Potential to Improve Fiber Digestion in the Rumen

by

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ABSTRACT

Ruminants have the capacity to utilize fibrous plant materials as substrates to provide energy for maintenance and growth. However, digestion of poor quality forage is incomplete in ruminants and varies among individual animals. Consequently, two studies were conducted in an attempt to elucidate differences among cattle, in terms of their abilities to digest forages. The effect of attempting to modify their microbiomes through inoculation with bison rumen contents, on forage digestibility was also examined.

The first experiment was conducted to determine whether ruminal digestibility of forage fiber by cattle could be increased by partially replacing rumen contents with bison rumen contents. Our secondary objective was to investigate differences in ruminal digestion kinetics among individual heifers, and differences in their response to inoculation with bison rumen contents. An *in situ* digestibility study was performed using 16 ruminally-cannulated heifers and four fiber sources: barley straw, canola straw, alfalfa hay, and timothy hay. Bags were incubated in duplicate, before and after inoculation, for 0, 4, 8, 12, 24, 48, 96, and 120 h and digestion kinetics were determined as washout fraction (a), potentially degradable fraction (b), rate of degradation (k_d), lag (L) and total potentially degradable fraction ($a+b$). No mean effect of inoculation with bison rumen contents was seen on effective ruminal degradability (*ERD*) for any feeds incubated ($P > 0.10$). However, a small decrease ($P > 0.05$) in k_d and $a+b$ fraction of barley straw was observed as a result of the inoculation, likely because cattle were already well adapted to a barley straw diet, while bison were not. A mean increase ($P < 0.05$) in $a+b$, with a concomitant decrease in k_d of alfalfa hay was observed. It was found that digestion kinetics

varied among heifers both before and after inoculation, and each heifer responded differently to inoculation with bison rumen contents.

Using the *in situ* digestibility kinetics measured in the first experiment, rumen inoculum was obtained from two heifers with the fastest k_d of barley straw NDF, and two heifers with the slowest k_d of barley straw NDF. Conventional barley straw, and barley straw treated by ammonia fiber expansion (AFEX) were used to examine differences between inoculum types. Two Rusitec apparatuses, with 8 fermenters in each, were used in a 4 × 4 Latin square design with 4 treatments and 4 fermenters per treatment. No effects of k_d or rumen inoculum were observed for digestibility of untreated barley straw. However, inoculum from heifers with a fast k_d digested AFEX straw to a greater extent ($P < 0.05$) than did inoculum from heifers with a slow k_d. These differences were believed to be due differences in the nature of the rumen microbiome between inoculum donors.

Together, these studies investigate individual variability among cattle in their ability to digest forages. They highlight how differences in rumen microbiome may impact fiber digestion in the rumen. Further investigation is needed to understand the exact microbial differences that are responsible for this variability in digestibility, and the potential to enhance the microbiome of cattle that exhibit less efficient ruminal fiber digestion.

Preface

The research project, which this thesis is a part of, received research ethics approval from the Lethbridge Research and Development Centre Animal Care Committee and cattle were cared for according to the guidelines of the Canadian Council on Animal Care.

Candace Griffith conducted all aspects of the studies presented in this thesis. Dr. Gabriel Ribeiro helped with research and data analysis and manuscript revisions for chapters 2 and 3. Drs. Masahito Oba, Karen Beauchemin, and Tim McAllister provided advice on protocols, data analysis and the final versions of chapters 2 and 3. The literature review in chapter 1, and discussion in chapter 4 are the candidate's original work.

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The Cow

By Robert Louis Stevenson

Thank you, pretty cow, that made
Pleasant milk to soak my bread,
Every day and every night,
Warm, and fresh, and sweet, and white.

Do not chew the hemlock rank,
Growing on the weedy bank;
But the yellow cowslips eat;
They perhaps will make it sweet.

Where the purple violet grows,
Where the bubbling water flows,
Where the grass is fresh and fine,
Pretty cow, go there to dine.

Acknowledgments

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Finally, I would like to thank my husband for his continuous encouragement and belief in me, and my parents for their encouragement and support throughout this process.

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List of Abbreviations

a: Washout fraction

a+*b*: Total ruminally degraded fraction

A:P: Acetate to propionate ratio

ADF: Acid detergent fiber

ADFD: Acid detergent fiber disappearance

AFEX: Ammonia Fiber Expansion

b: Degraded fraction

CH_4: Methane

DDGS: Dried distillers grains

DM: Dry matter

DMD: Dry matter disappearance

DMI: Dry matter intake

ERD: Effective ruminal degradability

F:C: Feed to concentrate ratio

FPA: Feed particle associated

FPB: Feed particle bound

FSG: Functional specific gravity

G:F: Gain to feed ratio

k_d: rate of degradation

L: lag

N: Nitrogen

NDF: Neutral detergent fiber

NDFD: Neutral detergent fiber disappearance

NH_3N: Ammonia

NPN: Non-protein nitrogen

OM: Organic matter

OMD: Organic matter disappearance

RFI: Residual feed intake

TDMD: True dry matter disappearance

uNDF: Undegradable NDF

VFA: Volatile fatty acid

Chapter 1: Literature Review

1.1. Introduction

The rumen has co-evolved a complex relationship with microbial communities that enable the ruminant to digest fibrous feed sources (Hoffman, 1989). However, fiber digestion is incomplete, mainly due to inaccessibility of substrate to the microbial community (McAllister et al., 1994) and limited ruminal retention time (Orskov and McDonald, 1979). As it is difficult to increase ruminal retention time, the desire to improve access of ruminal microorganisms to nutrients in hard to digest forages has resulted in a number of physical and chemical processing techniques for crop residues. Some of these processes are commonly used, such as grinding, while use of other techniques such as enzymes, steam or chemical treatments is limited (Taherzadeh et al., 2008). Attempting to improve ruminal digestion has also generated a significant amount of research into composition and function of the rumen microbiome. It has been discovered that there are differences in the species composition of the microbial microbiome (Henderson et al., 2015) and fiber digestion capacity among ruminant species as well as among individual animals of the same species (Jami and Mizrahi, 2012). Cross inoculations have been performed among ruminants both within (Satter and Bringe, 1969; Bond et al., 1983; Cole, 1991; Weimer et al., 2010) and across species (Dehority et al., 1999; Pamungkas et al., 2004, Pamungkas et al., 2006) in attempts to characterize these differences. With all of these factors in mind, the aim of this review is to examine the literature on fiber and its digestibility in the rumen of cattle and bison, treatment techniques to improve nutritive value of fiber, *in vitro* and *in vivo* techniques for measuring ruminal digestion, and inter- and intra-species ruminal cross inoculation to identify potential knowledge gaps for investigation.

1.2. Fiber structure

Fiber is a very important source of digestible energy in ruminant diets. In addition to providing energy, having proper amounts of fiber in the diet improves rumen health by decreasing acidosis (Erdman, 1988; Krause and Oetzel, 2006). Appropriate amounts of fiber can also increase milk fat concentration (Emery, 1988). Chemically, fiber is made up almost completely of hemicellulose, cellulose and lignin, which together create a physical matrix that provides structure to the plant (Figure 1). Pectin is also present, but does not contribute to resistance to digestion so this section will be limited to the effects of hemicellulose, cellulose and

lignin. Fiber content is assessed using neutral detergent fiber (NDF) and acid detergent fiber (ADF) analysis. Neutral detergent fiber content indicates total hemicellulose, cellulose and lignin content, while ADF analysis quantifies cellulose and lignin.

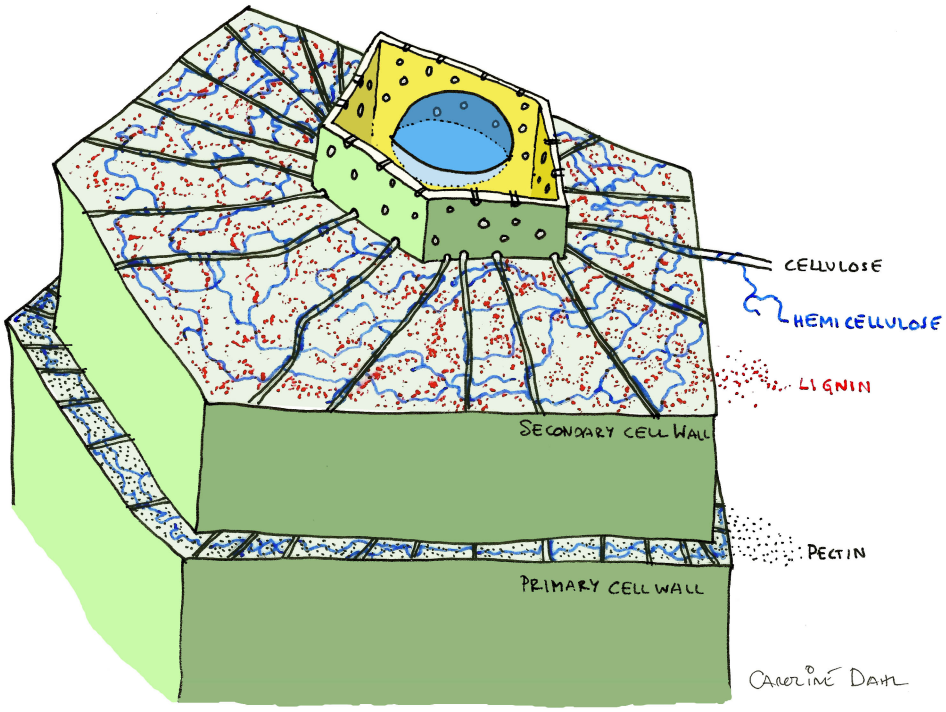

Figure 1.1 Plant cell wall composition and structure.

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1.2.1 Hemicellulose

Hemicelluloses are defined by their extraction methodology as being insoluble in water, but extractable with aqueous alkali (Giger-Reverdin, 1995). They comprise 10-25% dry matter (DM) of forages, up to 50% of the lignocellulosic biomass (Saha, 2003) and in their free form are the most accessible and easily digested components of fiber because of their amorphous organization. Hemicelluloses are made up of various pentose and hexose sugars linked in a variety of ways (Saha, 2003). Those found in grasses are xylans, and xyloglucans, while mannans can be found in leguminous forages (Schädel et al., 2010). Of these hemicelluloses, xylans and mannans are found in the secondary cell wall of plants, and as such are often more

difficult to access due to greater lignification. While hemicellulose is easily digested, when it is complexed with lignin digestion can be significantly inhibited.

1.2.2 Cellulose

Cellulose is associated with hemicellulose by hydrogen bonds (Morrison, 1979) and is often the most abundant component of the plant cell wall, comprising 10-45 % dry matter (DM) (Giger-Reverdin, 1995; Saha, 2003). It is made up of repeating glucose monomers connected by β(1->4) linkages**.** Naturally occurring cellulose I is most commonly found in a crystalline form (French and Johnson, 2007), but this is not the case when cellulose microfibrils are synthesized in the lab from cellulose chains (O'Sullivan, 1997). *In vitro* organization of cellulose chains thermodynamically favours cellulose II. The difference in organization between cellulose I and cellulose II is a result of the mode of synthesis by the terminal complex proteins that manufacture cellulose I. The terminal complexes synthesize and organize multiple strands of cellulose at the same time, rather than synthesizing complete strands and then forming them into microfibrils as is done *in vitro* (Okuda et al., 1994, O'Sullivan, 1997). Terminal complex synthesis results in crystalline cellulose I, which is much more difficult to degrade than amorphous cellulose (French and Johnson, 2007). This is because of the close alignment and hydrogen bonding of cellulose microfibrils within crystalline cellulose. In the plant, cellulose chains are highly structured within the secondary cell wall where they are densely packed into microfibrils, whereas in the primary cell wall cellulose chains run in random directions (O'Sullivan, 1997)

Cellulose crystallinity, and the organization of cellulose, hemicellulose and lignin in the fiber complex can limit physical access of microbial enzymes (Chesson, 1988; Hall et al., 2010). However, cellulose crystallinity can be disrupted by physical or chemical processing, and rumination (Sun and Cheng, 2002).

1.2.3 Lignin

Lignin is the third most abundant component of fiber. It is made up of polymers of aromatic alcohols and contains many different molecules and bonds, and as such is very difficult to enzymatically degrade. Lignin can be classified as core lignin and noncore lignin. Core lignin is composed of highly condensed cinammyl alcohol polymers (Grisbach, 1981) and is the component extracted in acid detergent lignin. Noncore lignins are comprised of p-coumaric

acids, which are largely associated with core lignin and ferulic acids, which act as a bridge between lignin and hemicellulose (Jung, 1989).

Lignin is covalently bonded to hemicellulose, but it is not bonded to cellulose, rather, it has a branched structure that fills the space between hemicellulose and cellulose, acting as a barrier to fiber digestion (Jung, 1989). Lignin is found in higher concentration in the primary cell wall, but because this wall is thinner compared to the secondary cell wall it is more abundant in the secondary cell wall of plants and increases as plants age (Wardrop, 1981). Over the course of a plant's life, lignin content can increase from <5% to up to 12% DM (Giger-Reverdin, 1995), or higher for low quality crop byproduct feeds. The structure of lignin impacts digestibility by physically and biochemically inhibiting microbial enzymes due to the presence of hydrophobic phenylpropanoids present (Besle et al., 1994). Two tests have been developed for lignin analysis (Stafford, 1962): phloroglucanol reacts with coniferaldehydes but no syringyl units and chlorine-sulfite binds to syringyl units (Jones and Jensen, 1964). Plant tissues typically react to only one of these lignin tests, indicating that different tissues differ in their lignin composition (Jones and Jensen, 1964). Lignin content in plant stems increases as they mature, due to more lignified sclerenchyma tissues and vascular bundles which contribute to structural integrity of the plant (Akin, 1989; Kong et al., 2013). Compared to leaves, this makes stems more recalcitrant to digestion, as they mature and this difference is seen more strongly in grasses which also have undegradable parenchyma in stem tissues (Terry and Tilley, 1964; Akin, 1989). In contrast the parenchyma in legume stems are completely degraded (Akin, 1989).

1.2.4 Fiber and plant tissue organization

Plant cell structure varies by functional tissue type, but all plant cells have a primary cell wall, which is composed of cellulose, hemicellulose and pectin. In some plant tissues, such as the chlorenchyma, no lignification occurs in the primary cell wall (Wilson, 1993). In structural tissues, such as xylem and parenchyma tissues, a secondary cell wall is formed inside the primary cell wall. Cells that contain secondary cell walls become lignified from the middle lamella, through to the primary cell wall and secondary cell wall (Wilson, 1993). There are exceptions to the lignification process, for example, collenchyma and phloem tissues in legumes, are not lignified, even when they are mature (Wilson, 1993).

In recent years, there has been a great degree of success in manipulating lignin content in a variety of forages by down regulating genes responsible for lignin synthesis (Zhong et al., 1998; Guo et al., 2001; Chen et al., 2004). The down regulation of genes coding for lignin synthesis results in plants with increased nutritive value. However, it also simultaneously reduces the structural integrity of the plant it and often comes at the expense of plant fitness, with increased lodging of the crop (Casler et al., 2002; Pederson et al., 2005).

1.2.5 Structural differences between C3 grasses, C4 grasses and legumes

Legumes and C3 grasses exist in cool climates and their photosynthetic pathway uses a 3-carbon intermediate, which becomes inefficient in hot weather due to an increase in energy lost to cellular respiration. Warmer climate grasses use a 4 carbon intermediate in their photosynthetic pathway and are thus termed "C4" grasses. C4 grasses, such as corn, evolved a more efficient photosynthetic pathway that can operate even when stomata are closed, reducing respiration. This reduction in respiration energy loss allows C4 grasses to thrive in warmer, dryer climates but makes them almost nonfunctional in cool climates (Pearcy and Ehlinger, 1984). C4 plants have a kranz anatomy (the tissues that allow C4 photosynthesis to occur) and as such have increased bundle sheath and sclerenchyma which lead to reduced dry matter degradability (DMD) as compared to C3 grasses (Wilson et al., 1983; Jung and Vogel, 1986).

Grasses and legumes differ in cell shape, tissue structure, lignin deposition (Wilson, 1993), lignin content and lignin composition (Jung, 1989). These differences in tissue organization, structure and lignin deposition cause differences in ruminal degradability. Core lignin content is larger in legumes than grasses (Buxton and Russell, 1988) but lignin in grasses usually inhibits digestion to a greater extent (Mowat et al., 1969). This may be because legumes sequester lignin in the xylem and interbundular cells without lignification of the parenchyma, which makes it easier to breakdown via mastication as compared to C3 grasses (Demarquilly and Jarrige, 1974). Thornton and Minson (1973) found that voluntary intake of legumes was 28% higher compared to grasses due to shorter retention time and the higher percentage of organic matter. Van Soest (1965) found that as lignin increased, voluntary intake of C3 grasses decreased, but this response was not observed with legumes, which generally have higher lignin content and higher intake than grasses (Jung et al., 1993). The higher intake is believed to be because legumes have more highly degradable cell wall contents, likely due to unlignified

parenchyma, which allows legume cell walls to be degraded more rapidly (Donefer et al., 1960). In support of this hypothesis, Jamot and Grenet (1991) found that regardless of maturity leguminous alfalfa stems were fully degraded *in vitro* after 24 h with the exception of xylem tissues. In contrast, it required 48 h to degrade all the unlignified stem tissues in ryegrass, with these authors concluding that these differences were responsible for the higher voluntary intake of alfalfa in ruminants.

1.2.6 Digestibility of forages

Because of differences in structure, forages can have a wide range of digestibilities depending on their maturity and family, with significant differences between C3 grasses, C4 grasses and legumes. The huge range in nutritive value and digestibility among different forages has led to hundreds of studies examining these differences. Following are a few examples illustrating the diversity of degradability among forages. Coblentz et al. (1998) found nitrogen (N) degradation *in situ* occurred more rapidly in alfalfa than gamagrass, despite both having similar N content, indicating that N in alfalfa is more accessible to rumen microorganisms. Hoffman et al. (1993) investigated *in situ* degradation of various forages and found that the DM in legume hay was degraded significantly faster and to a greater extent than grasses, despite having greater lignification. Colucci et al. (1992) compared the *in situ* degradability of a number of wheat, barley and oat straw varieties and found large differences in organic matter degradability (OMD), with wheat straw being the most recalcitrant (35.7% effective ruminal degradability (*ERD*)), followed by barley straw (48.7% *ERD*) and then oat straw (54.0% *ERD*). Orskov et al. (1990) found large year-to-year variation in nutritive value among cereal straws, indicating that environmental growing conditions also have a large effect on the nutritive value of forage.

The studies presented investigate ruminal digestion of an array of forages including C3 grasses differing in lignification; barley straw (*Hordeum vulgare*), canola straw (*Brassica rapus*) and timothy hay (*Phleum pratense*)) and leguminous alfalfa hay (*Medicago sativa*), chosen because of their differences in nutritive values (Table 1.1).

Table 1.1. Nutritional comparison of barley straw, canola straw, alfalfa hay and timothy hay.

	CP (%DM)	NDF (% DM)	Lignin (% DM)	TDN (% DM)
Barley straw[1]	6.1	71.6	5.2	48.3
Canola straw[2]	3.5	71.7	12.8	20.0
Alfalfa hay[1]	19.8	41.7	6.8	55.2
Timothy hay[1]	9.4	63.8	4.8	57.0

[1]Values obtained from Beef Cattle Nutrient Requirements, 2016.
[2]Values of CP and TDN obtained from Lardy and Anderson, 2009; values of NDF and Lignin obtained from Garmakhany et al. (2014).

1.3. Techniques for measuring ruminal fiber degradation

For a summary of these techniques see Table 1.2.

1.3.1 *In vivo*

In vivo techniques represent the techniques deployed to measure digestion within the individual animal. Total tract digestibility measures the digestibility in all sections of the gastrointestinal tract; therefore, rumen and post-ruminal digestion are not differentiated. This technique involves measuring intake and total fecal output over a period of days (usually 4 to 8 days) to measure the total input and output of nutrients by the ruminant. Alternatively, markers applied to feed or markers internal to feed can be used in conjunction with spot fecal samples to measure total tract digestion (Owens and Hanson, 1992). It is possible to measure differences in ruminal digestion *in vivo* using animals that are cannulated in the small intestine, or by collecting samples at the omasal orifice in the rumen (i.e., site that feed residues exit the rumen). However, these methods are expensive, laborious, and impose animal care issues.

1.3.2 *In situ*

The *in situ* (or *in sacco*) technique involves placing polyester bags containing feedstuffs into the rumen of a cannulated animal, and measuring disappearance of feed from the bags over time. It is an intermediate process between *in vivo* and *in vitro* as it involves controlling the amount of time the bags are in the rumen, and is therefore unaffected by rate of passage of digesta, but still affected by all other ruminal digestion processes. It is a valuable tool for measuring digestibility in the rumen, and comparing digestibility among feeds (Dewhurst et al., 1995). There are a number of factors that can be standardized which can influence disappearance of feed from the bags. For example, porosity of fabric can affect microbial access and washout fraction with small pore size reducing washout, and microbial access to incubated feed, but also

being subject to clogging by fine particles, and large pore size increasing washout and increasing microbial access to feed (Uden and Van Soest, 1994). However, even if these factors are standardized differences in the diets fed among research studies, making it difficult to compare among studies as higher concentrate diets may reduce pH leading to reduction in fiber degrading microbes.

Using the *in situ* technique, ruminal fiber digestion in the rumen has been mathematically characterized by Orskov and MacDonald (1979), with a subsequent update to include lag (McDonald, 1981), resulting in the equation $P = a + b\ (1 - e^{-kd(t-L)})$, where P is extent of degradation at time t, a is the soluble or washout fraction, b is the potentially digestible fraction, k_d is the rate of digestion, and L is the lag time. The a and b fraction combine to give the total digestible fraction. Rate and extent of digestion of fiber are affected by inherent characteristics of the fiber source, individual animal physiological differences and differences in the rumen microbiome. Effective ruminal degradability (*ERD*) can be calculated as: $ERD = a + b(k_d/(k_d+k_p)$ where k_p is the rate of passage and the other variable are measured *in situ*.

1.3.3 *In vitro*

The purpose of *in vitro* techniques is to simulate natural digestive conditions of the animal in a lab. There are three main techniques used to measure digestibility of forages *in vitro*: batch culture, semi-continuous culture, and continuous culture. These technologies can control, to varying degrees, physiological differences among individual animals that cause variation, such as ruminal retention time, mastication and saliva production, allowing for potential examination of differences in the microbiome among animals.

Commercially available enzymes can be used *in vitro* to degrade forage, which allows for comparison between studies, but is not an accurate representation of *in vivo* digestibility. Therefore, to mimic *in vivo* conditions all three techniques mentioned above often use rumen fluid; however, *in vitro* results can depend upon when the rumen fluid is collected in relation to feeding time and the diet the donor animals are consuming (Cone et al., 2002). Differences in rumen fluid conditions make it difficult to compare results across different studies; however, relative ranking of feeds across studies are unaffected by these factors (Richards et al., 1995; Cone et al., 2002).

The earliest iteration of batch culture was presented by Tilley and Terry (1963) and involved incubating feed in a test tube with strained rumen fluid and buffer under anaerobic conditions for 48 hours, followed by an additional 48 hour incubation in an acid solution containing pepsin. In more recent years, various modifications have been made to the traditional Tilley and Terry (1963) method, including omission of the second incubation step, and use of shorter incubation times (7, 24, 30 h) to represent ruminal conditions and retention time in high producing ruminants. This technique has been found to be a good predictor of *in vivo* values of digestibility because it mimics ruminal and abomasal digestion. However, batch culture techniques are only useful for short-term (a matter of hours) or medium term (a matter of days) investigations as end products accumulate causing a decrease in pH, and steady state conditions cannot be reached. Cellulolytic bacteria are particularly sensitive to low pH, so in studying fiber digestion pH control is especially important (Therion et al., 1982).

Commercial systems are available that automate the batch culture technique. For example, Daisy II (Ankom Technology, 2052 O'Neil Road, Macedon NY 14502) is similar to batch culture, but samples are weighed into small bags and groups of bags are incubated in large bottles containing rumen fluid and buffer, which are rotated in an incubator. This technique is less laborious than the Tilley and Terry (1963) technique, and can analyze up to 100 samples at the same time with comparable results for roughage analysis (Mabjeesh et al., 2000). Results from the Daisy II correlate well with *in situ* results making it a useful technique for measuring digestibility of feeds. A drawback to the batch culture technique is that it uses only strained rumen fluid, without any solid rumen inoculum. Inclusion of particle-associated microbes from solid inoculum has been found to increase the effectiveness of *in vitro* fiber digestion (Craig et al., 1987).

The rumen simulation technique (Rusitec) is a semi-continuous culture system that is time and labour consuming relative to batch culture, but appropriate for studying medium and long-term experiments (days to weeks) because end products are continuously removed and buffer inflow allows the microbial population to stabilize. The Rusitec system is composed of a water bath containing individual fermenters, each equipped with an input for artificial saliva (McDougall, 1948) and exit for effluent (Czerkawski and Breckenridge, 1977). Bags containing feed are incubated in the individual fermenters for a set amount of time (often 24 or 48 hours) and then removed and disappearance of feed is measured.

Table 1.2. Comparison of *in vivo* and *in vitro* ruminal fiber digestion techniques.			
Technique	Length of time	Useful for	Not useful for
In vivo / Total tract digestibility	Short term (days). Actual length of time it takes for feed to pass through ruminant, start to finish.	Measuring total tract digestion by individual animal. Rate of passage measurements.	Measuring ruminal digestion.
In situ	Short term (days).	Measuring ruminal digestion in animal.	Measuring total tract digestion. Rate of passage or retention time measurements.
In vitro			
Batch culture	Short term (days).	Measuring in vitro fiber digestibility. Measuring fermentation end products.	Examining microbial populations – microbial populations die off as end products accumulate.
Semi-continuous	Medium term (weeks) to long term (months).	Examining microbial populations – end products are removed allowing microbial populations to achieve equilibrium. Measuring fermentation end products. Examining microbial protein synthesis. Measuring 48 hour in vitro digestibility.	Labour and time intensive.
Continuous	Medium term (weeks) to long term (months).	Examining microbial populations – end products are removed allowing microbial populations to achieve equilibrium. Measuring fermentation end products. Examining microbial protein synthesis.	Cannot measure digestibility of feeds as they stratify and large particles remain in the system longer than small particles.

During bag removal it is important to continuously gas with CO_2 as failure to do this can significantly increase lag time and decrease rate of DM disappearance of incubated substrates (Grant and Mertens, 1992). Gas, VFA and ammonia production can be measured, and bacterial species can be characterized. Microbial nitrogen production can be measured by adding labeled nitrogen ^{N15} to the McDougall buffer. Buffer infusion, rate of passage, pH and retention time can all be controlled.

Dual flow continuous culture systems have an inlet and outlet for both fluid and solid components, and allow solids to stratify, mimicking the conditions of the rumen. This method does not allow for control of the time solid feeds spend in the system. This technique is ideal for measuring microbial populations as end products are removed and the dual flow environment closely imitates the rumen, but is not suitable for measuring digestion of feedstuffs over time, as retention time of large particles is indefinite.

1.4. Ruminal degradation of fiber

Ruminants possess a large forestomach known as the reticulo-rumen that contains a complex microbiome responsible for their ability to utilize high-fiber feeds (Hoffman, 1989; Nagaraja, 2016). Ruminants harvest and physically breakdown fibrous feedstuffs through mastication and rumination, while ruminal bacteria, protozoa and fungi produce enzymes that degrade the fiber by hydrolyzing bonds to release the sugars that are subsequently fermented (Owens and Basalan, 2016). Fermentation in the rumen occurs in two steps: first polysaccharides are broken down to monosaccharides via hydrolysis; second these monosaccharides are converted to volatile fatty acids (VFA), gases, and heat (Chesson and Forsberg, 1997). Hydrolysis of polysaccharides is often the rate-limiting step as it requires many enzymes working together to sequentially liberate monosaccharides (Chesson and Forsberg, 1997). The fermentation end products (VFA and gases) are toxic to the ruminal microbes when present in excessive quantities, thus if allowed to accumulate they would inhibit fermentation (Owens and Basalan, 2016). However, because the rumen is a semi-continuous fermentation system, VFA are absorbed across the rumen epithelium and used as energy while gases are lost to the environment during eructation (Owens and Basalan, 2016).

Nitrogen is presented in the ruminant diet in the form of protein and non-protein nitrogen (NPN). Non-protein nitrogen sources include urea and other soluble components of feed. Urea or NPN is converted to ammonia in the rumen, which is subsequently incorporated into amino acids which are used to synthesize microbial protein. Ammonia can also be absorbed into the blood stream where it can be transported to the liver and used for synthesis of urea. Urea produced in the liver can either be recycled into the rumen in saliva or through the rumen epithelium. Excess urea that is not recycled can be excreted in the urine (Huntington and Archibeque, 2000; Reynolds and Kristensen, 2008). The ruminal bacterial populations that breakdown fiber, and incorporate the nitrogen from both protein (in the form of peptides and amino acids) and NPN sources, subsequently act as a dietary source of protein for the ruminant.

1.4.1 Differences in rate and total degradable fraction of various forages

As rate of digestion (k_d) and potentially digested fraction ($a+b$) are influenced by lignin content these parameters vary among and within forages depending on maturity, growing conditions, part of the plant (leaf vs. stem) and processing with more mature forages containing greater lignin, and having reduced $a+b$. For a summary of digestion kinetics see table 1.3.

Hoffman et al. (1993) investigated digestion kinetics of a variety of perennial forages *in situ* in cows fed 56:44 forage to concentrate (F:C), across a range of maturities. They found total $a+b$ for late vegetative, late bud, and midbloom alfalfa of 73.4%, 60.3% and 55.2% respectively with rates of digestion of 15, 16 and 13 %/h. Coblentz et al. (1998) examined leaf, stem and total plant DM degradation kinetics of alfalfa at 10% bloom, and found $a+b$ of leaf was the greatest (89.6%) with the fastest rate of degradation (20%/h), followed by whole plant $a+b$ (76.6%) with 15.2%/h rate of degradation and finally alfalfa stem had 57.1% total $a+b$ DM at a rate of 12.8%/h. This trend holds for NDF $a+b$ as well: 75.4%, 38.8%, and 53.2% respectively for leaf, stem and total plant, with degradation rates of 9.1%/h, 5.3%/h, and 7.5 %/h (Coblentz et al., 1998). Dado and Allen (1996) found *in situ*, total $a+b$ of NDF was 47.9% for high NDF digestible alfalfa silage compared with 45.4% for low NDF digestible alfalfa silage, with respective rates of digestion of 11.4%/h and 11.2%/h. Total mixed rations formulated with highly digestible alfalfa silage had $a+b$ of 50.5% that was degraded at a rate of 11.3%/h, while low digestibility alfalfa silage had an $a+b$ of 47.1% with a rate of digestion of 12.6%/h.

Hoffman et al. (1993) found that early timothy had an *a*+*b* (DM) of 68.1% and rate of degradation of 9%/h, mid maturity timothy had an *a*+*b* (DM) of 52.5% and rate of degradation of 5%/h, and mature timothy had an *a*+*b* (DM) of 43.6% and rate of degradation of 4%/h. They found similar trends of decreasing *a*+*b* (DM) and rate of digestion for quackgrass (early: 62.7%, 7%/h; mid: 43.3%, 3%/h; mature: 35.0%, 4%/h), perennial ryegrass (early: 75.8%, 11%/h; mid: 69.4, 9%/h; mature:52.7%, 5%/h), red clover (early: 78.1%, 18%/h; mid: 68.7%, 13%/h; mature:64.2%, 14%/h), and a number of other forages (Hoffman et al., 1993).

Coblentz et al., (1998) similarly examined an *a*+*b* (DM and NDF) for red clover leaf, stem and total plant tissues. They found *a*+*b* for leaf, stem and total red clover plant of 88.0%, 71.5% and 85.8% DM, and 73.8%, 52.8% and 72.1% NDF respectively, with corresponding rates of digestion of 15.6%/h, 11.6%/h and 11.4%/h, for DM and 11.4%/h, 8.9%/h and 7.2%/h for NDF. They also looked at gamagrass degradability and found *a*+*b* (NDF) degradable fraction of 77.4% at the bootstage decreasing to 66.8% at maturity, with corresponding rates of degradation of 5.6%/h and 3.2%/h.

Coblentz et al., (2000) examined differences in digestion kinetics among grain forages at different stages of development. They found mature oat forage (stalk and grain) had the largest *a*+*b* at maturity (73.8%) compared with wheat (70.3%) or rye (61.1%).

1.4.2 Factors affecting fiber digestion in the rumen

There are a myriad of factors affecting fiber digestion in the rumen, including inherent properties of the substrate, dietary interactions, individual animal differences in buffering, rumination, intake, rate of passage, microbiome composition and VFA absorption, along with interactions between diet and the rumen environment.

1.4.2.1 Substrate factors

The undegradable fraction of forage, which can be described as the highly lignified portion of the plant, is the largest limitation imposed on fiber degradation in the rumen (Jung and Allen, 1995) and is an inherent characteristic of each feed type (Tamminga, 1993). Crystallinity of cellulose also represents a barrier in highly fibrous feeds (O'Sullivan, 1997; Hall et al., 2010).

Table 1.3. Digestion kinetics ($a+b$, k_d) of various forages at different stages of maturity.

	DM		NDF		
Forage	$a+b$[1] (%)	k_d (%/h)	$a+b$ (%)	k_d (%/h)	Reference
Alfalfa					
Late vegetative	73.4	15.0	47.9	11.0	Hoffman et al.,
Late bud	60.3	16.0	32.0	9.0	1993
Midbloom	55.2	13.0	28.6	7.0	
Alfalfa 10% bloom					
Leaf	89.6	20	75.4	9.1	Coblentz et al.,
Stem	57.1	12.8	38.8	5.3	1998
Total	76.6	15.2	53.2	7.5	
Alfalfa silage					
Low digestible fiber	N/A	N/A	45.4	11.2	Dado and
High digestible fiber	N/A	N/A	47.9	11.4	Allen, 1995
Red clover					
Late vegetative	78.1	18.0	23.4	5.0	Hoffman et al.,
Late bud	68.7	13.0	19.2	3.0	1993
Midbloom	64.2	14	22.3	8.0	
Red clover 10% bloom					
Leaf	88.0	15.6	73.8	11.4	Coblentz et al.,
Stem	71.5	11.6	52.8	8.9	1998
Total	85.8	11.4	72.1	7.2	
Timothy					
Second node	68.1	9.0	46.8	2.0	Hoffman et al.,
Boot stage	52.5	5.0	27.6	3.0	1993
Full inflorescence	43.6	4.0	26.4	4.0	
Gamagrass					
Boot stage	89.6	4.8	77.4	5.6	Coblentz et al.,
Anthesis	82.3	3.5	74.1	3.8	1998
Mature	67.7	4.2	66.8	3.2	
Oat grain forage					
Pre-boot state	92.2	8.6	83.8	8.6	Coblentz et al.,
Heading	82.6	4.6	77.1	4.5	2000
Mature	73.8	3.5	60.6	3.8	
Wheat grain forage					
Pre-boot state	92.5	11.1	87.4	10.8	Coblentz et al.,
Heading	88.0	5.6	80.0	5.6	2000
Mature	70.3	4.0	50.0	3.9	
Rye grain forage					
Pre-boot state	94.9	9.9	89.6	9.7	Coblentz et al.,
Heading	78.8	4.3	73.5	4.2	2000
Mature	61.1	3.4	43.6	3.1	

[1.] $a+b$ is the total degradable fraction, and k_d is the rate of degradation for dry matter (DM) and neutral detergent fiber (NDF).

Water soluble carbohydrate content of a substrate, such as glucose, fructose, or sucrose, can influence microbial composition in the rumen as well (Leng 1990), which can have a significant effect on ruminal degradation of fiber as increasing these components can cause a decrease in ruminal pH leading to inhibition of fiber digesting bacteria and protozoa.

Generally, lignin increases as plants mature and rate of degradation of a substrate decreases as maturity increases (Tamminga, 1993). This negative relationship between maturity and degradation is much more pronounced in stems than leaves (Terry and Tilley, 1964; Akin, 1989) and does not consistently occur in legume leaves (Hendrickson et al., 1981).

Within grass and legume leaves, certain tissue types are more easily degraded. For example grass and legume leaves, mesophyll and phloem cells (Akin and Burdick, 1975; Chesson et al., 1986) as well as epidermis tissues of cool season grasses (Akin, 1989) are often degraded within 12 hours of incubation in the rumen. Degradability of other tissue types varies depending on biotic and abiotic stressors applied to the plant (i.e., fungal or microbial infection, low or high environmental temperature, mineral deficiency, and so forth (Moura et al., 2010).

Functional specific gravity (FSG) is another inherent substrate property that affects digestibility in the rumen. While decreased particle size is important for increased FSG in the rumen, different substrates respond differently to ruminal fermentation resulting in different rates of changes in FSG depending on particle size and feed (Hooper and Welch, 1985; Siciliano-Jones and Murphy, 1991). For example, rate of change in FSG is higher in legumes than grasses (Hooper and Welch, 1985). This is important because FSG of forage particles, along with particle size will influence their ability to exit the rumen as small particles with increased FSG will sink to the reticulo-omasal orifice, and larger particles and particles with a lower FSG will float until they are broken down further and have increased FSG causing them to have a longer retention time which allows for further digestion of the substrate (Welch, 1986).

1.4.2.2 Microbial factors

Ruminants have evolved considerable symbiosis with ruminal microbes including bacteria, protozoa, fungi and Archaea. In exchange for digestion of fiber which releases

nutritious endproducts (VFA and nitrogen) to the host, the host contributes to breakdown of feedstuffs via mastication, and provides an anaerobic, buffered environment in which the microbes can subsist, and toxic endproducts either flow out of or are absorbed across the rumen epithelium. Ruminal contractions ensure mixing of rumen contents and facilitate microbial contact and subsequent attachment to feedstuffs (Nagaraja, 2016). Protozoa, fungi and bacteria have evolved to fill different niches within the rumen, and work together to ferment fiber.

Rumen protozoa have been classified into two main Orders: Holotrichs and Entodiniomorphids. Holotrichs do not digest fiber, but some Entodiniomophids, such as the large *Ophyroscholecidae, Epidinium*, *Polyplastron* and *Eudiplodinium* have been found to be very fibrolytic (Williams and Coleman, 1992; Tamminga, 1993; Takenaka et al., 2004). Based on defaunation studies in which protozoa are removed from the rumen, it has been proposed that these eukaryotes are responsible for 17-30% of ruminal fiber digestion (Dijkstra and Tamminga, 1995; Lee et al., 2000 Newbold et al., 2015). While protozoa contribute to fiber digestion directly, they may also contribute indirectly by selectively preying upon amylolytic bacteria, which encourages subsequent propogation of fibrolytic bacteria (Ushida et al., 1991). Unfortunately this predation can reduce microbial protein by up to 30% (Newbold et al., 2015), which represents a significant loss of microbial protein to the ruminant due to the establishment of futile protein cycles within the rumen.

Rumen fungi produce some of the most active fibrolytic enzymes found in the rumen (Wang and McAllister; 2002) and use rhizomes to penetrate lignified tissues, providing access points for microbial colonization, (Bauchop, 1981; Akin, 1989). However, fungi have a much slower growth rate than bacteria (Bauchop, 1981; Forsberg and Cheng, 1992) limiting their functional fiber degradation in the rumen by retention time.

The three known, major cellulolytic ruminal bacteria are *Ruminococcus albus, Ruminococcus flavefaciens,* and *Fibrobacter succinogens.* These three bacteria have been found to have low amino acid requirements, which allows them to survive solely on ammonia (Leng, 1990). The presence of either the non-core lignin, p-coumaric or ferulic acid, reduces cellulolytic activity (Chesson et al., 1982) and growth rate of all of these bacteria, and reduces total extent of growth of *F. succinogens* (Varel and Jung, 1984) reinforcing the fact that high lignin content is a

limiting factor in substrate availability both due to physical obstruction and chemical inhibitory effects.

In order for cellulolytic bacteria to degrade fiber, attachment is essential. Attachment of bacteria usually occurs at sites of epidermal damage, where carbohydrates are most accessible (McAllister et al., 1994). The highly cellulolytic bacteria *R. flavefaciens* and *R. albus* have both been found to use cellulosomes, which are membrane bound multi-enzyme complexes which attach the bacteria to the fiber source and degrading cellulose (Ding et al., 2001; Wang and McAllister, 2002). Recent investigation has suggested that *F. succinogens* uses a "third way" to degrade fiber that is more efficient than *R. albus* and *R. flavefaciens*. *Fibrobacter succinogens* binds to fibrous substrates using fibro-slime proteins and selectively cuts and imports chains of cellulose into its periplasmic space where the chains of cellulose are further degraded by endocellulases (Ransom-Jones et al., 2012).

While it is known that all of these microbes contribute to ruminal fiber digestion, there have been a number of *in vitro* attempts to quantify relative contribution of fiber degradation by individual group of microbes (i.e., protozoa, bacteria, fungi). Lee et al. (2000) found fungi alone to be responsible for most fiber digestion of switchgrass *in vitr.,* Zhang et al. (2007) found synergistic effects between bacteria and protozoa and bacteria and fungi, and all three together, on degradation of corn stover *in vitro.* They found that individually there were no differences in *in vitro* degradation of corn stover between individual microbial group of protozoa, bacteria or fungi. However *in vitro* studies are confounded by our inadequate ability to culture all ruminal microbes. Even given the limitation on *in vitro* propagation of rumen microbes there is no doubt that synergism and antagonism between types of microbes contribute to fiber degradation in the rumen (Wang and McAllister, 2002).

1.4.2.3 Dietary factors

There are many interactions between specific dietary components and fiber digestion. For example, in order to optimize digestibility of poor quality substrate in the rumen environment sulfur, phosphorus, magnesium, and ammonia must be provided to the animal (Leng, 1990). Sulfur is needed at a minimal level of 1 ug/mL in order for rumen bacteria to synthesize sulfur containing amino acids for microbial protein synthesis (Bray and Till, 1975), and is also essential for the growth of rumen fungi (Gordon and Phillips, 1989). A deficiency in phosphorous or

magnesium can reduce microbial growth resulting in slowed ruminal digestibility and reduce intake (Wilson and Minson, 1980; Durand et al., 1988).

Perhaps the most important supplement to provide when looking to improve the digestive efficiency of poor quality forage is nitrogen in the forms of protein and non-protein nitrogen sources. Without an abundant source of nitrogen that can be converted to amino acids, peptides and ammonia in the rumen and used for microbial protein synthesis, microbial growth is severely inhibited. Source of dietary nitrogen can also have an effect on ruminal degradation of feed. Stritzler et al. (1992) found that supplementing with fishmeal led to significantly improved *in situ* degradability of fiber than supplementation with urea. Nitrogen can also be obtained by the ruminant from lysed ruminal microbes, and sloughed-off epithelial cells as well as from nitrogen-recycled urea in saliva and across the rumen wall, but availability of this is dependent on dietary nitrogen (Obara et al., 1991).

While crude protein supplementation increases fiber digestion, lipid supplementation at over 5% of the diet has been found to decrease ruminal fiber digestion due to toxic effects on some ruminal fibrolytic bacteria, methanogens, and protozoa (Henderson, 1973). Similarly, supplementing with other high energy feeds, such as grain or starch reduces digestibility of fiber by increasing the rapid production of VFA leading to a fall in ruminal pH (Hoover, 1986; Tamminga and VanVuuren, 1988). Low pH inhibits cellulolytic bacteria, causing a shift in rumen microbial composition away from fibrolytic microorganisms to amylolytic and proteolytic bacteria (Orskov and Fraser, 1975). Extent of inhibition of fiber digestion can vary with starch source, for example, barley has been found to inhibit fiber digestion to a greater extent than corn (Tamminga, 1993) because starch in corn is more difficult to access in the rumen due to the dense protein matrix present in the endosperm (McAllister et al., 1993). Order of feeding can also affect fiber digestibility when feeding a mixed diet, with fiber digestibility increasing when grain is fed prior to forage (Beauchemin and Buchanan-Smith, 1990).

1.4.2.4 Animal factors

Fiber digestion has been found to be more efficient in larger ruminant species (Van Soest, 1994); this is thought to be due to reduced maintenance metabolism in proportion to body size (Demment and Van Soest, 1984). Likewise fiber particles have a longer retention time in cattle than sheep (Tamminga, 1993) perhaps because they chew less frequently than sheep (Ulyatt et al,

1986). Variation in chewing frequency is also seen among individual cattle (Grummer et a., 1987; Dado and Allen, 1994). Mastication is an important physiological process for improved fiber digestibility as it works to break up large particles and increase surface area for microbial attachment.

Dry matter intake (DMI) can have a significant effect on ruminal fiber digestibility, with increased intake leading to increased rate of passage, and reduced fiber digestibility (Owens and Goetsch, 1986). Dry matter intake is affected by palatability of feeds (Van Soest, 1965) and rumen fill capacity of the feed, which is based on fiber content and particle size (Balch and Campling, 1962). Relationships between intake, chemical composition of substrate and digestibility are quite variable and dependent on plant species (Van Soest, 1965).

Efficiency can be assessed in a number of ways, including gain to feed ratio (G:F; Koch et al., 1963), which is the amount of weight gained per unit of feed, and residual feed intake (RFI) which is the difference between the actual and predicted feed intake of an animal based on its growth and body weight (Arthur and Herd, 2008). There are a number of factors that affect digestive efficiency including feed intake, environmental conditions (i.e. weather) which may challenge thermoregulation, retention time, and rumen microbial composition and level of activity of the animal (Herd et al., 2004; Herd and Arthur, 2009; Carberry et al., 2012).

Animals with a slower rate of passage have been found to have more complete digestion and increased efficiency (Balch, 1952) and slower rate of passage optimizes bacterial growth on cellulose (Maeng et al., 1989) providing an advantage for fiber digestion to animals. Tamminga et al. (1989) found that increasing feeding level of dairy cattle caused a >10 % reduction in ruminal degradation of fiber largely due to increased dilution rate. They also found that rate of passage can be affected by environmental factors and diet, for example cold stress can increase feed intake, which in turn increases rate of passage (Tamminga et al., 1989). In addition to increasing rate of passage, increasing DMI also increases rumen fill (Shaver et al., 1986), which can increase accumulation of VFA's when cattle are fed concentrate in their diet, leading to decreased pH and reduce activity of cellulolytic bacteria (Tamminga and Van Vuuren, 1989). Ruminal pH is another important factor in relation to ruminal fiber degradation; it varies depending upon diet, buffering capacity of the rumen, saliva production (Maekawa et al., 2002),

and rate of ruminal VFA absorption (Penner et al., 2009). Lower ruminal pH inhibits cellulolytic bacteria and their enzymatic activity reducing ruminal degradation of fiber.

1.5. Forage and crop residue treatments

Crop residues are much less expensive than grass hay on a DM basis, currently costing between or $40-$60 per tonne for straw, compared to $85 or more per tonne for hay (Alberta Agriculture and Forestry; 2016). While crop residues such as straw represent a relatively untapped fiber source, and are much cheaper than hay, the nutritive value of crop residues is very low. There have been a number of treatments developed for biofuel production that make hemicellulose and cellulose more accessible. These technologies may have implications for improving crop residues for ruminant feed as well.

1.5.1 Physical processing

Physical processes to improve the nutritive value of poor quality feedstuffs include chopping, grinding, and pelleting, and involve reducing particle size to increase surface area for microbial attachment, ultimately increasing intake by reducing rumen fill and increasing rate of passage (Owen 1978). Greenhalgh and Reid (1973) found increased intake for pelleted vs. non-pelleted feeds in both cows and sheep, with greater increases in intake for pelleted low quality forages than high quality forages. The increased intake in pelleted low quality forage compared with pelleted high quality forage is attributed to the significantly lower intake of low quality forage compared with high quality forage before pelleting. This is in line with the findings of Jarrige et al. (1973) that intake of mature forage is increased as particle size is decreased. While pelleting increased intake, it also decreased DMD, likely due to reduced residence time in the rumen (Greenhalgh and Reid, 1973).

Hot water or steam explosion have been effectively used to process crop residues. Both of these treatments remove hemicellulose and alter lignin structure. Steam explosion works by applying high-pressure steam to recalcitrant materials and then rapidly releasing pressure and flash cooling (Mosier et al., 2005) while hot water treatments involve heating a slurry of crop residue under pressure for 15-20 minutes. The major appeal to using a water-based pretreatment is that no chemicals are needed for neutralization, which reduces processing costs and environmental concerns, however it is not as effective as many chemical treatments as it does not disrupt crystalline cellulose or solubilize lignin (Mosier et al., 2005).

1.5.2 Biological/Enzymatic treatments

Biological pretreatments involve application of bacteria or fungi cultures or fiber degrading enzymes to forage and allowing them to incubate for hours, days or weeks (Beauchemin et al., 2003; Alvira et al., 2010). White-rot fungi have been employed as an environmentally friendly, biological pretreatment to lignocellulosice crop residues and have proved very effective at degrading lignin, however they must be left for weeks in order to have significant impact (Kumar et al., 2009). Concentrated fibrolytic enzymes can also be added directly to feeds to improve digestibility (Beachemin et al., 1995) but there is significant variability in efficacy among available products. Therefore the use of enzyme mixtures does not necessarily improve the growth performance of beef or dairy cattle (Beauchemin et al., 2003). Success of enzymatic pretreatment depends on a number of factors including: type and quantity of enzyme, specificity of enzyme(s) for the feed they are applied to, and method of enzyme inclusion (i.e. whether enzyme is applied to forage immediately prior to feeding or left for a period of time to incubate prior to feeding) (Beauchemin et al., 2003).

1.5.3 Chemical treatments

Chemical treatments for crop residues include alkaline and acid pretreatments such as sodium hydroxide and sulfuric acid, which work by altering the structure of lignin, hydrolyzing hemicellulose and/or inducing swelling in cellulose, causing disruption of its crystalline structure by breaking hydrogen bonds (Mosier et al., 2005).

Acid pretreatments using inorganic acids like sulfuric acid, have drawbacks because strong acids can corrode equipment. It can also be difficult to recover excess acid after treatment, and acid treatment can result in byproducts that inhibit fermentation (Wymann, 1996). Diluted acids offer an effective alternative to strong acid pretreatment as they are gentler on equipment, have reduced inhibitory byproducts, and can be used along with high temperatures to expedite xylan hydrolysis (Alvira et al., 2010)

Alkali pretreatments solubilize lignin and increase cellulose digestibility by disrupting its crystalline structure (Carvalheiro et al., 2008) making it very effective for use on crop residues (Kumar et al., 2009). Ammoniation of crop residues has been implemented on farm by placing bales under a large plastic sheet and gassing them with anhydrous ammonia. This method has certain drawbacks, such as volatilization and loss of excess ammonia to the surrounding

environment (Freney et al., 1983; Rasby et al., 1989) and potential health hazards of anhydrous ammonia exposure if proper protective equipment is not worn.

Graham and Aman (1984) found ammoniation of barley straw increased the potentially degradable fraction by 23% by reducing ferulic acid, which is a non-core lignin associated with hemicellulose (Jung, 1989). P-coumaric content of ammoniated barley straw, which is closely associated with core lignin, was increased in the residues indicating that it is unaffected by ammonia treatment. Xiao et al. (2001) found > 68% solubilization of lignin in highly lignified crop residues following treatment with 1M NaOH. Urea treatment of barley straw was found to be more effective at higher temperature and moisture content than cooler dry conditions (Cañeque et al. 1998).

Fahmy and Orskov (1984) examined the effect of various chemical treatments and combinations of these treatments on degradability of barley straw in sheep. They found that ammonia and sodium hydroxide treatments were effective at increasing *in situ* degradability of barley straw in sheep, with additive effects when barley straw was treated with both. They also observed increased intake in sheep fed ammonia treated straw, likely because of increased N availability.

Colucci et al. (1992) treated wheat, barley, and oat straw with urea and found that oat straw, which was the most recalcitrant in this study, responded most favourably, indicating the treatment had a larger effect on substrates with a lower initial organic matter degradability (OMD). Tuah et al. (1986) similarly found an increased effect of ammoniation on barley, oat and wheat crop residues with lower nutritive value compared to the same straw with a greater nutritive value. These studies highlight ammoniation as a powerful technology to increase nutrient availability of recalcitrant feedstuffs in ruminants.

1.5.4 Physicochemical treatments

Physicochemical pretreatments manipulate the physical state of water or use solvents to disrupt the fiber matrix. These usually involve manipulation of pressure and can be used in conjunction with alkaline or acid pretreatments, such as ammonia fiber expansion (AFEX). The AFEX treatment involves packing the crop residue into a reactor, sealing the reactor and pumping in gaseous ammonia. The reactor is then heated to 80 degrees Celsius and pressure is

increased to maximum 200 psi for 30 m with quick pressure release. The quick depressurization disrupts fiber structure by decrystallizing cellulose, altering lignin, and pre-hydrolyzing hemicellulose (Yang and Wyman, 2008). The AFEX process occurs in an enclosed silo and has an ammonia extraction step, which allows for recovery of most of the ammonia, making it economically more viable and safer, with reduced environmental contamination (Campbell et al., 2013).

1.6. Cattle and bison evolutionary history

Ruminant species have evolved a variety of dietary preferences and feeding behaviours based on their environmental niches, and as such, they can be classified on a spectrum from concentrate selectors to roughage eaters (Pérez-Barbería et al, 2004). Concentrate selectors, or browsers do not eat large amounts of fiber, but rather selectively eat the more concentrated, low fiber portions of plants, while roughage eaters primarily consume fiber-rich parts of the plant.

North American cattle (*Bos taurus*) are believed to have originated from domesticated wild *Bos primigenius* around 10,000 years ago in Southeast Asia (Perkins, 1969; Bruford et al., 2003) as humans realized the potential of ruminants to turn plant fiber into high quality milk and meat protein. Humans actively artificially selected for specific characteristics in cattle which presumably included docility, growth performance, aesthetic preference including horn size and coat color (Kelly, 1994). European cattle were introduced into North America during colonialism, circa 1640 (Bowling, 1942). Despite human involvement in their recent evolutionary development, cattle have continued to be roughage eaters. It is only more recently with the advent of feedlots that their diet has shifted to include increased amounts of concentrate. However, fibrous feeds continue to comprise a large portion of cattle diets.

Bison bison bison (plains bison) and *bison bison athabascae* (wood bison*)* are North America's largest herbivore (Hudson and Frank, 1987) and evolved in the late Pleistocene, during the Wisconsin glaciation (Guthrie, 1970). It seems logical to infer due to their ability to survive harsh Canadian winters as roughage eating ruminants, that they could have a superior capacity to digest poor quality forages as compared to domesticated cattle. In order to define the ecological niche of bison a number of studies have focused on defining their dietary preferences and eating behaviours. Hudson and Frank (1987) observed the feeding behaviour of plains bison during summer and autumn and found that their feeding behaviour and intake varied by season

with grazing time and DMI increasing in the autumn. Fortin et al. (2003) found the diet of plains bison in Prince Albert National Park, Saskatchewan was dominated by slough sedge (*Carex atherodes*) in the summer while, in the winter their diet was supplemented with thistle (*Cirsium arvense*) and rivergrass (*Scolochloa festucacea*). Slough sedge seemed to be the forage of choice for plains bison, regardless of season even when wheatgrass was widely available and consumption of wheatgrass would maximize long term gains (Fortin et al., 2002). Availability of plant species does seem to have some influence on bison's grazing diet: Larter and Gates (1991) observed wood bison's seasonal dietary variation in the Mackenzie Bison Sanctuary in the Northwest Territories and found bison diet in the winter consisted of >96% sedge, while in the summer they diversified their diet by consuming grass (*Graminae*) and willow (*Salix*). Similarly, Bergmann et al. (2015) found that plains bison intake of woody shrubs increased in spring and fall. Reynolds et al. (1978) examined feces of plains bison from the Slave River lowlands in the Northwest Territories and found regardless of season, slough sedge and reedgrass were the most abundant feed sources. Regardless of seasonal and locational variation in the diet of bison, the dependence on sedge as a feed source for bison is apparent. Neutral detergent fiber content of slough sedge ranges from 65-75% DM over the course of the plants' maturity, ADF from 30-40% and acid detergent lignin from 2.5% to 5.5% (Hubbard, 1988) with it having a chemical composition similar to barley straw.

While cattle (*Bos taurus*) and bison (*Bison bison*) are both roughage eaters, bison have been found to be more efficient at digesting fiber than cattle (Hawley et al., 1981; Larter, 1991; Plumb and Dodd, 1993) and tend to select more lignified roughage when grazing compared to cattle (Peden, 1974). However the maximum feed intakes of bison and cattle consuming forages are similar (Arthur, 1986; Hudson and Frank, 1987). Richmond et al. (1977) compared digestion coefficients between bison, yak and cattle and found that, while bison and cattle had similar feed intake, bison exhibited less selectivity, and digested all forages to a greater extent than cattle. However, the increased digestibility in bison did not result in enhanced rates of gain, except for alfalfa, suggesting that bison have a higher maintenance energy requirement. Yak and bison had increased gain on alfalfa, compared to other forages fed, while cattle did not (Richmond et al., 1977). Similarly, Schaefer et al. (1978) examined digestive function in bison, yak and cattle fed a pelleted diet and found that bison and yak had longer retention times in the gastrointestinal tract than cattle, which contributed to improved apparent nitrogen absorption.

1.7. Species and individual differences in rumen microbiome

Differences in diet have led to differences in enzymatic capacity among ruminant species (Hoffman, 1989; Perez-Barberia et al, 2004) due to selection for a specific microbiome suited to specific dietary preferences. While it has been found that there are similarities in the genera of ruminant microbiomes when compared within and across ruminant species both regionally and globally (Henderson et al., 2015), differences exist at the microbial species level among individual ruminants, within and across ruminant species. These differences in ruminal microbial species can have important effects on animal metabolism. For example, Varel and Dehority (1989) found that bison have increased numbers of cellulolytic *F. succinogens, R albus* and *R. flavefaciens*, compared to cattle when fed the same diet.

Despite the fact that there are broad similarities in genera among ruminants, individual animals have unique and quite resilient, microbiomes which are influenced and maintained due to differences in individual ruminant genetic composition (Cole, 1991; Weimer et al., 2010) termed their "hologenome" (Rosenberg and Zilber-Rosenberg, 2011). These differences in hologenome have been highlighted by Shabat et al. (2016) who measured feed efficiency in 146 milking cows and from those selected 78 extreme (low or high feed efficiency) animals to examine differences in microbiome. They found that those cows with lower microbial species richness had increased feed efficiency due to increased VFA production along with decreased acetate: propionate ratio, which is associated with increased energy harvest and reduced methane production. They found that more efficient cattle had fewer, more dominant, microbial species, which had fewer metabolic pathways than inefficient cattle. They also found that inefficient cattle produced significantly more end products such as methane, that could not be used by the animal, or that had a negative impact on energy harvest by the animal. These results indicate individual microbiome can have significant impact on ruminant energy metabolism.

Despite the resilience in individual hologenome, modifications to the rumen microbiome have been achieved using methods such as supplementation with direct fed microbials (Yoon and Stern, 1995) or inoculation with rumen fluid with novel characteristics (Jones and Megarrity, 1986) indicating the possibility of modifying the rumen microbiome when an unfilled niche is available (Weimer, 2015).

1.7.1 Cross inoculation studies

Cross inoculation has been done within species in an attempt to mitigate the effects of fasting (Bond et al., 1983; Cole, 1991) with mixed effects. Bond et al. (1983) found increased feed intake post-fasting in steers inoculated with rumen fluid from fed steers, compared with steers that did not receive inoculation. Conversely, Cole (1991) found no difference in DMI between fasted lambs that received inoculation from fed lambs compared to those that did not. Weimer et al. (2010) performed two cross inoculations between cattle. In their initial cross inoculation the rumen microbiomes of each cow returned to pre-inoculation state after 14 and 61 days, respectively. After the second cross inoculation the microbiome shifted back toward its pre-inoculation composition, but there were some lasting changes in rumen pH and VFA production in one of the cows up to 62 days later. Similarly, Jones and Megarrity (1986) managed to transfer the ability to degrade the toxin, 3, 4 dihydroxy pyridine, found in leucana, to goats by inoculating them with goat rumen fluid containing microbes with the enzymatic ability to do so.

Inter-species cross inoculations have been performed in an attempt to imbue cattle with novel traits. For example, Dehority et al. (1999) performed a cross inoculation in the form of a single complete replacement of rumen contents of pregnant cattle fed pine needles with rumen contents from elk, in an unsuccessful attempt to mitigate the abortive effects of pine needles in cattle. The failure to transfer novel characteristics from elk to cattle does not preclude possible benefits of transferring novel traits from other ruminant species to cattle. There is greater overlap in diet and foraging pattern between bison and cattle than between cattle and elk (Hartnett et al., 1997). This niche similarity between bison and cattle may allow for the microbial dissemination between these two ruminant species that was unsuccessful between cattle and elk. For example, Pamungkus et al. (2004, 2006) transferred rumen contents between cattle and water buffalo, and found that it decreased the ruminally degraded fraction of leguminous leucana, but increased the degradability of Napier grass in water buffalo using an *in situ* technique. In cattle, after cross-inoculation with water buffalo rumen contents, they found reduced rumen pH and increased ammonia and VFA concentration in the rumen resulting in a decrease in bacterial populations (Pamungkus et al., 2004). Unfortunately, after inoculating cattle with water buffalo rumen contents, they did not test digestibility parameters in cattle.

1.8. Knowledge gap and proposed research

Based on the literature reviewed here, the following are knowledge gaps I intend to address with my research.

Using the *in situ* technique I will evaluate:

1) How much variation is there among individual animals in terms of ability to digest forage in the rumen? Is this variation similar among forages?

2) Does inoculating cattle with bison rumen contents improve their ability to digest various forages?

3) What are individual animal responses to inoculation with bison rumen contents?

With the corresponding hypothesis:

1) There will be variation among individual animals in terms of ability to digest forage, and individual heifer's ability to digest different forages will be similar for all forages.
2) Inoculating cattle with bison rumen contents will improve their ability to digest various forages.
3) Individual heifers will respond positively to inoculation, but will vary in their degree of response.

Using the Rusitec system, and based on information gathered in the *in situ* study, I will try to answer the following questions:

4) Do differences in rumen inoculum contribute to differences in rate of digestion among heifers?

5) How does rumen inoculum chosen from animals with a fast or slow rate of digestion differ in its ability to digest fiber from an easily accessible fiber source compared to a recalcitrant fiber source (AFEX vs. untreated barley straw)?

With corresponding hypothesis:

4) Differences in rumen inoculum, reflecting differences in microbiome composition among cattle will contribute to differences in rate of digestion among heifers.
5) Inoculum from heifers with a fast rate of ruminal digestion will degrade AFEX and untreated barley straw to a greater extent than inoculum from heifers with a slow rate of ruminal digestion.

To investigate these knowledge gaps I will employ the *in situ* technique to measure digestibility of various forages in the rumen. These measurements will allow me to compare digestibility parameters between animals as well as among forage types. I will also employ the Rusitec system to examine differences in rumen fluid selected from animals with fast or slow k_d. I will incorporate AFEX barley straw as a substrate in the Rusitec system to compare digestion capacities of rumen fluid from heifers with a fast or slow k_d on AFEX straw, a more easily digested fiber source, and untreated barley straw, a more recalcitrant fiber source.

Chapter 2: Potential for improving fiber digestion in the rumen of cattle (*Bos taurus)* through microbial inoculation from bison (*Bison bison): In situ* fiber degradation

2.1. INTRODUCTION

With increasing demand for beef and increasing costs of feeding, there is interest in improving the feed efficiency of cattle. While cattle have the ability to digest fiber through a symbiotic relationship with anaerobic microorganisms that reside in the digestive tract, digestion of hemicellulose and cellulose is incomplete (Varga and Kolver, 1997). Crop residues such as straw are abundant agricultural byproducts and an improvement in the ability of cattle to digest hemicellulose and cellulose would effectively improve the utilization of these feedstuffs.

Cross inoculation of rumen contents within species has been utilized as a method of alleviating low feed intake and digestive diseases with mixed results (Bond et al., 1983; Cole, 1991; Weimer et al., 2010). Similarly, cross inoculation between species (elk to cattle) has been attempted with some elk-adapted species of protozoa establishing in the rumen of cattle post-inoculation (Dehority et al., 1999). Inoculation of cattle with bison rumen contents has not been previously attempted, it is not known whether it is possible to improve digestibility in cattle by introducing a foreign microbiome into their rumen. As bison have been shown to be more efficient at digesting cellulose and hemicellulose than cattle (Hawley et al., 1981), we hypothesized that inoculation of cattle with rumen contents from bison would improve their ability to digest forage.

It is well known that there is variation among individual animals for many traits, including feed conversion efficiency (e.g., Carberry et al., 2012; Rius et al., 2012). Reasons for animal variation are related to animal behavior, physiology, genetics, the rumen microbiome (Hernandez-Sanabria et al., 2011; Carberry et al., 2012; Rius et al., 2012), andmong other factors. We hypothesized that cattle would differ in their ability to digest fiber in the rumen, and respond differently to inoculation with bison rumen contents when compared with each other.

The objectives of this experiment were to determine if inoculating cattle with bison rumen contents would improve potential fiber degradation of various forages and to examine individual variation among cattle in their ability to digest forage and their responses to inoculation with bison rumen contents through the use of the in situ technique.

2.2. MATERIALS AND METHODS

This experiment was conducted at Agriculture and Agri-Food Canada in Lethbridge, Alberta. Animals used in this experiment were cared for in accordance with Canadian Council on Animal Care guidelines (CCAC, 2009). All procedures and protocols used in this study were reviewed and approved by the Animal Care Committee at the Lethbridge Research and Development Centre.

2.2.1 Experimental Design, Animal Management and Diet

Sixteen ruminally cannulated Angus × Hereford beef heifers weighing 461 kg (± 21 kg) were used in the experiment. Cattle were blocked in 2 groups of 8 allowing measurements to be made in 8 animals at a time (Fig 1). Before the study, the cattle were treated for parasites (1% w/v, Ivomec®, Merial Canada Inc., Baie D'Urfé, Québec, Canada). The study was conducted over 88 d, with the first 46 d as the baseline period, the next 14 d as the rumen inoculation period, 12 d as the recovery period and then a final 16 d to test the effect of the inoculation. During the rumen inoculation period cattle rumens were emptied and inoculated with bison rumen digesta twice. Each animal was considered to be an experimental unit, with every animal receiving the same rumen inoculation treatment. The *in situ* ruminal degradability of barley straw, canola straw, alfalfa hay, and timothy hay were measured before and after rumen inoculation with bison rumen contents, as outlined in Figure 1.

The cattle were housed in tie stalls and bedded with wood shavings on top of rubber mats and exercised daily for 2 h. Cattle were adapted to a diet of 700 g/kg barley straw and 300 g/kg pelleted concentrate (DM basis; Table 1) for 28 d prior to the start of the experiment. The diet was formulated using NRC (2000) to meet protein, vitamin and mineral requirements for a 1.0 kg/d gain for heifers weighing 450 kg. The amount of concentrate fed was adjusted at the beginning of each week and provided as 30% of the total DM intake (DMI) of the previous 7 d. Concentrate was fed daily at 0930 h, 30 min before barley straw. Barley straw was chopped to 6

-10 cm and provided *ad libitum*, with 10 to 20% orts. The amount of feed offered and orts were weighed daily to calculate DMI of individuals.

2.2.2 Rumen Inoculation

Thirty-two rumens from bison were collected from a local abattoir for each of the two inoculations with 64 rumens in total collected over the course of the study. Inoculations were performed in the morning on d 46 and in the morning of d 60 of the study. Esophageal and pyloric sphincters of each bison rumen was sealed with plastic zip ties and the entire rumen was placed into an insulated container and immediately transported in under 30 min to the Lethbridge Research and Development Centre in a heated truck. Upon arrival rumens were cut open and contents from all bison rumens were evacuated into a 39°C holding tank, and mixed under circulating O_2-free CO_2 to maintain anaerobic conditions. The bison were reared on a commercial farm and for at least 6 weeks prior to slaughter they were fed a diet of 75:25 barley silage:oats (DM basis).

Meanwhile, the rumen of each heifer was evacuated, and the contents emptied into insulated containers (1 container per heifer) which were sealed and weighed. Thirty percent of the rumen contents (by wet weight) were returned to the host heifer, and the remaining 70% was replaced with pooled bison rumen contents from the holding tank. The entire procedure was completed within 4 h of the time of collection of bison rumens from the abattoir.

2.2.3 In Situ Measurements

Alfalfa hay, timothy hay, barley straw and canola straw (Table 2) were ground through a 2 mm screen using a Wiley mill (standard model 4; Arthur H. Thomas Co., Philadelphia, PA, USA) and incubated in duplicate in the rumen of each heifer for 0, 4, 8, 12, 24, 48, 96, and 120 h. Bags used for incubation were 10 × 20 cm Ankom bags (R1020, ANKOM Technology, Macedon, NY, USA; 50 μm porosity) with 6.0 g (± 0.05 g) of forage per bag. To avoid fraying of Ankom bags the tops were folded inside the bag, ironed and then sealed (Impulse heat sealer, 120v 50/60HZ, Ankom Technology, Macedon, NY, USA) approximately 1 cm from the top.

In situ incubations were performed on d 1 to 13 and again after inoculation with bison rumen contents on d 71 to 83 (Fig. 1; i.e, starting 13 d after the second inoculation). Bags were incubated in all 16 cattle; as stated earlier, incubations were performed in 2 groups of 8 cattle,

one week apart. Measurements for each group took place over 6 d, with a 1 d break between groups. Bags were inserted into the rumen 1 h after feeding, and removed after the designated amount of time. Duplicate Ankom bags were placed inside larger mesh bags (30 × 30 cm) with 3 equal sized channels sewn into them to keep the smaller Ankom bags from aggregating together. Three Ankom bags were placed in both outside channels of the larger mesh bags and 2 in the middle channel of the mesh bags. There were a total of 8 Ankom bags per mesh bag, and 1 mesh bag per time point. Input and output of bags were scheduled so that there were never more than 3 mesh bags (24 Ankom bags) inside each heifer at any given time to prevent overcrowding in the rumen. Ten min prior to insertion of bags into the rumen they were placed in buckets filled with 39°C water so the contents were wet prior to insertion in the rumen, in order to allow for immediate colonization by ruminal microbes.

Upon removal of bags from the rumen they were immediately submerged in ice water to arrest microbial activity. Once removed from the rumen, all Ankom bags remained grouped by animal for the duration of their washing and drying. Mesh bags were emptied of Ankom bags, and Ankom bags were rinsed, placed into mesh laundry bags and washed in a large top-loading washing machine on a cold, gentle cycle (approximately 5 min, no spin cycle). The washing procedure was repeated once, after which the water ran clear and the bags were removed from the washing machine. The bags were removed from the mesh bags, briefly rinsed under cold running water to move the remaining contents to the bottom of the bag and checked for broken seams. Two Ankom bags with broken seams were discarded. Bags were gently squeezed to remove excess water and placed in foil trays in a forced air oven at 55°C for 72 h, cooled in a desiccator, and weighed.

The 0 h bags were placed in a 2000 mL beaker of water on a hotplate at 39°C for 30 mins, to determine the washout fraction. Bags were stirred every 10 min and after 30 min they were washed and dried as previously described.

The duplicate samples were composited, ground through a 1 mm screen, and analyzed sequentially for neutral detergent fiber (NDF, Van Soest et al., 1991) and acid detergent fiber (ADF; AOAC 2005, method 973.18) with modifications to each procedure for use in a fiber analyzer (Ankom 200, Ankom Technologies), with heat-stable alpha amylase and sodium sulfite used in the NDF procedure. Initial forages were analysed for DM by drying the samples at 135°C

for 2 h (AOAC, 2005, method no. 930.15) followed by hot weighing, nitrogen (crude protein = N × 6.25) by flash combustion with gas chromatography and thermal conductivity detection (AOAC, 2005, method no. 990.03; Carlo Erba Instruments, Milan, Italy), and NDF and ADF as previously described.

Undigestible NDF (uNDF; Van Amburgh et al., 2015) was measured by incubating each forage in triplicate for 244 h in three additional heifers that were not part of the original group of 16. And total degraded fraction after 244 h was determined as 100-uNDF. Disappearance of NDF and ADF at each time (t) was calculated and the non-linear fitting procedure in SAS (SAS Inc., Cary, NC, USA) was used to simultaneously estimate the degradation parameters in the model as follows (McDonald, 1981):

$$\boldsymbol{Disappearance = a + b(1 - e^{-k_d(t-l)})}$$

where a is the washout fraction, b is the potentially degradable fraction, k_d is rate of digestion (/h) of b, and (a+b) is the total potentially degradable fraction, and l is the lag. Effective rumen degradability (*ERD*) was then calculated as $ERD = a + b(k_d/(k_d+k_p)$ where k_p is the rate of passage (%/h) of the individual animal (ranging between 1.74 to 2.71). Passage rates of digesta within each heifer were determined using Yb-labelled NDF barley straw and reported elsewhere (Ribeiro et al., 2016).

Observed 24 and 48 h disappearance and *in situ* degradation parameters were analyzed using the MIXED procedure of SAS (SAS Inc., Cary, NC, USA) using a model that included inoculation treatment (pre-inoculation, post-inoculation), group, and treatment × group. Heifer was considered the experimental unit. Inoculation was treated as a repeated measure to account for effects of inoculation within animal. Effects of group and the group × inoculation period were later removed from the model as they were not significant. For each variable analyzed, various covariance structures among compound symmetry, heterogeneous compound symmetry, autoregressive, heterogeneous autoregressive, Toeplitz, unstructured and banded were evaluated and the one resulting in the lowest corrected Akaike information critical value was chosen. Significance was declared at $P \leq 0.05$ and a trend was considered at $0.05 < P \leq 0.10$. Linear regressions were performed to assess relationships between changes in k_d and a+b.

2.3. RESULTS

Overall, inoculation had no effect ($P > 0.10$) on *ERD* of NDF (Table 3) or ADF (Table 4) in any of the incubated forages. All effects of inoculation of the rumen of cattle with pooled bison rumen contents depended on the forage and the degradation variable. For barley straw, k_d of NDF was decreased ($P < 0.05$) after inoculation, while both lag time and *a* fraction of NDF were increased ($P = 0.05$) and *b* fraction of NDF was decreased ($P < 0.01$; Table 3). Mean observed 24 h and 48 h NDF disappearance were both decreased ($P < 0.05$) for barley straw. For canola straw, only lag time of NDF degradation ($P = 0.01$) was increased by inoculation, but there was a tendency for a reduced *b* fraction of NDF ($P < 0.10$). The k_d of alfalfa hay NDF decreased ($P < 0.01$) after inoculation, but the *b* fraction of NDF ($P < 0.05$) increased. There were no effects on disappearance of NDF at 24 h or 48 h. There were very minor effects of inoculation on timothy hay NDF as lag time tended to increase ($P < 0.10$) and 48 h disappearance tended to decrease ($P < 0.10$).

Results for degradation of ADF (Table 4) followed similar trends in most cases to those of NDF. Overall there were no beneficial effects of inoculation on ADF degradation, except for alfalfa hay, where lag time decreased ($P = 0.01$), *a* fraction decreased ($P = 0.01$), and *b* fraction increased ($P = 0.001$). Even with these responses, *ERD* of ADF and disappearance of ADF at 24 and 48 h were not affected ($P > 0.10$).

The 100-uNDF fraction was considerably greater than the *a*+*b* fraction for all forages both before and after the rumen transfer (Table 5). The transfer decreased mean *a*+*b* of NDF in barley straw ($P = 0.001$) and tended to decrease it for canola straw ($P = 0.10$), but increased mean *a*+*b* of alfalfa hay ($P < 0.001$), with no effect ($P > 0.10$) for timothy hay. The results for mean *a*+*b* of ADF mirrored those of NDF for each forage (data not reported). While it was not possible to statistically evaluate maximum, minimum, and SD numerically it appears that the decrease in mean *a*+*b* for barley straw and canola straw corresponded to a decrease in minimum *a*+*b* and an increase in the SD. For alfalfa hay where mean *a*+*b* increased, both maximum and minimum *a*+*b* numerically increased as well.

Over the experiment, changes in *a*+*b* due to inoculation were negatively correlated with change in k_d for all forages except barley straw (Figure 2). Thus, within forage, heifers with lower k_d had a relatively greater *a*+*b* compared to pre-inoculation values, except in the case of

barley straw where these two parameters were not related. The overall decrease in $a+b$ of barley straw NDF and ADF due to inoculation was the result of $a+b$ decreases in most heifers (Figure 3A). The overall decrease in k_d of barley straw NDF and ADF due to inoculation resulted from a decrease in 12 of the 16 heifers for NDF and 7 of the 16 heifers for ADF (Figure 3B). The overall increase in $a+b$ of NDF and ADF in alfalfa hay was due to an increase in almost all heifers (Figure 4A). The overall decrease in k_d of NDF and ADF of alfalfa hay due to inoculation was the result of a decrease in 12 heifers for NDF and 13 heifers for ADF (Figure 4B).

Within heifers, there was no consistency across forage substrates for changes in k_d, $a+b$, or *ERD* due to inoculation (Figure 5). Before inoculation the relative ranking of individual heifers for k_d (from fastest to slowest k_d; Table S.1) and $a+b$ (from largest to smallest; Table S.2) varied by forage. Overall, heifers 7, 5, 12 and 13 had relatively fast k_d for all forages, while heifers 3, 4, and 16 had greater $a+b$ for at least 3 of the 4 forages. Animal 14 ranked at the bottom for both variables. After inoculation ranking changed among heifers with 9, 5, and 11 having the fastest k_d and 13, 12 and 15 the slowest k_d and 12, 1, 15 with the largest $a+b$ while 11, 5, 6 had the smallest $a+b$ (Table S. 3; Table S. 4.).

2.4. DISCUSSION

The *in situ* technique has been used extensively in ruminants to evaluate the rumen degradability of forages. Substrates are incubated in several animals to account for variability in the rumen environment among individuals. In contrast to its typical use in feed evaluation, the *in situ* technique was used in the present study to investigate variability in rumen degradation of selected forages as caused by changes in the rumen microbiome. While useful for studying the effects of rumen inoculation on changes in ruminal fiber degradation, one limitation of the *in situ* technique is the pore size of the bags. The 50 μm pore size used in the study was selected to minimize washout of fine particles while allowing protozoa entry into the bags. However, cellulolytic protozoa that are larger than 50 μm (Coleman et al., 1976) would have been excluded from contributing to fiber degradation in the current study.

The study investigated if bison rumen inoculum could potentially complement the rumen microbial community of cattle fed a low quality diet of barley straw, possibly enhancing ruminal fiber digestion. Bison are known to have greater DMI and digestibility in cooler months

compared to cattle (Peden et al., 1974, Hudson and Frank, 1987) making them more adaptable to winter pastures than cattle (Bergmann, 2015). Bison rumen fluid has been found to increase 48 h *in vitro* digestibility of graminoids compared to cattle rumen fluid (Plumb and Dodd, 1993). This increase in digestibility by bison rumen fluid is possibly a reflection of the bison rumen microbiome having evolved to be more proficient at digesting lower quality forages as compared to cattle (Peden et al., 1974). The bison rumen also houses a greater proportion of the cellulolytic bacteria *Fiberbacter succinogens, Ruminococcus albus* and *R. flavefaciens*, compared with cattle (Varel and Dehority,1989) indicating that bison dietary niche may be more favourable for bacterial populations associated with ruminal fiber digestion.

Weimer et al. (2010) transferred rumen contents between two cattle that differed in their rumen microbiome, with both microbiomes reverting back to the original hosts within 14 d for one cow and 61 d for the other. However, after rumen transfer between two different cattle they found that some differences in microbiomes remained after 62 d, suggesting that not all components within the microbiome are static within the original host. Similarly, Jones and Megarrity (1986) rendered goats insensitive to 3, 4-dihyroxy pyridine by inoculating them with rumen contents from goats that were capable of degrading this compound. In our study inoculation of cattle with bison rumen contents failed to improve *ERD* in any of the four forages incubated. Had we measured *in situ* degradability of the various forages immediately after inoculation, we may have seen more differences in *ERD* and other degradability parameters. However, the intent of this study was to evaluate if inoculation with bison rumen contents conferred a lasting ability on cattle to exhibit improved forage digestion.

Extent and rate of degradation of fiber from barley straw, canola straw, and timothy hay were not improved, but there was an increase in the $a+b$ fraction of fiber from alfalfa hay. This result is in line with the findings of Richmond et al. (1977) that bison fed alfalfa hay had increased gain compared with cattle. Similarly, Pamungkas et al. (2006) reported that after transferring rumen contents from cattle into water buffalo the $a+b$ fraction of leguminous leucana decreased, but k_d increased.

Alfalfa hay had the lowest fiber content (47.9% NDF, 35.7% ADF) of the feeds incubated, but it had an uNDF similar to that of timothy hay (21.9 versus 20.1%, respectively). As expected the barley and canola straws were considerably more recalcitrant than the hays,

based on their uNDF fraction (67.6 and 56.8%, respectively). It is possible that differences in the cell wall chemistry between legumes (alfalfa hay) and graminaceous plants (grasses and cereals; Buxton and Russell, 1988; Jung, 1989; Wilson, 1993), led to the observed differences in degradation changes for alfalfa hay versus the other substrates. Lignin content and composition is different in legumes than grasses (Jung, 1989) with legumes having a larger core lignin content than grasses (Buxton and Russell, 1988).

It is impossible to determine from this experiment whether the bison microbiome effects on cattle forage degradability were due to differences in their respective diets or host specific symbiosis. It is possible that the decrease in $a+b$ of NDF and ADF and k_d of NDF in barley straw occurred because the cattle were previously adapted to a barley straw diet, while the bison were fed barley silage and oats. It has been well established that dietary adaptation causes shifts in the microbial composition in the rumen (Tajima et al., 2001; Fernando et al., 2010; Belanche et al., 2012). Because the bison had not consumed barley straw before inoculation, it is likely that the microbiome in their rumen was less able to digest barley straw than that of heifers.

In contrast to the lack of effect on barley straw, the increase in the b fraction of NDF and ADF and $a+b$ of NDF for alfalfa hay may indicate that inoculation of heifers with bison rumen contents improved the extent of alfalfa fiber degradation because the microbiome was not previously adapted to leguminous forage. The lack of an effect of inoculation on canola straw and timothy hay (feeds to which animals were also not adapted) may also suggest that bison rumen inoculum also exhibited higher digestive activity for legumes. Richmond et al. (1977) found that the weight gain in bison fed alfalfa was higher than cattle, a response that they attributed to increased efficiency of nitrogen use (Peden et al., 1974, Schaefer et al., 1978) along with a slower rate of passage of solid digesta (Schaefer et al., 1978).

The concomitant decrease in k_d and lag time with increase in the b and $a+b$ fractions of alfalfa hay NDF may indicate a potential trade-off in metabolic pathways of microbes involved in fiber degradation. Shabat et al. (2016) found greater feed efficiency, based on residual feed intake, in cattle with a less diverse rumen microbiome and they postulated that this was due to less complex metabolic pathways. However, their study did not measure the degradation kinetics of feeds with the rumen. It is possible that less complex metabolic pathways increase k_d due to more rapid access to nutrients, while more complex metabolic pathways have greater

potential for more extensive degradation, but at a slower rate. The negative correlations between change in k_d and change in $a+b$ of NDF observed for canola straw and timothy hay may be explained in a similar manner.

The increased lag time for barley straw NDF and canola straw NDF and ADF due to rumen transfer, may indicate that microbes from the bison donor required more time to initiate digestion of these recalcitrant substrates as the bison donors were not well adapted to these feeds. In contrast, the decreased lag time of ADF in alfalfa hay may indicate that the microbiome from the bison was more suited to the digestion of alfalfa, possibly because it was a less recalcitrant fiber source or because the heifers were adapted to a barley straw diet while the bison were not.

The uNDF represents a uniform feed fraction of NDF that is indigestible by rumen microbes, and thus is considered an inherent characteristic of the plant fiber (Van Amburgh et al., 2015). Theoretically, given adequate exposure time, rumen microorganisms can degrade all NDF except uNDF. In our study, there were considerable differences between the theoretical potentially degradable fraction of NDF (1-uNDF) and $a+b$ of each forage, demonstrating that fiber digestion in the rumen is limited by retention time.

Ranking of heifers based on k_d or $a+b$ showed that some individuals had an overall faster or slower k_d or larger or smaller $a+b$ for most or all of the forages examined. It is evident from the rankings that heifers varied in their ability to degrade fiber, even when fed the same diet. One notable example was animal 14, which had a consistently lower k_d and $a+b$ for all forages before transfer. While there was no overall change in *ERD* due to rumen transfers, many individuals exhibited substantial changes in *in situ* kinetics after rumen transfer with the direction and magnitude of these responses differing among forage types (Figure 5). It is worth noting that both positive and negative responses to inoculation occurred within an individual depending upon substrate, and there were some large changes in *ERD* within individual animals despite no overall mean effect of the transfer of *ERD*. Therefore, to understand the true impact of inoculation it might be worth exploring the change in rumen microbiome of individuals that had large positive responses to the inoculation for specific recalcitrant feedstuffs. For example, heifers 1, 10 and 11 had a large increase in *ERD* of canola straw, and detailed examination of the microbiome may shed light on the biological factors that led to this response. Such information

may provide valuable insight into how the rumen microbiome may be potentially manipulated to improve the digestibility of recalcitrant feeds in ruminants.

In conclusion, there was no benefit to inoculating cattle with bison rumen contents on the *ERD* of barley straw, canola straw, alfalfa hay, or timothy hay. Similarly, this practice caused no increase in the extent of degradation of barley straw, canola straw or timothy hay. There was, however, an increase in extent of degradation of alfalfa hay that occurred along with a decrease in the rate of degradation. It is not possible from the study design to ascertain whether the change in alfalfa degradation was due to differences in the rumen microbiomes of bison versus cattle or a reflection that cattle were adapted to a barley straw diet for a prolonged period prior to the start of the experiment.

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Tables

Table 2.1. Ingredient and chemical composition of diet

Item (g/kg)	% of DM
Ingredient	
Barley straw[1]	70.0
Concentrate[2]	30.0
Corn DDGS	20.0
Canola meal	8.0
Calcium carbonate	1.250
Urea	0.300
Dicalcium phosphate	0.247
Salt	0.150
Feedlot premix[3]	0.050
Vitamin E premix	0.003
Chemical composition	
DM, %	92.1
OM	92.0
CP	15.9
NDF	65.9
ADF	37.4

[1]Composition (mean ± SD; % DM basis): 92.0 ± 0.89 DM, 92.8 ± 1.59 OM, 6.3 ± 1.17 CP, 78.0 ± 3.89 NDF and 46.6 ± 3.46 ADF.
[2]Composition (mean ± SD; % DM basis): 92.4 ± 0.41 DM, 90.1 ± 0.36, 38.3 ± 0.37 CP, 37.6 ± 1.41 NDF and 16.0 ± 0.65 ADF.
[3]Supplied per kg of diet DM: 65 mg Zn, 28 mg Mn, 15 mg Cu, 0.7mg I, 0.2 mg Co, 0.3 mg Se, 6000 IU Vitamin A, 600 IU Vitamin D, 47 IU Vitamin E.

Table 2.2. Chemical composition of incubated substrates, pre-incubation.

Item (g/kg)	Substrates			
	Barley straw	Canola straw	Alfalfa hay	Timothy hay
DM[1]	899	945	917	948
OM	905	903	902	941
N	8	10	27	14
CP	49	60	169	85
NDF	782	743	479	690
ADF	481	573	357	389

[1]DM = dry matter; OM = organic matter; N = nitrogen; CP = crude protein; NDF = neutral detergent fiber; ADF = acid detergent fiber.

Table 2.3. *In situ* neutral detergent fiber disappearance parameters of barley straw, canola straw, alfalfa hay and timothy hay before and after inoculation with bison rumen contents.

		Inoculation			*P*-value
Substrate	Variable[1]	Before	After	SEM	Inoc[2]
Barley straw	k_d	0.0394	0.0364	0.0014	0.04
	lag	1.72	2.78	0.446	0.03
	a	0.906	1.33	0.195	0.05
	b	59.0	57.2	0.458	0.002
	ERD	38.7	37.5	0.884	0.18
	24 h	34.9	31.7	1.040	0.04
	48 h	50.0	47.9	0.386	<0.001
Canola straw	k_d	0.0305	0.0323	0.0021	0.39
	lag	2.19	3.66	0.548	0.01
	a	0.627	0.611	0.109	0.89
	b	36.8	35.7	0.605	0.09
	ERD	21.9	22.0	0.654	0.90
	24 h	18.3	17.6	0.661	0.30
	48 h	27.2	26.8	0.693	0.53
Alfalfa hay	k_d	0.0806	0.0625	0.0052	0.003
	lag	3.00	1.97	0.706	0.16
	a	3.97	4.00	0.443	0.94
	b	48.4	50.3	0.811	0.02
	ERD	40.7	40.1	0.778	0.42
	24 h	42.4	40.6	1.542	0.32
	48 h	49.5	50.2	0.387	0.11
Timothy hay	k_d	0.0376	0.0354	0.002	0.29
	lag	2.22	3.05	0.480	0.09
	a	4.00	4.37	0.326	0.27
	b	65.2	64.2	0.618	0.14
	ERD	45.0	44.4	1.056	0.60
	24 h	39.3	37.1	1.579	0.19
	48 h	56.3	54.8	0.776	0.06

[1]k_d = rate of degradation; a, washed out fraction; b, potentially degradable fraction; *ERD*, effective ruminal degradability; 24 h, actual NDF disappearance at 24 h; 48 h, actual NDF disappearance at 48 h.
[2]Inoc=Inoculation with bison rumen contents; no group or inoculation × group effects were observed ($P > 0.05$).

Table 2.4. *In situ* acid detergent fiber degradation barley straw, canola straw, alfalfa hay and timothy hay before and after inoculation with bison rumen contents.

		Inoculation			*P*-value
Substrate[1]	Variable[2]	Before	After	SEM	Inoc[3]
Barley straw	k_d	0.0390	0.0360	0.0020	0.21
	lag	1.52	2.21	0.377	0.09
	a	0.116	0.297	0.151	0.25
	b	58.0	56.1	0.591	0.007
	ERD	37.0	35.5	1.060	0.26
	24 h	33.3	30.1	1.131	0.05
	48 h	48.3	45.9	0.543	0.004
Canola straw	k_d	0.0342	0.0318	0.0032	0.46
	lag	2.79	4.69	0.760	0.02
	a	2.10	1.87	0.269	0.42
	b	34.8	35.1	1.213	0.85
	ERD	23.2	22.2	0.783	0.20
	24 h	19.3	17.9	0.957	0.16
	48 h	28.3	26.7	0.782	0.06
Alfalfa hay	k_d	0.0771	0.0598	0.0049	0.003
	lag	3.23	1.72	0.563	0.01
	a	3.63	2.98	0.305	0.04
	b	47.2	49.5	0.659	0.001
	ERD	39.2	38.3	0.850	0.29
	24 h	40.6	38.9	1.673	0.32
	48 h	47.9	48.4	0.453	0.29
Timothy hay	k_d	0.0351	0.0322	0.0017	0.09
	lag	2.29	3.22	0.802	0.26
	a	1.04	1.15	0.184	0.58
	b	66.7	65.7	0.541	0.08
	ERD	41.9	40.9	1.148	0.39
	24 h	35.4	31.9	1.813	0.07
	48 h	53.4	51.6	0.954	0.07

[2]*ERD*, effective ruminal degradability; k_d = rate of degradation; a, soluble and washed out fraction; b, potentially degradable fraction; 24 h, actual NDF disappearance at 24 h; 48 h, actual NDF disappearance at 48 h.

[3]Inoc=Inoculation with bison rumen contents; no group or inoculation × group effects were observed ($P > 0.05$).

Table 2.5. *In situ* NDF degradability summary statistics of barley straw, canola straw, alfalfa hay and timothy hay before and after inoculation with bison rumen contents.

Substrate	Variable[1]	Inoculation		*P*-value
		Before	After	
Barley straw	uNDF	32.4		
	Mean *a*+*b*	59.9	58.5	0.001
	Min *a*+*b*	58.7	56.3	
	Max *a*+*b*	61.2	61.1	
	SD	1.14	1.29	
Canola straw	uNDF	43.2		
	Mean *a*+*b*	37.4	36.3	0.09
	Min *a*+*b*	35.8	33.3	
	Max *a*+*b*	40.6	41.4	
	SD	1.31	2.2	
Alfalfa hay	uNDF	21.9		
	Mean *a*+*b*	52.2	54.1	<0.001
	Min *a*+*b*	51.0	52.1	
	Max *a*+*b*	54.5	56.1	
	SD	1.19	1.52	
Timothy hay	uNDF	20.1		
	Mean *a*+*b*	69.2	68.6	0.22
	Min *a*+*b*	66.9	66.0	
	Max *a*+*b*	74.4	71.2	
	SD	1.66	1.50	

[1]uNDF, undigestible NDF; *a*+*b*, potentially degradable fraction; Min, minimum; Max, maximum; NDF, neutral detergent fiber; SD, standard deviation

Figures

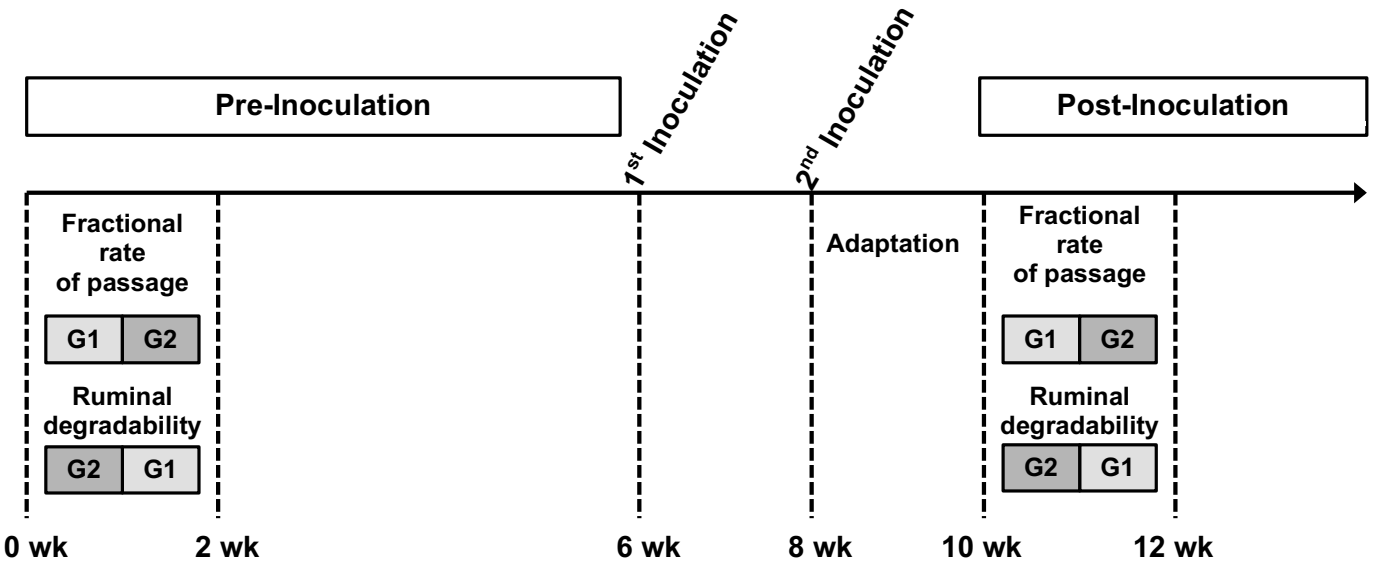

Figure 2.1. Experimental design. Each group contains 8 of 16 animals

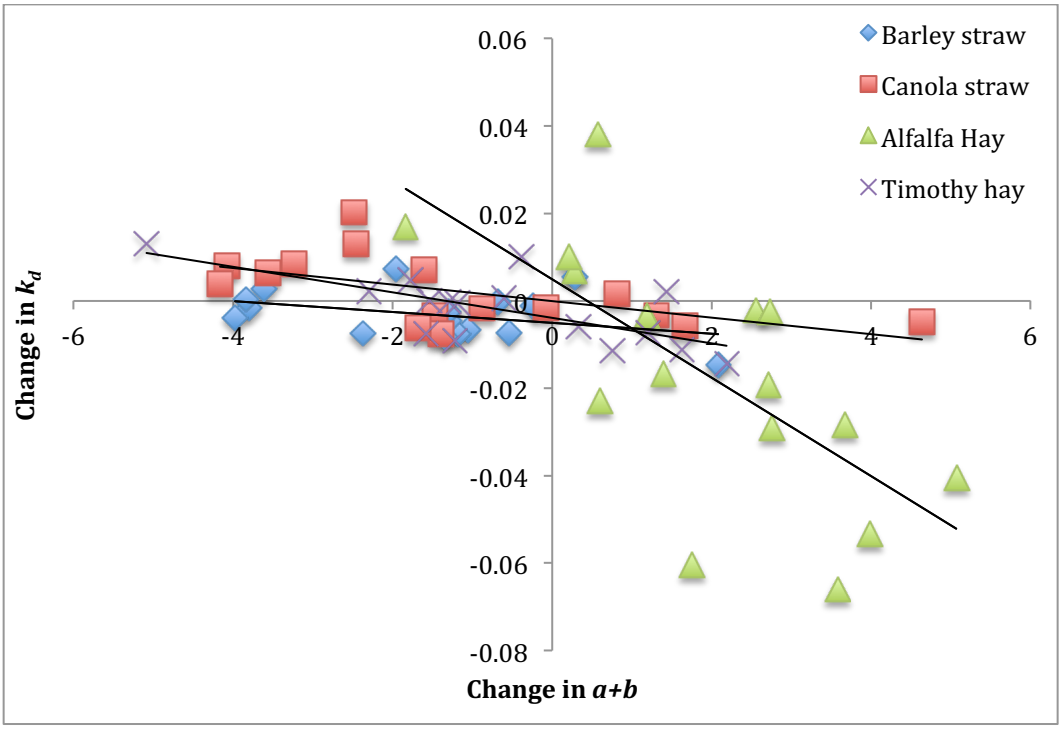

Figure 2.2. Regression analysis of change in rate of digestion (Δk_d) and change in potentially degradable fraction ($\Delta a+b$) of NDF for barley straw (y = -0.0012x - 0.005; $r^2 = 0.138$), canola straw (y = -0.0019x - 4E-05; $r^2 = 0.323$), alfalfa hay (y = -0.0113x + 0.0049; $r^2 = 0.469$), and timothy hay (y = -0.0029x - 0.0039; $r^2 = 0.467$).

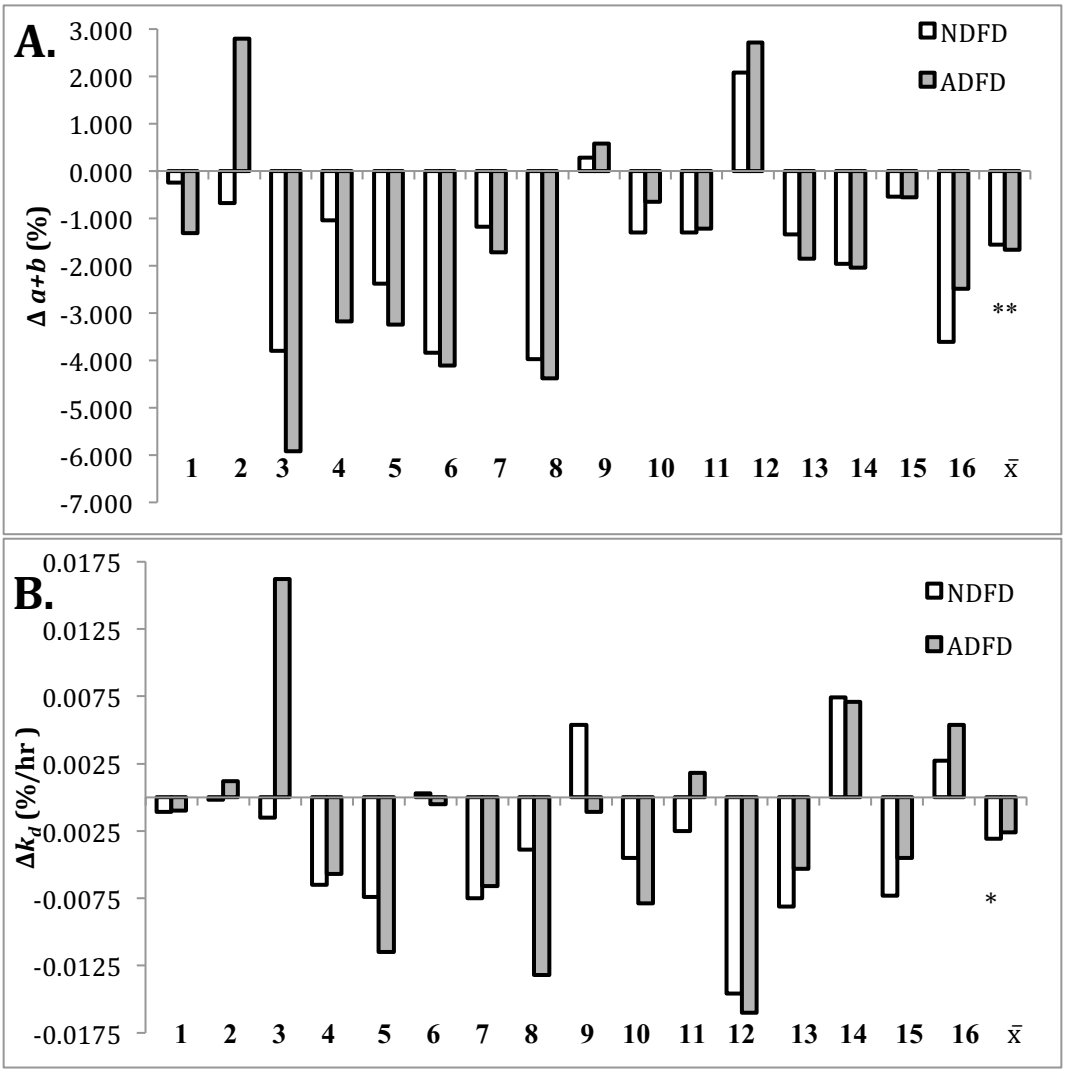

Figure 2.3. A) Change in potentially degradable fraction (a+b) of barley straw due to inoculation. * Denotes $P < 0.05$. **B**) Change in rate of digestion (k_d) of barley straw due to inoculation. * Denotes $P < 0.05$. Shown by animal 1 to 16 from left to right with mean values on the far right.

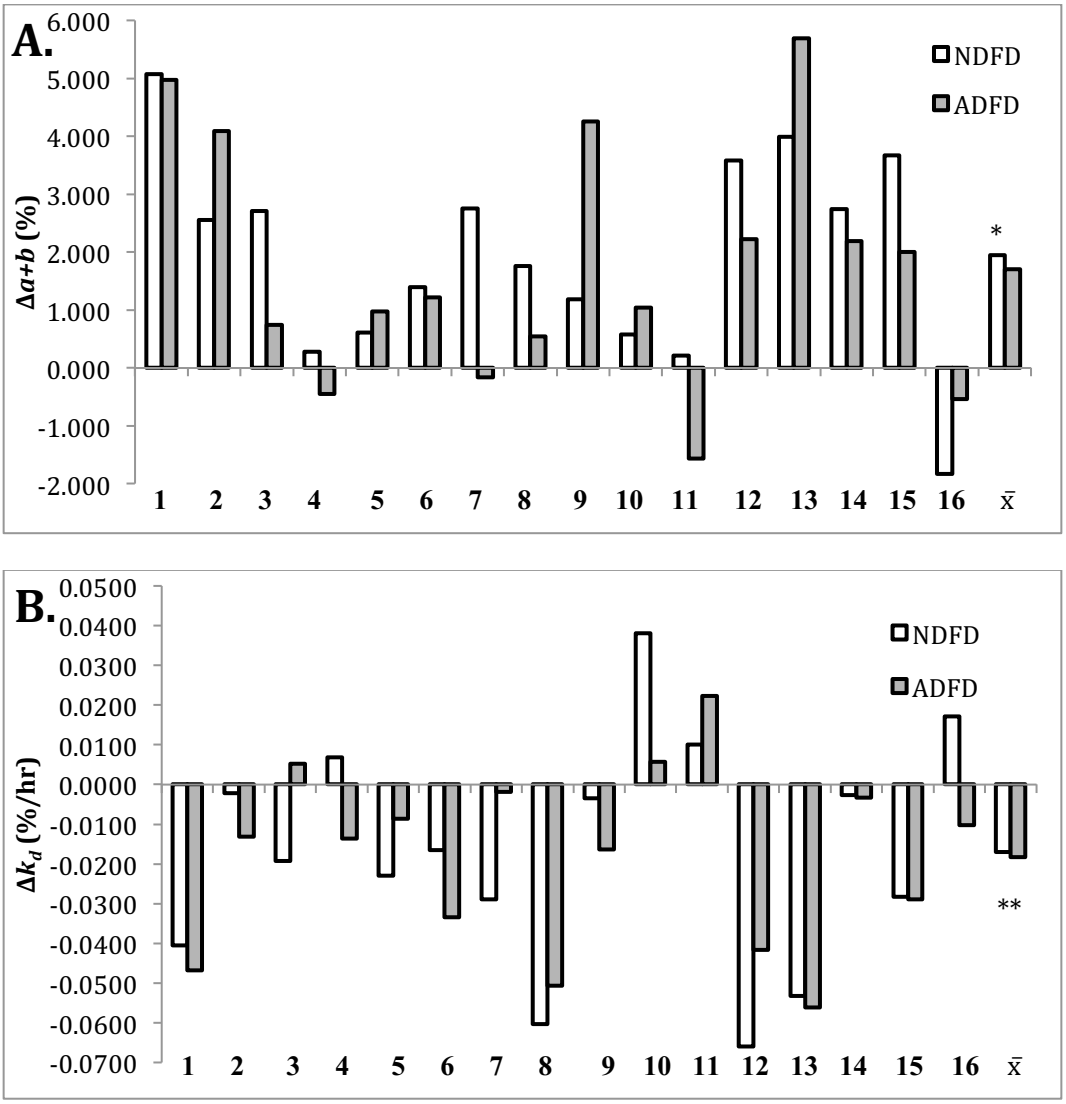

Figure 2.4. A) Change in potentially degradable fraction ($a+b$) of alfalfa hay due to inoculation. * Denotes $P < 0.05$ **B**) Change in rate of degradation (k_d) of alfalfa hay after ruminal inoculation with bison rumen contents. * Denotes $P < 0.05$. Shown by animal 1 to 16 from left to right with mean values on the far right.

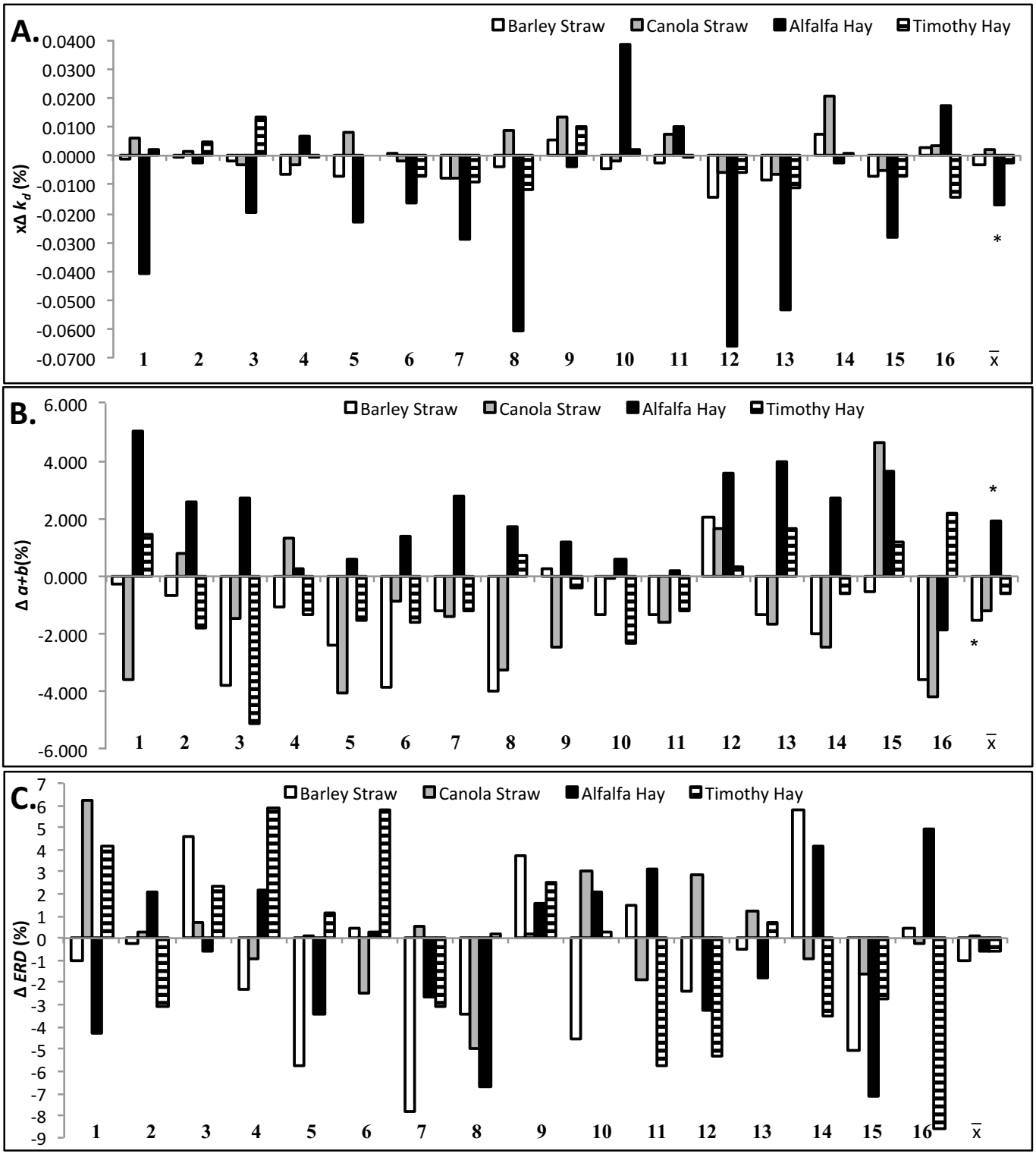

Figure 2.5. Change in **A**) rate of degradation (k_d), **B**) potentially degradable fraction (a+b), and C) effective ruminal degradability of NDF for barley straw, canola straw, alfalfa hay, timothy hay. Shown by animal 1 to 16 from left to right with mean values on the far right.

Supplemental tables

Table S.2.1. Pre-inoculation ranking of heifers based on *in situ* degradability values of k_d for all feeds.

Animal Number	Barley	Canola	Alfalfa	Timothy	Total
7	7	1	3	1	12
5	1	3	5	6	15
12	3	5	1	8	17
13	2	9	4	2	17
11	5	10	10	4	29
10	10	7	8	5	30
8	8	12	2	10	32
3	6	2	9	16	33
4	4	11	13	7	35
6	15	4	7	9	35
16	13	8	16	3	40
2	11	6	12	12	41
9	12	13	11	13	49
15	9	14	15	11	49
1	14	15	6	15	50
14	16	16	14	14	60

[1]This value denotes the rank of heifer for k_d of each individual forage. Number 1 indicates largest k_d, or fastest digester and 16 denotes smallest k_d or slowest digester for a particular feed.
[2]This number is the sum of the k_d ranking values for all forages.

Table S.2.2. Pre-inoculation ranking of heifers based on *in situ* ***p***otentially degradable fraction (*a*+*b*) for all feeds.

Animal Number	Barley	Canola	Alfalfa	Timothy	Total
4	4	6	2	4	16
16	2	2	1	15	20
3	7	11	3	1	22
1	9	1	11	5	26
8	5	5	12	6	28
9	15	7	4	2	28
5	6	8	6	10	30
12	14	9	7	3	33
2	13	13	5	8	39
10	8	4	14	14	40
11	10	10	8	13	41
15	12	12	10	7	41
7	1	15	15	11	42
13	11	3	13	16	43
6	3	16	16	12	47
14	16	14	9	9	48

[1]This value denotes the ranking of each heifer for *a*+*b* by individual forage. Number 1 indicates largest *a*+*b* and 16 denotes smallest *a*+*b* for a particular feed.

[2]This number is the sum of the *a*+*b* ranking values for all forages.

Table S.2.3. Post-inoculation ranking of heifers based on *in situ* degradability values of k_d for all feeds.

Animal Number	Barley	Canola	Alfalfa	Timothy	Total
9	1	3	6	1	11
5	2	1	5	5	13
11	6	4	2	3	15
2	7	8	8	4	27
3	4	5	11	7	27
10	13	11	1	2	27
14	3	2	10	12	27
4	8	14	4	6	32
16	5	7	9	15	36
7	14	13	3	8	38
6	12	9	7	13	41
1	11	10	14	11	46
8	9	6	15	16	46
13	10	15	13	10	48
12	16	12	12	9	49
15	15	16	16	14	61

[1]This value denotes the rank of heifer for k_d of each individual forage. Number 1 indicates largest k_d, or fastest digester and 16 denotes smallest k_d or slowest digester for a particular feed.
[2]This number is the sum of the k_d ranking values for all forages.

Table S.2.4. Post-inoculation ranking of heifers based on *in situ* ***p***otentially degradable fraction (*a*+*b*) for all feeds.

Animal Number	Barley	Canola	Alfalfa	Timothy	Total
12	1	2	3	4	10
1	4	6	1	1	12
15	7	1	4	3	15
3	3	11	2	6	22
4	5	3	10	9	27
7	2	8	9	11	30
2	8	5	6	12	31
9	6	12	8	5	31
13	11	7	5	8	31
16	13	9	11	7	40
8	16	14	12	2	44
10	9	4	16	16	45
14	15	15	7	10	47
11	10	10	14	14	48
5	12	16	13	13	54
6	14	13	15	15	57

[1]This value denotes the ranking of each heifer for *a*+*b* by individual forage. Number 1 indicates largest *a*+*b* and 16 denotes smallest *a*+*b* for a particular feed.

[2]This number is the sum of the *a*+*b* ranking values for all forages.

Chapter 3: Fermentation of Ammonia Fiber Expansion Treated and Untreated Barley Straw in a Rumen Simulation Technique using Rumen Inoculum from Cattle with Slow Versus Fast Rate of Fiber Disappearance[1]

3.1. Introduction

Variation among beef cattle in residual feed intake (Koch et al., 1963; Herd et al., 2004), feed efficiency, feeding behaviour, metabolic rate and methane production (Nkrumah et al., 2006) has been well documented and thus it is logical to infer that variability in rumen fermentation occurs as well. It has recently been established that around the world, the rumen of cattle has the same core microbiome at the genus level (Jami and Mizrahi, 2012; Henderson et al., 2015), with abundance and types of microbial species varying among individual animals. Although there is variation among microbial species, there seem to be overall functional similarities of rumen microbial communities (Galbraith et al, 2004; Jami and Mizrahi, 2012). Weimer et al. (2010) reported that when >95% rumen contents were transferred between two cattle fed the same diet, with differing host-specific microbial populations, the populations reverted back to those possessed by the original host within 14 and 61 days. This suggests the existence of a hologenome, where interactions between host and microbial genetic components result in the establishment of a unique microbiota that helps regulate host physiological responses (Rosenberg et al., 2010). For example, Jami et al. (2014) reported that increased milk fat production in dairy cows was strongly correlated to an increase in the ratio of *Firmicutes* to *Bacteroidetes* in rumen contents. A decrease in *Bacteroidetes* relative to *Firmicutes* has been found in obese mice and is connected to an increase in blood and tissue fat (Turnbaugh et al., 2006). Similarly, greater feed efficiency has been reported in cattle with a less diverse rumen microbiome due to less complex metabolic pathways (Shabat et al., 2016). Therefore exploration of the differences between cattle due to their inherent gut microbiomes and the potential differences in digestive capacity is of interest. There is a paucity of information that links individual variation in digestion efficiency and the rumen microbiome. Optimizing the ruminal microbiome of individual animals to

[1] This chapter has been published as Griffith CL, Ribeiro GO Jr, Oba M, McAllister TA and Beauchemin KA (2016) Fermentation of Ammonia Fiber Expansion Treated and Untreated Barley Straw in a Rumen Simulation Technique Using Rumen Inoculum from Cattle with Slow versus Fast Rate of Fiber Disappearance. *Front. Microbiol.* 7:1839. doi: 10.3389/fmicb.2016.01839.

improve digestive function could improve fibre digestion in the rumen and decrease cost of animal production.

Another potential avenue for mitigating feed costs is the use of less costly agricultural residues as ruminant feed sources. Straw is one such abundant byproduct, but its total digestible nutrient (TDN) content is low (40 to 46% of dry matter [DM]; Kopp, 2003), limiting its use in ruminant diets. To this end, much research has examined the possible use of alkali pre-treatments such as ammoniation as a means of enhancing the digestibility of NDF in the rumen (Hendriks and Zeeman, 2009; Alvira, 2010; Talebnia et al., 2010; Abdel-Aziz, 2015). Ammoniation of straw has been shown to disrupt hemicellulose-lignin bonds and cellulose crystallinity to allow enzymes access and increase hydrolysis of hemicellulose and cellulose. However, traditional ammoniation methods pose potential health hazards and a large portion of the ammonia is volatilized (Freney, 1983; Rasby et al, 1989). Efficiency of ammoniation treatment has been improved with the advent of ammonia freeze explosion (Dale and Moreira, 1982), later termed Ammonia Fiber Expansion (AFEX™). AFEX uses moisture and high pressure during ammonia treatment, with a subsequent pressure release and ammonia removal (Campbell et al., 2013). Bals et al. (2010) found that AFEX was far more effective as it increased digestion of late harvest switchgrass by 206% as compared to a 56% increase with traditional ammoniation methods. Using AFEX and untreated barley straw in the study allowed us to examine the effect inoculum source on digestion of easily accessible fiber source and a more complex fiber source, while using the same feed source.

The *in situ* method (Orskov and McDonald, 1979) is widely used to characterize fiber digestion in the rumen. As this method involves measuring fiber digestion at different time points, it is possible to estimate the rate of fiber digestion in the rumen. Rates of fiber degradation vary among animals and may be influenced by a number of host factors such as rate of passage, rumen capacity, and saliva production. Therefore the rumen simulation technique (Rusitec; Czerkawsk and Breckenridge, 1977) is well suited to determine if differences in rate of digestion are related to differences in microbial populations, the rumen simulation technique (Rusitec; Czerkawsk and Breckenridge, 1977) is well suited. The Rusitec affords strict control of saliva infusion, amount of feed, time of feeding, and temperature, while allowing for measurement of rumen fermentation end products, such as methane (CH_4), volatile fatty acids (VFA), microbial populations, and pH. Controlling for physiological components such as saliva

production and rate of passage allows for a focused investigation of differences in microbial populations (i.e. inoculum sources) while allowing multiple runs simultaneously, simulating multiple cows with the same inoculum.

The objective of this study was to use the Rusitec system to determine whether AFEX treatment improves the ruminal digestibility of barley straw, and whether the extent of this improvement varies among heifers with fast or slow rate of degradation of untreated straw neutral detergent fiber (NDF). It was hypothesized that AFEX treatment would increase digestibility of barley straw and that inoculum from heifers with fast rate of degradation would degrade both straws more completely in a 48 h time period than those with a slow rate of degradation.

3.2. Materials and methods

The experiment was conducted at Agriculture and Agri-Food Canada in Lethbridge, Alberta. The experiment was approved by the Lethbridge Research and Development Centre Animal Care Committee and cattle were cared for following the guidelines of the Canadian Council on Animal Care (CCAC, 2009).

3.2.1 Experimental design and treatments

Two Rusitec apparatuses, each equipped with 8 fermenters, were used (n = 4 fermenters per treatment) and the experiment was conducted over a period of 15 days with 8 days of adaptation and 7 days of sample collection. The experiment was a completely randomized block design with a 2 × 2 factorial arrangement of treatments; two sources of inoculum (slow or fast rate of NDF disappearance) and two substrates (untreated or AFEX treated barley straw diet). Inoculum from heifers with slow and fast rate of NDF disappearance was obtained by pooling rumen inoculum from 2 heifers each chosen based on their rate of NDF disappearance (k_d) of barley straw as measured *in situ*.

Inoculum donors were preselected by incubating untreated, ground (2-mm) barley straw in duplicate in the rumen of 16 cannulated Angus × Hereford beef heifers fed 700 g/kg untreated barley straw and 300 g/kg concentrate (dry matter [DM] basis) consisting of 600 g/kg dried distillers grains (DDGS), 223 g/kg canola meal, 167 g/kg supplement and 10 g/kg urea. Barley straw was incubated in the rumen of each heifer for 0, 4, 8, 12, 24, 48, 96, and 120 h during a single incubation period. Bags used for incubation were 10 × 20 cm Ankom bags (R1020,

ANKOM Technology, Macedon, NY, USA, 50 micron porosity) with 6.0 g (± 0.05g) of feed per bag. Ten minutes prior to insertion into the rumen bags were submerged in 39 ° C water. Bags were inserted into the rumen 1 h after feeding, and removed after the appropriate amount of time. Duplicate Ankom bags were placed inside larger mesh bags (30×30 cm) which were placed into the rumen through the cannula and fully submerged. No microbial contamination correction was performed, as this contamination was assumed to be similar between heifers. Disappearance of NDF was calculated for each time point for each heifer and the rate of NDF disappearance in percent per hour (k_d) was estimated by fitting the data to the following model (McDonald, 1981):

$$P = a + b\,(1 - e^{-kd(t-L)}),$$

where P is extent of degradation at time t, a is the soluble or washout fraction, b is the potentially digestible fraction, and L is the lag time. Lag time measurements are subject to error, and retention time varies by animals, therefore, k_d was chosen as the variable for animal selection. Heifers were then ranked from slow to fast based on k_d and the two animals with the fastest and the two with the slowest rates of disappearance were chosen for this study (Fast > 4.18% h^{-1} vs. Slow < 3.17% h^{-1}; Table 1).

AFEX treatment was performed by Michigan Biotechnology Institute (Lansing, Michigan, USA) using a pair of packed bed AFEX reactors as described by Campbell et al. (2013). Briefly, barley straw was ground through a 30.5 mm screen and packed into stainless steel baskets at a density of 100 kg/m^2. Baskets were then inserted into a reactor tube where they were pre-steamed in order to displace air and raise the temperature to between 80-85 °C. Vapor ammonia was applied at a rate of 80-100 g/min to a level of 1 kg ammonia per kilogram dry straw and a maximum pressure of 200 psi and left for 30 min to soak. Pressure was released, and residual ammonia was stripped by steam stripping and vaporized at atmospheric pressure before being re-pressurized and charged to the next reactor by an ammonia compressor.

3.2.2 Substrate processing

Substrates (untreated and AFEX barley straw) were ground through a 4-mm screen using a Wiley mill (standard model 4; Arthur H. Thomas Co., Philadelphia, PA, USA) and particle size distribution was assessed by sieving 50 g of feed for 5 min on a Ro Tap particle separator (model RX-29; W.S Tyler, Mentor, OH, USA) equipped with four screens (1,180 μm, 850 μm, 600 μm, 300 μm) and a bottom pan. Because AFEX straw had a greater percentage of smaller particles as

it shattered more than untreated straw, the untreated straw was further ground through a 2-mm screen. To ensure that both substrates had the same particle size distribution each substrate was reconstituted from the sieved fractions to have the following particle size distribution: 100 g/kg > 1,180 μm; 200 g/kg < 1,180 μm and > 850 μm; 350 g/kg < 850 μm and > 600 μm; and 350 g/kg < 600 μm and > 300 μm. The fines (< 300 μm) were removed from both substrates to prevent wash out from the bags in fermenters. The same concentrate that was fed to the heifers was ground through a 2-mm screen. Samples were mixed thoroughly and weighed separately into bags with a pore size of 50 μm. Bags used for concentrate were 5 × 10 cm (R510, ANKOM Technology, Macedon, NY, USA); bags used for straw were 10 × 20 cm (R1020, ANKOM Technology, Macedon, NY, USA).

3.2.3 Rumen simulation technique

Inoculum was collected one month after k_d was measured. Animals were maintained on the same diet of 700 g/kg barley straw and 300 g/kg pelleted concentrate (DM basis) in the interim. Inoculum was obtained from the four selected ruminally cannulated beef heifers 2 h after feeding. Rumen fluid and solid contents were pooled for the 2 heifers with fast, and for the 2 with slow rates of NDF disappearance. Rumen fluid was filtered through four layers of cheesecloth into insulated thermoses and transported to the laboratory.

Treatments were randomly assigned to 900-mL fermenters so that both Rusitec systems had two replicates per treatment with four replicates per treatment overall. Each fermenter had a buffer input and effluent output port. Fermenters were maintained at 39°C by immersion in a water bath. Each fermenter was filled with 180 mL pre-warmed artificial saliva (pH = 8.2, McDougall, 1948) modified to contain 0.3 g/l of $(NH_4)_2SO_4$, and 720 mL of strained rumen fluid. Three labeled bags were placed in each fermenter, one containing 10 g of solid rumen digesta, one containing 7 g of barley straw (AFEX or untreated) and one containing 3 g of concentrate. The relative amounts of straw and concentrate were similar to that in the diets fed to the donor heifers. After 24 h, the bag containing rumen digesta was removed and replaced by two bags, one containing 7 g barley straw, and the other containing 3 g concentrate. Thereafter one bag containing concentrate and one bag containing straw were replaced at the same time daily so that each bag remained in the fermenter for 48 h. Bags were exchanged under a stream of O_2-free CO_2. The artificial saliva was continuously infused into the fermenters at a rate of 2.9%/hour (replacing 70% of the fermenter volume each day). Effluent was collected in a 1 L

flask, and gas was collected in a 2 L bag (Curity®; Conviden Ltd, Mansfield, MA) attached to the effluent flask. Every day at the time of feed bag exchange, rumen fluid pH, total gas production and effluent volume were measured.

3.2.4 Dry matter and organic matter disappearance

Dry matter disappearance (DMD) and organic matter (OM) disappearance (OMD) at 48 h was determined on days 9 to 11 and 13 to 15. Feed bags were removed from each fermenter, washed in cold running water until the water was clear, and dried at 55°C for 48 h. To ensure sufficient sample for chemical analysis, concentrate samples were pooled in groups of 3 days by fermenter for days 9 to 11 and 13 to 15. Forage and pooled concentrate samples were ground through a 1-mm screen prior to chemical analysis.

3.2.5 Fermentation metabolites

Just prior to feed bag exchange, total gas production from each fermenter was measured daily on days 9 to 15 using a gas meter (Model DM3A, Alexander-Wright, London, UK). Gas samples (20-mL) were collected from the septum of the collection bags using a 26-gauge needle and transferred to 6.8-mL evacuated exetainers (Labco Ltd, Wycombe, Bucks, UK). Samples were stored at room temperature until the end of the experiment when they were analyzed for CH_4.

At the time of feed bag exchange, 2.5-mL subsamples of liquid were collected for VFA and NH_3N analysis from fermenters on days 11 to 14. Samples were placed in 5-mL scintillation vials containing 0.5 mL of 25% (w/w) metaphosphoric acid and immediately frozen at -20°C until VFA analysis. For NH_3N analysis, subsamples were placed in scintillation vials containing 0.5 mL of 1% sulfuric acid for NH_3N, and then frozen at -20°C until analysis. The concentrations of VFA and NH_3N (mmol/L) were multiplied by the outflow rate of fluid infused to the vessels (L/day) to determine VFA and NH_3N production (mmol/d)

3.2.6 Microbial protein synthesis

From day 7 until the end of the experiment, the McDougall's buffer was modified by replacing $(NH_4)_2SO_4$ with 0.3 g/L ^{15}N-enriched $(NH_4)_2SO_4$ (Sigma Chemical Co., St Louis, MO; minimum ^{15}N enrichment 10.01 atom%; Pilgrim et al., 1970). On days 13 to 15, the 24 h accumulation of effluent in each flask was preserved with 20% (wt/vol) sodium azide (3 mL) and 40 mL of effluent was subsampled for isolation of bacteria associated with the liquid fraction.

The 48-h bag residues were processed to obtain feed particle associated (FPA) and feed particle bound (FPB) bacterial fractions. Bags were removed from the fermenter, gently squeezed and then placed into a plastic bag with 20 mL of McDougall's buffer and processed for 60 s at 230 rpm in a Stomacher 400 laboratory paddle blender (Seward Medical Ltd., London, UK). Processed liquid was gently squeezed out, decanted and retained in a 50 mL falcon tube. Bags were washed twice more with 10 mL of buffer in each wash and each time the buffer was excised, pooled and retained to estimate the FPA bacterial fraction. Washed solid feed residues were considered to represent the FPB bacterial fraction

The effluent liquid samples were then processed by centrifuging at 20,000 × g for 30 min at 4°C and the resulting pellet was centrifuged three times at 20,000 × g for 30 min at 4°C after washing with McDougall's buffer. Pellet was re-suspended in distilled water, frozen at -20°C, lyophilized and ball-ground for N and ^{15}N analysis. The FPA bacterial samples collected after stomaching were centrifuged (500 × g, 10 min, 4°C), with the resulting supernatant subsequently centrifuged (20 000 × g, 30 min, 4°C). The resulting pellet was washed three times as described for effluent pellets. The pellet was re-suspended in distilled water, frozen at -20°C, lyophilized and ball-ground for N and ^{15}N analysis and 16S rRNA quantification. The washed, solid feed residues, containing the FPB bacterial fraction were dried at 55°C for 48 h, weighed for DM determination and then ground and analyzed for N and ^{15}N concentrations.

3.2.7 Protozoa

Protozoa counts were determined in the fermenters on days 9, 12 and 15. Rumen fluid from each fermenter was collected by gently squeezing the 48-h forage and concentrate bags. The fluid from the forage and concentrate bags were pooled by fermenter and 5.0 mL of the rumen fluid was preserved in 5.0 mL of methyl green formalin-saline solution (Ogimoto and Imai, 1981). Protozoa samples were stored in the dark at room temperature until counted. Protozoa were enumerated by light microscopy using a Levy-Hausser hemacytometer (Hausser Scientific, Horsham, PA). Each sample was counted twice and if the duplicates differed by more than 10%, counts were repeated. Protozoa genera were not characterized as protozoa numbers were very low in the Rusitec making it difficult to accurately evaluate protozoa populations.

3.2.8 DNA extraction and 16S rRNA copy quantification

DNA was extracted from all ground FPA samples using a Qiagen QIAmp Stool Mini kit (Qiagen Inc., Valencia, CA, USA), slightly modified to improve DNA extraction from Gram-positive bacteria. Briefly, 30 mg of sample was added to 1.4 mL Buffer ASL, stool lysis buffer, and vortexed until thoroughly homogenized (approx. 1 min). The solution was then pipetted into a new tube containing sterile zirconia beads (0.3 g, 0.1 mm; 0.1 g, 0.5 mm) and homogenized for 3 min at 30/s on a Qiagen Tissue Lyser II (Yu and Morrison, 2004). Samples were then mixed at 700 rpm while heated at 95° C for 5 min. Samples were vortexed briefly and centrifuged at 13,200 rpm for 1 min. The supernatant was separated, added to an inhibitEX tablet and the Qiagen Stool Mini Kit protocol was followed. Total DNA was quantified using PicoGreen with a NanoDrop 3300 fluorometer, normalized to 20 ng/uL, and run on a gel to check for quality.

Using previously described primers and annealing temperatures, qPCR was performed to determine the relative abundance of the following fibrolytic bacteria: *Ruminococcus albus* (Wang et al, 1997)*, Fibrobacter succinogens, Ruminococcus flavefaciens, Selenomonas ruminantium, Prevotella bryantii* (Tajima et al., 2001), and total bacterial 16S rRNA (Oss et al., 2016).

3.2.9 Sample analysis

Samples of feed and feed fermentation residues were analyzed for analytical DM by drying 1.0 g (+/- 0.05 g) of each sample for 2 h at 135° C using a forced air oven. Samples were ashed at 550° C for 5 h to estimate OM. Neutral detergent fiber inclusive of ash (NDF) and acid detergent fiber (ADF) were analyzed by the sequential method with the ANKOM200 Fiber Analyzer using reagents as described by Van Soest et al. (1991). Sodium sulphite and α-amylase were used during NDF determination. Total N concentration and atom per cent excess (APE) of ^{15}N was determined using a mass spectrometer (Ribeiro et al., 2015). Concentration of CH_4 in the gas samples was determined using a Varian gas chromatograph equipped with a GS-Carbon-PLOT 30 m × 0.32 mm × 3 μm column and thermal conductivity detector (Agilent Technologies Canada, Inc. Mississauga, ON, Canada). The oven temperature was set at 35°C with an injector temperature of 185°C (1:30 split, 250 μL injector volume) a detector temperature of 150°C and helium (27 cm/s) as the carrier gas.). Ammonia was analyzed using the modified Berthelot

method as described by Rhine et al. (1998) and VFA were analyzed by gas chromatography as described by Wang et al. (2001).

3.2.10 Calculations and statistical analysis

True dry matter disappearance was determined as DMD adjusted for microbial DM: initial sample weight – (final sample weight - microbial DM)/initial sample weight.

Total effluent microbial N (MN) production (mg/day) was calculated using the N concentration (%) determined for the microbial pellet, multiplied by the microbial weight in the total effluent (mg/day). Microbial weight in the total effluent was calculated by multiplying daily effluent production (mL) by the microbial density (mg/mL) in the 40 mL subsample. Microbial N production from feed particle associated (FPA) fraction was calculated by multiplying the N concentration (%) in the FPA microbial pellet by the microbial weight of the FPA fraction (mg/day). Feed particle bound (FPB) MN production (mg/day) from straw and concentrate fractions were calculated using the following equation:

$$MN = \frac{APE\ in\ RN}{APE\ in\ MN} \times RN$$

where APE in RN = the percent excess of ^{15}N in the fraction analyzed, and APE in FPA microbial pellet was used as the source of APE in MN. Total MN production (mg/day) was calculated as the sum of microbial production in the effluent, FPA, FPB of straw residues and FPB of concentrate residues.

Totals presented in Table 3 were calculated as [(concentrate + straw before incubation) – (concentrate + straw after incubation)]/(concentrate +straw before incubation).

Relative bacterial populations were calculated as (total copy number of species in a given fermenter on a given day/total bacterial copy number in the same fermenter on the same day) × 100.

All data were analyzed using the MIXED procedure of SAS (SAS Inc., Cary, NC, USA). Individual fermenter was considered the experimental unit with day of sampling treated as a repeated measure. Straw, inoculum, straw × inoculum were considered fixed effects while apparatus was considered a random effect. For each parameter analyzed a covariance structure among compound symmetry, heterogeneous compound symmetry, autoregressive, heterogeneous

autoregressive, Toeplitz, unstructured and banded was chosen based on the lowest corrected Akaike information critical values. Significance was declared at $P < 0.05$ and a trend was considered at $0.05 \leq P \leq 0.10$. Differences among treatments were determined using Fisher's protected ($P < 0.05$) LSD test using the PDIFF option in SAS for straw × inoculum interactions.

3.3. Results

3.3.1 Disappearance and fermentation characteristics

AFEX treated straw had greater DMD, OMD, TDMD, aNDFD and ADFD ($P < 0.001$) than untreated straw (Table 3). The straw × inoculum interactions ($P < 0.05$) for these variables indicate that Fast inoculum increased ($P < 0.05$) disappearance of AFEX straw, but had no effect on untreated straw. The NDFD and ADFD of concentrate was lowered with Fast inoculum with untreated straw ($P < 0.05$), but was not affected by the other treatments.

The N disappearance was greater ($P < 0.001$) for AFEX straw than for untreated straw (Table 3). N disappearance of untreated straw increased with Slow inoculum (P=0.008), but inoculum source had no effect on N disappearance from AFEX. Microbial N production was greater for Fast inoculum in the effluent and overall ($P < 0.05$).

Untreated straw produced more CH_4 per gram of DMD then did AFEX straw ($P = 0.046$; Table 4). No other CH_4 variable was affected by straw or inoculum source. AFEX straw decreased pH compared to untreated straw ($P < 0.001$). AFEX straw and Slow inoculum promoted greater NH_3N production than untreated straw ($P < 0.001$) and Fast inoculum ($P = 0.015$), with no interaction between the two. AFEX straw resulted in more total VFA production than untreated straw ($P < 0.001$), and the straw × inoculum interaction indicated that more VFA was produced with AFEX straw incubated with Fast inoculum ($P = 0.035$) whereas Fast inoculum had no effect on VFA from untreated straw. Interactions were also observed for the proportions of acetate, butyrate and caproate ($P < 0.05$). Fast inoculum decreased the proportion of acetate for untreated straw ($P < 0.001$) and caproate for AFEX straw ($P < 0.001$). Fast inoculum increased the molar proportion of butyrate for untreated straw, yet it decreased it for AFEX straw ($P < 0.001$), although proportions were greater for untreated than AFEX straw. AFEX increased ($P < 0.001$) the molar proportion of propionate, but reduced ($P < 0.001$) that of valerate, isobutyrate, and isovalerate. AFEX also reduced the A:P ratio ($P < 0.001$), with the

effect of inoculum dependent on straw type; Fast inoculum reduced ($P = 0.029$) A:P ratio for untreated, but not AFEX straw.

3.3.2 Microbial populations

AFEX had no effect on total protozoa counts, nor was there an effect ($P > 0.10$) on bacterial populations (Table 5). Copy numbers of *R. albus* were increased ($P = 0.035$) for Fast inoculum. Total bacterial 16S rRNA after adaptation tended to be greater ($P = 0.10$) for AFEX straw, with Fast inoculum increasing copies for AFEX but not untreated straw (interaction, $P = 0.013$).

3.4. Discussion

Ammoniation is known to increase DMD and N content of various straws including wheat (Horton, 1981; Herrera-Seldana et al., 1982; Givens et al., 1988; Kondo, et al., 1992), oat (Horton, 1981; Givens et al., 1988), and barley (Horton, 1981; Hadjipanayiotou, 1982; Dryden and Kempton, 1983; Givens et al., 1988). Compared to traditional ammoniation treatments, AFEX is an advanced ammoniation technology that has been shown to result in an even greater increase in the digestibility of crop residues, by cleaving the hemicellulose-lignin ester linkages, or lignin carbohydrate complexes more efficiently (Chundawat et al., 2010). Ammoniation treatment disrupts the crystalline structure of cellulose I converting it to cellulose III (Mitall, 2011), which allows for much faster hydrolysation of ß1-4 glycosidic bonds by microbial enzymes (Fan, 1980; Igarashi, 2007; Hall, 2010). Dale et al. (1997) found even low levels of enzymes digested AFEX to near theoretical yields. Thus, the 26% greater DMD and 21% greater NDFD of AFEX compared with untreated barley straw observed in the present study is consistent with the previous literature, and highlights the potential of AFEX technology to improve nutritive value of straw for feed. While AFEX may be impractical to implement on farms, Campbell et al. (2013) are working on developing this technology for regional depots, which would greatly increase access to this technology.

Observed differences between Fast and Slow inoculums may be attributed to differences in microbial populations within the inoculum, as the Rusitec system removes variation in physiological factors such as saliva production, rumen fill, rumination time, rate of passage and rate of absorption, that contribute to individual variability in fiber digestion observed in vivo. The increase in DMD, TDMD, and ADFD of AFEX straw when incubated with Fast inoculum in the Rusitec, with no effect on disappearance of untreated straw, indicates that heifers selected

based on faster rate of NDF digestion of untreated barley straw were able to more thoroughly digest AFEX straw in 48 hours compared to animals selected for slower rate of digestion. Rumen inoculum selected on the basis of a faster rate of NDF disappearance would likely contain greater populations and activity of microorganisms that degrade cell wall, which is consistent with the observation that *R. albus* was more abundant in Fast rumen inoculum than in Slow rumen inoculum. *R. albus* has long been known to be one of the most cellulolytic organisms in the rumen (Graham, 1985). The lack of effect of rumen fluid on the populations of the other four bacteria measured may simply indicate that they were not responsible for the differences in digestibility observed. There are many fibrolytic bacteria such as *Butyrivibrio fibrosolvens*, *Clostridium longisporum*, *Clostridium lochheadii*, *Eubacterium cellulosolvens, and Prevotella spp* (Stewart *et al*., 1997) that were not characterised in this study. This study did not look at interactions among bacteria, for example *Prevotella spp*. are known to be very effective at digesting hemicellulose in alfalfa, as well as contributing to increased digestion of cellulose when cultured with other cellulolytic bacteria (Dehority, 1967). There are also many, as of yet unculturable bacteria, that may also contribute to differences in ruminal degradation. Pooling the rumen fluid from two animals may have also eliminated some of the differences between individual Fast and Slow inoculum in relative population size of the selected bacteria due to potential antagonistic differences between bacteria from each donor animal. In addition, differences in methanogens, fungi, and protozoa species that may have contributed to differences in digestion observed were not assessed in the current study.

The increase in N disappearance of barley straw seen with AFEX was likely due to increased accessibility of cell wall contents due to enhanced NDFD (Graham and Aman, 1984). Ammoniated straw also contained more N than untreated barley straw because N from the ammoniation treatment is sequestered by the forage during treatment. While this excess is reduced by the ammonia recovery step in AFEX treatment (Chundawat et al., 2013), some of the N remains bound to the substrate accounting for the greater initial N content of AFEX compared with untreated straw (99 versus 43 g/kg DM). This agrees with the findings of Bals et al. (2010) who found increased N compared to untreated substrate for corn stover and switchgrass, but reduced N compared to traditional ammoniation. The increase in N available for use in the rumen, along with the increase in degradability of AFEX straw, make it appealing as a potential feedstuff for cattle.

The reduction in CH_4 when expressed relative to digested DM for AFEX compared with untreated straw was likely due to greater propionate and decreased molar proportions of butyrate, and a lower acetate to propionate ratio. Propionate acts as an alternative hydrogen sink in the rumen diverting hydrogen away from CH_4 synthesis while the production of butyrate and acetate promote methanogenesis (Moss, 2000). The increase in VFA production caused by the AFEX treatment was likely responsible for the slight, but significantly lower pH measured in those fermenters compared with those fed untreated straw.

This study focuses on differences in rumen inoculum, but it is well known that other characteristics of individual animals, such as rumination time, saliva production, rumen fill, rate of passage, and rate of absorption can impact their ability to digest forage. As we continue to demystify the interactions between host animals and their microbiome, improving the ability of individual animals to digest forages will become more tenable.

3.5. Conclusions

AFEX was found to increase digestibility of barley straw DM by more than 30%. As AFEX technology becomes more widely available, it has potential to increase the use of straw as feed. Further to this, research is ongoing on microbiome contributions to variations in metabolic efficiency among animals (eg. Hernandez-Sanabria et al, 2011; Khaiosa-ard and Zebeli, 2014). These differences may one day be exploited to improve individual efficiency. In working toward this, our study showed that rate of digestion due to rumen fluid source can be an important differentiating factor among ruminants, and contribute to significant differences in their ability to digest forage. This is likely due to differences in microbial populations, although this cannot be confirmed based on this study due to the limited number of bacterial species examined. In trying to improve the ability of ruminants to digest fiber it will be important to explore both physiological and microbiome characteristics of individual animals, and their interactions.

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Tables

Table 3.1. Rate of *in situ* NDF disappearance digestion of Fast[1] and Slow heifers

Animal	k_d[2] (%/h)	Lag (h)	a (%)[3]	b (%)	24h NDFD[4]	48h NDFD
Fast 1	4.32	2.80	0.90	58.5	38.6	49.1
Fast 2	4.18	1.67	0.90	56.7	35.7	48.5
Slow 1	3.17	4.13	2.60	56.1	27.4	46.7
Slow 2	2.88	1.11	0.90	61.1	24.4	48.4

[1] Fast refers to inoculum from animals with fast rate of NDFD; slow refers to animals with slow rate of NDFD.
[2]k_d=rate of disappearance per hour based on disappearance of Barley Straw measured *in sacco.*
[3]a fraction is the percentage of washout from the initial substrate, b is the percentage degraded in the rumen over 120 h.
[4]24 h and 48 h observed NDFD

Table 3.2. Ingredient and chemical composition of substrates

Item (g/kg DM)	Ingredients		
	AFEX Barley Straw[1]	Untreated Barley Straw	Concentrate[2]
DM	935	924	905
OM	940	928	905
N	16	7	59
CP	99	43	366
NDF	666	804	357
ADF	488	456	145

[1]Values for sieved and reconstructed AFEX and untreated straw.
[2]Comprised of 66.7% dried distillers grains solids, 26.6% canola meal, 5.7% supplement, 1% urea.

Table 3.3. Effect of inoculum and ammoniation (trt) of barley straw on DMD, OMD, NDFD, ADFD, TDMD, N disappearance, and microbial N production[1]

Item	Treatment[2]				SEM	P-value		
	Untreated		AFEX			Trt[3]	Inoculum	Int[3]
	Slow	Fast	Slow	Fast				
DMD (g/kg DM)								
Barley straw	461[c4]	464[c]	612[b]	636[a]	5.9	< 0.001	< 0.001	0.002
Concentrate	846	816	848	848	15.2	0.079	0.11	0.11
Total	624	618	720	733	8.2	< 0.001	0.46	0.052
OMD (g/kg DM)								
Barley straw	466[c]	467[c]	615[b]	639[a]	4.7	< 0.001	< 0.001	< 0.001
Concentrate	875	854	879	875	12.7	0.12	0.14	0.31
Total	586[b]	580[b]	694[a]	707[a]	7.7	< 0.001	0.32	0.021
TDMD (g/kg DM)								
Barley straw	500[c]	503[c]	633[b]	666[a]	5.6	< 0.001	< 0.001	< 0.001
Concentrate	860	828	858	859	14.0	0.081	0.12	0.092
Total	607[c]	601[c]	700[b]	725[a]	7.3	< 0.001	0.015	< 0.001
NDFD (g/kg DM)								
Barley straw	455[c]	451[c]	559[b]	593[a]	9.4	< 0.001	< 0.001	< 0.001
Concentrate	785[a]	740[b]	769[ab]	785[a]	21.7	0.23	0.23	0.022
Total	507[c]	498[d]	599[b]	627[a]	10.8	< 0.001	0.004	< 0.001
ADFD (g/kg DM)								
Barley straw	427[c]	441[c]	534[b]	577[a]	12.3	< 0.001	< 0.001	0.008
Concentrate	689[a]	649[b]	674[ab]	685[a]	18.2	0.35	0.20	0.028
Total	460[c]	465[c]	551[b]	585[a]	12.4	< 0.001	< 0.001	< 0.001
N Disappearance (g/kg DM)								
Barley straw	681	643	773	759	10.9	< 0.001	0.029	0.27
Concentrate	931	915	925	940	16.8	0.33	0.94	0.13
Total	852	827	846	849	14.4	0.33	0.19	0.11
Microbial N production (mg/d)								
Effluent	31.1	34.5	29.7	36.2	2.50	0.94	0.048	0.52
FPA[5]	5.6	5.6	5.7	5.1	0.20	0.39	0.12	0.25
FPB straw	19.9	18.7	18.2	18.8	0.56	0.19	0.61	0.14
FPB concentrate	1.3	1.4	1.2	1.4	0.19	0.62	0.22	0.94
Total	57.6	60.2	54.8	63.3	2.73	0.95	0.045	0.27

[1]DM = dry matter; DMD = dry matter disappearance; OMD = organic matter disappearance; NDFD = neutral detergent fiber disappearance; ADFD = acid detergent fiber disappearance; TDMD = true dry matter disappearance.

[2] Fast refers to inoculum from animals with fast rate of NDFD; slow refers to animals with slow rate of NDFD.

[3] Trt refers to straw treatment: AFEX or untreated; Int refers to interaction between treatment and inoculum.

[4] a,b,c,d Values within a row with the same letter do not differ ($P > 0.05$).

[5]FPA = feed particle associated; FPB = feed particle bound

Table 3.4. Effect of inoculum and ammoniation (trt) of barley straw on gas production and fermentation variables (pH, total VFA and individual VFA)

Item	Treatment[1]				SEM	P-value		
	Untreated		AFEX			Trt[2]	Inoculum	Int[2]
	Slow	Fast	Slow	Fast				
Gas production								
Total, L/d	1.54	1.61	1.59	1.60	0.135	0.87	0.76	0.79
CH_4, %	6.20	6.37	6.14	6.11	0.534	0.66	0.88	0.79
CH_4, mL/d	94.2	101	94.1	105	14.4	0.83	0.23	0.83
CH_4, mg/d	61.2	66.0	61.2	67.6	9.30	0.87	0.26	0.86
CH_4, mg/g incubated DM	6.66	7.19	6.65	7.32	0.973	0.91	0.26	0.88
CH_4, mg/g digested DM	11.7	12.9	9.67	10.8	1.49	0.046	0.22	0.98
pH	6.70	6.69	6.64	6.61	0.019	< 0.001	0.095	0.34
Ammonia, mg/d	110	98.3	137	131	5.29	< 0.001	0.015	0.45
Total VFA, mmol/d	50.6[c3]	49.0[c]	57.4[b]	59.6[a]	1.88	< 0.001	0.67	0.035
VFA, mol/100 mol								
Acetate (A)	66.1[a]	63.9[b]	63.7[b]	64.1[b]	0.618	0.001	0.006	< 0.001
Propionate (P)	22.6	23.8	25.9	26.2	0.603	< 0.001	0.021	0.21
Butyrate	7.22[b]	8.28[a]	6.74[c]	6.17[d]	0.098	< 0.001	0.025	< 0.001
Valerate	1.62	1.66	1.40	1.34	0.042	< 0.001	0.60	0.10
Isobutyrate	0.916	0.933	0.849	0.848	0.012	< 0.001	0.55	0.48
Isovalerate	1.51	1.44	1.33	1.30	0.035	< 0.001	0.19	0.52
Caproate ($\times 10^{-2}$)	4.52[c]	4.59[c]	5.19[a]	4.98[b]	0.011	< 0.001	0.21	0.018
A:P ratio	2.93[a]	2.69[b]	2.47[c]	2.45[c]	0.090	< 0.001	0.012	0.029

[1] Fast refers to inoculum from animals with fast rate of NDFD; slow refers to animals with slow rate of NDFD.

[2] Trt refers to straw treatment: AFEX or untreated; Int refers to interaction between treatment and inoculum.

[3] a,b,c,d Values within a row with the same letter do not differ ($P > 0.05$).

Table 3.5. Effect of inoculum and ammoniation (trt) of barley straw on rumen microbes and microbial protein synthesis[1]

Item	Treatment[2]				SEM	P-value		
	Untreated		AFEX			Trt[3]	Inoculum	Int[3]
	Slow	Fast	Slow	Fast				
Total protozoa, × 10^3 cells/mL	8.58	8.00	6.67	6.25	1.43	0.22	0.73	0.95
Total bacterial 16S rRNA copies after adaptation (× 10^9)[4]	107.9[ab5]	85.9[b]	96.8[b]	130.0[a]	14.5	0.10	0.56	0.013
F. succinogenes (%)	2.41	1.29	2.17	1.25	0.718	0.85	0.18	0.89
R. albus (%)	0.0286	0.0433	0.0258	0.0522	0.0087	0.74	0.035	0.51
R. flavefaciens (%)	0.0678	0.0538	0.0585	0.0610	0.0095	0.91	0.56	0.41
S. ruminantium (%)	0.0422	0.0685	0.0599	0.108	0.041	0.37	0.25	0.73
P. bryantii (%)	0.0186	0.0011	0.0156	0.0323	0.0165	0.40	0.98	0.31

[1]Populations calculated as percent of total bacterial 16S rRNA.
[2]Fast refers to inoculum from animals with fast rate of passage; slow refers to animals with slow rate of passage
[3]Trt refers to straw treatment: ammoniated or untreated; Int refers to interaction between treatment and inoculum.
[4]All bacteria quantified using FPA samples from each fermenter.
[5]Letters a,b,c,d denote significant difference, values with the same letter are not significantly different than each other.

Chapter 4: General Discussion

The process of artificial selection that has long been employed in agriculture highlights the inherent variation among individuals within a species. Historically cattle have been bred for increased muscle mass, milk production, docility, specific coat colour, etc. My research involved looking at a less obviously visible aspect of variation among cattle – their ruminal microbiome. Ruminant nutrition has been examining differences in rumen microbiome since long before the advent of high throughput genetic screening. Some of the earliest studies discovered different types of protozoal populations in individual sheep and cows (Eadie, 1962). Type A and type B populations of protozoa were observed by Eadie (1962), as well as antagonisms between the two groups with type A protozoan dominating type B protozoan in sheep, and the opposite occurring in cattle.

My work has continued in this vein of study, but rather than examining direct differences in composition of the microbiome I examined differences in the outcome of microbial activities among cattle, in the form of digestibility of various forages. I also examined the effect of modifying these microbiomes through inoculation with bison rumen contents. Because the results of each of my studies have been discussed in each chapter I will limit this discussion to an attempt to answer the questions posed at the outset, and to integrating the results and implications of the studies performed.

My goals with the first study were to examine individual variation in digestion of various forages *in situ*, and to determine if inoculation with bison rumen contents had an overall and/or individual effect on *in situ* ruminal digestibility of forages.

Prior to inoculation with bison rumen contents there was a wide range of variation among cattle in their ability to digest forage. Effective ruminal degradability of barley straw, a feed that heifers were fully adapted to, ranged from 31.6% to 44.9% pre-inoculation. Rate of degradation of barley straw *in situ* ranged from 2.7%/h to 4.9%/h, and the total degraded fraction ($a+b$) ranged from 58.7% to 63.3% after 120 hours. A similarly large range in digestion parameters was seen among all feeds incubated in the rumen indicating a large range of variation in digestion among cattle. Most animals that had a high or low ranking for a single variable (*ERD*, k_d, or $a+b$), had a similar ranking for most or all feeds incubated, indicating that some heifers were better at digesting most or all forages than other heifers, and conversely some heifers were

worse at digesting most forages. Because bags are anchored in the rumen during the *in situ* technique they are not subject to rumination or mastication, thus any degradation that occurs within the bag is due to microbial activity. Therefore variability in individual animal's ability to digest different fiber sources in this case may indicate the uniqueness of each individual animal's microbiome, reinforcing the notion of the "hologenome", or the interactive effects of individual animal genetic composition on the composition of their microbiome (Rosenberg and Zilber-Rosenberg, 2011). However it is important to note that due to the pore size of the bags incubated not all microbes were represented in this study, for example large protozoa and fungal spores would have been excluded.

Inoculating cattle with bison rumen contents did not improve their ability to digest forages in terms of *ERD*; there was, however, a mean decrease in k_d and $a+b$ of barley straw NDF and ADF. There was also a mean increase in $a+b$ with a simultaneous decrease in k_d for alfalfa hay after 120 h due to inoculation with bison rumen contents. The increase in $a+b$ of alfalfa hay NDF occurred in 15 of 16 heifers and the decrease in k_d of alfalfa hay NDF occurred in 12 of the 16 heifers. Pearson correlation analysis indicated a negative correlation between change in $a+b$ and change in k_d due to inoculation with bison rumen contents in alfalfa hay, canola straw and timothy hay. This pattern was not seen in barley straw, probably because heifers were well adapted to a barley straw diet, allowing for optimum digestion of that diet before the transfer. Consequently, the transfer of rumen contents from bison tended to hinder rather than enhance the digestion of barley straw. The negative correlation between change in $a+b$ and change in k_d seen in 3 of the 4 forages incubated may indicate competing microbiome metabolic pathways with a trade-off between rate and extent of degradation. Shabat et al. (2016) found more efficient dairy cattle had fewer, more dominant microbial species that produced fewer, more useful metabolites compared with less efficient cattle, which had greater microbial species diversity and produced more end products that could not be used by the animal. It is possible that the more efficient microbiome seen by Shabat et al. (2016) would result in a faster k_d, as observed in this study. In contrast, a less efficient microbiome may eventually degrade fiber to a greater extent as a result of a more complex metabolic pathway. These contrasting approaches could explain the negative correlation in changes in k_d with changes in $a+b$ observed in this study.

Individual response to inoculation with bison rumen contents varied by forage type. Because individual heifer response was so varied among animals and feeds it is difficult to parse what the characteristics of positive responders were. All individuals responded positively for some variables with some forages and negatively with other forages. In order to understand what allowed some animals to respond positively to the inoculation with bison rumen contents, it would be necessary to conduct a comparative analysis of the microbiome of negative and positive responders to inoculation in terms of forage digestion as reflected by (*ERD, kd, a+b*).

The *in situ* technique was employed, which permits observation of differences in digestion parameters (k_d, and $a+b$) that are not affected by rate of passage, and consequently allows us to examine fiber degradation in the rumen over a controlled time period. Digestive capacity of the rumen is opposingly influenced by k_d and rate of passage (k_p; Allen and Mertens, 1988), therefore, to optimize digestion in the rumen an ideal ruminant would have a slow k_p with a fast k_d. As k_p can be influenced by intake (Owens and Goetsch, 1986) animals with a fast or slow k_d were chosen as donors for subsequent investigation of the digestive ability of their rumen contents *in vitro*.

My objectives with the second study were to identify whether the differences in k_d observed *in situ* were a result of differences in microbes present in rumen fluid, and whether rumen fluid chosen from heifers with fast or slow k_d would differ in their ability to digest ammonia fiber expansion treated (AFEX) barley straw and untreated barley straw *in vitro*.

There was wide variation in *in situ* k_d estimates seen among heifers. This difference was examined using the Rusitec system in a study to investigate whether there were functional differences in rumen microbiome of animals with greatly differing k_d. Digestion of AFEX barley straw was more complete than untreated barley straw regardless of inoculum source, highlighting the benefit of AFEX treatment to improve nutritive value of recalcitrant feedstuffs (Bals et al., 2010). The result that rumen inoculum from heifers with a fast k_d had the ability to digest AFEX barley straw to a greater extent in 48 hours than inoculum from heifers with a slow k_d indicates there are functional differences between these extremes in k_d that become more relevant when digesting a less recalcitrant fiber source.

These studies examined two main areas of variation: 1) variation among heifers in their ability to digest forage and 2) variation in response to inoculation by bison rumen contents. In

further examining the first of these I discovered functional differences between individuals with fast or slow rate of degradation when digesting a more accessible fiber source (AFEX barley straw). In examining the second of these (animal variation in response to inoculation) I discovered that some animals responded positively, some animals responded negatively, and some had little to no response to inoculation. Those animals that responded positively warrant further investigation to elucidate the microbiome shift that occurred, and the potential for improving degradation of fiber in the rumen.

4.1 Limitations

As with any study a number of weaknesses existed in my studies. One such weakness was the pore size of Ankom bags used (50 μm), which precludes access of any large fibrolytic protozoa (Coleman, 1976; Carro et al., 1995), and fungal spores. Thus, these studies likely underestimate ruminal fiber degradation to a certain extent. However, the choice of 50 μm was made deliberately in order to maximize retention of fine particles while allowing access of most microbes to the forage.

It could be construed that another weakness of this study was that animals were chosen for the Rusitec study based on k_d, and not *ERD*. Effective ruminal degradability is confounded by k_p of individual animals, and therefore affected by factors not directly related to the microbiome and was excluded on this basis. It was thought that, of the variables measured, differences in k_d would have the greatest effect on ruminal substrate digestibility within the limited time of incubation (48 h), so this was the variable chosen for further investigation.

This study, while examining some meaningful sources of variation in ruminal degradability, does not elucidate much about efficiency of individual animals. While it seems logical to assume that animals with a faster k_d and slower k_p would be more efficient in terms of gain to feed ratio (G:F), this was not found to be the case (data not shown). However calculation of G:F was affected by low intakes of all animals due to the poor quality of the maintenance diet.

This research contributed to furthering our understanding of differences among individual animals in terms of digestibility in the rumen. This research doesn't present a practical way to select individuals with a faster k_d, or greater *ERD*, in part due to the lack of connection between *in situ* digestibility studies and RFI or G:F efficiency studies.

Lastly, a number of confounding factors exist in this study. Namely, it is impossible to disentangle effect of stress caused by the transfer on *in situ* digestibility. It is also impossible to disentangle the change in k_p of individual animals from fluctuations in intake.

4.2 Future Research

No research has linked *in situ* digestibility parameters with animal efficiency. Measures of efficiency like RFI and G:F indicate energy harvested from the total digestive tract compared with energy used by the individual animal. However, there is some flexibility within the total digestive tract for partitioning energy. For example, it has been found that a decrease in ruminal digestibility due to defaunation, resulted in an increase in postruminal digestion. Therefore, it may be valuable to examine whether there is an explicit link in k_d in the rumen, to animal efficiency, as to my knowledge this has not been attempted. I calculated G:F for the animals in this study (data not shown) but there was no correlation between animals that had both high k_d and low k_p with increased G:F. This may be because diet intake was quite low varied largely among cattle, due to the high-fiber nature of the feed, which affected k_p.

Another potential future study, based on the research presented here, is to examine the microbiome of heifers who responded positively, and those that did not respond at all to inoculation for each variable and feed type in an attempt to determine which microbes were responsible for the increases in digestibility observed. For example, to examine factors that led to improved digestion of recalcitrant feedstuffs such as canola straw a future study can examine the microbiomes of those animals that responded positively to inoculation, as well as the pre- and post-inoculation microbiomes of those individuals that showed no response in terms of *ERD,* k_d or $a+b$ of canola straw.

4.3 Industry implications

The broader goal of this research was to discover an enzyme or combination of enzymes to improve fiber degradation in the rumen to increase digestibility of fiber in low quality forages during the time fiber is retained in the rumen. If the limitation of individual heifers to digest low quality forages is due to a common gap in a metabolic pathway, an enzyme or microbial additive may help improve digestibility of cattle. An additive may be in the form of an enzyme cocktail that will improve digestibility of all or most cattle, or as we learn more about the specificity of the microbiome of individual ruminants, increasingly tailored enzyme additives may be a useful

strategy to meet the needs of the individual animal to improve its performance. This individualized management of cattle has started to emerge in the dairy industry, with the emergence of precision dairy farming, in which concentrate rations are fed on an individual animal basis to optimize milk production, based on genetic or microbiome data collected from individual animals using a transponder, and a robotic feeding system (Maltz, 2015). Similar technology exists to supply supplements to cattle on pasture (Thomas and Buckmaster, 2003). This technology is getting continually more nuanced and has potential to be implemented in the beef industry to optimize nutrition on an individual animal basis.

4.4 Conclusions

My research directly examined variation among cattle in their ability to digest various forages, and their response to inoculation with rumen contents from bison. Inoculation with bison rumen contents had no effect on mean *ERD* for any of the feeds, but it did decrease mean k_d and $a+b$ of barley straw NDF and ADF, and increase mean $a+b$ but decrease k_d of alfalfa hay NDF and ADF. Differences were found among heifers for all *in situ* digestibility parameters, as well as in their response to inoculation. Extreme differences in k_d were found to have functional differences *in vitro* when digesting AFEX barley straw, but not when digesting untreated barley straw, indicating digestibility of less recalcitrant fiber sources may be affected to a greater extent by differences in k_d of animals due to limited retention time. The functional differences in k_d are thought to be a result of differences in rumen microbiome metabolic pathways, or differences in the number of microbes present in the rumen. This research showed that there was potential to modify the ability of individual animals to digest forages, but the results were variable, highlighting the fact that we cannot yet predict the effects of such modifications.

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