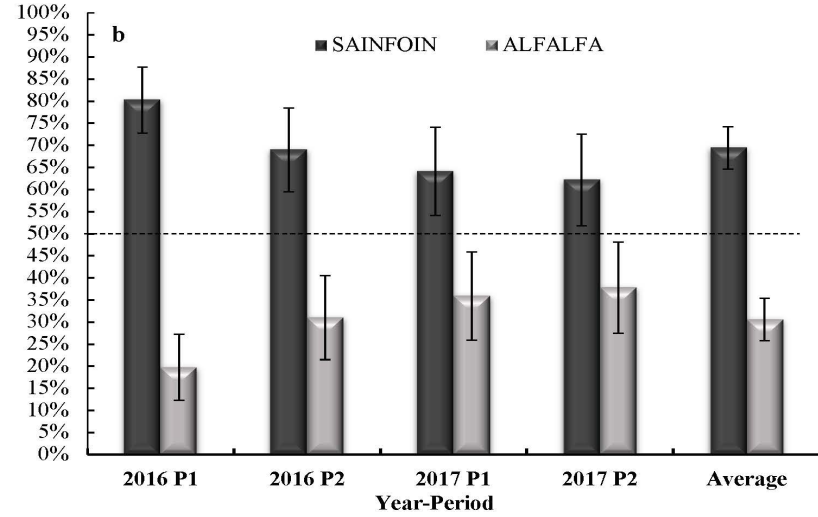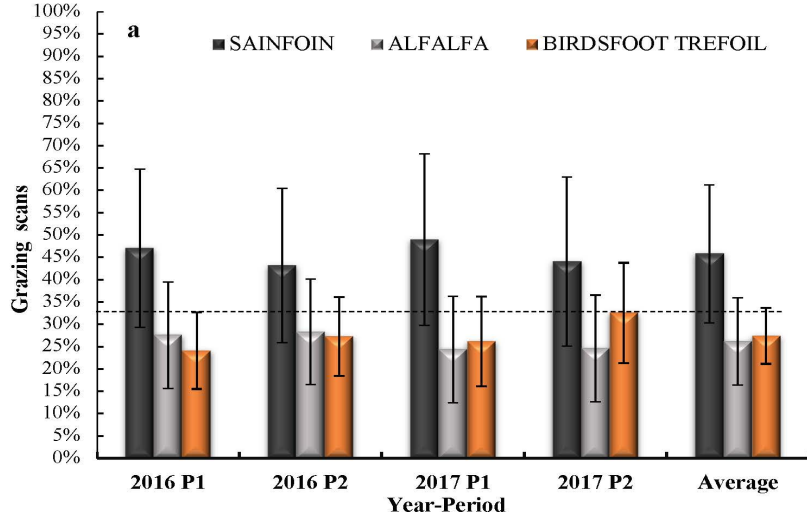
Figure 5-1. Percentage of grazing scans where heifers recorded a preference for a legume species in 3- or 2-way choices among alfalfa, sainfoin and birdsfoot trefoil, during 2 grazing periods (P1 and P2) in 2016 and 2017. Values are means for 3 spatial replications. Bars represent upper and lower values of 90% confidence intervals. Dashed lines indicate indifference or no preference (33% and 50% for 3- and 2-way choices respectively) for any species. A legume species was considered "preferred" or "not preferred" when the confidence interval for the mean did not include the indifference threshold.

Table 5-3. Percentage of grazing scans, behavioral levels of activity, hair cortisol concentration, and average daily gains [LS means (SEM)] by heifers grazing single legumes, and 2- and 3-way choices of legumes: Alfalfa (ALF), Birdsfoot trefoil (BFT) and Sainfoin (SF).

Treatments	Grazing scans, % of total scans	Standing Time, % of total daily time	Steps, number/d	Motion Index	Hair Cortisol[d], ng/g	ADG[e], kg/d
ALF	48.2[a] (2.0)	44.2 (1.4)	1511 (148)	5033 494)	2.00 (0.34)	0.930[c] (0.075)
BFT	42.4[b] (1.9)	44.3 (1.7)	1447 (172)	4550 (576)	1.44 (0.24)	1.136[ab] (0.075)
SF	50.4[a] (2.0)	46.2 (1.4)	1634 (145)	5180 (483)	1.86 (0.31)	0.927[c] (0.075)
ALF-SF	52.6[a] (2.0)	47.6 (1.4)	1731 (145)	5726 (483)	1.81 (0.31)	0.893[c] (0.075)
ALF-BFT	49.3[a] (2.0)	45.2 (1.4)	1566 (148)	5652 (494)	1.90 (0.32)	0.972[bc] (0.075)
SF-BFT	47.8[a] (2.0)	44.1 (1.4)	1648 (145)	5132 483)	2.36 (0.40)	1.033[bc] (0.075)
ALF-SF-BFT	48.7[a] (2.1)	45.7 (1.6)	1653 (162)	6222 (538)	2.12 (0.40)	1.268[a] (0.083)
			P-values			
Treatment effect	0.063	0.534	0.877	0.423	0.584	0.054
Period Effect[a]	0.001	0.192	0.208	0.312	0.001	0.009
Year Effect	0.093	0.341	0.083	0.051	0.024	0.768
Treatment × period[b]	0.679	0.604	0.544	0.515	0.761	0.198
Treatment × year	0.255	0.593	0.982	0.999	0.793	0.139
2-way vs monocultures[c]	0.080	0.548	0.359	0.172	0.337	0.618
3-way vs monocultures	0.478	0.682	0.521	0.052	0.391	0.014
3-way vs 2-way choice	0.588	0.994	0.979	0.250	0.814	0.007

[a-b] LSmeans in a column with different letters differ (P<0.10). Values are means for 3 spatial replications (blocks), two years, and two periods within each year of study.
[a] Period effect for percentage of grazing scans is nested within each year of study.
[b] Interaction between treatment and period for percentage of grazing scans is nested within each year of study.
[c] Indicate that these are pre-planned contrasts between 2-way, 3-way choices and monoculture treatments.
[d] Hair cortisol concentration. Reported values are back transformed LSmeans across 2 grazing periods of two consecutive years and 3 spatial replications.
[e] ADG = Average daily gain. Treatment values for each spatial replication (blocks) are the average of two heifers in each combination treatment*block.

Feeding patterns

When grazing events were analyzed across daily 17-h sessions in 1-h time intervals (from 0500 to 2200 h), no treatment × time intervals interactions were detected for both periods of 2016 (P=0.133 and P=0.707; for P1 and P2, respectively; Fig. 5-2a-b)

and during P1 of 2017 (P=0.274; Fig. 5-2c), showing that all treatments followed similar grazing patterns throughout the day. It can be observed that all animals in P1 (mid-June – mid-July) during both years started to graze at the same time (0500 to 0600 h; i.e., dawn), and by the next hour (0600 to 0700 h) 69.2 (2016) and 64.9% (2017) of the scans recorded represented grazing events (Fig. 5-2a-c). After this interval, and from mid-day to afternoon, heifers showed grazing events that alternated between 40 and 50% of the total scans recorded, ending at dusk with the greatest percentages of grazing events (70 to 90%) between 1900 and 2200 h.

Due to differences in photoperiod, animals during P2 (Mid-August- Early September) started to graze approximately one hour later (0600-0700 h) and showed their first peak of daily grazing events between 0700-0800 h (Fig. 5-2b). Consistent with P1, although 1 h earlier, animals showed a second peak of grazing events at dusk, with percentages ranging between 75 to 87% of all the scans recorded. In contrast to the rest of the periods, a treatment × time interval interaction was observed for P2 in 2017 (Fig. 5-2d), driven by a sharp decline in grazing events for all treatments except for the 3-way choice treatment during the 0800 to 0900 h time interval, and for the high percentage of grazing events (79.2% of all scans) observed at noon for the same treatment.
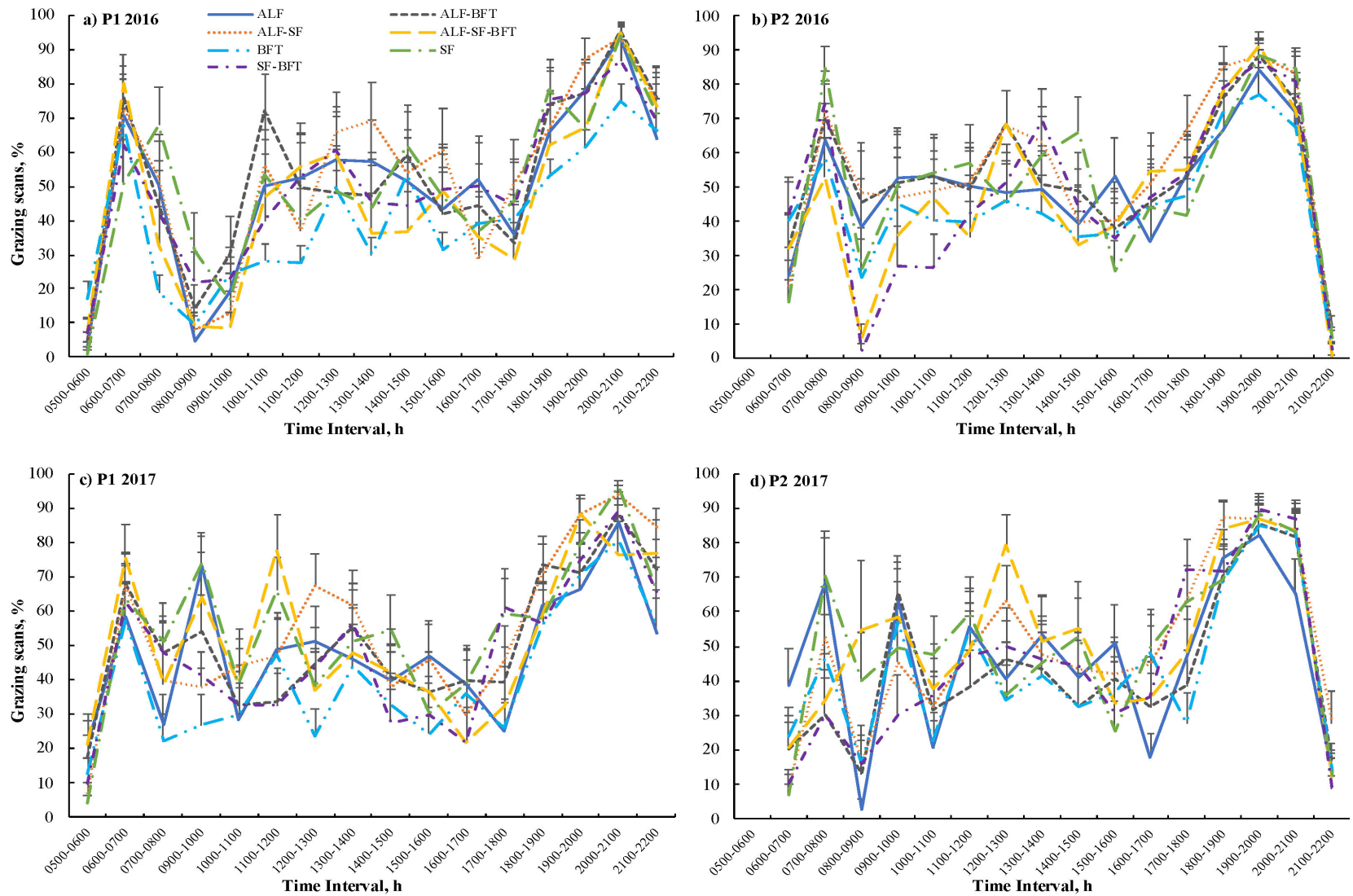
Figure 5-2. Grazing patterns (percentage of grazing scans recorded at each hour of the day) by heifers grazing single forages, 2- or 3-way choices of Alfalfa (ALF), Birdsfoot trefoil (BFT) and Sainfoin (SF) during two grazing periods in 2 consecutive years. Values represent the average of 6 heifers across 4 days in each period of 2016 and 2017. Time interval 0800-0900 was dropped from the analysis in P1 2017 due to missing values. Time intervals 0500-0600 were dropped from the analysis in P2 2016 and P2 2017 because most of the observed grazing percentages were zero.

Behavioral levels of activity

Averaged across periods and years in the study, no differences among treatments were observed for the number of steps taken by heifers on a daily basis (P=0.877; Table 5-3). On average across treatments, the number of steps was also similar for both periods in 2016 (P=0.110) and in 2017 (P=0.447; data not shown), although 2017 heifers took more steps than 2016 heifers (1707 vs. 1490; SEM=80; P=0.083).

No differences among treatments were observed for levels of activity measured as a motion index (P=0.423; Table 5-3). However, when contrasting the 3-way choice against the average of the three-single species in pre-planned contrasts, the former showed a motion index 26.4% greater than the average value recorded for single species treatments (6222 vs. 4921; P=0.052). In contrast, no differences in motion index were detected between 2-way choices and single-legume species (5504 vs. 4921; P=0.172). Consistent with number of steps, the motion index also showed greater values during 2017 than during 2016 (5764 vs. 4949; SEM=18; P=0.051).

There were no differences among treatments in the percentage of time heifers spent standing (P=0.534; Table 5-3). The same response applies to the percentage of time animals spent lying down, as both variables are linear combinations (i.e., standing time = total time – time lying down). No interactions were detected between treatments and periods or treatments and years, with similar standing times across grazing periods and years (P>0.10).

Figure 5-3 shows the percentage of time that heifers spent standing in each treatment at each hour (time interval) for each grazing period in each year. No interactions between treatments and time intervals were observed for both periods of

2016 (P=0.307 and P=0.979; for P1 and P2, respectively) and 2017 (P=0.164 and

P=0.107). Consistent with the pattern observed for the percentage of grazing scans,

animals during P1 and between 0600 and 0700 h (73.2 to 79.4% of the time recorded)

and 1900 and 2200 h (70 to 98.6%) spent most of the time standing. During P2, due to

differences in photoperiod, peaks shifted to the 0700 to 0800 h interval in 2016 (89.4%)

and to the 0900 to 1000 h interval in 2017 (90.1%). At dusk, peaks of standing time

occurred earlier in P2 than in P1, from 1800 to 2100 h, both during 2016 (67.7 to 96.5%)

and 2017 (70.0 – 97.9%).
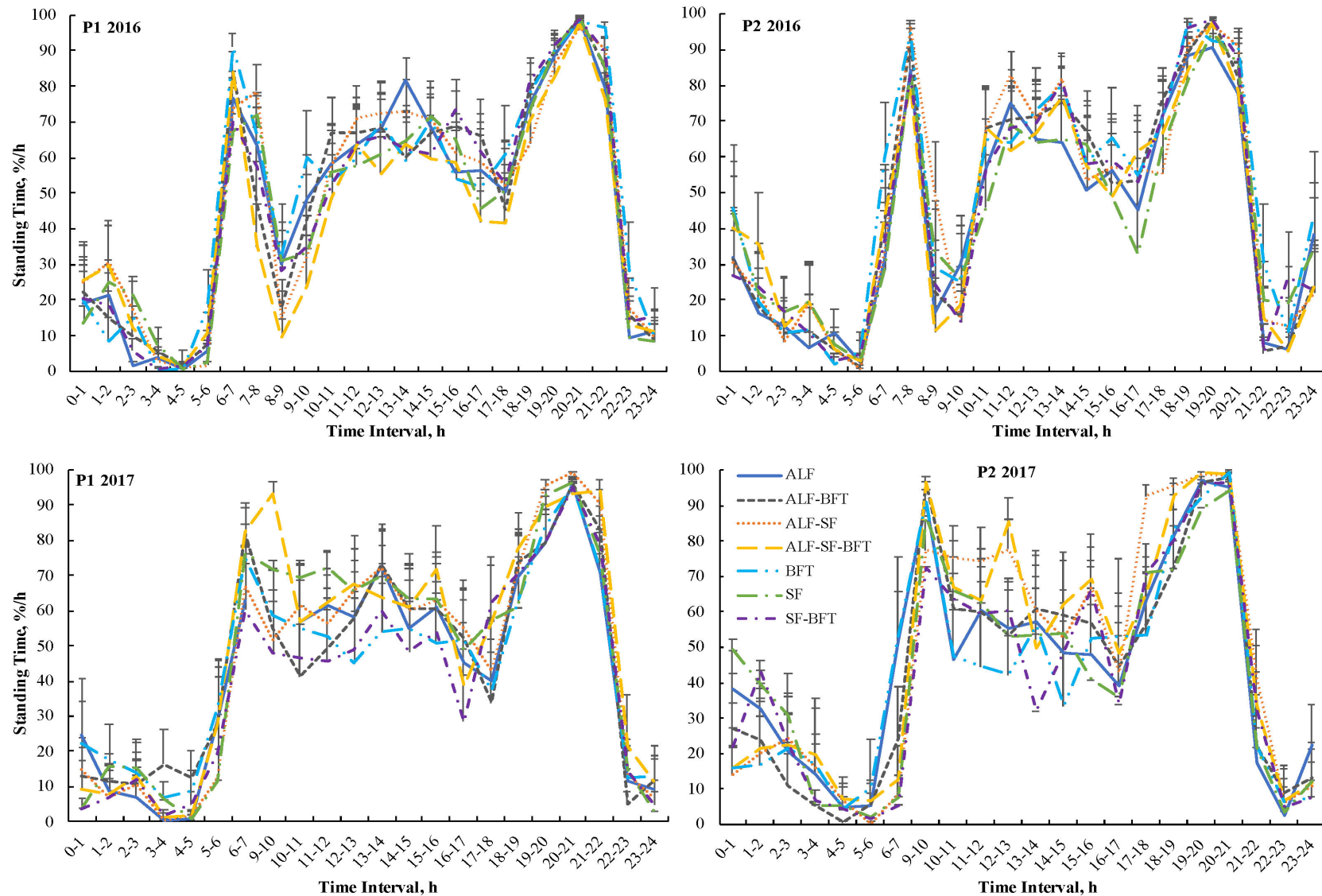
Figure 5-3. Percentage of standing time recorded in each hour of the day of heifers grazing single forages, 2- or 3-way choices of those forages: Alfalfa (ALF), Birdsfoot trefoil (BFT) and Sainfoin (SF) during two grazing periods in two consecutive years. Values represent the average of 3 heifers across 7 days in each period of 2016 and 9 days in each period of 2017. Time interval 8-9 and 9-10 were dropped from the analysis in both periods of 2017 due to missing values.

Cortisol concentrations

Cortisol concentrations in the hair of the heifers during the study is reported in Table 5-3. No differences in the levels of cortisol were observed among treatments (treatment effect; P=0.584), and no treatment × period or treatment × year interactions were detected (P=0.761 and P=0.337, respectively). Similarly, no differences were observed for the pre-planned contrasts between the 3-way choice (ALF-SF-BFT) and the average cortisol values for monocultures (ALF, SF, and BFT) (P=0.391), or averages between 3-way and 2-way choices (P=0.814). Averaged across treatments, the levels of cortisol were greater at the end of P1 than at the end of the P2 (2.35 ±0.21 vs. 1.55 ±0.14 ng/g; P=0.001), and they were greater during the first than during the second year of the study (2.25 ±0.20 vs. 1.62 ±0.15 ng/g; P=0.024 for 2016 and 2017, respectively).

Average daily gains

Averaged across periods and years, cattle grazing monoculture BFT gained more BW than cattle grazing monoculture ALF or SF (P=0.077 and P=0.073, respectively); no differences were observed between SF and ALF treatments (P=0.980; Table 5-3). When sainfoin or birdsfoot trefoil were offered with alfalfa in 2-way choices (ALF-BFT or ALF-SF), BW gains did not differ from those observed in animals under the ALF treatment (P>0.10). In contrast, when the three species were offered in the 3-way choice (ALF-SF-BFT), heifers gained 27% more BW than the average of all monoculture (ALF, SF, and BFT) treatments (P=0.014), and 30.0% more than the average of all 2-way choice treatments (P=0.007). In contrast, no differences were observed in ADG between the average of 2-way choices and the average of all monoculture treatments (P >0.10). Heifer

BW gain during different periods and years were presented in a previous study (Lagrange el al., 2020).

DISCUSSION

Foraging behavior by cattle grazing choices of legumes

When heifers were allowed to choose among strips of different legume species, they selected a diverse diet, which was consistent with behaviors typically observed in generalist herbivores (Provenza, 1996; Provenza et al., 2003). The frequent moves to fresh paddocks and the high forage allowances in each paddock prevented restrictions in selectivity, as confirmed by the low levels of legume utilization apparent from high post-grazing pasture DM (Table 5-2). Despite the high biomass availability for all forages in 2- and 3-way choices, where heifers could have selected the preferred species, significant amounts of all legumes were incorporated into the diet, consistent with previous studies were diverse forage alternatives are presented to cattle (Maughan et al., 2014; Villalba et al., 2015a)

In addition to choosing a diverse diet, forage selection by heifers was not random. Based on scan sampling data, sainfoin was the preferred species in 3-way (almost 50% of all grazing events), and 2- way (70% of all grazing events) choices. Previous studies have also reported a preference for sainfoin over alfalfa by cattle grazing strips of these legumes and tall fescue (Villalba et al., 2015a), and sheep fed in confinement showed a greater (2.41 X) preference index for sainfoin hay over alfalfa hay (Khalilvandi-Behroozyar et al., 2010).

Several explanations have been provided for selection of varied diets by

herbivores. Some contend that no single forage species is capable of providing all the nutrients and the appropriate proportions that herbivores need (Westoby, 1978). Others proposed the need to minimize the ingestion of foods with plant toxins (i.e., the toxin dilution hypothesis; Freeland and Janzen, 1974). Finally, the satiety hypothesis states that varied diets are the consequence of transient food aversions caused by flavors, nutrients, and toxins ingested too frequently or in excess (Provenza, 1996). These hypotheses are not mutually exclusive, and it is likely that they all contributed to the heifers' foraging decisions in this study.

It is likely that heifers reduced their grazing time in alfalfa, despite the fact that this species showed the greatest concentration of CP and the lowest contents of ADL, in order to reduce the potentially toxic effects of rapid protein breakdown and ammonia accumulation in the rumen and blood (Provenza, 1995). It is also likely that the lower preferences for alfalfa are partially explained by the incidence of sub-acute frothy bloat caused by the ingestion of this legume (Wang et al., 2012). As an example of negative influences of CP and bloat on preference, sheep develop aversions to forages associated with high levels of ammonia in the rumen (Villalba and Provenza, 1997), and they learn to avoid foods that cause rumen distension and to prefer foods that attenuate this effect (Villalba et al., 2009). In contrast, sainfoin had a lower concentration of CP and a greater concentration of fiber and ADL, and yet it was preferred over alfalfa or birdsfoot trefoil. The presence of relatively high concentrations of CT was likely significant in the observed preference for sainfoin, as CT reduces the incidence of bloat and sainfoin is a non-bloating legume (Wang et al., 2012). Thus, incorporation of high levels of sainfoin in the diet did not cause, or could have even alleviated, the discomfort caused by sub-acute

levels of frothy bloat caused by the consumption of alfalfa (McMahon et al., 1999; Wang et al., 2006). The greater ADG observed in heifers grazing BFT and the reduced percentage of grazing scans recorded for this species suggest that less birdsfoot trefoil was more satisfying, probably because the primary nutrients were more concentrated, particularly since BFT contains CT that reduce protein concentrations in the rumen.

Condensed tannins have the property of binding with proteins with high affinity (Jones and Mangan, 1977), which can subsequently provide a better quality of protein and thus a better profile of dietary amino acids to the small intestine. Condensed tannins also reduce the rate of proteolysis in the rumen and thus the accumulation of ammonia in the animal's tissues (Waghorn, 2008), which prevents the extra energy cost needed for ammonia detoxification (Lobley and Milano, 1997). Consistent with this notion, significantly greater concentrations of BUN and UUN were observed in heifers grazing alfalfa than in those grazing sainfoin monocultures (Lagrange et al., 2020). Preference for sainfoin over birdsfoot trefoil could also be explained by lower rates of proteolysis and ammonia formation with sainfoin consumption (e.g., 4 X the concentration of CT in sainfoin relative to birdsfoot trefoil). In addition, greater concentrations of BUN have been observed in animals consuming birdsfoot trefoil than in those consuming sainfoin (Lagrange and Villalba, 2019). Alternatively, the lower CP concentration in sainfoin than in alfalfa or birdsfoot trefoil may have contributed to dilute total protein ingestion and thus balance the ratio of energy to soluble protein ingested by heifers (Hill et al., 2009). Finally, the type of CT present in sainfoin do not appear to cause toxic effects in ruminants; on the contrary, sheep prefer high- to low-tannin-containing sainfoin pellets after a period of conditioning where they experience the post-ingestive effects of both

feeds (Costes-Thiré et al., 2018).

In contrast to results found in this study, sheep preferred alfalfa to sainfoin or birdsfoot trefoil (55:33:14), or alfalfa to sainfoin (70:30 ratio) in 2-way choices (Lagrange and Villalba, 2019). Alfalfa was the legume of greatest nutritional quality and fermentation rate in that study, although the mix selected by sheep produced fermentation rates similar to those observed for pure alfalfa (Lagrange et al., 2019). Nevertheless, the CP concentration of alfalfa (17.7%) was much lower than those observed in this study (28.3%), which likely reduced the negative post-ingestive effects of alfalfa described above. Additionally, sheep may be more tolerant to excesses of dietary N (Constable et al., 2017) or frothy bloat (Colvin and Backus, 1988).

When sainfoin was not present in the choice (i.e., ALF-BFT treatment), heifers preferred birdsfoot trefoil over alfalfa in a 60:40 ratio. The presence of CT -even when at lower concentrations than in sainfoin- could also explain this pattern as described above. In addition, the concentration of non-structural carbohydrates in birdsfoot trefoil may be greater than in alfalfa which could improve the imbalance of high protein/energy ratios typical of legume diets (Christensen et al., 2015). Finally, differences in sward structure (i.e., that lead to a greater bite sizes for BFT; see below) may also contribute to explain a preference for birdsfoot trefoil over alfalfa.

Levels of activity by cattle grazing monocultures vs. choices of legumes

Total grazing events were similar among treatments, except for BFT which showed lower values across periods and years. Likewise, grazing patterns were not influenced by treatment, suggesting that grazing activity was not constrained by the

availability of forage alternatives in choice treatments relative to monocultures. Given that animals in choice treatments selected a diverse diet (see previous section), a reduced number of grazing events in 3- and 2-way choice treatments could have been expected relative to monocultures due to an increased investment in searching and forage switching activities that reduce foraging efficiency (Laca, 1998). Nevertheless, the spatial distribution of legumes in the present study (i.e., in patches), typically reduce searching activities relative to mixed swards as the manifestation of a preference occurs automatically after the selection of a specific feeding station (Chapman et al., 2007).

Searching activities may also be minimized given that cattle manifest spatial memory, which contributes to increased foraging efficiency (Bailey et al., 1996; Laca, 1998). Heifers in our study were familiar with the distribution of strips in their paddocks, which were fixed, a design that reduces searching time (Soder et al., 2007) relative to random distribution. Switching activities from one strip to the next may also reduce the number of grazing bouts as animals need to move among feeding stations, but this outcome was likely minimized by the proximity and size of the legume strips relative to the body size of the heifers. Given this context, it is likely that the time invested in switching between strips in choice treatments was similar to the time invested in switching between feeding stations by animals grazing monocultures during the process of moving along the grazing pathway (Bailey et al., 1996).

Grazing efficiency is the ratio between grazing and walking time (Owen-Smith et al., 2010), which increases with increments in short-term herbage intake rates and in residence time per feeding station (Gregorini et al., 2009). Consistent with grazing scans and patterns, no differences among treatments were observed regarding number of daily

steps, suggesting that the spatial distribution of patches in choice treatments led to similar grazing efficiencies to those in animals grazing monocultures, with the added benefit of building a diverse diet, typical of generalist herbivores. These benefits could be summarized as the incorporation of beneficial (i.e., antioxidant, antiparasitic, nutraceutical) secondary compounds like CT (Waghorn, 2008; Gourlay and Constabel, 2019), improved ADG (Lagrange et al., 2020) and lower levels of excretion of urinary N (Lagrange et al., 2020). Previous research also shows no differences in walking bouts by cattle due to differences in plant diversity, attributed in that case to lower sensitivity to changes in vegetation structure relative to smaller body-size animals like sheep or goats (Cuchillo Hilario et al., 2017).

Despite all treatments showing similar numbers of daily steps, the motion index for the 3-way choices was 26.4% greater than the average observed for monocultures. This suggests that heifers in the treatment with highest diversity moved faster, likely to maintain their foraging efficiency when more legume species were available for selection. Consistent to number of steps taken or grazing scans, no differences among treatments were detected regarding total standing time (Table 5-3), suggesting similar residence time per feeding station across all treatments, which further supports the idea that grazing efficiency was similar for choice or no-choice treatments.

A possible explanation for the lower number of grazing events by the BFT treatment entails sward structure. Birdsfoot trefoil plants present a more prostrate growth habit relative to alfalfa or sainfoin (Grabber et al., 2014), with greater biomass per unit of area (see Table 5-2) and higher bulk density (i.e., herbage weight per unit of canopy volume), which is correlated with a greater leaf area index (Gibb and Orr, 1997). These

characteristics might have led to a greater bite mass, a fundamental variable determinant of intake rate which is dependent on sward structure (Laca et al., 1992). Thus, heifers on this treatment likely invested longer times per bite in order to process and swallow a greater bolus (Laca et al., 1994), but possibly with greater intake rates that led to lower grazing times. In contrast, heifers in treatments containing sainfoin and/or alfalfa with quite different sward structure, with an erect growth habit, larger stems and lower bulk density in the upper layers likely promoted a lower bite size (Carvalho, 2013). These characteristics might have involved more time invested in handling activities, and consequently greater likelihoods of being captured by scan sampling in a grazing position. In addition, such differences in forage structure and bite size may partially explain the greater proportion of grazing scans recorded for sainfoin in the SF-BFT treatment.

The daily grazing pattern followed by heifers on different diets was analyzed by grazing period due to the observed differences in photoperiod, which affects the time that animals spend eating, ruminating and resting (Gregorini et al., 2006). The grazing and standing activities of cattle appear to be synchronized for all treatments (Figs. 2a-d and 3a-d). Internal motivations for synchrony induced by daylight may be stronger than external factors like feeding time in dairy cows (Flury and Gygax, 2016). The proximity of animals from different treatments using contiguous plots separated by an electrical fence might have also induced heifers to mimic behaviors of cattle allocated to other treatments, thus leading to synchrony (Stoye et al., 2012). Heifers showed a typical grazing pattern with two major grazing events during the day, as reported in previous research (Gregorini et al., 2006), one early in the morning 1 h after sunrise and one in the

evening, with greater numbers of grazing events towards the last 3 hours before dark.

Shorter photoperiod by Period 2 shifted the peaks of grazing activity to 1 h later in the

morning and 1 h earlier in the afternoon than in Period 1, "compressing" the grazing

activity within those limits. In-between peaks of grazing activity, heifers were less

synchronous than during dawn or dusk grazing events, reflecting what other authors have

reported in previous studies (Stoye et al., 2012). It is likely that factors imposed by

different treatments, like motivation to consume diverse diets vs. reduction in feeding

bouts due to monotony, influenced feeding during those in-between periods, like the

sharp decline in grazing events during the 0800 to 0900 time interval for all treatments

except for the 3-way choice treatment, and for the high percentage of grazing events

observed at noon for the same treatment of greatest diversity. Nevertheless, such pattern

only occurred for period 2 in 2017, and thus they were not consistent for all periods or

years.

***Performance and cortisol levels by cattle grazing
monocultures vs. choices of legumes***

Heifers grazing BFT performed better than animals grazing ALF or SF, gaining

an average of 22.5% more BW across periods and years of study. This effect may be

attributed in part to an increase in the absorption of essential amino acids from the small

intestine due to the presence of moderate concentrations of CT in birdsfoot trefoil and

their particular molecular weight and chemical structure (McAllister et al., 2005;

Waghorn, 2008). Alternatively, a greater proportion of non-structural carbohydrates in

birdsfoot trefoil that reduces the N/energy imbalance typically observed in legumes may

also explain the greater BW gains by heifers in the BFT treatment (Chail et al., 2016), as

well as potential greater bite sizes in BFT swards as discussed above. In contrast, the effects of subclinical bloat and excess protein may explain the lower ADG in the ALF treatment.

When both tanniferous legumes were consumed together along with alfalfa in the 3-way choice, ADG was greater than for ALF or SF. This may be explained by increments in DM intake when heifers were exposed to a greater degree of forage diversity, which is consistent with previous studies where sheep were exposed to a diversity of flavors Villalba et al., (2011) or feeds (Catanese et al., 2012). In addition, by consuming a mixed diet, animals obtain a more balanced mixture of nutrients allowing for greater growth rates than grazing a monoculture (Provenza et al., 2007). Thus, chemical complementarities induced by the incorporation of forages like birdsfoot trefoil and sainfoin with high concentrations of soluble carbohydrates (Christensen, 2015; Chail et al., 2016; Stewart et al., 2019) and moderate levels of CT may have allowed for an improved utilization of the high contents of rumen-degradable protein in alfalfa and therefore, greater animal performance.

Frequent or excessive exposure to the same orosensorial or postingestive stimuli, like those experienced when ruminants consume monotonous diets or forages can be stressful (Provenza, 1996). On the other hand, animals grazing monocultures may experience frustration after unsuccessful attempts at solving the challenge of building a nutritionally balanced diet, which is more likely to happen when alternatives are available (Meehan and Mench, 2007; Manteca et al., 2008; Villalba et al., 2010). Consistent with this notion, a diversity of food items offered to sheep in confinement reduces plasma cortisol levels relative to animals fed monotonous rations (Villalba et al., 2012; Catanese

et al., 2013), and reduces lymphocyte counts (Catanese et al., 2014) and stress-induced hyperthermia in open field tests (Villalba et al., 2012). Nevertheless, no differences in hair cortisol levels were observed in this study for animals exposed to choice or no-choice treatments. It is likely that the level of frustration due to exposure to monotonous diets is different in grazing ruminants from those consuming diets in confinement (Higashiyama et al., 2007). In fact, dairy cows in confinement are willing to perform work to gain access to pasture (von Keyserlingk et al., 2017). In addition, hair cortisol may be different than plasma cortisol, as hair values represent the sum of multiple events occurring during the period of hair growth, in contrast to cortisol values that are taken from a blood sample (Davenport et al., 2006). These factors in addition to differences among species could explain the lack of responses in cortisol levels observed in this study versus the positive responses observed by sheep fed monotonous vs. diverse diets in confinement. Alternatively, locomotor activities may promote increased levels of hair cortisol in cattle. Comin et al., (2011) observed increased levels of hair cortisol in dairy cows grazing in highland summer pastures after one month of being moved out from winter housing, which required greater daily activity. Results from our study shows no differences in number of steps among treatments, consistent with the lack of differences observed in hair cortisol.

CONCLUSIONS

Heifers offered a choice among tanniferous (sainfoin, birdsfoot trefoil) and non-tanniferous (alfalfa) legumes preferred tanniferous legumes (particularly sainfoin) over alfalfa, although they selected significant amounts of all three species in the 3-way choice

treatment, thus building a diverse diet. Such selection by heifers led to greater BW gains in the 3-way choice treatment, but it did not differ from the BFT monoculture treatment. Average daily gain on both the 3-way and the BFT monoculture treatment was greater than monoculture ALF and SF. Forage diversity in this study did not influence grazing events or other types of activities like walking or time spent lying down. Thus, heifers presented with a choice of legumes segregated in patches did not need to invest additional time or modify their daily grazing patterns in order to build a diverse diet, compared with animals grazing monocultures. Likewise, no differences in hair cortisol were observed between animals grazing diverse or single pastures. Collectively, this study suggests that diverse landscapes presented in patches have the potential to enhance animal performance in legume-based finishing systems without influencing grazing time, grazing patterns or other activities such as standing, walking, moving or resting. The incorporation of a diverse array of chemicals into the diet, like the ingestion of different types and concentrations of CT or soluble carbohydrates may promote synergisms that benefit animal nutrition and health.

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CHAPTER 6

SUMMARY

My research suggests that tanniferous legumes like sainfoin and birdsfoot trefoil have the potential to reduce environmental impacts and enhance the nutrition of ruminants when presented in a diverse legume diet in addition to alfalfa. When offered choices, both lambs and heifers selected a varied diet typical of generalist herbivores, incorporating significant amounts of all species into their diets. The particular spatial arrangement of forages in segregated strips (i.e., patches) rather than intermingled mixtures likely represented an important factor influencing foraging behavior. This was evident as heifers did not invest additional time in searching and forage switching activities, or modified their daily grazing patterns in order to build their diverse diet. A different aggregation of species could have reduced foraging efficiency relative to animals grazing monocultures.

Both lambs in confinement (Chapter 2) or heifers in the grazing study (Chapter 5) selected forages in a non-random pattern, but proportions of the legumes selected were different in the different animal species. For instance, Chapter 5 shows that sainfoin was the preferred species by heifers over alfalfa and birdsfoot trefoil in 3- (46:27:27) and 2-way choices (70:30). Additionally, birdsfoot trefoil was preferred over alfalfa (62:38). In contrast, Chapter 2 shows that lambs preferred alfalfa over sainfoin and birdsfoot trefoil in 3- (53:33:14) and 2-way choices (70:30), and they preferred sainfoin over birdsfoot trefoil (70:30). Different nutritional composition and concentrations of condensed tannins (CT) among the forages utilized in both experiments might have influenced legume

preferences between animal species. The selection performed by lambs in choice treatments allowed for high intake values, comparable to those observed for pure alfalfa, while incorporating bioactive compounds to the diets. When substrates of the same forages, and their proportions consumed by the lambs were incubated *in vitro* (Chapter 3), the proportions selected resulted in greater gas production rates and lower times to reach half of the potential gas production than mixtures formed with equal parts of each of the species (i.e., indifferent selection), indicating that animals were able to build a diet that enhanced fermentation kinetics relative to random selection. In fact, the selection performed by lambs in 2- and 3-way choices led to positive associative effects that increased dry matter and fiber digestibility relative to lambs consuming pure alfalfa diets (Chapter 2).

Considering the greater concentration of CP observed in alfalfa and birdsfoot trefoil in the grazing study, it is likely that heifers' preference for sainfoin (a tanniferous species) over other legumes represented the need to attenuate the accumulation of ammonia in the rumen through the ingestion of CT (Chung et al., 2013), particularly given that excesses of ammonia in the rumen and blood are aversive and may limit the ingestion of forages high in CP (Provenza, 1995). In support of this, greater concentrations of BUN and UUN were observed in heifers grazing alfalfa (Chapter 4), and in lambs fed alfalfa or birdsfoot trefoil (Chapter 2) than in animals grazing sainfoin monocultures. In addition, the lower preference observed for alfalfa may be partially explained by the incidence of sub-acute frothy bloat, typically observed in ruminants grazing this legume (Wang et al., 2012).

One of the most relevant implications of this work entails the finding showing that co-grazing a diversity of legumes enhances BW gains in finishing cattle relative to

grazing monocultures of the same legumes (Chapters 4 and 5). When the three species were offered in a 3-way choice (ALF-SF-BFT), heifers gained 27% more BW (1.27 kg/d) than the average of all monoculture treatments (1.00 kg/d), and 30.0% more than the average of all 2-way choice treatments (0.97 kg/d), suggesting positive associative effects among tanniferous and non-tanniferous legumes. This may be explained by increments in DM intake in heifers exposed to a greater degree of forage diversity (Provenza et al., 2007), and a more balanced proportion of ingested nutrients and bioactive compounds, i.e., a greater proportion of non-structural carbohydrates that improved the ratio of ammonia-N to energy in the rumen. Additionally, a moderate supply of CT to the rumen likely allowed for a more efficient utilization of the high concentration of rumen-degradable protein in alfalfa (Waghorn, 2008). This was supported by the observed reduction (20%) in the partitioning of dietary N to urine and the increase (43%) in the proportion of retained N relative to the average observed for monocultures, as shown in Chapter 4. In fact, when both tanniferous legumes were ingested together (SF-BFT), the effect in the reduction of urinary N concentration was even greater than the observed for the single tanniferous species, likely due to a synergistic effect between different chemical structures of CT in both legumes. In addition, the reduced concentration of urinary N by heifers grazing sainfoin was contrasted by a greater partitioning of N into feces (30%) than in animals grazing birdsfoot trefoil (23%), suggesting a lower disassociation of CT-protein complexes in the abomasum, due to the greater precipitation capacity of CT in sainfoin (McAllister et al., 2005). Thus, the addition of this legume is positive to attain reductions in environmental impacts as organic N in feces is metabolized at a slower rate than N in urine, representing less potential for ammonia and

N_2O volatilization from soil and manure (Cai et al., 2017).

Although no significant differences among treatments were observed for the amounts of enteric CH_4 emitted daily, emissions by grazing heifers in this study (Chapter 4) were much lower than those typically observed for grass-finishing diets (36–37 g/kg DMI). This highlights the high nutritional value of all legumes in this study relative to grass, regardless of the presence of CT. In addition, greater BW gains in cattle fed diverse legumes (1.27 kg/d) vs. legume monocultures (1.00 kg/d), or in animals finished on legumes vs. animals finished on grasses (~0.6 kg/d), imply reductions in the number of days to slaughter, and thus lower levels of CH_4 production over the animal's lifetime. Thus, this study shows that legume diversity contributes to enteric CH_4 abatement and a "cleaner" finishing phase relative to legume monocultures or grass-finishing systems. Consequently, improving the nutrition of animals through the strategic use of different legume species that allow the consumption of balanced diets has the potential to increase animal productivity with lower environmental impacts, leading to more sustainable beef production systems.

Although I explored the finishing phase of the beef production systems, hay of these legumes might be used for cow-calf production in the U.S intermountain west in order to improve body condition score, pregnancy rates and reduce nutrient excretions of mother cows, which usually graze low quality forages in the mountain ranges. Likewise, the use of these legumes as hay or direct grazing might provide benefits for producers retaining calves in "backgrounding" dry lots or small pastures, as increments in body conditions and stocker's growth rates. In fact, there are many local producers around northern Utah and Southern Idaho which are currently using legume forages as the

unique diet or in combination with grasses for stocking or finishing cattle

(https://laufamilyfarm.com; www.etcherrycreekfarms.com), and recently, a remote

sensing study estimated that more than 412,000 ha in the state of Utah is considered

agricultural land under irrigation and could be destined to the establishment of improved

perennial pastures for livestock production (Guevara-Ballesteros, 2019).

Future research should focus on the mechanisms by which different chemical

structures of diverse sources of CT affect methanogenesis and the minimum

concentration required for each source of CT in order to reduce CH_4 production *in vivo*,

without affecting the rate and extent of nutrient digestion. In addition, new research needs

to explore how CT from different tanniferous legumes interact with proteins from other

non-tanniferous forages. Such effort may lead to improvements in N utilization and

concomitant reductions in urinary N excretions, likely through synergistic effects.

Additionally, it is important to investigate how different sources of condensed and

hydrolysable tannins complement each other in order to improve N utilization and reduce

CH_4 emissions in livestock production systems.

New avenues of research should focus on the role of non-structural carbohydrates

in forages with the aim of improving synchronies between energy and ruminal degradable

protein and their effects on the efficiency of nutrient utilization and fermentation profiles

in ruminants. There is a need for exploring how the combined ingestion of different

legumes with high contents of ruminal degradable proteins and legumes or grasses with

elevated concentrations of non-structural carbohydrates affect animal performance and

environmental impacts in beef production systems.

Finally, improvements in the agronomic characteristics of some tanniferous

legumes like sainfoin and other "non-traditional" forage species, such as regrowth

capacity after grazing, biomass yield, persistence and adaptability under different

environmental conditions may give producers a broader range of options under different

ecological sites to create more sustainable grazing environments. Combining the benefits

of high-producing and resilient forages with diverse and complementary contents of

nutrients and bioactive compounds will help create more efficient beef production

systems with a better quality of the product and increased efficiencies that reduce

environmental impacts.

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APPENDICES

APPENDIX A

PICTURES AND DIAGRAM OF THE EXPERIMENTAL STUDIES

Figure A-1. *Forty-two commercial Columbia-Polypay-Suffolk crossbred lambs penned individually.*

Figure A-2. *(Left) Lambs having free access to culinary water and trace mineral salt blocks. **Figure A-3**. (Right)Lambs receiving the 3-way choice with each legume in separate buckets.*

Figure A-4. *Measurement of the head-space gas pressure of the flasks with an USB output pressure transducer.*
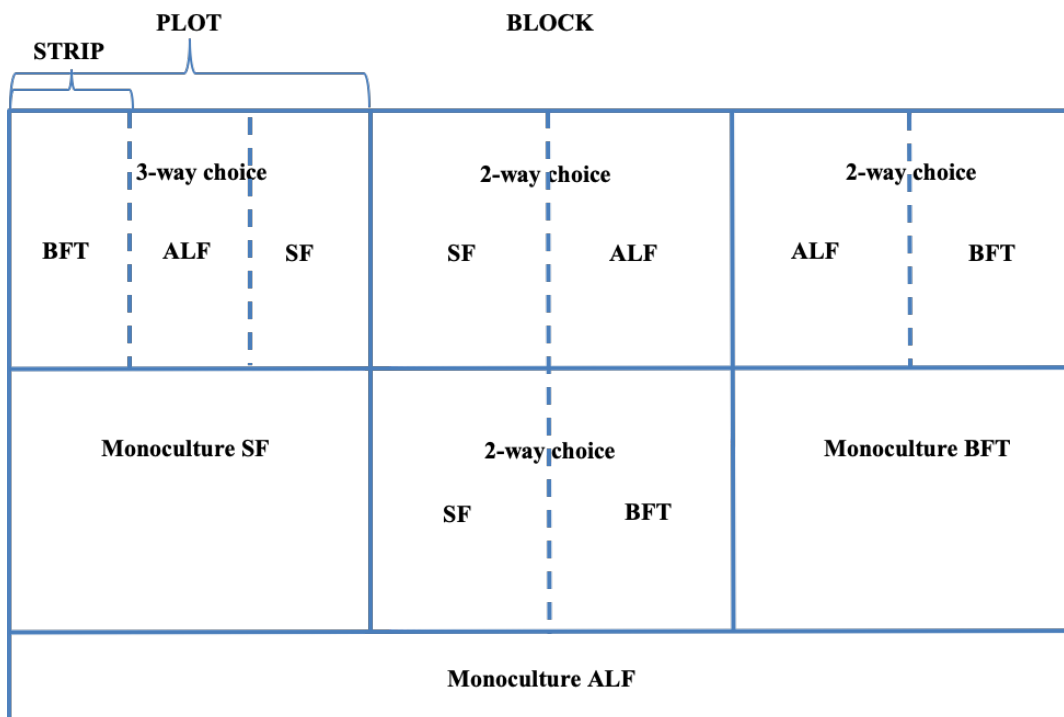
Figure A-5. *An illustration of the experimental design. There were three blocks (spatial replications) of the design presented.*

Figure A-6. *Heifers grazing the 3-way choice treatment. From the bottom to the top: Birdsfoot trefoil, Alfalfa and Sainfoin.*

Figure A-7. *Example of a plot with a 2-way choice treatment. Left (Sainfoin), right (Alfalfa).*
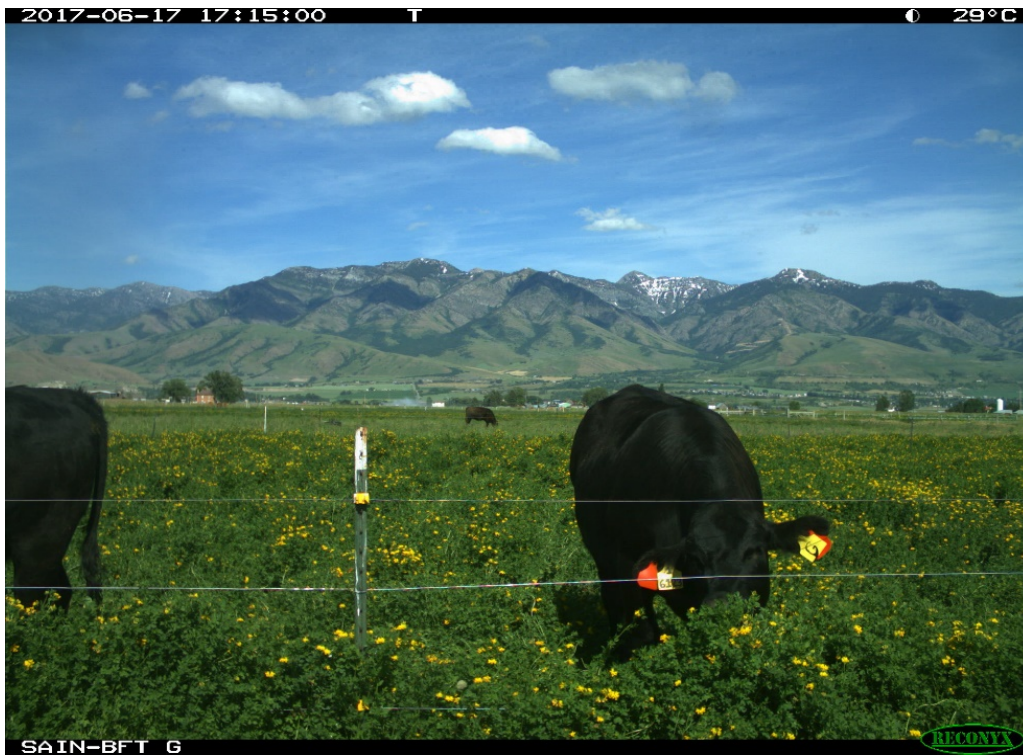
Figure A-8. Heifers grazing the birdsfoot trefoil monoculture treatment. Electric fence limiting the experimental plots.

Figure A-9. Heifer fitted with a halter and evacuated canister for enteric methane collection.

Figure A-10. Dr. Juan Villalba observing heifer fitted with a methane collection canister in the alfalfa monoculture treatment.

Figure A-11. Heifer grazing the sainfoin monoculture treatment while using the enteric methane collection canister.

APPENDIX B

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CURRICULUM VITAE

Sebastian P. Lagrange

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Positions Held:

- Instituto Nacional de Tecnología Agropecuaria (INTA EEA Bordenave), Argentina. Feb 2003-current.

    - Researcher and Extension Agent: Ruminant nutrition, pasture and forage management, and beef cattle production.

    - Chief of the Beef Cattle Management and Production lab at INTA Bordenave Experimental Station (2012-2014).

    - Chief of the Forage and Feed analytical laboratory at INTA Bordenave Experimental Station (2012-2014). '

    - Manager of the Animal Production Experimental Unit from 2003 to 2014.

    - Regional coordinator of the livestock production project for the southwestern region of Buenos Aires province (2009-2012).

    - Participation in national projects in the area of grass-fed beef production systems, ruminant metabolism and meet quality.

    - Participation in national projects in the area of forage and pastures: Introduction, evaluation and management of alternative forages species and cultivars.

    - Scholarship in Beef Cattle Management and Production Systems at INTA Bordenave Experimental Station (2003-2007).

Skills:

- Native Language: Spanish. Second Language: English.
- Excellent oral and written skills.
- Effectively present technical information to wide variety of audiences-academic, farmer and ranchers, general public.
- On-farm communication with livestock owners/operators.

- Data Analysis and Data Management.
- Beef Cattle Nutrient Requirement Software (Calculations of dietary supply and requirements).
- Statistical analysis software, SAS 9.4 proficiency, Excel, Word, Power point.
- Conduct of field agricultural research.
- Proximate analysis of feeds and forages, lab analysis and interpretation of feed samples

Education:

- PhD, Utah State University, August 2014 – April 2020;

  Major emphasis: Range Animal Nutrition; Major Professor: Dr. Juan Villalba

  - Projects: Evaluate effects of forage diversity and bioactive compounds as condensed tannins on livestock foraging behavior, productivity and environmental impacts.

  - Interactions among different nutrients and plant secondary compounds on ruminal digestion process and fermentation parameters.

  - Increase nutrient utilization efficiency in ruminants grazing different combinations of tanniferous (sainfoin, birdsfoot trefoil) and non-tanniferous legumes (alfalfa) and reduce methane emissions and nitrogen excretions in forage-based production systems.

  - Apply research to improve sustainability of forage-based beef finishing systems.

  - Mitigation of greenhouse gas emissions in ruminant production systems.

  - Evaluation of non-traditional forages legumes in livestock production systems.

- MS: Universidad Nacional del Sur, 2009;

  Major emphasis: Ruminant Nutrition; Major Professor: Dr. Hugo Arelovich

  - ➢ Project: Effects of Protein Supplementation on Growth Rate and Fermentation Parameters of Steers Grazing stockpiled Grain Sorghum.

- BS: Universidad Nacional del Sur, 2001

Major: Agricultural Engineer (Animal Production).

Membership and Leadership:

- Member, Argentinian Association of Animal Production (AAPA), 2003 – current.

- Member, American Association of Animal Science (ASAS), 2016 – current.

Publications:

1- Lagrange, Sebastian, and Juan J. Villalba (2019). "Tannin-containing legumes and forage diversity influence foraging behavior, diet digestibility and nitrogen excretion by lambs." *Journal of animal science*, 97:3994–4009. https://academic.oup.com/jas/advance-article-abstract/doi/10.1093/jas/skz246/5542826

2- Lagrange, Sebastian, S. Lobón, and Juan Jose Villalba (2019). "Gas production kinetics and in vitro degradability of tannin-containing legumes, alfalfa and their mixtures." *Animal Feed Science and Technology,* 253:56-64. https://doi.org/10.1016/j.anifeedsci.2019.05.008

3-Arelovich, H. M., Lagrange, Sebastian, Torre, R., Martinez, M. F., & Laborde, H. E. (2018). "Feeding value of whole raw soya beans as a protein supplement for beef cattle consuming low-quality forages". Journal of animal physiology and animal nutrition, 102:421-430. https://onlinelibrary.wiley.com/doi/full/10.1111/jpn.12761

4- DiLorenzo, N., Dubeux J.C., Garcia, L., Guevara, R.D, Lagrange, Sebastian and J. J. Villalba. 2020. Legumes as a strategy for reducing greenhouse gas emissions of forage-livestock systems. International Grasslands Congress-International Rangelands Congress in Nairobi, Kenya.

5-Lagrange, Sebastian, Guevara Ballesteros, R., Beauchemin, K., MacAdam, J., & Villalba, J. (2018). PSXI-31 Methane emissions by cattle grazing tannin-containing legumes. Journal of Animal Science, 96(suppl_3), 223-224.

6-Lagrange, Sebastian, Beauchemin, K. A., MacAdam, J. W., & Villalba, J. J. (2017). 290 Effects of grazing diverse combinations of sainfoin, birdsfoot trefoil, and alfalfa on beef cow performance and environmental impacts. Journal of Animal Science, 95(suppl_4), 143-144.

7-Lagrange, S., & Villalba, J. J. (2016). Influence of forage diversity on feeding behavior and diet digestibility in lambs. Journal of Animal Science, 94, 289-289.

8-Lagrange, S., Arelovich, H.M, Vasicek, J.P; Bravo, R.D, y Martinez, M.F. (2013). Yield and nutritional quality of deferred sorghum hybrids. Journal of Animal Science, 91. (suppl_1), 555.

9-Lagrange, S., Arelovich, H.M, Frache, F.X, Bravo, R.D, Martinez M.F y Amela M, I. 2013. Biomass yield and quality of barley forage prior to ensiling as affected by fertilizer rate and harvest date. Journal of Animal Science, 91. (Suppl_1), 556.