# Assessment of rumen microbiota in beef heifers with different feed efficiency and managed under different feeding systems

by

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**Abstract**

The linkage between rumen microbiota and feed efficiency has been studied in cattle managed under the drylot system, especially in the feedlot with high-grain diets. However, pasture-based beef operations still dominate the beef production system around the world. In Alberta and other provinces of Canada, most cow-calf producers keep their cattle on summer pasture to lower the labor and feed costs. Similarly, backgrounding beef cattle are mainly operated under the extensive feeding system, mostly on grazing. To date, very limited studies focused on the rumen microbiota of beef cattle under grazing system. To fill this knowledge gap, the present study was performed and is presented in this thesis. This study investigated the interactions of two feeding systems (drylot vs grazing) and feed efficiency (measured with residual feed intake (RFI)) (H-RFI, n=8; L-RFI, n=8) on microbial populations and fermentation products. This study also explored whether the rumen microbiota in heifers with divergent feed efficiency have different change patterns when the feeding system changed. The results showed that the feeding system had significant effects on butyrate and isovalerate concentrations, and acetate to propionate ratio in the rumen. During the transition from drylot to grazing system, heifers with divergent RFI had different patterns of changes in microbial taxonomic compositions. For example, the relative abundance of two predominant phyla *Firmicutes* and *Bacteroidetes* were significantly increased and decreased, respectively, only in H-RFI heifers when the feeding system changed from drylot to grazing. These results suggest that these inefficient heifers had more diverse rumen microbial communities than efficient heifers. Rumen microbial functions were also predicted using PICRUSt2 package. In total, 10 major bacterial MetaCyc pathways were predicted, and 7 of them significantly increased only in the rumen of H-RFI heifers when the feeding system changed. This indicates that L-RFI (efficient) heifers have

more stable rumen microbiota when the feeding system changed, which also suggests that efficient heifers may maintain their efficiency in different feeding systems through maintaining feed efficiency-related microbes and microbial metabolic pathways. This work provides fundamental knowledge to help to understand the rumen microbiota of cattle under different feeding systems and support the development of potential methods to manipulate rumen microbiota to improve the feed efficiency of beef cattle under grazing.

## Preface

This thesis is an original work by Junhong Liu with collaborations led by Dr. Leluo Guan at the University of Alberta.

The thesis work includes one animal study, which was received ethics approval from the Animal Care and Use Committee University of Alberta (Protocol No. AUP00001284). All animals were cared following the guidance of the Canadian Council on Animal Care (1993).

Animal experiments in Chapter 2 were conducted at the Lacombe Research and Development Centre (Lacombe, Alberta, Canada) and at the Mattheis Research Ranch, University of Alberta, in collaboration with Gemstone Cattle Company, a local beef cattle producer.

**Dedication**

I would like to delicate this thesis to my family members
Sincerely appreciate your support and encouragement all the time

**Acknowledgement**

First, I would like to express my sincere thanks to my supervisor Dr. Leluo Guan! Thanks for giving me the opportunity to be your student from January 2018. It is my great honor to study my MSc program under your supervision, and thank you for your supports, patience, and encouragement with my project, especially when I made mistakes and had difficulties throughout the program. Your guidance helped me face the challenges and weaknesses in my project. Additionally, your motivation and energy in both research and life always inspired me to be a successful person.

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# List of abbreviations

AAFC: Agriculture and Agri-food Canada

ADF: acid detergent fiber

ADG: average daily gain

ANOVA: analysis of variance

A:P: acetate to propionate ratio

ASV: amplicon sequence variant

BCRC: Beef Cattle Research Council

BCS: body condition scores

BTA: *Bos taurus* autosome

BW: body weight

CP: crude protein

DADA2: deficiency of adenosine deaminase 2

DM: dry matter

DMI: dry matter intake

FCR: feed conversion ratio

FDR: false discovery rate

GC: gas chromatography

GF: gain:feed ratio

GHG: greenhouse gases

H-RFI: high residual feed intake

IMG: Integrated Microbial Genomes

ITS: internal transcribed spacer

L-RFI: low residual feed intake

MBW: metabolic body weight

ME: metabolizable energy

NDF: neutral detergent fiber

$P_{adj}$: adjusted *P* value

PCoA: Principal-Coordinate Analysis

PERMANOVA: permutational multivariate analysis of variance

PICRUSt2: phylogenetic investigation of communities by reconstruction of unobserved states 2

QIIME2: quantitative insights into microbial ecology 2

qPCR: quantitative real-time PCR

RCF: relative centrifugal force

RFI: residual feed intake

RFID: radio-frequency identification

RIM-DB: rumen and intestinal methanogens database

SNP: single nucleotide polymorphism

SOP: standard operating procedure

TDN: total digestible nutrients

TMR: total mixed ration

VFA: volatile fatty acid

WGCNA: weighted correlation network analysis

# Chapter 1. Literature review

## 1.0 Introduction

Alberta is the largest beef cattle production province in Canada with a continuous increase in beef cattle inventory (Statistics Canada, 2019). Alberta beef is a provincial symbol and heritage because of its prime quality and authentic feature (Blue, 2008; Pogue et al., 2018). Beef production in the North American commercial beef production cycle includes three phases: cow-calf stage, backgrounding phase, and finishing period (Lupo et al., 2013; Alemu et al., 2016). In Alberta and other provinces of Canada, most cow-calf producers herd their cattle on summer pasture for lower labor and feed costs, and it has been reported that extending the grazing period of beef cattle can lower the costs for farms in Western Canada (McGeough et al., 2017). However, due to the urbanization patterns and the change of farm types, 845,200 hectares of agricultural land were lost, and 1,379,300 hectares of pastureland were shifted to cropland in Alberta from 2000 to 2012 (Haarsma et al., 2014). Such impacts of urbanization have also happened in other countries, like the rapidly developing country, China (Li et al., 2014), and developed country like the United States (Rashford et al., 2011). Therefore, developing a sustainable beef production system with improved feed efficiency when there are decreasing natural resources is necessary for the long-term success of the Alberta beef industry and all over the world.

In addition to the loss of pastureland, another challenge of developing a sustainable beef production system is the methane emission from the rumen microbial fermentation of cattle. Methane released from cattle contributes to 17 and 3.3% of the global methane and the total greenhouse gases (GHG) emissions, respectively (Knapp et al., 2014), and production of enteric

methane in cattle also results in 2 to 12% of gross energy loss in the host (Johnson and Johnson, 1995). Studies have shown that efficient beef cattle could produce less methane than inefficient ones (Basarab et al., 2013; Dini et al., 2018). Therefore, reducing enteric methane produced in cattle is a win-win strategy for reducing GHG of the environment and improving gross energy utilization for cattle. In the rumen, volatile fatty acids (VFAs) are also produced by the rumen microbial fermentation and absorbed through the rumen wall and carried by the blood stream to the liver, which meet 70% of the energy requirement of cattle (Flint and Bayer, 2008). Such pathways indicate the importance of studying the rumen microbiota in understanding the feed efficiency of cattle.

Therefore, the following literature review aims to describe the current knowledge of feed efficiency in different beef cattle production systems, functions of the rumen and rumen microbial fermentation, factors affecting rumen microbiota, factors affecting feed efficiency, and the relationship between the rumen microbiota and feed efficiency in beef cattle.

## 1.1 Feed efficiency and production system

### 1.1.1 Beef cattle production system in Canada

Beef cattle production system in Canada can be classified through two different aspects, by the phases in the beef production cycle and by the density of cattle herds. The three phases in the North American commercial beef production cycle are: 1) cow-calf stage for breeding purpose and producing weaned calves for further grazing and/or feeding, 2) backgrounding phase for increasing body weight of steers or heifers and prepare yearlings for the feedlot, and 3) finishing phase in which cattle gain body weight dramatically for slaughter (Alemu et al., 2016; Beauchemin et al., 2010; Lupo et al., 2013; Pogue et al., 2018). The operations of beef cattle can

also be divided into the extensive system and intensive system by the level of density: Beef cattle in an extensive system can walk freely on open rangeland and graze different types of vegetations, whereas those in the intensive system are in confined drylot with less varieties in the feed (Alemu et al., 2016; Pearson et al., 2019). In Canada, most cow-calf and backgrounding beef cattle are kept in extensive grazing systems while finishing beef cattle are fed in intensive drylot systems (Endres and Schwartzkopf-Genswein, 2018).

In Canada, most beef farms practiced some forms of grazing during the warm or cold season to maintain the profitability since animal feed represents the highest input cost for beef cattle farms (Finneran et al., 2012; Sheppard et al., 2015). Sheppard et al. (2015) reported that for these operations with on-farm summer pasture from May to October, most pasturelands were used for replacement heifers (62%), followed by backgrounding steers or heifers (26%), and only 5.6% pasturelands were used for finishing cattle. In addition to summer grazing, many farms practiced extended grazing in the cold season for the considerable economic and environmental benefits, such as reducing the soil erosion under sustainable grazing system with reducing cultivation, and depositing of excreta on pasture (Baron et al., 2014; McMillan et al., 2018; Pogue et al., 2018). These cold season grazing strategies including rolled or processed forages, bales, stockpiled forages, swathed cereal crops, and standing corn (Sheppard et al., 2015).

As mentioned previously, Alberta is the largest beef producing province in Canada, and the recent beef farm inventory reports showed that there are 1.9 million heads of steer and heifers for the market in Canada, with 1.1 million of these grown in Alberta (AAFC, 2019). Together with Saskatchewan and Manitoba, these three prairie provinces in Western Canada produced roughly 80% of the national beef cattle (AAFC, 2019). In these three provinces, there were 35,034 cow-calf operations with an average of 145.9 head of cattle, but only 397 feedlots with an

average of 2597.7 heads of cattle (AAFC, 2019). Even though there were much more cow-calf operations than feedlots, the cow-calf farms had a much smaller scale.

Although about more than half of cow-calf beef farms in Alberta, Saskatchewan, and Manitoba extended their summer grazing to cold seasons, some producers concerned about watering and excess snow may reduce the cattle's growth performance since the winter grazing activities combined with low temperature result in higher energy requirement than those in the intensive confined system with a heated watering system (Aasen et al., 2004; Baron et al., 2014; Sheppard et al., 2015). Therefore, cow-calf producers may also keep beef cattle in the intensive confined drylot during winter and feed them with silage, forage, or hay, which may result in higher labor and fuel cost than those in extensive winter grazing systems. A five-year study in Alberta compared the changes of body condition scores (BCS) of beef cattle under different winter feeding methods, and the results showed that winter swath grazing with triticale, corn, or barley may lead to a slightly reduced BCS of cows but still within the recommended range (Baron et al., 2014). In the meantime, winter swath grazing benefits the cow-calf producers with significantly lower feed, diesel fuel, and labor cost (Pogue et al., 2018).

Backgrounding is the key phase for the beef production system in Canada to let heifers and steers build up frame size for finishing in feedlot operations (Pogue et al., 2018). Although heifers and steers can grow in either extensive grazing system or intensive drylot system during the backgrounding stage, a recent study in Western Canada showed that using extensive winter grazing of swath grazing or stand corn grazing could significantly decrease the cost per steer in backgrounding without affecting the steer's performance afterwards in the finishing period (McMillan et al., 2018). The most common feedlot operations in Canada are managed by large specialized companies that purchase heifers or steers from cow-calf and backgrounding

operations and feed them with highly concentrated diet in an intensive production system to let them grow rapidly for slaughter (Endres and Schwartzkopf-Genswein, 2018; Pogue et al., 2018). In addition to the large intensive feedlot operations, there are also a small portion of farms in Canada that finished their cattle with summer grazing on pasture for the minor grass-fed beef market (Sheppard et al., 2015).

In summary, beef cattle production in Canada is dominated by the grazing system for most cow-calf operations and backgrounding farms. The cold season swath grazing is also widely accepted by more cow-calf farms today.

### 1.1.2 Measuring feed efficiency of beef cattle

Feed efficiency nowadays is a very popular topic for beef cattle producers and researchers. Improving feed efficiency of beef cattle can increase profitability and lower the environmental footprint of beef production simultaneously (Kenny et al., 2018; Moore et al., 2009). Feed efficiency in beef cattle has been studied for many decades (Koch et al., 1963), which is a complex multifaceted trait that has an association with diet, breed, behavior, environment, and rumen microbiota, and so on. (Berry and Crowley, 2013; Fitzsimons et al., 2014; Guan et al., 2008; Herd and Arthur, 2009; Owens et al., 1997). To date, several different methods have been practiced in measuring feed efficiency of beef cattle, including feed conversion ratio (FCR) and its mathematical inverse gain:feed ratio (G:F), and residual feed intake (RFI) (Kenny et al., 2018), which are summarized below.

#### 1.1.2.1 Feed conversion ratio (FCR)

The FCR is calculated by the feed intake (dry matter intake; DMI) divided by the average daily gain (ADG) during the measurement period, which is one of the most traditional methods for measuring feed efficiency in beef cattle. Cattle with a lower FCR consume less feed per kilogram of body weight gain than those higher FCR cattle, hence low FCR cattle are efficient, and high FCR cattle are inefficient because of the lower cost in the feed. Although FCR or G:F is commonly used by the beef industry for its efficient evaluations of production, the beef industry is moving away from FCR in recent years because these measures are undesirable in improving feed efficiency genetically (Cantalapiedra-Hijar et al., 2018; Moore et al., 2009). The negative correlation between FCR and ADG indicated that breeding strategies based on FCR might result in faster growing cattle with a large mature body size (Arthur et al., 2001). Therefore, selecting with FCR is likely leading to produce beef cattle with increased maintenance requirement for the large body size and therefore increase the feed cost in the long run.

#### 1.1.2.2 Residual feed intake (RFI)

The RFI was defined as the difference between the actual feed intake and the expected required feed intake for supporting both maintenance and growth during a test period (Koch et al., 1963). Low RFI animals are efficient since they eat less than expected, and those with high RFI are inefficient because they eat more than expected. The calculation of RFI is based on DMI, ADG, and metabolic body weight (MBW) with a linear regression (Basarab et al., 2011; Nkrumah et al., 2006). Compared with FCR, RFI is mathematically independent with the component traits of animal production and DMI was more strongly correlated with RFI, therefore, measuring feed efficiency with RFI may represent cattle's inherent variation in basic

metabolic processes that determine production efficiency (Archer et al., 1999; Arthur et al., 2001; Cantalapiedra-Hijar et al., 2018; Kenny et al., 2018). With such advantages, RFI has become the preferred measurement in recent years with its advantages in breeding.

Selecting beef cattle with RFI can genetically improve feed efficiency and reduce feed cost in the production cycle through the breeding procedure because of the moderate heritability of RFI in beef cattle (Kenny et al., 2018). Berry and Crowley (2013) reviewed 39 scientific publications estimating RFI in growing cattle and summarized a moderate heritability of 0.33 ± 0.01 (range of 0.07 to 0.62) for RFI, which suggested that breeding for improved RFI could cumulatively improve feed efficiency. The repeatability of a trait is also important in the beef cattle industry for consistent feed efficiency in different production phases. Studies found that RFI was moderately repeatable between two different feeding stages with the same diet (Gomes et al., 2012; Kelly et al., 2010), as well as with different but similar diet (grass silage followed by fresh grass) (Coyle et al., 2016). However, when cattle were fed with significantly different types of diet, such as low and high energy diet, re-ranking of RFI was observed (Durunna et al., 2011; Kelly et al., 2010). Such re-ranking results suggest a genotype and diet interaction may exist in the trait of RFI.

#### 1.1.2.3 Measuring RFI in different feeding systems

Measuring RFI requires expensive and specialized equipment for recording DMI and body weight (BW) with several weeks in a testing trial (Culbertson et al., 2015; Moore et al., 2009). Currently, the radio-frequency identification (RFID) along with automatic specialized feed stations, such as GrowSafe System® (GrowSafe Systems Ltd., Airdrie, Alberta, Canada) and Calan Broadbent Feeding System (American Calan, Inc, Northwood, New Hampshire, USA),

have been widely used for the RFI test in beef cattle (Culbertson et al., 2015; Schaefer et al., 2018; Wang et al., 2006). Considering the RFI test is expensive, the optimum test duration could reduce the cost in measuring RFI without affecting its accuracy. Earlier studies suggested that a 70 days test of RFI with bi-weekly body weight measurements were required for RFI estimation (Archer et al., 1997), which could be shortened to 63 days under the GrowSafe System® with BWs measured weekly (Wang et al., 2006). Although the shorter measurement of 56 days could provide reliable RFI values (Culbertson et al., 2015), the current test duration of RFI is commonly 70-76 days following a 21-28 days adaptation period to let cattle adapt to the testing station environment and diet because the longer test duration resulted in increased accuracy and may eliminate the effects of data interruptions (Culbertson et al., 2015; Higgins et al., 2019; Mukiibi et al., 2019; Thompson et al., 2018).

Compared with the vast majority of feed efficiency studies under confinement, studies on the feed efficiency of cattle grazing on pasture are limited, despite that the extensive grazing system dominates the beef production system, especially for cow-calf and backgrounding phases (Kenny et al., 2018). The challenge of studying the feed efficiency of cattle grazing on pasture derives mainly from the difficulty in estimating individual DMI (Cottle, 2013; Smit et al., 2005). For estimating DMI of grazing cattle, numerous methods have been developed, including indigestible plant markers, animal characteristics and performance, animal behavior based modelling, on intake capacity and herbage fill value, and on sward and pasture conditions (Hellwing et al., 2015). Indigestible plant markers, such as *n*-alkane technique (Mayes et al., 1986), have been widely used for estimating the feed intake of grazing cattle for the advantages in applying with a wide range of pasture (Undi et al., 2008). Basically, the *n*-alkane pellets with synthetic alkane were offered twice daily on pasture for grazing, and the fecal samples of each

cattle were collected afterwards, then the feed intake during grazing could be estimated (Manafiazar et al., 2015).

Since it is difficult to estimate feed intake on pasture, it would be very useful if the RFI tested in drylot could be applied on pasture. Although a few efforts have made in such area, the studies have shown contrasting results (Kenny et al., 2018). Several studies observed that cattle did not show constant feed efficiency in different feeding systems, specifically, cattle with low and high RFI when tested in drylot had similar DMI in the grazing system (Lawrence et al., 2013; Meyer et al., 2008). However, Manafiazar et al. (2015) reported that efficient cattle (low-RFI) tested in drylot consumed less forage on pasture, and similar results were also reported the pre-tested high RFI cattle had higher DMI when grazed on a predominantly perennial ryegrass grassland (McDonnell et al., 2016). Comparing the experimental designs of these studies, the average age at the beginning of the RFI test varied. The average age of cattle in Meyer at al. (2008) and Lawrence et al.'s (2013) studies were 1.9 and 2.8 years old, respectively, but about 1 and 1.3 years old in Manafiazar et al. (2015) and McDonnell et al. (2016)'s studies, respectively. In fact, The Beef Cattle Research Council (BCRC) suggested that RFI should be measured in young cattle (7-10 months of age) (BCRC, 2017), therefore, the age difference in these studies may result in such contrasting results. In summary, in order to get higher repeatability of RFI tested in drylot and applied the RFI ranking in the grazing system, young cattle herd (7-10 months of age) should be used.

### 1.1.3 Factors affecting RFI

Several factors can influence RFI, and there were at least five major physiological processes, including 1) feed intake, 2) feed digestion, 3) body composition and metabolism, 4)

animal activity levels, and 5) thermoregulation contribute to the total RFI variations in cattle (Herd et al., 2004; Richardson and Herd, 2004). Overall, these five physiological processes explained 73% of the variation in RFI. Specifically, biological mechanisms of protein turnover, tissue metabolism and stress, digestibility, heat increment and fermentation, physical activity, body composition, and feeding patterns contributed 37%, 10%, 9%, 9%, 5%, and 2% to the variation in RFI, respectively (Herd and Arthur, 2009). The most recent study indicated that seven traits, including body composition, animal movement, digestive function, hematology, temperament, immune competence, and heat production explained 57% of the variation in RFI measured in feedlot (Herd et al., 2019). In addition to these factors contributed to 73% of the variation in RFI, there are also 27% due to other factors, such as the immunology system because the unhealthy animal may have lower feed efficiency.

Although the effects of these complex biological processes in affecting RFI have been clearly proved, the molecular basis of the regulation principle behind this trait has not been clearly defined. Recently, host transcriptomics studies with low and high RFI beef cattle have identified several differentially expressed genes from different tissues related to the RFI trait, including rumen epithelium, liver, muscle, and backfat. (Kong et al., 2016; Mukiibi et al., 2019; Sun et al., 2018). In these studies, Kong et al. (2016) identified 122 differentially expressed genes from rumen epithelial tissues and indicated the differences in nutrients absorption capacities between high and low RFI steers; Sun et al. (2018) generated 20 gene modules (co-expressed genes) significantly associated with feed efficiency traits across rumen epithelium, liver, skeletal muscle and backfat tissues in beef cattle; Mukiibi et al. (2019) reported that immune response related genes were differentially expressed in liver tissues with divergent feed efficiency component traits.

In addition to the host gene regulations in feed efficiency, rumen microbial activities also play important roles in determining feed efficiency of cattle and there were significant differences in the association between rumen microbiota and feed efficiency variation in cattle (Huws et al., 2018). Therefore, it is necessary to review the current studies of rumen microbiota and its roles in feed efficiency.

### 1.2 Rumen fermentation

The rumen is the largest chamber of the digestive system in ruminant animals, which provides a complex anaerobic environment for its symbiotic microbial activities. To reveal these activities in the rumen, the pioneering work by Dr. Hungate using the culture technologies with a revolutionary Hungate roll tube approach to study strictly anaerobic rumen bacteria was a milestone of identifying the rumen microbiota (Hungate, 1950). Today, researchers have found that there are bacteria, archaea, fungi, and protozoa in the rumen, with an estimated population density of $10^{10\text{-}11}$, $10^{7\text{-}9}$, $10^{3\text{-}6}$, $10^{4\text{-}6}$ cells per ml rumen fluid, respectively (Sirohi et al., 2012). During the microbial fermentation in the rumen, VFAs (mainly acetate, propionate, and butyrate), microbial proteins, and B group vitamins are produced for the host animals to meet the daily energy and nutritional requirement (Beaudet et al., 2016; Flint and Bayer, 2008; Snelling and Wallace, 2017). However, methane is also produced during rumen fermentation at the same time, which can cause energy losses of the host animals. Therefore, studying rumen microbial compositions and functions is the key to understand and improve feed digestive efficiency for ruminant animals.

### 1.2.1 The rumen microbial composition

Similar to the human gut, the cattle rumen is sterile at birth and the establishment of the microbial community in rumen happens immediately after birth (Yáñez-Ruiz et al., 2015). Among the four groups of rumen microbiomes, bacteria and archaea are the first "residents" in the rumen from day zero (Jami et al., 2013; Rey et al., 2014; Yáñez-Ruiz et al., 2010), then anaerobic fungi and protozoa are late rumen colonizers (Fonty et al., 1987; 1988), which indicated that rumen fungi and protozoa colonization and composition could be affected by the external environment.

Bacteria are the most abundant ruminal microorganisms (Sirohi et al., 2012). Despite the differences in ruminant species, animal breed, diet, environment, and geographic locations, the recent studies by sequencing bacterial 16S rRNA genes showed that there is a group of core bacteria in the rumen, with *Firmicutes* and *Bacterioidetes* the most predominant bacterial phyla (Guo et al., 2018; Liu et al., 2016; Mayorga et al., 2016; Sun et al., 2019). At the genus level, *Prevotella*, *Butyrivibrio*, and *Ruminococcus*, as well as unclassified *Lachnospiraceae*, *Ruminococcaceae*, *Bacteroidales*, and *Clostridiales* were the most abundant bacteria (Henderson et al., 2015).

The second most abundant ruminal microorganisms are archaea and these rumen archaea are strictly methanogens, which account for up to 99% of all archaea (Moissl-Eichinger et al., 2018). In the rumen, the most common methanogens are from genus *Methanobrevibacter*, which could be divided into two subgroups, one is the SGMT clade: *Methanobrevibacter smithii*, *Methanobrevibacter gottschalkii*, *Methanobrevibacter millerae* and *Methanobrevibacter thaueri*, and the other one is the RO clade: *Methanobrevibacter ruminantium* and *Methanobrevibacter olleyae* (Janssen and Kirs, 2008; Kittelmann et al., 2013). The other two methanogen genera in

the rumen are *Methanomicrobium* and *Methanomassiliicoccus* (formerly referred to as *Thermoplasmatales* or Rumen Cluster C (RCC)) (Iino et al., 2013; Janssen and Kirs, 2008).

In addition to prokaryotic bacteria and archaea, eukaryotic protozoa and fungi are also living in the rumen. The ciliated protozoa in the rumen have a larger size than bacteria and archaea, accounting for up to 50% of the rumen microbial biomass (Sylvester et al., 2004). Recent studies using amplicon sequencing of ciliate protozoal 18S rRNA genes found that protozoal communities were more variable than bacteria and archaea, with 12 genera protozoal groups and *Entodinium* and *Epidinium* being the two most predominant genera in the rumen (Henderson et al., 2015). The rumen anaerobic fungi all belong to phylum *Neocallimastigomycota*, which accounts for up to 20% of the microbial biomass (Edwards et al., 2017). Based on previous studies using microscopic approaches of directly examining colonies and cultures, ruminal fungi have been classified into six genera: bulbous (*Caecomyces*, *Cyllamyces*), hyphael monocentric (*Neocallimastix* and *Piromyces*), and hyphael polycentric (*Orpinomyces* and *Anaeromyces*) (Griffith et al., 2009). Recently, three new genera of anaerobic fungi including *Buwchfawromyces*, *Oontomyces*, and *Pecoramyces* (formerly known as *Orpinomyces* sp. C1A) have been described (Callaghan et al., 2015; Dagar et al., 2015; Hanafy et al., 2017; Youssef et al., 2013). With the development of classification techniques, more rumen microbes and previous unidentified microbial groups have been revealed, which can help researchers to study the role of rumen microbiota in animal performance.

### 1.2.2 The rumen microbiota functions

The rumen microbial fermentation is a complex systematic activity with rumen microbiota interacting with each other to degrade the plant cell wall carbohydrates and produce

fermentation products for the host (Huws et al., 2018). The rumen microbial fermentation products, including VFAs, proteins, and vitamins play important roles in the host metabolism (Beaudet et al., 2016; Flint and Bayer, 2008; Snelling and Wallace, 2017).

The VFAs produced by rumen microbiota can meet 70% of the host energy requirement (Flint and Bayer, 2008). Among the four microbial groups, bacteria are responsible for the production of VFAs, and hydrogen, formate, and methanol methylamines are also produced by bacteria, which contribute to the production of methane by methanogens (Henderson et al., 2015; Seshadri et al., 2018). Besides the production of VFAs, another function of rumen microbiota is to produce microbial protein using the amino acids that are converted from ammonium. In ruminants, microbial protein is considered as an important protein resource because more than 80% of rumen microbial protein can be digested in the small intestine, which accounts for more than half of the total absorbable protein in the small intestine (Storm et al., 1983; Tas et al., 1981).

In addition to microbial protein, studies of rumen microbial vitamins have been conducted for decades and rumen fermentation could synthesize B group vitamins, including thiamine, riboflavin, niacin, vitamin B6, folates, and vitamin B12 for mature ruminants (Beaudet et al., 2016; Castagnino et al., 2016; Seck et al., 2017). Seck et al. (2017) suggested that the differences in feed ingredients and nutrient composition may play an important role in the difference in vitamins production. The production of vitamins may also vary among animals, and Lima et al. (2019) observed that efficient cattle (Low RFI) had a higher relative abundance of microbial genes involved in vitamin B12 production than inefficient (High RFI) cattle. The vitamin B12 producers in the rumen were members of the genera *Anaerovibrio, Mitsuokella, and Selenomonas* within the Firmicutes (Seshadri et al., 2018).

The rumen protozoa and fungi were not the main protagonists in producing VFAs, protein, and vitamins, but they have important roles. By summarizing several defaunation studies to understand the effect of removing rumen protozoa, Newbold et al. (2015) found that small *Entodinium* are responsible for bacterial protein turnover and holotrich protozoa play an important role in methane production. Therefore, study of the relationship among rumen microbes can provide fundamental knowledge to improve feed efficiency. Although defaunation could increase microbial protein by up to 30% and reduce methane emission by up to 11%, a decrease in feed digestibility was also happened with these animals (Hristov et al., 2013; Newbold et al., 2015). The decrease in feed digestibility could lead to negative effects on feed efficiency, therefore, defaunation is not a practical procedure for methane mitigation strategy and should not be considered as the main strategy to improve the feed efficiency of cattle. Anaerobic fungi are the most active microorganisms in the rumen by penetrating plant structure with the extensive set of enzymes and benefiting the microbial fermentation of other microbes, therefore, several studies have verified the benefits of anaerobic fungi for the host animals with improved feed intake, feed digestibility, feed efficiency, more ADG and milk production (Lee et al., 2000; Paul et al., 2004; Saxena et al., 2010; Tripathi et al., 2007).

To date, a total of 336 rumen bacteria and archaea are characterized according to the Hungate1000 project, which account about 75% of prokaryotic taxa at the genus level in the rumen. The eukaryotic groups of rumen microbiota have been studied as well, including 12 ciliate protozoal genera and 9 anaerobic fungal genera have been described. The development of omic studies have helped to reveal more previously unidentified rumen microbiomes, however, the functions of rumen eukaryotic microbiomes still need to be studied and the interactions are not well defined.

### 1.3 Factors affecting rumen microbiota and its functions

Many factors can affect rumen microbiota, including diet, host, environment, and so on. Understanding how rumen microbiota change with different conditions is the key for researchers to improve feed efficiency and reduce the methane emission of cattle.

#### 1.3.1 Diet

Diet has been considered as the major factor affecting the bacterial abundance in the rumen (Henderson et al., 2015). For example, forage and concentrate diets resulted in different groups of bacterial communities in the rumen (Ellison et al., 2014; Petri et al., 2013), and the ratio of forage to concentrate change in diet led to a significant change in bacterial composition (Li et al., 2019b). Similarly, the rumen methanogenic communities also varied between cattle fed with high-energy diet and low-energy diet (Zhou et al., 2010). The high-energy diet with high ratio of grain could result in lower bacterial diversity and less fibrolytic microbes, such as protozoa and fungi in the rumen (Huws et al., 2018). Another study found that cattle fed with the same type of diet but different feed formula (corn silage versus grass silage) also altered the compositions of bacteria, methanogen, and protozoa, which could be due to the different feed nutritional compositions in corn silage and grass silage (Lengowski et al., 2016). An earlier study also indicated that feeding red clover silage resulted in a different rumen microbial diversity compared with feeding perennial ryegrass silage (Huws et al., 2010). In summary, different diets with varied energy levels, feed types, or grass species may alter rumen microbiota significantly.

### 1.3.2 Feed additives

In order to improve feed digestibility or reduce methane emission by manipulating rumen microbiota, several feed additives or unique feed sources have been studied. Active dry yeast is a widely used probiotic in lactating cows to increase milk yield by stimulating bacterial activities in the rumen (Jiang et al., 2017; Meller et al., 2019; Uyeno et al., 2017). Several studies with garlic oil, ginkgo fruit, and cashew nutshell showed the potency of these feed additives in modifying rumen microbiota (Busquet et al., 2005; Ma et al., 2016; Oh et al., 2017a; 2017b). Another recent study found that feeding rams with different levels of air-dried brown seaweed, the rumen microbial communities differed among dietary treatments (Zhou et al., 2018a). However, the plant extracts may be difficult to standardize for altering rumen microbiota (Henderson et al., 2016). Furthermore, a recent study noticed that feeding dairy cows with garlic could affect the milk and thus change the color, texture, aroma and flavor of ripened cheese (Polizel et al., 2018), which could impact the use of garlic for the beef industry. Therefore, apply feed additives to manipulate rumen microbiota still needs a long way to go.

### 1.3.3 Host

Although Henderson et al. (2015) indicated that rumen microbiota was mainly affected by diet, their results also found that rumen bacterial groups were divergent among different host species. Such a finding suggests that the host may play an important role in affecting rumen microbiota. A previous microbial transplantation experiment with two pairs of dairy cows by exchanging whole rumen contents found that rumen bacterial community and microbial fermentation may return to their original host status (Weimer et al., 2010), which suggests the importance of the host in the composition of the rumen microbiota. Furthermore, a recent rumen

microbial transfaunation study using nine pairs of beef steers with divergent RFI ranks revealed a highly individualized effect on the re-establishment of the bacterial community (Zhou et al., 2018b). Analogously, transplanting rumen microbiomes from donor cows to 3-6 weeks old calves did not show significant alteration in the rumen bacterial communities in calves (Cersosimo et al., 2019). These rumen transplantation studies together indicate the power of host effect on rumen microbiota.

To date, several studies of the host effect on gut microbiota composition have revealed the heritability of gut microbiota in humans, mice, and pigs (Camarinha-Silva et al., 2017; Davenport et al., 2015; Lim et al., 2017; Org et al., 2015). However, there were limited studies of the heritability of rumen microbiota in cattle, with only one in dairy cows and one in beef cattle (Difford et al., 2018; Li et al., 2019a). Difford et al. (2018) first reported the heritability of rumen bacteria and archaea communities in dairy cows. More recently, Li et al. (2019a) indicated several host factors including breed (Angus, Charolais, and Kinsella composite hybrid) and sex (bulls, heifers, and steers) may also affect rumen microbiota. Their study detected a moderate heritability ($h^2 \geq 0.15$) of several microbial parameters, including the diversity indices, the relative abundance of ~34% of bacterial and archaeal taxa (59 out of 174), and the copy number of total bacteria, as well as divergent heritabilities among different phyla (Li et al., 2019a). Li et al. (2019a) also reported that the variance in single nucleotide polymorphisms (SNPs) of host cattle may also shape the rumen microbiota with associations of 19 SNPs located on BTA (*Bos taurus* autosome 1, 2, 3, 5, 7, 10, 12, 13, 16, 19, 26, and 27) with microbial taxonomic features were observed. In summary, studies by Difford et al. (2018) and Li et al. (2019a) suggest that host plays an important role in the rumen microbiota of cattle, and manipulating selected

heritable microbiomes with genetic selection and breeding could be a possible strategy for improving feed efficiency of cattle and reducing methane emission from ruminants.

### 1.3.4 Environment

As previously reviewed in section 1.1.1, the beef cattle production system in Canada involves grazing system (summer grazing and winter grazing) and drylot system. Such differences in feeding system and grazing seasons may affect the rumen microbiota in cattle. An early study compared the rumen microbiota during two weeks total mixed ration (TMR) diet in drylot and two weeks on pasture grazing of dairy cows (de Menezes et al., 2011) indicated bacterial and archaeal communities were significantly affected by the diet in a different environment. Similarly, ewes housed in drylot with ryegrass hay supplemented with commercial concentrate versus ewes grazing on a perennial ryegrass pasture could result in an increased microbial population and diversity for bacteria, methanogen, and fungi in the rumen (Belanche et al., 2019).

Compared with concentrated feed in drylot, fresh plants on pasture contain phenolic compounds tannins, which could affect rumen protozoa due to its toxicity (McMahon et al., 2000; Vasta et al., 2010). Previous studies have suggested that tannins could reduce the rumen protozoa population *in vitro* (Bhatta et al., 2009) and *in vivo* (Cieslak et al., 2012). As previously reviewed in section 1.2.2, lower rumen protozoa could improve cattle's performance with lower methane emission, less bacterial protein turnover, and higher feed efficiency. However, inconsistent results were also reported, which may due to the different sources of natural tannins (Aboagye et al., 2018). Such inconsistencies suggest that grazing on a natural pastureland with various forage types can result in a much more diverse rumen microbiota within a herd.

For extensive grazing systems, the differences in forage may result in divergent rumen microbiota since the forage qualities could be varied due to the different grazing seasons (Ma et al., 2019; Noel et al., 2017). Noel et al. (2017) compared the rumen bacteria of dairy cows in New Zealand grazed on a ryegrass and clover mixed pasture during four seasons: winter (August), spring (November), summer (February), and autumn (May). Their results showed that different bacterial communities (beta-diversity) in the rumen were observed among seasons, which may be due to the seasonal differences in diets, including fiber and crude protein (CP) concentrations (Noel et al., 2017). Ma et al. (2019) also noticed significant differences in beta-diversity of yak rumen bacteria communities grazed with different forage growth stages, including re-green stage (May), grassy stage (July), and withered stage (December) on a natural alpine meadow pastureland. Although the predominant phylum *Bacteroidetes* was consistent among all three forage growth stages, there were significant shifts of relative abundance of *Firmicutes*, *Verrucomicrobia*, *Proteobacteria* detected in the rumen of yak grazed on different seasons (Ma et al., 2019). Ma et al. (2019) indicated that such changes in rumen microbiome may due to the effects of temperature on plant productivity.

To date, studies of the environmental effect on rumen microbial compositions and functions mainly focus on the differences of diets in different feeding systems. However, cattle on natural grazing system have access to natural water, soil, and different cattle may have different eating behavior in selecting vegetation, which could also affect rumen microbiota.

### 1.4 Linkage between rumen microbiota and cattle feed efficiency

The linkage between rumen microbiota and cattle feed efficiency (high and low RFI) was first proposed by Guan et al. (2008). Subsequently, studies of rumen microbiota and their

association with cattle feed efficiency have been conducted. The current research mainly focuses on identifying particular rumen microbial taxa related to feed efficiency. Such associations between rumen bacteria and feed efficiency in beef cattle have been reported at different taxonomic levels, such as phylum (*Firmicutes*, *Lentisphaerae*, and *Chloroflexi*), and genus (*Succiniclasticum*, *Lactobacillus*, *Ruminococcus*, and *Prevotella*) (Li et al., 2019; McCann et al., 2014; Myer et al., 2015). Similar linkages also have been found with rumen archaea, including *Methanobrevibacter* sp. strain AbM4, *Methanosphaera stadtmanae*, *Methanobrevibacter ruminantum*, *Methanobrevibacter smithii* (Li et al., 2019a; Zhou et al., 2009; 2010). Compared to the prokaryotic group, there are much more limited studies with eukaryotic groups of rumen microbiota and the linkage with feed efficiency. Since some protozoa may prey on bacteria and result in microbial protein recycling and decreased microbial protein for the animal, current knowledge of the association of rumen protozoa and feed efficiency indicated that eliminating rumen protozoa can increase the microbial protein supply for animal (Newbold et al., 2015). Overall, the current understanding of rumen microbiota and its association with feed efficiency mainly focus on rumen bacteria and archaea, with fewer studies on rumen protozoa and fungi, despite their importance in fiber and starch degradation.

### 1.5 Knowledge gaps in relation to research objectives and hypotheses

Although the rumen microbiota and its association with different parameters, including feed, host, and feed efficiency have been explored in the recent decade with the development of molecular techniques, most findings only considered ruminants in confined drylot system and only very limited attention was focused on the rumen microbiota of beef cattle in the extensive grazing system, despite that the grazing system still dominated the feeding system in the world,

especially for cow-calf and backgrounding phases (Kenny et al., 2018). It is important to study the rumen microbiota and its association with feed efficiency in different production systems (drylot and grazing) to provide more complete knowledge of rumen microbiota in beef cattle for further improving the feed efficiency of cattle through manipulating rumen microbiota.

We hypothesized that different feeding systems (drylot and grazing) can result in different rumen microbial communities and their fermentation products in heifers due to the differences in diet and environment, and heifers with low residual feed intake (L-RFI) have less diverse changes of rumen microbiota than heifers with high residual feed intake (H-RFI) when the feeding system changed from drylot to grazing. The objective of the present study was to test if the rumen microbiota of efficient heifers (L-RFI) and inefficient heifers (H-RFI) have divergent changes when the environment changes from drylot to grazing. Specifically, we aimed to investigate how the rumen microbiota, rumen microbial fermentation products, and predicted rumen microbial functions would change during the transition of the feeding system from drylot to grazing. The long-term objective of this thesis study is to provide fundamental knowledge of rumen microbiota of cattle under the grazing system for future strategies to reduce the methane emission and improve feed efficiency by manipulating rumen microbiota.

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# Chapter 2. Investigation of the rumen microbiota of L-RFI and H-RFI heifers during the transition from drylot to grazing

## 2.0 Introduction

Improvement of feed efficiency is an urgent task for the development of a more sustainable beef cattle industry, because raising beef cattle with higher feed efficiency can significantly improve productivity and reduce global greenhouse gas emission (Moore et al., 2009; Kenny et al., 2018). Feed cost represents up to three-quarters of total direct costs (Nielsen et al., 2013), which is one of the major factors influencing the profitability of beef cattle farms. In the meantime, cattle with higher feed efficiency produce less methane than that have lower feed efficiency (Nkrumah et al., 2006; Hegarty et al., 2007). Feed efficiency is a complex and multi-trait phenotype, and FCR, G:F, and RFI are commonly used measures of feed efficiency in beef cattle (Kenny et al., 2018). RFI is calculated as the difference between actual and expected feed intake for growth (Koch et al., 1963). Cattle with lower RFI are considered to be efficient because they have less feed intake than expected, conversely, cattle with higher RFI are inefficient. In recent years, RFI has gained popularity as it is independent of BW gain and animal size (Cantalapiedra-Hijar et al., 2018) as well as having moderate heritability in beef cattle (Berry and Crowley, 2013). Therefore, selecting efficient cattle with lower RFI may improve the feed efficiency of the herd in the long run.

Recently, the linkage between rumen microbiota and feed efficiency of beef cattle has been reported widely (Guan et al., 2008; Zhou et al., 2009; Hernandez-Sanabria et al., 2010; Li and Guan, 2017). Bacteria, archaea, protozoa, and fungi in the rumen interact with each other to break down ingested feed through microbial fermentation and produce VFAs, which can meet about 70% of the energy requirement of the host (Flint and Bayer, 2008). Therefore, it is

important to study rumen microbiota to help better understand feed efficiency of cattle. To date, several studies have revealed that efficient and inefficient cattle have different relative abundance of bacterial taxa such as *Butyrivibrio*, *Lactobacillus*, *Prevotella*, *Ruminococcus*, and *Succinivibriowere* genera (Guan et al., 2008; Hernandez-Sanabria et al., 2010; Carberry et al., 2012; Myer et al., 2015). Similarly, the association between feed efficiency and archaeal species *Methanobrevibacter smithii*, *Methanosphaera stadtmanae*, and *Methanobrevibacter* sp. strain AbM4 have been found in beef steers and heifers (Zhou et al., 2009; Carberry et al., 2014). However, the studies of linkage between rumen microbiota and feed efficiency only focused on beef cattle in drylot or feedlot under confinement feeding systems, it is not clear whether such relationships also exist when cattle are grazing on a natural pasture.

Grazing on pasture is a common practice and widely used by small family-owned cow-calf and backgrounding beef operations in Western Canada (Endres and Schwartzkopf-Genswein, 2018). Despite the importance of grazing system for the beef cattle industry, especially small scaled family-owned farms, only a few studies investigated feed efficiency of cattle under the grazing system (Lawrence et al., 2012; 2013; Manafiazar et al., 2017; Oliveira et al., 2018). Besides, very limited studies explored the rumen microbiota shift in ruminants (including cattle, yak, and sheep) when the feeding system changed from drylot to grazing (Pitta et al., 2010; Mohammed et al., 2014; Zhou et al., 2017; O'Callaghan et al., 2018; Belanche et al., 2019). However, none of these studies investigated the relationship between feed efficiency and rumen microbiota under the grazing system. Therefore, it is not clear whether efficient cattle can maintain their efficient rumen microbial fermentation in both drylot and grazing systems.

In the present study, we hypothesized that the rumen microbiota of L-RFI heifers is more stable than H-RFI heifers when they were transited from drylot to grazing system. Therefore, the

present study assessed rumen microbial populations, compositions, and their fermentation products of cattle bred for different classes of RFI (high and low) under both drylot and grazing, aiming to identify whether rumen microbiota is associated with grazing as reported in the feedlot, and whether heifers with H-RFI and L-RFI have different patterns of changes in the rumen microbiota.

## 2.1 Materials and methods

### 2.1.1 Ethical statement

The animal study was conducted in 2015 and 2016 at the Lacombe Research and Development Centre (Lacombe, Alberta, Canada) and at the University of Alberta Mattheis Research Ranch, in collaboration with the Gemstone Cattle Company, a local beef cattle producer. All animals' handling procedures and protocols were reviewed and approved by the University of Alberta Animal Care and Use Committee (AUP00001284), following the guidance of the Canadian Council on Animal Care (1993).

### 2.1.2 Animal trial and sample collection

In 2015, a total of 60 replacement Hereford-Angus crossbred heifers were born at Mattheis Research Ranch from April 18$^{th}$ to June 24$^{th}$. These 60 heifers were selected from low RFI cows (n=80) bred with low RFI bulls (n=2), medium RFI cows (n=290) bred with medium RFI bulls (n=27), and high RFI cows (n=80) bred with high RFI bulls (n=2) (Figure 2.1) in order to produce a group of heifers with different phenotypes and genetic background for RFI. Therefore, 30 Heifers were predicted to have high RFI values with associated cows and 30 heifers were predicted to have low RFI values. In the first period of this study (from February

19th, 2016 to April 26th, 2016), all 60 heifers were raised in a drylot feeding system at the Lacombe Research and Development Centre and fed with 100% barley silage diet to validate the RFI ranking by measuring individual feed intake (GrowSafe Systems Ltd., Airdrie, Alberta, Canada). Simultaneously, observations of individual animal $CH_4$ production were collected using a GreenFeed Emissions Monitoring System (GEMS) (C-Lock Inc., Rapid City, South Dakota, USA). The individual methane emission (g per day) and methane yield (g $kg^{-1}$ DMI) data were extracted from Nicky Lansink's MSc thesis (Lansink, 2018). Woodchips and shavings were added for bedding in drylot and water access was ad libitum. Individual body weight was measured before and after the drylot trial. Rumen fluid samples were collected from each animal after 67 days in the drylot (on April 27th, 2016) by introducing flexible plastic tubing into the rumen and sucking up 45 mL rumen fluid. One heifer was excluded from the study due to the failure to collect rumen fluid after the first two attempts according to the Standard Operating Procedure (SOP). Therefore, a total of 59 rumen fluid samples were collected and were stored at -80°C until further processing. Barley silage samples were collected and analyzed by Cumberland Valley Analytical Services (CVAS Inc., Maugansville, Maryland, USA).

Based on the RFI value tested in drylot, 16 heifers (8 H-RFI and 8 L-RFI) were selected for the continuing grazing trial at the Mattheis Research Ranch. Heifers were grazed on a 100% forage oats (*Avena sativa*; cv. CDC Baler) pastureland for 22 days from mid-June to early-July 2016. The body weight of each heifer was measured before and after the grazing period. Similarly, rumen fluid samples were collected on July 5th from each heifer after the grazing trial via orogastric tubing as described above. Rumen fluid samples were stored at -80°C until further processing. Oak forage samples were collected and analyzed by Cumberland Valley Analytical Services (CVAS Inc., Maugansville, Maryland, USA). The nutritional compositions of both diets

are shown in Table 2.1. The barley silage diet in drylot and forage oats under grazing have similar metabolizable energy (ME), neutral detergent fibre (NDF), and total digestible nutrients (TDN), but barley silage has higher acid detergent fibre (ADF) and lower CP than forage oats.

### 2.1.3 DNA extraction and qPCR analysis

Total DNA was extracted from 4.5mL of each rumen fluid sample using bead beating and phenol-chloroform extraction methods (Guan et al., 2008). Briefly, 4.5 mL of rumen fluid sample was transferred to a new tube and washed with 1 mL TN150 (10 mM Tris-HCl [pH 8.0], 150 mM NaCl) buffer by vortex and centrifugation at 200 × g for 5 minutes at 4°C to remove particles. Then, 1.5 mL supernatant was transferred to a new centrifuge tube containing 0.3g of zirconium beads (0.1 mm diameter) and the cells were lysed by physical disruption using bead beating with a BioSpec Mini Bead-Beater (BioSpec, Bartlesville, OK, USA) at 4,800 rpm for 3 minutes. The supernatant was obtained from each sample and transferred to a new tube for phenol-chloroform-isoamyl alcohol (25:24:1) extraction. The extracted DNA was precipitated with cold 100% ethanol and 3M $CH_3COONa$ and after precipitation, it was resuspended in nuclease-free water. The concentration and quality of total DNA of each sample were measured at A260 and A280 with ND-1000 spectrophotometers (Thermo Fisher Scientific, Wilmington, DE).

### 2.1.4 Estimation of microbial populations with qPCR

Total DNA was used to estimate total bacteria, total archaea, total fungi and total protozoa population through measuring the copy numbers of their respective marker genes using quantitative real-time PCR (qPCR). The primers targeting each mark gene are listed in Table 2.2.

Specifically, the qPCR reaction of each sample was performed with 10 µl of Fast SYBR Green Master Mix (Applied Biosystems), 1 µl of forward primer (20 pmol/µl), 1 µl of reverse primer (20 pmol/µl), 7 µl nuclease-free water, and 1 µl DNA template, and each reaction was performed in triplicate. For the total population of bacteria and archaea, the standard curves were made using serial dilutions of plasmid DNA containing a full-length 16S rRNA gene of *Butyrivibrio hungatei* and a partial 16S rRNA gene of *Methanobrevibacter* sp. strain AbM4, respectively. The partial 18S rRNA gene of *Entodinium longinucleatum* and partial internal transcribed spacer (ITS) rRNA gene of *Punctularia strigosozonata* was used for making standard curves for total protozoa and fungi population calculation, respectively. The same qPCR program was used for archaea, protozoa and fungi as following: initial denaturation at 95℃ for 20 seconds, followed by 40 cycles of 95℃ for 3 seconds, annealing at 60℃ for 30 seconds. For bacteria, a different program was applied: 95℃ for 5 minutes, followed by 40 cycles at 95℃ for 20 seconds and 60℃ for 60 seconds. Melting curves were generated for all qPCR assays by using the following program: 95°C for 15 seconds and then 15 seconds at each interval with a temperature increase of 0.3°C every 20 seconds from 60°C to 95°C. Then, the results were calculated into copy number per milliliter rumen fluid to estimate the microbial populations using the formula described by Zhou et al. (2009). Specifically, the copy numbers of marker genes of targeted microbes (per ml rumen fluid were calculated using the formula $(M_Q \times C \times V_D)/(S \times V)$), where $M_Q$ is the quantitative mean of the copy number, C is the DNA concentration of each rumen fluid sample, $V_D$ is the dilution volume of extracted DNA, S is the DNA amount (ng) applied to qPCR, and V is the rumen fluid volume subjected to DNA extraction.

### 2.1.5 Measurement of ruminal volatile fatty acids (VFA) concentration

Ruminal VFA measurement followed the method described by Guan et al. (2008) using gas chromatography (GC). Specifically, for each sample, 7 ml of rumen contents were transferred to a 15 ml tube and then centrifuged at 4,500 rpm for 5 minutes at 4 ℃ to obtain rumen fluid. After centrifugation, 3-5 ml rumen fluid was transferred to a 5 ml tube and centrifuged at 13,000 rpm for 5 minutes at 4 ℃ to obtain clear rumen fluid. Then, the supernatant was transferred to a new 5 ml tube and well mixed with 1ml 25% phosphoric acid, then the 5 ml tubes with samples were centrifuged at 13,000 rpm for 5 minutes at 4 ℃. After that, 1 ml of the mixture was transferred to a 1.5 ml microcentrifuge tube and 0.2 ml internal standard solution added. After overnight incubation at -20 ℃, samples were centrifuged at 19,000 relative centrifugal force (RCF) for 5 minutes at 4 ℃ and then transferred to a 1.8 ml GC vial for processing at the Chromatography Facility of University of Alberta. The concentrations of total VFA, acetate, propionate, butyrate, valerate, isobutyrate, and isovalerate were measured and calculated by μmol VFA per milliliter rumen fluid.

### 2.1.6 Profiling of the rumen microbiota using amplicon-sequencing

Paired-end sequencing (2×300 bp) of amplicon DNA was performed using the Illumina MiSeq PE300 at Genome Quebec (McGill University, Montreal, QC, Canada). Briefly, the primers Bac9F (5'- GAGTTTGATCMTGGCTCAG) and Bac515R (5'- CCGCGGCKGCTGGCAC) were used to amplify the bacterial V1-V3 region of 16S rRNA genes, primers Arc915aF (5'- AGGAATTGGCGGGGGAGCAC) and Arc 1386R (5'- GCGGTGTGTGCAAGGAGC) were used to amplify the archaeal V6-V8 region of 16S rRNA genes, and RP841F (5'- GACTAGGGATTGGARTGG) and Reg1302R (5'-

AATTGCAAAGATCTATCCC) primers were used for ciliate protozoal 18S rRNA genes amplification (Table 2.2) (Henderson et al., 2015). In the present study, the amplicon sequencing of the fungi group was excluded in the present study due to unsuccess to produce the amplicon from all samples after making attempts with six different pairs of fungal primers (shown in Table 2.3). At Genome Quebec, two-step PCR was used to produce PCR amplicons and add barcodes separately. Specifically, the same PCR programs were applied for bacteria, archaea, and protozoa to produce PCR amplicons using the following cycle program: initial denaturation at 94℃ for 2 minutes, followed by 33 cycles of 94℃ for 30 seconds, annealing at 58℃ for 30 seconds, elongation at 72℃ for 30 seconds, followed by a final elongation step of 72℃ for 7 minutes. Then, a second PCR was performed with the amplicons produced in the first step to add barcodes with the following cycle conditions: initial denaturation 95℃ for 10 minutes, followed by 15 cycles of 95℃ for 30 seconds, 60℃ for 30 seconds, 72℃ for 60 seconds, followed by a final elongation step of 72℃ for 3 minutes.

### 2.1.7 Amplicon sequencing data analysis

The sequencing data were analyzed using Quantitative Insights Into Microbial Ecology 2 (QIIME 2) 2019.7 version (Bolyen et al., 2019). Specifically, the data were demultiplexed, then the sequence reads were filtered, denoised, and merged. After quality control, the Deficiency of Adenosine Deaminase 2 (DADA2) plugin in QIIME2 was used to remove chimeric sequences and the amplicon sequencing variants (ASVs) table was generated (Callahan et al., 2016). The representative sequences were aligned to the Rumen and Intestinal Methanogens Database (RIM-DB) for archaea and to the SILVA 132 Small Subunit rRNA Database for protozoa and bacteria (Quast et al., 2013; Seedorf et al., 2014). Alpha diversity and beta diversity were calculated

based on the ASV table using R and the MicrobiomeAnalyst tool (Dhariwal et al., 2017). For α-diversity, Chao1 and Shannon indices were calculated to evaluate the richness and evenness of bacteria, archaea, and protozoa. Significant differences in α-diversity indices across groups were determined using a non-parametric paired Wilcoxon test. For β-diversity analysis, the dissimilarity and distance among rumen microbiota in each group were calculated with Permutational Multivariate Analysis of Variance (PERMANOVA) based on the unweighted UniFrac (Lozupone et al., 2011). Principle Coordinate Analysis (PCoA) was performed to visualise these distance matrices in 2-dimensional space with the MicrobiomeAnalyst tool (Dhariwal et al., 2017).

### 2.1.8 Microbial function predictions

Microbial functions were predicted using a phylogenetic investigation of communities using the reconstruction of unobserved states 2 (PICRUSt2) package in QIIME2 based on amplicon sequencing data (Douglas et al., 2019). The PICRUSt2 genome database is based on the recent Integrated Microbial Genomes (IMG) database (Markowitz et al., 2012), and it was used to predict MetaCyc metabolic pathways for bacterial, archaeal, and protozoal ASVs (Caspi et al., 2015).

### 2.1.9 Statistical analysis

Copy numbers of total bacteria, archaea, protozoa and fungi from qPCR were used to evaluate microbial population changes under different feeding systems. Copy numbers per milliliter rumen fluid values were transformed using base-10 logarithm to meet the normal distribution assumptions of statistical analysis. The ruminal VFA concentrations (μmol per

milliliter rumen fluid) were also used to study fermentation activities. For heifers in drylot, the relationship among RFI, ADG, DMI, methane yield, methane emissions, microbial populations, and VFA profiles were analyzed using the weighted correlation network analysis (WGCNA) package version 1.67 in R (Langfelder and Horvath, 2008). Pearson's correlation coefficient was performed to explore the relationship, and only correlations with coefficient > 0.3 or < -0.3 and with adjusted *P* value < 0.1 were then visualised using Cytoscape (v3.7.2) software (Shannon et al., 2003). To reveal the interaction between RFI groups (H-RFI vs L-RFI) and production system (drylot vs grazing) on microbial populations and VFA concentrations, the Linear mixed-effect models were performed in this study using R. The statistical model included animal as a random effect and RFI (H-RFI or L-RFI) and feeding system (drylot or grazing) as fixed effects. The comparison of the relative abundance of the detected microbial taxa and the relative abundance of predicted MetaCyc pathways were performed with the non-parametric Kruskal-Wallis test.

In order to reveal the general networks of 16 heifers under different feeding systems, the WGCNA (v1.67) package in R was also used for exploring the relationships between ADG, RFI, VFA concentrations, microbial population, predicted microbial functions and relative abundance of detected rumen microbiome of 8 H-RFI heifers and 8 L-RFI heifers under drylot and grazing (Langfelder and Horvath, 2008). The Spearman's rank correlation was performed with WGCNA package and further filtered to select only correlations with coefficient > 0.3 or < -0.3 and with adjusted *P* value < 0.05, which were then visualised using Cytoscape (v3.7.2) software (Shannon et al., 2003). The Benjamini-Hochberg method was used to adjust all the *P* values obtained during data analysis into the false discovery rate (FDR) (Benjamini and Hochberg, 1995). For all

comparisons, adjusted *P* value ($P_{adj}$) of less than 0.05 was considered as a statistical significance, and $P_{adj}$ between 0.05 and 0.1 was considered as a trend.

## 2.2 Results

### 2.2.1 The relationship between microbial populations and rumen fermentation of heifers in drylot

The ruminal VFA and microbial abundance are shown in Table 2.4. When the relationships between rumen measures and phenotypes ((obtained from Nicky Lansink's thesis (Lansink, 2018)) were analyzed, no significant correlations between VFA profiles (total VFA, acetate, propionate, butyrate, valerate, isovalerate, and isobutyrate concentrations) methane ($CH_4$ emissions, $CH_4$ yield) and microbial populations (bacteria, archaea, protozoa, and fungi) or phenotypic dataset (RFI) for heifers in drylot system were identified. However, as shown in Figure 2.2, the archaeal population and ADG showed a positive correlation ($r = 0.48$, $P_{adj} = 0.0183$). For the fermentation parameters, CH4 emission (g per day) was positively correlated with ADG ($r = 0.4$, $P_{adj} = 0.0821$), DMI ($r = 0.44$, $P_{adj} = 0.0362$), and CH4 yield (g $kg^{-1}$ DMI) ($r = 0.59$, $P_{adj} = 0.0014$); while CH4 yield (g $kg^{-1}$ DMI) was negatively correlated with DMI ($r = -0.46$, $P_{adj} = 0.0293$), and RFI ($r = -0.59$, $P_{adj} = 0.0016$). There was no correlation between RFI and CH4 emissions ($r = -0.05$, $P_{adj} = 0.9320$).

### 2.2.2 The average daily gain of heifers in drylot and under grazing

During the first period of the animal trial in the drylot system, the ADG of the selected L-RFI and H-RFI heifers was 0.64 ± 0.15 kg and 0.60 ± 0.10 kg, respectively. However, when heifers grazed on the forage oats, the ADG of L-RFI and H-RFI animals was 1.91 ± 0.49 kg and

2.10 ± 0.36 kg, respectively. Both L-RFI and H-RFI heifers had significantly increased ($P < 0.05$) ADG during grazing. No interactions of RFI and feeding systems were found on ADG ($P > 0.05$). The RFI values of eight H-RFI heifers ranged from 0.3 to 1.15, and from -0.83 to -0.31 for eight L-RFI heifers (Figure 2.3)

### 2.2.3 Interaction of feeding systems and RFI with microbial populations and VFA profiles

For rumen microbial populations of bacteria, archaea, protozoa and fungi, the effect of interaction between RFI and feeding system effects on rumen microbiota was found for protozoa with a tendency ($P = 0.07$), and the system tended to have effects on total archaea in the rumen ($P = 0.09$) (Table 2.4). For VFA concentrations in the rumen, there were only significant system effects on butyrate, isobutyrate, and acetate to propionate ratio (A:P) ($P < 0.05$), while the effect of interaction between RFI and feeding system on VFA concentrations was not observed (Table 2.4).

### 2.2.4 General amplicon sequencing results

For rumen bacteria, a total of 7532 ± 2814, 11089 ± 3840, 10264 ± 3003, 8984 ± 1135 high quality sequences were generated for H-RFI heifers in drylot, H-RFI heifers under grazing, L-RFI heifers in drylot, and L-RFI heifers under grazing, respectively. Each group of these sequences were assigned into 32 ± 5, 28 ± 8, 35 ± 9, and 31 ± 10 ASVs respectively. Similarly for rumen archaea, 9773 ± 6581, 10478 ± 3570, 10881 ± 1654, 11356 ± 2910 high quality sequences, and 50 ± 12, 50 ± 10, 54 ± 5, 47 ± 13 ASVs were obtained for H-RFI heifers in drylot, H-RFI heifers under grazing, L-RFI heifers in drylot, and L-RFI heifers under grazing, respectively. As for protozoa, 22443 ± 10606, 18438 ± 11097, 11065 ± 1024, 32155 ± 25049

high quality sequences, and 46 ± 10, 46 ± 8, 47 ± 8, 43 ± 7 ASVs were obtained for H-RFI heifers in drylot, H-RFI heifers under grazing, L-RFI heifers in drylot, and L-RFI heifers under grazing, respectively (Table 2.5).

### 2.2.5 The alpha and beta diversity of rumen microbiota under two feeding systems

The alpha diversity (richness and evenness) of rumen bacteria, archaea, and protozoa did not differ between heifers in drylot and under grazing for both H-RFI and L-RFI groups (Table 2.6) ($P > 0.1$). For the beta diversity of rumen microbiota, PCoA plots showed that bacterial profiles of both H-RFI (PERMANOVA, $P = 0.002$) and L-RFI heifers (PERMANOVA, $P = 0.002$), archaeal profiles of L-RFI heifers (PERMANOVA, $P = 0.027$) clustered according to feeding systems. However, the archaeal profiles of H-RFI heifers, protozoal profiles of both H-RFI and L-RFI heifers were not separated between the two feeding systems (PERMANOVA, $P > 0.1$) (Figure 2.4).

### 2.2.6 Comparisons of microbial compositions of H-RFI and L-RFI heifers between two feeding systems

For rumen bacteria, a total of 10 phyla, 69 families, and 197 genera were assigned. To identify microbial phylotypes, only those with a relative abundance of 0.1% and present in more than half the number of the total animals (4 out of 8) and at least in one group of heifers were considered as detected and maintained for downstream analyses. After filtration against these criteria, 10 phyla and 80 genera of bacteria were detected. Overall, *Firmicutes* (69.78±3.25%) and *Bacteroidetes* (24.55±2.82%) accounted for the predominant phyla detected in the rumen of these heifers. At genus level, the predominant bacteria were *Ruminococcaceae* NK4A214 group

(13.85±1.33%), followed by *Christensenellaceae* R-7 group (9.00±0.79%). As for archaea, 4 genera and 12 species were assigned, and 6 species belonging to 2 genera were detected using the same cutoff criteria. *Methanobrevibacter gottschalkii* clade (94.24±1.81%) was the predominant archaea. Similarly, 5 out of 7 ciliate protozoa were detected at genus level, and *Entodinium* (32.45±4.57%) and unclassified *Trichostomatia* (52.91±5.46%) were the predominant rumen protozoa in the rumen of heifers.

When the feeding system changed from drylot to grazing, six and three bacterial phyla changed significantly in the rumen of H-RFI and L-RFI heifers, respectively. To be specific, the relative abundance of *Firmicutes* and *Actinobacteria* were increased, while *Bacteroidetes*, *Proteobacteria*, *Patescibacteria*, and *Fibrobacteres* were decreased in H-RFI heifers when feeding system was changed from drylot to grazing. For heifers with L-RFI, only the relative abundance of *Actinobacteria* was increased, while *Proteobacteria* and *Patescibacteria* were decreased when the feeding system changed from drylot to grazing (Table 2.7). At the genus level, there were 26 bacterial genera that showed significant changes with 15 increased and 11 decreased in their relative abundance for H-RFI heifers. By contrast, only 15 bacterial genera showed significant changes with 9 increasing and 6 decreasing in their relative abundance for L-RFI heifers ($P_{adj} < 0.05$) (Table 2.8). As for rumen archaea, only H-RFI heifers had a trend of increase in the relative abundance of uncultured *Methanosphaera* ($0.05 < P_{adj} < 0.1$) and a significant increase in the relative abundance of unidentified archaea ($P_{adj} < 0.05$) (Table 2.9). H-RFI heifers also had a significant increase of unidentified protozoa ($P_{adj} < 0.05$) in their rumen when the feeding system changed (Table 2.10). However, the relative abundance of archaeal species and protozoal genera did not differ in L-RFI heifers ($P_{adj} > 0.1$) when the feeding system

changed. Overall, the rumen bacteria of L-RFI heifers were more stable than H-RFI heifers when the feeding system changed from drylot to grazing.

### 2.2.7 Comparison of major predicted functions of rumen microbiota under two feeding systems

In total, 331, 82, and 331 MetaCyc pathways were predicted based on identified bacteria, archaea and protozoa amplicon sequences using PICRUSt2 packages in QIIME2. In this study, the 10 most abundant pathways were considered as the major predicted functions of rumen microbiota. Specifically, they were predicted bacterial MetaCyc pathways including the pentose phosphate pathway (non-oxidative branch), L-isoleucine biosynthesis II, L-isoleucine biosynthesis IV, superpathway of pyrimidine nucleobases salvage, adenosine ribonucleotides *de novo* biosynthesis, gondoate biosynthesis (anaerobic), L-isoleucine biosynthesis I, L-valine biosynthesis, pyruvate fermentation to isobutanol pathway, and *cis*-vaccenate biosynthesis. For archaeal, incomplete reductive TCA cycle, L-methionine biosynthesis III, methanogenesis from $H_2$ and $CO_2$, coenzyme B biosynthesis, L-isoleucine biosynthesis IV, guanosine ribonucleotides de novo biosynthesis, coenzyme A biosynthesis I (prokaryotic), L-isoleucine biosynthesis II, L-isoleucine biosynthesis I, and pyruvate fermentation to isobutanol pathways were the main predicted MetaCyc pathways. In addition, there were also main predicted protozoal MetaCyc pathways including pyruvate fermentation to isobutanol, superpathway of pyrimidine nucleobases salvage, adenosine ribonucleotides de novo biosynthesis, superpathway of adenosine nucleotides *de novo* biosynthesis I, guanosine ribonucleotides de novo biosynthesis, UMP biosynthesis I, 5-aminoimidazole ribonucleotide biosynthesis I, 5-aminoimidazole ribonucleotide biosynthesis II, superpathway of 5-aminoimidazole ribonucleotide biosynthesis,

and L-isoleucine biosynthesis II pathways (Figure 2.5). Among these top 10 major predicted functions in H-RFI heifers and L-RFI heifers when feeding system changed from drylot to grazing, only H-RFI heifers had 7 significantly increased bacterial MetaCyc pathways ($P < 0.05$) (Figure 2.5 A). There were no differences in predicted archaeal and protozoal MetaCyc pathways for both H-RFI and L-RFI heifers under two feeding systems (Figure 2.5 B; C).

### 2.2.8 Correlation between major predicted microbial functions and rumen microbiota

A total of 8 major predicted bacterial MetaCyc pathways had strong positive correlations with five bacterial genera including *Lachnospiraceae* NK3A20 group (R = 0.51, $P_{adj}$ = 0.037), *Kandleria* (R = 0.5 ~ 0.54, $P_{adj}$ = 0.0261 ~ 0.0446), *Acetitomaculum* (R = 0.52 ~ 0.57, $P_{adj}$ = 0.0151 ~ 0.035, *Coprococcus* 1 (R = 0.501 ~ 0.58, $P_{adj}$ = 0.0124 ~ 0.0395), *Ruminococcaceae* UCG-005 (R = 0.5 ~ 0.58, $P_{adj}$ = 0.0117 ~ 0.0475); two archaeal species including unassigned Archaea (R = 0.52 ~ 0.62, $P_{adj}$ = 0.0046 ~ 0.0351) and unassigned *Methanosphaera* (R = 0.63 ~ 0.79, $P_{adj}$ = 0.00001 ~ 0.003), and one unassigned Protozoal genus (R = 0.5 ~ 0.64, $P_{adj}$ = 0.0027 ~ 0.0437). There were no negative correlations between rumen microbiota and predicted bacterial MetaCyc pathways. For the major predicted archaeal MetaCyc pathways, only two positive correlations were found. Specifically, *Methanobrevibacter gottschalkii* were positively correlated with coenzyme A biosynthesis I (prokaryotic) (R = 0.77, $P_{adj} < 0.0001$) and *Succiniclasticum* were positively correlated with L-methionine biosynthesis III (R = 0.54, $P_{adj}$ = 0.0245). Fifteen negative correlations between rumen microbiota and major predicted archaeal MetaCyc pathways were observed (Figure 2.6).

### 2.2.9 Correlation between VFA profiles, ADG and rumen microbiota

A total of nine bacterial genera showed strong positive correlations with ADG (R = 0.5 ~ 0.73, $P_{adj}$ = 0.0001 ~ 0.0469), while 12 bacterial genera had strong negative correlations with ADG (R = -0.67 ~ -0.52, $P_{adj}$ = 0.0012 ~ 0.0349). For A:P ratio, strong positive correlations with six bacterial genera were detected (R = 0.5 ~ 0.6, $P_{adj}$ = 0.0068 ~ 0.0437), while strong negative correlations were found with nine bacterial genera (R = -0.62 ~ -0.51, $P_{adj}$ = 0.0046 ~ 0.0423) and one undetected protozoa (R = -0.53, $P_{adj}$ = 0.03). Among these rumen microbiota, five of them had positive correlations with ADG and negative correlations with A:P at the same time, including *Kandleria* (R = 0.73, $P_{adj}$ = 0.0001 for ADG; R = -0.59, $P_{adj}$ = 0.0094 for A:P), *Acetitomaculum* (R = 0.56, $P_{adj}$ = 0.0171 for ADG; R = -0.53, $P_{adj}$ = 0.0311 for A:P), *Olsenella* (R = 0.64, $P_{adj}$ = 0.0026 for ADG; R = -0.59, $P_{adj}$ = 0.0089 for A:P), [Eubacterium] *cellulosolvens* group (R = 0.68, $P_{adj}$ = 0.0009 for ADG; R = -0.52, $P_{adj}$ = 0.0351 for A:P), and [Eubacterium] *nodatum* group (R = 0.57, $P_{adj}$ = 0.0136 for ADG; R = -0.51, $P_{adj}$ = 0.0423 for A:P). Another group of 3 bacterial genera showed negative correlations with ADG and positive correlations with A:P at the same time, there were *Prevotellaceae* UCG-001 (R = -0.57, $P_{adj}$ = 0.0142 for ADG; R = 0.6, $P_{adj}$ = 0.0068 for A:P), *Ruminococcaceae* V9D2013 group (R = -0.56, $P_{adj}$ = 0.0182 for ADG; R = 0.5, $P_{adj}$ = 0.0437 for A:P), and uncultured *Bacteroidales* (R = -0.61, $P_{adj}$ = 0.0053 for ADG; R = 0.5, $P_{adj}$ = 0.0437 for A:P). For VFA concentrations,propionate and isobutyrate concentrations were only negatively correlated with bacterial genus *Ruminococcus* 2 (R = -0.5, $P_{adj}$ = 0.0457 for propionate; R = -0.55, $P_{adj}$ = 0.0197 for isobutyrate). Butyrate and isovalerate concentrations had strong negative correlations with bacterial genera *Ruminococcus* 2 (R = -0.58, $P_{adj}$ = 0.0126 for butyrate; R = -0.54, $P_{adj}$ = 0.0249 for isovalerate), uncultured *Lachnospiraceae* (R = -0.55, $P_{adj}$ = 0.0211 for butyrate; R = -0.54, $P_{adj}$ = 0.0236 for isovalerate),

and uncultured *Rickettsiales* (R = -0.51, $P_{adj}$ = 0.0432 for butyrate; R = -0.51, $P_{adj}$ = 0.0424 for isovalerate) (Figure 2.7).

## 2.3 Discussion

In the present study, the diets of barley silage fed in drylot and forage oats grazed on pasture had similar energy and NDF levels but with different ADF and CP (Table 2.2). It was previously reported that the RFI of beef cattle tested in drylot with silage diets had positive correlations (r = 0.4, $P < 0.01$) with the RFI estimated on fresh grasses (Coyle et al., 2016). This suggests that RFI are repeatable traits when beef cattle are fed with only forage diets. In addition to the similar diets in energy and NDF levels applied in the present study, the breeding strategy of cows with divergent RFI rankings crossed with associated RFI ranking bulls would generate a group of heifers with the distinct genetic background of feed efficiency (Figure 2.1). Since RFI has moderate heritability in beef cattle (Berry and Crowley, 2013), together with the similar energy and NDF of diets used in the present study, it could be speculated that the ranking of RFI would be consistent within these 16 heifers in drylot and under grazing.

In the present study, both L-RFI and H-RFI heifers had significantly faster growth on pasture comparing to in drylot, which may due to the differences in the feed intake of heifers under different feeding systems, as well as the temperature differences in seasons. The drylot period in this study was performed from January to April, and the grazing period was from June to July. Due to the lower temperature during the drylot period, heifers may have a faster metabolic rate to increase heat production and maintain body temperature (Christopherson et al., 1979). With similar energy levels of barley silage fed in drylot and forage oats grazed on pastureland, both L-RFI and H-RFI heifers had much higher ADG under grazing than in drylot.

During the first animal trial period in the drylot system, interestingly, RFI had a negative correlation with $CH_4$ yield and no relationship with $CH_4$ emissions. The overall production of $CH_4$ is affected by DMI and cattle with lower DMI may have lower $CH_4$ production (Johnson and Johnson, 1995; Grainger et al., 2007). However, Fitzsimons et al. (2013) reported that although the L-RFI heifers with lower DMI had lower $CH_4$ emissions, after standardizing the weight of DMI, L-RFI heifers had similar $CH_4$ yield compared to H-RFI heifers on grass silage diets. Similar results were also found in steers fed with concentrate diets (Hegarty et al., 2007). In contrast, Nkrumah et al. (2006) indicated that L-RFI cattle had less $CH_4$ yield than H-RFI cattle. However, the present study together with the findings of McDonnell et al. (2016) suggest that heifers with L-RFI may have higher $CH_4$ yield than heifers with H-RFI. Such inconsistent relationships between $CH_4$ yield and RFI of cattle suggest that there are inherent mechanisms together with RFI that contribute to the differences in $CH_4$ production. The $CH_4$ in the rumen of cattle is produced by archaea during microbial fermentation using the CO2, or formate and $H_2$ produced by rumen bacteria (Seshadri et al., 2018). Therefore, the assessment of rumen microbiota of cattle with L-RFI and H-RFI can provide knowledge of inherent mechanisms of cattle to develop a mitigation strategy for methane emission.

When the feeding system changed from drylot to grazing, strong system effects were observed on butyrate and isovalerate concentrations, as well as A:P ratio. The system effects on butyrate and isovalerate concentrations in the present study were in agreement with those of Mohammed et al. (2014) who reported increased butyrate and isovalerate from orchardgrass hay based drylot to orchardgrass pasture for the grazing system or when compared these two feeding systems. An *in vitro* study indicated that feeding cattle with high starch diets could result in increased butyrate in the rumen (Vallimont et al., 2004). The increased butyrate concentration in

the present study may due to the higher ADF and similar NDF in barley silage than in forage oats. The ADF measures the portion of lignin and cellulose of plant materials and the NDF measures the portion of lignin, cellulose, and hemicellulose (Beauchemin, 1996). Therefore, compared to the barley silage diet, the forage oats diet had a higher portion of hemicelluloses, which are plant cell-wall polysaccharides (BeMiller, 2019). Butyrate was produced by *Lachnospiraceae* and *Butyrivibrio* during the plant polysaccharide degradation (Seshadri et al., 2018). Therefore, the higher ADF and similar NDF in barley silage than in forage oats could lead to higher butyrate production in the rumen of heifers under the grazing system. Isovalerate belongs to branched-chain fatty acids derived from branched-chain amino acids, and early studies of isobutyrate and isovalerate indicated their importance in the synthesis of amino acids and lipids (Allison et al., 1962; Allison and Bryant, 1963). The increase of isovalerate from protein degradation in the rumen of heifers under grazing could due to the higher CP level in the oat forage. The feeding system also had strong effects on A:P ratio, and the A:P ratio in the rumen has a relationship with gluconeogenesis. Lower A:P indicated a more efficient rumen fermentation in cattle when more hydrogen is used to produce propionate which is mainly used for hepatic gluconeogenesis in the liver (van Houtert, 1993), and the shift of fermentation products from acetate to propionate indicated more $H_2$ and less $CH_4$ formation (Janssen, 2010). Therefore, the lower A:P ratio in the grazing system indicates a more efficient rumen fermentation of heifers on pasture compared to in drylot.

In order to investigate how the rumen microbiota in H-RFI and L-RFI heifers changed during the transition from drylot to grazing system, taxonomic analysis was performed. For the system effects on detected rumen bacteria at the phylum level, H-RFI heifers had more changes than L-RFI heifers. The relative abundance of two predominant phyla *Firmicutes* and

*Bacteroidetes* significantly increased and decreased respectively in H-RFI heifers but not in L-RFI heifers when the feeding system changed from drylot to grazing. Both *Firmicutes* and *Bacteroidetes* phyla play important roles in the degradation and metabolism of plant structural carbohydrates to produce VFA in the rumen (Seshadri et al., 2018). Seshadri et al. (2018) also reported that *Lachnospiraceae* and *Butyrivibrio* genera in *Firmicutes* phylum were key members of rumen microbiota who produce butyrate. In the present study, a seven-fold increase in *Lachnospiraceae* NK3A20 genus (previously described as *Butyrivibrio*) explained the significant increase of butyrate in the rumen of H-RFI heifers when the feeding system changed from drylot to grazing. Overall, such different patterns of changes suggest that H-RFI heifers have a less stable rumen bacterial community compared with L-RFI heifers when the feeding system changed.

Comparing the changes of bacterial genera in heifers during the feeding system changes, different patterns of changes were observed in heifers with H-RFI and L-RFI. For example, the predominant genus *Ruminococcaceae* NK4A214 had more than 2-fold increase during the feeding system changed from drylot to grazing in H-RFI heifers only. It has been reported that the relative abundance of *Ruminococcaceae* NK4A214 in the rumen of yak were lower on forage diets than concentrate *Ruminococcaceae* NK4A214 diets (Liu et al., 2019). The different results observed in the present study may be due to the differences in yak and cattle, along with the diets differences. However, due to the limited information of *Ruminococcaceae* NK4A214, it is unclear why this taxon increased only in H-RFI heifers only when the feeding system changed from drylot to grazing. Although the relative abundance of *Lachnospiraceae* NK3A20 genus were significantly increased in heifers with both H-RFI and L-RFI from drylot to grazing, there were 7-fold increase in H-RFI heifers but only 2-fold increase in L-RFI heifers. *Lachnospiraceae*

NK3A20 genus plays an important role in polysaccharide degradation and mainly for the xylan component of plant materials (Seshadri et al., 2018). Therefore, we speculate that both H-RFI and L-RFI heifers had better capacity in degrading polysaccharides of the plant under the grazing system, but H-RFI heifers had much more improvement with a much higher increased *Lachnospiraceae* NK3A20 genus when the feeding system changed from drylot to grazing. However, the mechanism is unclear, which needs further study. The genus *Coprococcus* 1 had 10-fold increase (from 0.09% to 0.86%) in the rumen of H-RFI heifers but did not change in the rumen L-RFI heifers. It has been reported this feed efficiency related bacterial genus remained stable in L-RFI cattle even if being challenged by transfaunation of the rumen content of H-RFI cattle (Zhou et al., 2018), and cattle with high feed efficiency had a higher abundance of *Coprococcus* (Myer et al., 2016). Therefore, the environment changes in the present study did not affect the *Coprococcus* 1 genus in L-RFI heifers, but influence H-RFI heifers significantly. This suggests that H-RFI heifers have less stable rumen microbiota, which indicates that researchers may manipulate certain feed efficiency related bacteria in the rumen of inefficient heifers to improve their feed efficiency. For rumen protozoa and archaea, only H-RFI heifers had significant increases in undetected archaea and undetected protozoa. However, due to the limitation of the database, it was unknown which archaeal species and protozoal genera were involved. Therefore, future studies using metagenomics are needed to explore the patterns of changes of archaea and protozoa in the rumen during the feeding systems changed from drylot to grazing.

In this study, we further used the PICRUSt2 package to predict MetaCyc pathways in order to understand how these changes in the rumen bacteria, archaea, and protozoa can affect the microbial metabolism functions (Caspi et al., 2015; Douglas et al., 2019). Previously,

PICRUSt version 1 and PICRUSt based CowPI were widely used by researchers to predict the bacterial functions based on the 16S rRNA gene sequencing and Greengene database (Langille et al., 2013; Wilkinson et al., 2018). The PICRUSt2 package has been developed recently and expanded its power to predict protozoal, and fungal functions based on 18S rRNA gene and ITS sequences using the recent IMG database (Markowitz et al., 2012; Douglas et al., 2019). In the present study, the changes of major predicted MetaCyc pathways under different feeding systems were similar to the patterns of changes in microbial population and taxonomy analysis. Only heifers with H-RFI had a significant increase in 7 out of 10 major bacterial MetaCyc pathways when the feeding system changed from drylot to grazing. Li and Guan (2017) studied the metatranscriptomic profiles of beef cattle fed with a high energy diet and revealed that inefficient cattle may have more diverse activities of rumen microbiomes than those of efficient cattle. Although different types of diets were applied in the present study, the predicted microbial functions by PICRUSt2 also suggest that H-RFI heifers had more diverse rumen microbial activities when the feeding system changed from drylot to gazing and L-RFI heifers had the ability to retain their functions under different feeding systems. For predicted protozoal MetaCyc pathways, even though no statistical differences were found between two feeding systems for H-RFI and L-RFI heifers, the large standard deviation in each pathway (data not shown) suggests a strong host effect existed in determining the protozoal activities.

The 7 major bacterial MetaCyc pathways that significantly increased in the rumen of H-RFI heifer belong to 4 different categories, including proteinogenic amino acid biosynthesis pathways, nucleoside and nucleotide biosynthesis pathway, pentose phosphate pathways, and fermentation of pyruvate pathway (Caspi et al., 2017). In addition, these 7 MetaCyc pathways had positive correlations with five bacterial genera *Lachnospiraceae* NK3A20, *Acetitomaculum,*

*Kandleria, Coprococcus* 1, *Ruminococcaceae* UCG-005, which all belong to *Firmicutes* phylum. For these 5 bacterial genera, *Lachnospiraceae* NK3A20, *Acetitomaculum,* and *Kandleria* were also positively correlated with ADG. This suggests that these bacterial genera may play very important roles in microbial functions for the host and are beneficial for the growth of cattle. The functions of these key rumen microbiota have been reported recently, for example, the *Lachnospiraceae* NK3A20 genus has the ability to degrade cellulose, hemicellulose (xylan/xyloglucan) and pectin (Seshadri et al., 2018). Due to the limitation of the previous database, the *Lachnospiraceae* NK3A20 together with some other genera were defined as *Butyrivibrio* for their abilities in producing butyrate in the past (Russell and Rychlik, 2001; Henderson et al., 2019). Myer et al. (2015) reported that *Butyrivibrio* were more abundant in high ADG beef cattle than those with low ADG. In the present study, a strong positive correlation between *Lachnospiraceae* NK3A20 and ADG was also noticed. Although there was no correlation between *Lachnospiraceae* NK3A20 and butyrate concentrations found in the present study, our results together with previous studies suggested that *Lachnospiraceae* NK3A20 group (previously defined as *Butyrivibrio*) play a very important role in body weight gain of beef cattle. Another VFA producer *Acetitomaculum* genus is categorized as acetogenic bacteria, which involves the production of acetate (Greening and Leedle, 1989). Although there was no correlation between *Acetitomaculum* and acetate concentrations found in the present study, *Acetitomaculum* was positively correlated with ADG and negatively correlated with A:P ratio, which also suggest the importance of *Acetitomaculum* in animal performance. No correlations between *Lachnospiraceae* NK3A20 and butyrate concentrations, as well as *Acetitomaculum* and acetate concentrations were found in the rumen may due to the VFA concentrations measured in the present study were the VFA left in the rumen after host

absorption, not the actual VFA production by rumen microbiota. The genus *Kandleria* is a member of the family *Erysipelotrichaceae* (Salvetti et al., 2010), this family has been recently reported to have a strong association with host lipid metabolism and immunology in the human gut (Kaakoush, 2015). Together with a positive correlation with ADG and a negative correlation with A:P, the genus *Kandleria* may also be an important group of rumen bacteria in determining animal growth. In summary, these relationships between *Lachnospiraceae* NK3A20, *Acetitomaculum*, *Kandleria* and fiber degradations, VFA productions, host immunology indicate these key bacteria play important roles in ADG of beef cattle.

For *Coprococcus* 1, no correlations between this genus and ADG or VFA were observed. However, *Coprococcus* 1 was positively correlated with 5 of the 10 major MetaCyc pathways. *Coprococcus* has been reported to have a high abundance in efficient cattle (Myer et al., 2016), and efficient steers may maintain their high abundance of *Coprococcus* 1 genus to preserve their efficient microbial fermentation (Zhou et al., 2018). In the present study, *Coprococcus* 1 genus in H-RFI heifers increased from 0.09% in drylot to 0.86% under grazing while it did not change in L-RFI heifers. This result suggests that the *Coprococcus* 1 genus is much more unstable in H-RFI heifers than in L-RFI heifers when the feeding system changed, which may also due to the L-RFI heifers had a relatively high abundance of *Coprococcus* 1 in both feeding systems. This result together with the findings of Zhou et al. (2018) suggests that efficient cattle may have certain mechanisms to maintain their efficiency-related bacteria and keep their efficient microbial fermentation. Furthermore, these results suggested that it is possible to improve the feed efficiency of H-RFI heifers through applying a targeted feeding management strategy to manipulate the rumen microbiota to increase the efficiency-related microbiota and therefore improve the feed efficiency of the whole herd.

In addition, positive correlations were also found between major predicted bacterial MetaCyc functions and unassigned *Methanosphaera,* unassigned archaea, and unassigned protozoa. This indicates that some microbes may share the functions, and some of these functions play an essential role in rumen function. The relationships between predicted archaeal MetaCyc functions and rumen microbiota were less complex than those predicted bacterial MetaCyc functions, and the primary relationships found were negative. The most abundant predicted major pathway, incomplete reductive TCA cycle was negatively correlated with bacteria *Atopobium* genus. The second most abundant predicted pathway L-methionine biosynthesis III was negatively correlated with *Coprococcus* 1, unassigned *Methanosphaera*, unassigned archaea, and *Entodinium*, and positively correlated with *Succiniclasticum*. The coenzyme A biosynthesis I (prokaryotic) pathway only had relationships with archaea, including negative correlations with unassigned archaea, unassigned *Methanosphaera*, *Methanobrevibacter ruminantium*, and positive correlation with *Methanobrevibacter gottschalkii*. The other 7 major pathways all negatively correlated with unassigned archaea. These suggest that both bacteria and protozoa contribute to the archaeal MetaCyc functions in the rumen. However, a large group of unassigned archaea in the current study indicates future studies on metagenomics and/or metabolomics are needed.

## 2.4 Conclusion

In conclusion, the present study investigated whether efficient (L-RFI) and inefficient (H-RFI) heifers had different patterns of changes when the feeding system changed from drylot to grazing. The results revealed that heifers with H-RFI may have less stable rumen microbiota, for example, the relative abundance of two predominant phyla *Firmicutes* and *Bacteroidetes*

significantly increased and decreased respectively only in H-RFI heifers when the feeding system changed from drylot to grazing. Besides, only heifers with H-RFI had a significant increase in 7 out of 10 major predicted bacterial MetaCyc pathways when the feeding system changed. Such easy changeable rumen microbiota and functions only found in H-RFI heifers suggest that these inefficient heifers had less stable rumen microbial communities than efficient heifers. This also indicates that L-RFI heifers have certain functions to maintain their efficient rumen microbiota to keep their efficient microbial fermentation. In summary, the present study provided new knowledge for the industry to potentially improve the feed efficiency in beef cattle in the future by manipulation of rumen microbiota and applying targeted management of beef cattle with different feed efficiency.

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**Table 2.1 Composition of diet nutritive value in drylot and under grazing.**

| | Drylot | Grazing |
|---|---|---|
| | **Barley silage** | **Forage oats** |
| Metabolizable energy (MJ $kg^{-1}$)* | 9.51 | 9.99 |
| Crude protein (%) | 10.93 | 26.8 |
| Acid detergent fibre (%) | 31.37 | 23.1 |
| Neutral detergent fibre (%) | 47.43 | 47.0 |
| Total digestible nutrients (%) | 63.03 | 66.2 |
| Calcium (%) | 0.39 | 0.26 |
| Phosphorus (%) | 0.26 | 0.38 |
| Magnesium (%) | - | 0.24 |
| Potassium (%) | - | 3.79 |

All values are on a dry matter basis.

*Metabolizable energy (ME), MJ $kg^{-1}$ DM = ((TDN, %/100) × 4.4 Mcal $kg^{-1}$ TDN) × 4.184 MJDE $Mcal^{-1}$ × 0.82 MJ ME $MJ^{-1}$ DE (NRC 1996).

**Table 2.2 Primers used to quantify and identify rumen microbes.**

| | Microbial group (marker genes) | Primers* | Sequence (5' to 3') | Amplicon size (bp) | Annealing temp (°C) | Reference |
|---|---|---|---|---|---|---|
| **qPCR** | Total bacteria (16S rRNA gene) | U2-F<br>U2-R | ACTCCTACGGGAGGCAG<br>GACTACCAGGGTATCTAATCC | 467 | 60 | (Stevenson and Weimer, 2007) |
| | Total archaea (16S rRNA gene) | uniMet1-F<br>uniMet1-R | CCGGAGATGGAACCTGAGAC<br>CGGTCTTGCCCAGCTCTTATTC | 160 | 60 | (Zhou et al., 2009) |
| | Total fungi (ITS rRNA gene) | fungi-F<br>fungi-R | GAGGAAGTAAAAGTCGTAACAAGGTTTC<br>CAAATTCACAAAGGGTAGGATGATT | 120 | 60 | (Denman and McSweeney, 2006) |
| | Total protozoa (18S rRNA gene) | P-SSU-316-F<br>P-SSU-539-R | GCTTTCGWTGGTAGTGTATT<br>CTTGCCCTCYAATCGTWCT | 223 | 60 | (Sylvester et al., 2004) |
| **Amplicon sequencing** | Bacteria (16S rRNA gene) | Bac9F<br>Bac515R | GAGTTTGATCMTGGCTCAG<br>CCGCGGCKGCTGGCAC | 525 | 58 | (Henderson et al., 2015) |
| | Archaea (16S rRNA gene) | Arc915aF<br>Arc1386R | AGGAATTGGCGGGGGAGCAC<br>GCGGTGTGTGCAAGGAGC | 492 | 58 | (Henderson et al., 2015) |
| | Protozoa (18S rRNA gene) | RP841F<br>Reg1302R | GACTAGGGATTGGARTGG<br>AATTGCAAAGATCTATCCC | 511 | 58 | (Henderson et al., 2015) |

* "F" designates the forward primer and "R" the reverse primer.

**Table 2.3 Exploring primers to identify rumen fungi.**

| Primers | Primers name | Sequence (5' to 3') | Target region | Annealing temperature (°C) | Reference |
|---|---|---|---|---|---|
| 1 | MN100F<br>MNGM2 | TCCTACCCTTTGTGAATTTG<br>CTGCGTTCTTCATCGTTGCG | ITS1 | 50 | (Tuckwell et al., 2005) |
| 2 | ITS1-F<br>ITS2 | CTTGGTCATTTAGAGGAAGTAA<br>GCTGCGTTCTTCATCGATGC | ITS1 | 53 | (Zhang et al., 2017) |
| 3 | ITS5<br>ITS2 | GGAAGTAAAAGTCGTAACAAGG<br>GCTGCGTTCTTCATCGATGC | ITS1 | 53 | (Ishaq et al., 2017) |
| 4 | ITS1-F_KYO2<br>ITS2_KYO2 | GCTTTCGWTGGTAGTGTATT<br>CTTGCCCTCYAATCGTWCT | ITS1 | 53 | (Toju et al., 2012) |
| 5 | ITS3<br>ITS4 | GCTTTCGWTGGTAGTGTATT<br>CTTGCCCTCYAATCGTWCT | ITS2 | 53 | (Toju et al., 2012) |
| 6 | ITS3<br>ITS4_KYO1 | GCTTTCGWTGGTAGTGTATT<br>CTTGCCCTCYAATCGTWCT | ITS2 | 53 | (Toju et al., 2012) |

**Table 2.4 ANOVA analysis of rumen microbial population and VFA concentrations**

| | | H-RFI | | L-RFI | | RFI | System | RFI × System |
|---|---|---|---|---|---|---|---|---|
| | | Drylot | Grazing | Drylot | Grazing | | | |
| **qPCR** | Bacteria | 10.51±0.14 | 10.81±0.11 | 10.79±0.20 | 10.80±0.21 | 0.4348 | 0.3776 | 0.3992 |
| | Archaea | 7.52±0.19 | 7.83±0.12 | 7.64±0.14 | 7.93±0.19 | 0.5207 | 0.0879 | 0.9598 |
| | Protozoa | 6.65±0.21 | 6.05±0.19 | 6.14±0.26 | 6.70±0.46 | 0.8204 | 0.9459 | 0.0748 |
| | Fungi | 5.19±0.23 | 5.22±0.41 | 5.00±0.30 | 5.51±0.35 | 0.8738 | 0.4310 | 0.4738 |
| **VFA** | Acetate | 86.13±8.90 | 88.74±6.70 | 94.16±12.91 | 69.08±10.73 | 0.5729 | 0.2834 | 0.1909 |
| | Propionate | 21.00±2.78 | 26.80±2.37 | 21.88±3.51 | 21.36±3.52 | 0.4720 | 0.4063 | 0.3222 |
| | Butyrate | 8.82±1.05 | 13.44±1.10 | 8.83±1.27 | 11.20±2.25 | 0.4717 | 0.0352 | 0.4658 |
| | Valerate | 1.23±0.25 | 1.16±0.12 | 1.16±0.27 | 0.91±0.17 | 0.4563 | 0.4642 | 0.6677 |
| | Isovalerate | 1.88±0.19 | 2.58±0.29 | 1.67±0.22 | 2.06±0.29 | 0.2084 | 0.0262 | 0.4845 |
| | Isobutyrate | 1.24±0.14 | 1.47±0.09 | 1.20±0.16 | 1.24±0.20 | 0.3994 | 0.4212 | 0.5542 |
| | A:P Ratio | 4.28±0.25 | 3.36±0.18 | 4.41±0.14 | 3.28±0.08 | 0.9183 | <0.0001 | 0.4758 |
| | Total VFA | 120.97±13.15 | 134.07±10.06 | 129.56±18.29 | 106.24±17.01 | 0.5311 | 0.7380 | 0.2442 |

Values are means ± SEM. The original data of qPCR results (copy number per mL rumen fluid) were transformed by $\log_{10}$. VFA concentrations were measured by μmol per mL rumen fluid sample.

**Table 2.5 Sequencing counts and amplicon sequence variants (ASV)**

| | H-RFI | | L-RFI | |
|---|---|---|---|---|
| | **Drylot** | **Grazing** | **Drylot** | **Grazing** |
| **Bacteria** | | | | |
| Sequencing counts | 7532 ± 2814 | 11089 ± 3840 | 10264 ± 3003 | 8984 ± 1135 |
| ASV | 32 ± 5 | 28 ± 8 | 35 ± 9 | 31 ± 10 |
| **Archaea** | | | | |
| Sequencing counts | 9773 ± 6581 | 10478 ± 3570 | 10881 ± 1654 | 11356 ± 2910 |
| ASV | 50 ± 12 | 50 ± 10 | 54 ± 5 | 47 ± 13 |
| **Protozoa** | | | | |
| Sequencing counts | 22443 ± 10606 | 18438 ± 11097 | 11065 ± 1024 | 32155 ± 25049 |
| ASV | 46 ± 10 | 46 ± 8 | 47 ± 8 | 43 ± 7 |

**Table 2.6 α-diversity in the rumen of heifers under different feeding systems**

| | H-RFI | | | | L-RFI | | | |
|---|---|---|---|---|---|---|---|---|
| | **Drylot** | **Grazing** | **SEM** | ***P* value** | **Drylot** | **Grazing** | **SEM** | ***P* value** |
| **Bacteria** | | | | | | | | |
| Chao1 | 39.13 | 47.50 | 2.67 | 0.1550 | 62.50 | 57.75 | 2.86 | 0.3719 |
| Shannon | 3.39 | 3.49 | 0.07 | 0.7985 | 3.80 | 3.59 | 0.07 | 0.1304 |
| **Archaea** | | | | | | | | |
| Chao1 | 35.13 | 36.13 | 1.73 | 1 | 38.44 | 35.19 | 1.83 | 0.5600 |
| Shannon | 2.29 | 2.30 | 0.09 | 0.7209 | 2.26 | 2.23 | 0.10 | 0.5054 |
| **Protozoa** | | | | | | | | |
| Chao1 | 35.29 | 33.88 | 1.46 | 0.6009 | 32.20 | 33.13 | 1.32 | 0.1709 |
| Shannon | 1.87 | 1.94 | 0.19 | 0.8665 | 2.15 | 1.74 | 0.22 | 0.7117 |

**Table 2.7 The bacterial phyla in the rumen of heifers with H-RFI and L-RFI had different responses to grazing**

| Phylum | H-RFI | | | | L-RFI | | | |
|---|---|---|---|---|---|---|---|---|
| | Drylot | Grazing | SEM | $P_{adj}$ | Drylot | Grazing | SEM | $P_{adj}$ |
| *Firmicutes* | 52.39 | 79.73 | 7.24 | 0.0197 | 67.78 | 79.23 | 5.60 | 0.2444 |
| *Bacteroidetes* | 38.54 | 16.61 | 5.98 | 0.0231 | 25.91 | 17.16 | 5.27 | 0.2740 |
| *Proteobacteria* | 3.24 | 0.34 | 1.47 | 0.0320 | 2.13 | 0.30 | 0.64 | 0.0085 |
| *Actinobacteria* | 0.00 | 1.89 | 0.52 | 0.0035 | 0.36 | 2.11 | 0.50 | 0.0145 |
| *Patescibacteria* | 1.96 | 0.58 | 0.47 | 0.0248 | 1.53 | 0.26 | 0.36 | 0.0194 |
| *Fibrobacteres* | 2.05 | 0.23 | 0.66 | 0.0033 | 0.87 | 0.08 | 0.28 | 0.0630 |
| *Chloroflexi* | 0.35 | 0.40 | 0.11 | 0.4928 | 0.72 | 0.28 | 0.19 | 0.7738 |
| *Lentisphaerae* | 0.47 | 0.08 | 0.21 | 0.2684 | 0.23 | 0.00 | 0.07 | 0.1120 |
| *Spirochaetes* | 0.29 | 0.08 | 0.07 | 0.4243 | 0.17 | 0.51 | 0.33 | 0.9907 |
| *Tenericutes* | 0.13 | 0.00 | 0.05 | 0.0774 | 0.01 | 0.02 | 0.02 | 0.9457 |

Data represented as the average relative abundance (%) in each feeding system.

**Table 2.8 Transition from drylot to grazing altered the rumen bacterial genera**

| Phylum | Family | Genus | H-RFI | | | | L-RFI | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Drylot | Grazing | SEM | $P_{adj}$ | Drylot | Grazing | SEM | $P_{adj}$ |
| ***Firmicutes*** | | | | | | | | | | |
| | *Ruminococcaceae* | *Ruminococcaceae* NK4A214 | 7.91 | 18.21 | 2.58 | 0.020 | 10.64 | 14.64 | 2.89 | 0.105 |
| | | *Ruminococcus* 2 | 3.2 | 1.47 | 0.72 | 0.165 | 5.48 | 3.45 | 0.98 | 0.012 |
| | | *Ruminococcaceae* CAG-352 | 1.13 | 0.21 | 0.33 | 0.042 | 1.32 | 0.74 | 0.41 | 0.053 |
| | | *Ruminococcaceae* UCG-014 | 1.45 | 0.10 | 0.30 | 0.031 | 1.12 | 0.72 | 0.31 | 0.325 |
| | | *Ruminococcaceae* UCG-005 | 0.10 | 0.61 | 0.17 | 0.021 | 0.16 | 0.42 | 0.16 | 0.055 |
| | | *Ruminococcaceae* UCG-002 | 0.05 | 1.07 | 0.28 | 0.011 | 0.08 | 0.35 | 0.11 | 0.405 |
| | *Lachnospiraceae* | *Lachnospiraceae* NK3A20 | 1.00 | 6.92 | 1.34 | 0.002 | 2.48 | 5.21 | 1.82 | 0.043 |
| | | *Acetitomaculum* | 0.00 | 1.63 | 0.44 | 0.015 | 0.40 | 1.32 | 0.72 | 0.016 |
| | | [Ruminococcus] *gauvreauii* group | 1.10 | 2.53 | 0.43 | 0.040 | 1.36 | 2.19 | 0.43 | 0.041 |
| | | [Eubacterium] *cellulosolvens* group | 0.00 | 0.72 | 0.20 | 0.016 | 0.09 | 0.54 | 0.31 | 0.018 |
| | | *Coprococcus* 1 | 0.09 | 0.86 | 0.20 | 0.009 | 0.38 | 0.55 | 0.10 | 0.346 |
| | | *Blautia* | 0.07 | 0.49 | 0.13 | 0.032 | 0.25 | 0.48 | 0.15 | 0.031 |
| | | *Shuttleworthia* | 0.00 | 0.22 | 0.07 | 0.041 | 0.00 | 0.14 | 0.09 | 0.136 |
| | | *Pseudobutyrivibrio* | 0.11 | 0.04 | 0.06 | 0.867 | 0.26 | 0.13 | 0.09 | 0.032 |
| | | *Oribacterium* | 0.18 | 0.94 | 0.21 | 0.015 | 0.38 | 0.65 | 0.17 | 0.037 |
| | *Erysipelotrichaceae* | *Kandleria* | 0.10 | 3.14 | 0.88 | 0.004 | 0.21 | 1.46 | 0.76 | 0.010 |
| | *Veillonellaceae* | *Schwartzia* | 0.00 | 0.18 | 0.07 | 0.053 | 0.00 | 0.13 | 0.08 | 0.012 |
| | Family XIII | [Eubacterium] *nodatum* group | 0.00 | 0.35 | 0.10 | 0.006 | 0.13 | 0.24 | 0.10 | 0.144 |
| ***Bacteroidetes*** | Rikenellaceae | *Rikenellaceae* RC9 gut group | 7.18 | 4.01 | 1.23 | 0.043 | 4.74 | 4.32 | 0.76 | 0.566 |
| | *Prevotellaceae* | *Prevotellaceae* UCG-003 | 0.73 | 0.14 | 0.16 | 0.006 | 0.36 | 0.23 | 0.11 | 0.134 |
| | | *Prevotellaceae* UCG-001 | 6.97 | 1.31 | 1.84 | 0.007 | 2.91 | 1.75 | 0.70 | 0.044 |
| | | *Paraprevotella* | 0.00 | 0.00 | 0.00 | 1.000 | 0.27 | 0.11 | 0.08 | 0.004 |
| | others | uncultured *Bacteroidales* RF16 | 1.73 | 0.23 | 0.60 | 0.020 | 0.41 | 0.28 | 0.19 | 0.218 |
| | | uncultured *Bacteroidales* UCG-001 | 0.33 | 0.08 | 0.09 | 0.047 | 0.10 | 0.10 | 0.07 | 0.856 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | uncultured *Bacteroidales* F082 | 0.59 | 0.20 | 0.16 | 0.267 | 0.94 | 0.63 | 0.28 | 0.022 |
| ***Actinobacteria*** | *Atopobiaceae* | *Olsenella* | 0.00 | 1.17 | 0.41 | 0.011 | 0.00 | 0.71 | 0.23 | 0.025 |
| | *Eggerthellaceae* | uncultured *Eggerthellaceae* | 0.00 | 0.09 | 0.03 | 0.027 | 0.03 | 0.04 | 0.02 | 0.672 |
| ***Patescibacteria*** | *Saccharimonadaceae* | uncultured *Saccharimonas* | 1.64 | 0.49 | 0.41 | 0.024 | 1.21 | 0.70 | 0.31 | 0.057 |
| | others | uncultured *Absconditabacteriales* (SR1) | 0.33 | 0.09 | 0.12 | 0.110 | 0.31 | 0.17 | 0.09 | 0.046 |
| ***Proteobacteria*** | others | uncultured *Rickettsiales* | 0.56 | 0.17 | 0.18 | 0.025 | 0.58 | 0.38 | 0.18 | 0.073 |
| ***Spirochaetes*** | *Spirochaetaceae* | *Sphaerochaeta* | 0.20 | 0.00 | 0.06 | 0.029 | 0.06 | 0.04 | 0.04 | 0.713 |
| ***Fibrobacteres*** | *Fibrobacteraceae* | *Fibrobacter* | 2.05 | 0.22 | 0.65 | 0.003 | 0.87 | 0.49 | 0.28 | 0.063 |

Among 80 detected genera, only these genera had significant changes least in one group of heifers (H-RFI and L-RFI) between two feeding system were shown. Data represented as the average relative abundance (%) in each feeding system.

**Table 2.9 Transition from drylot to grazing had limited effects on the rumen archaea**

| Species | H-RFI | | | | L-RFI | | | |
|---|---|---|---|---|---|---|---|---|
| | Drylot | Grazing | SEM | $P_{adj}$ | Drylot | Grazing | SEM | $P_{adj}$ |
| *Methanobrevibacter gottschalkii* | 89.72 | 94.52 | 5.04 | 0.60 | 95.69 | 97.05 | 0.79 | 0.59 |
| *Methanobrevibacter ruminantium* | 9.65 | 3.55 | 5.06 | 0.53 | 3.30 | 1.58 | 0.72 | 0.45 |
| *Methanobrevibacter smithii* | 0.03 | 0.16 | 0.05 | 0.22 | 0.21 | 0.11 | 0.05 | 0.32 |
| uncultured *Methanobrevibacter* | 0.07 | 0.08 | 0.03 | 1.00 | 0.08 | 0.03 | 0.02 | 0.49 |
| uncultured *Methanosphaera* | 0.02 | 0.37 | 0.11 | 0.07 | 0.06 | 0.35 | 0.11 | 0.11 |
| unassigned archaea | 0.50 | 1.32 | 0.21 | 0.04 | 0.67 | 0.88 | 0.24 | 0.67 |

Data represented as the average relative abundance (%) in each feeding system.

**Table 2.10 Transition from drylot to grazing had limited effects on the rumen protozoa**

| Genus | H-RFI | | | | L-RFI | | | |
|---|---|---|---|---|---|---|---|---|
| | **Drylot** | **Grazing** | **SEM** | **$P_{adj}$** | **Drylot** | **Grazing** | **SEM** | **$P_{adj}$** |
| *Entodinium* | 28.32 | 42.85 | 6.24 | 0.85 | 35.68 | 23.63 | 6.79 | 0.65 |
| *Ophryoscolex* | 10.72 | 0.90 | 4.74 | 0.88 | 18.17 | 5.30 | 7.28 | 1.00 |
| *Polyplastron* | 10.35 | 7.65 | 4.66 | 0.42 | 3.45 | 2.35 | 1.17 | 0.19 |
| unclassified *Trichostomatia* | 50.54 | 46.62 | 7.49 | 0.95 | 42.12 | 68.03 | 8.06 | 0.39 |
| unassigned protozoa | 0.07 | 1.98 | 0.81 | 0.01 | 0.58 | 0.69 | 0.29 | 0.24 |

Data represented as the average relative abundance (%) in each feeding system.

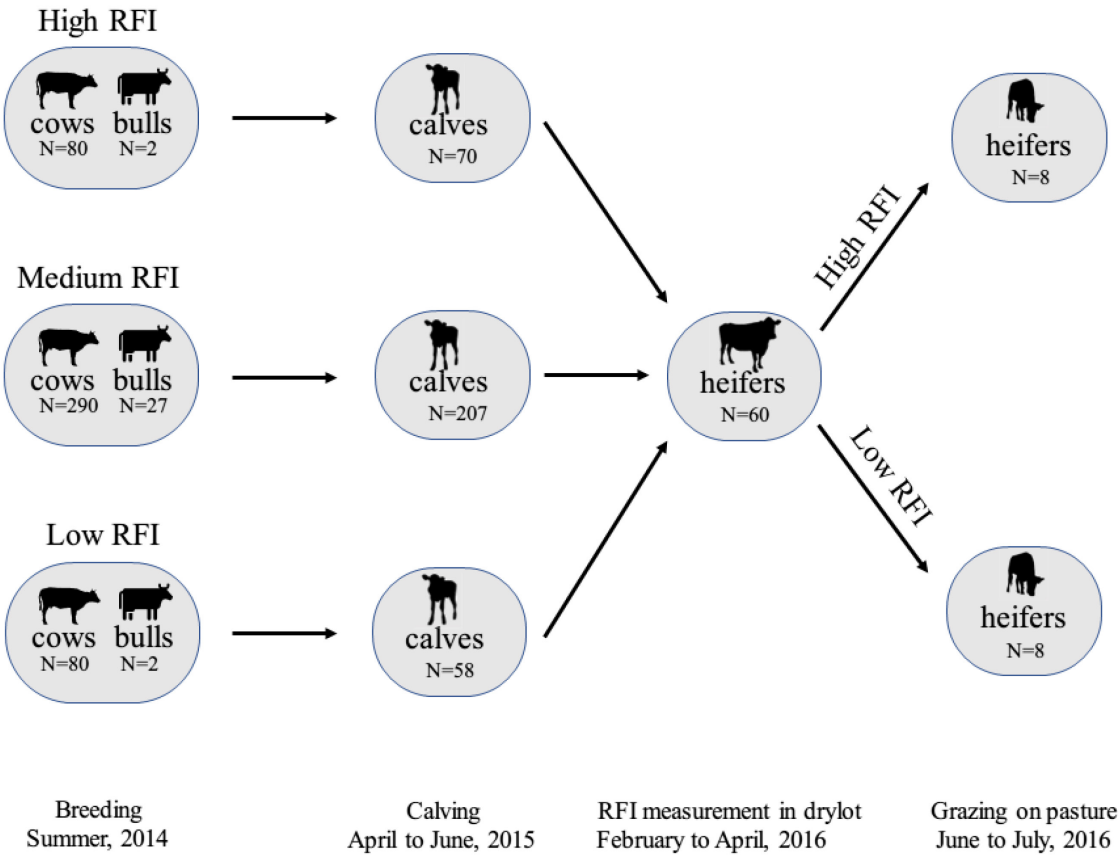



**Figure 2.1 A flow chart of the animal trials performed in this study.** The initial animal trials included 80, 290, and 80 cows with high RFI, medium RFI, and low RFI, respectively. Due to the culling by the owner and missing data of some animals, the number of cows in early 2015 were 74, 219, and 50 for high RFI, medium RFI, and low RFI, respectively.

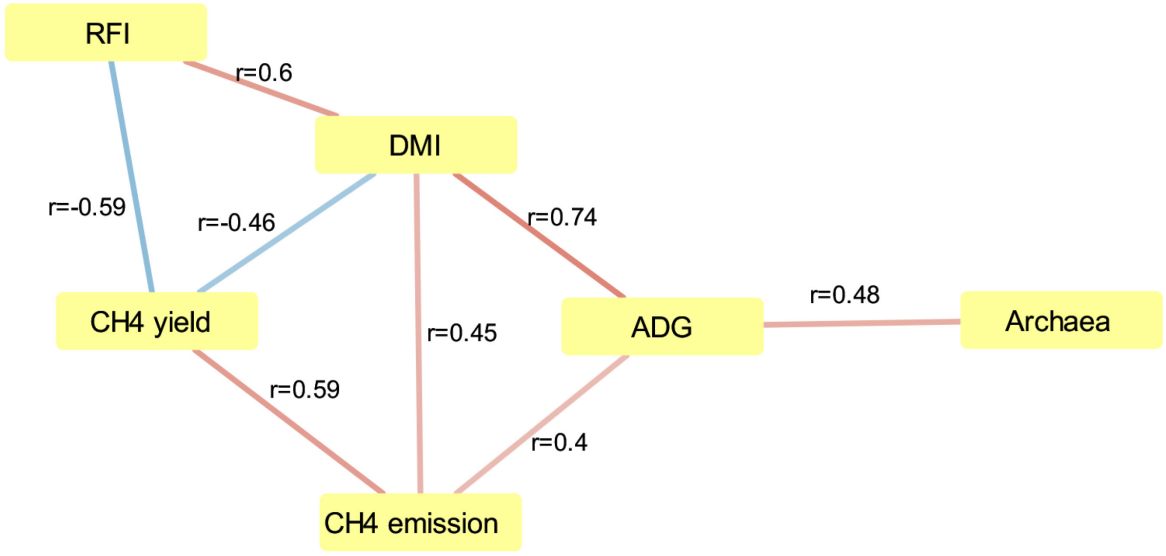



**Figure 2.2 Network of microbial population and phenotypic datasets of heifers in drylot.** Microbial populations (bacteria, archaea, protozoa, and fungi), microbial fermentation products (VFA concentrations and $CH_4$ productions), phenotypic datasets (ADG, DMI, and RFI) were analyzed using Pearson's correlation. $CH_4$ emission is the average $CH_4$ (g) production per day. $CH_4$ yield (g $kg^{-1}$ DMI) was calculated by $CH_4$ emission (g) divided by DMI (kg). Due to a lack of adequate $CH_4$ or RFI data, only 35 animals in drylot system were included in the analysis. The edge colour indicates the correlation relationship, with red indicates positive correlations and blue indicates negative correlations. Only these correlations with $|r|>0.3$ and $P_{adj}<0.1$ were presented.

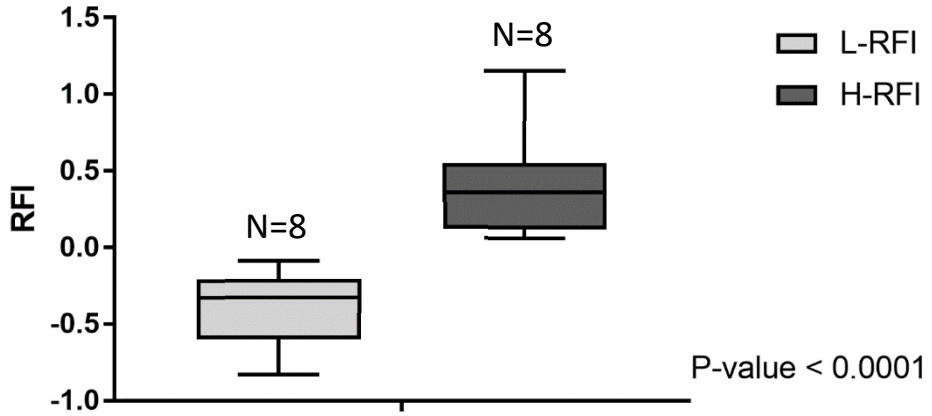



**Figure 2.3 The box plot of RFI values in 8 L-RFI heifers and 8 H-RFI heifers selected in drylot.** The RFI values of eight H-RFI heifers ranged from 0.3 to 1.15, and from -0.83 to -0.31 for eight L-RFI heifers

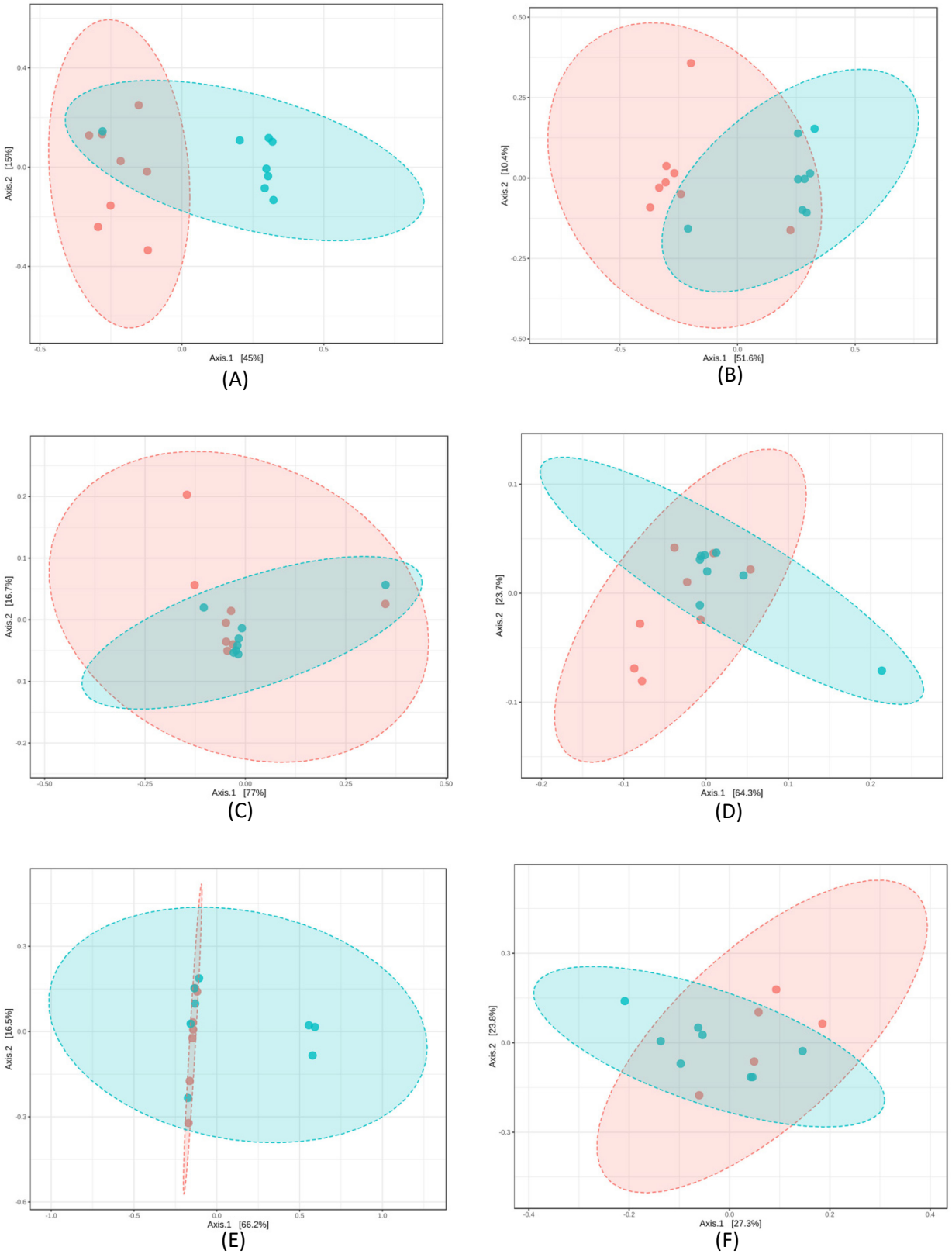
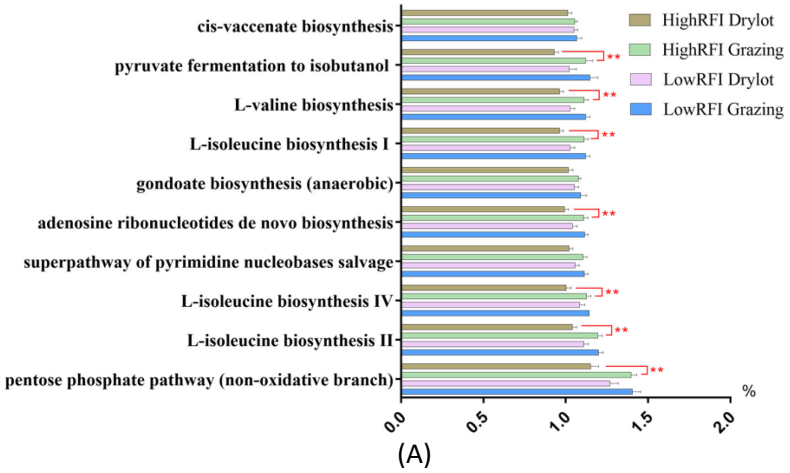
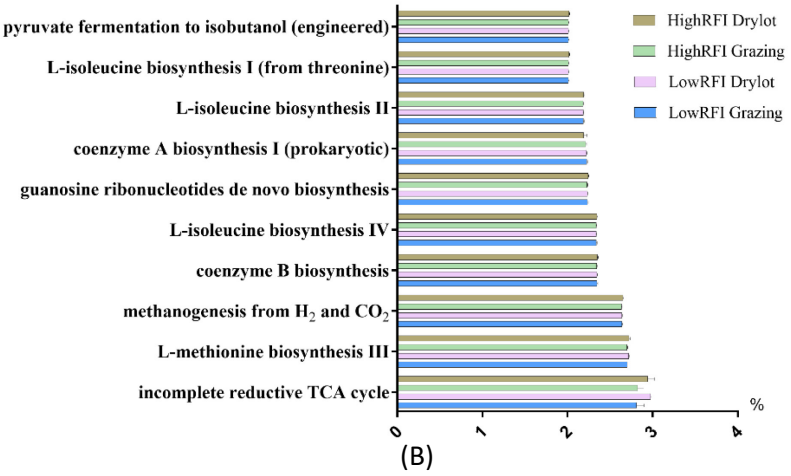



**Figure 2.4 β-diversity of rumen microbiota in drylot and under grazing rumen microbial profiles with PCoA.** Red and blue represented rumen microbiota of heifer in drylot and under

grazing respectively**.** (A) Bacterial profiles of H-RFI heifers are plotted along the first two principal component axis (PC1 and PC2), which explained 45% and 15% of the variance. (B) Bacterial profiles of L-RFI heifers are plotted along the first two principal component axis (PC1 and PC2), which explained 51.6% and 10.4% of the variance. (C) Archaeal profiles of H-RFI heifers are plotted along the first two principal component axis (PC1 and PC2), which explained 77% and 16.7% of the variance. (D) Archaeal profiles of L-RFI heifers are plotted along the first two principal component axis (PC1 and PC2), which explained 64.3% and 23.7% of the variance. (E) Protozoal profiles of H-RFI heifers are plotted along the first two principal component axis (PC1 and PC2), which explained 66.2% and 16.5% of the variance. (F) Protozoal profiles of L-RFI heifers are plotted along the first two principal component axis (PC1 and PC2), which explained 27.3% and 23.8% of the variance. The ellipses represent 95% confidence intervals.



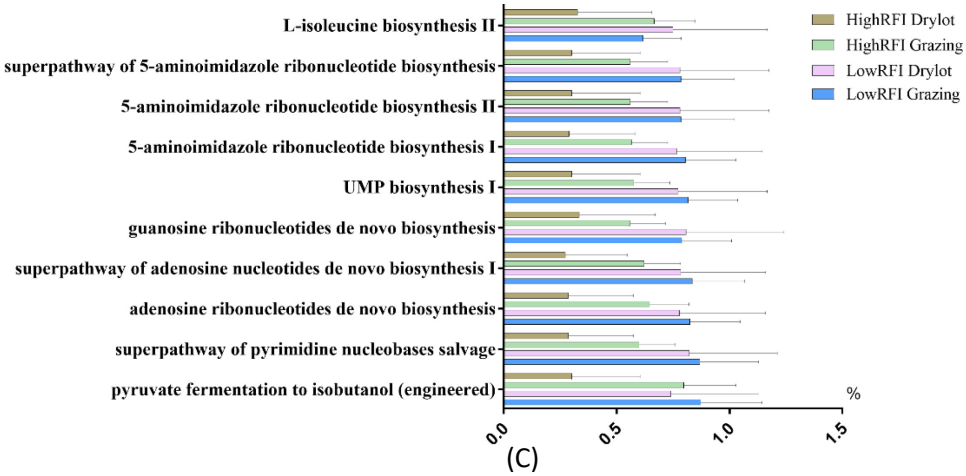
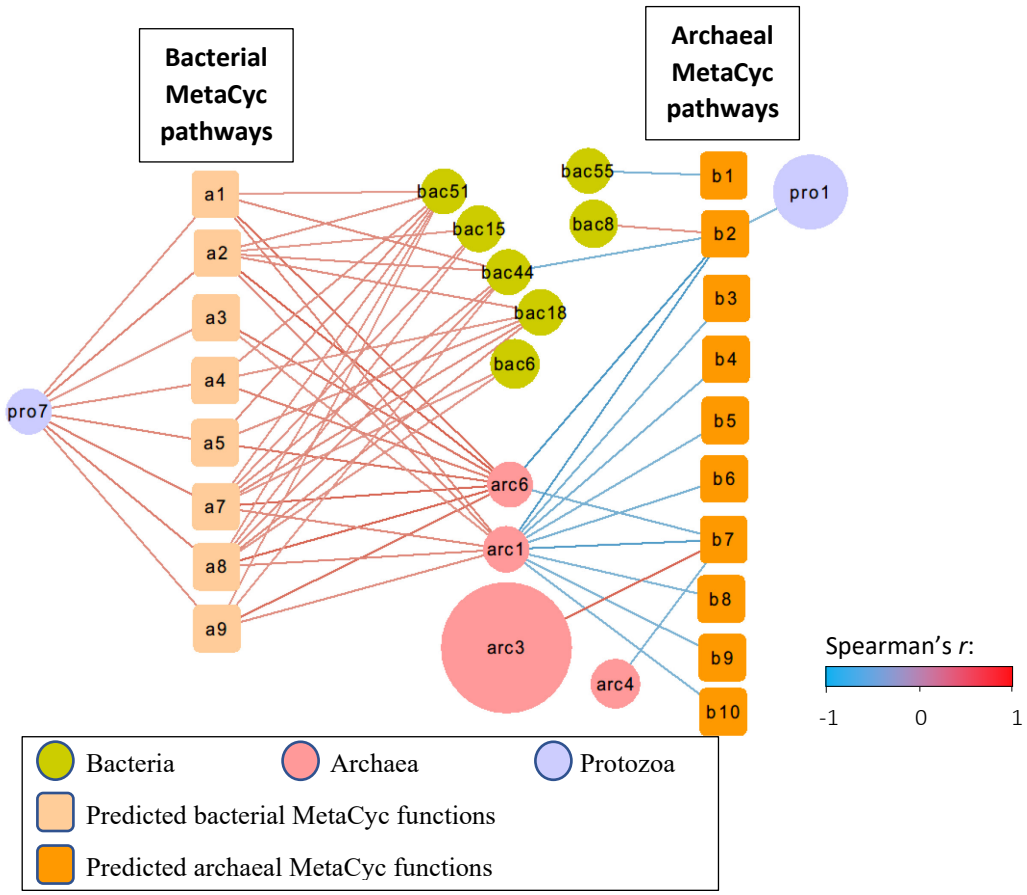




**Figure 2.5 Relative abundances of the major microbial MetaCyc metabolic pathways.** The values on the x axis indicate the proportions of the functions listed. (A) bacterial top 10 MetaCyc metabolic pathways. (B) archaeal top 10 MetaCyc metabolic pathways. (C) protozoal top 10MetaCyc metabolic pathways.

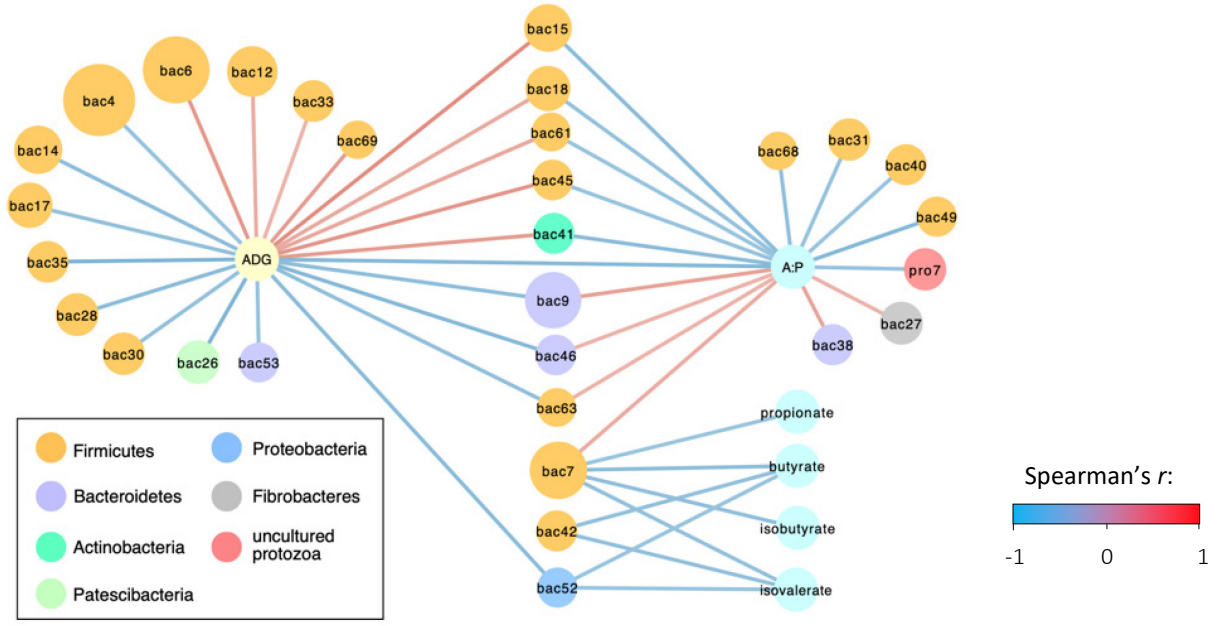



| ID | Bacteria genus | ID | Archaea species | ID | Protozoa genus |
|---|---|---|---|---|---|
| **bac6** | *Lachnospiraceae* NK3A20 | **arc1** | unassigned archaea | **pro1** | Entodinium |
| **bac8** | *Succiniclasticum* | **arc3** | *Methanobrevibacter gottschalkii* | **pro7** | unassigned protozoa |
| **bac15** | *Kandleria* | **arc4** | *Methanobrevibacter ruminantium* | | |
| **bac18** | *Acetitomaculum* | **arc6** | unassigned *Methanosphaera* | | |
| **bac44** | *Coprococcus* 1 | | | | |
| **bac51** | *Ruminococcaceae* UCG-005 | | | | |
| **bac55** | *Atopobium* | | | | |

| ID | Bacterial MetaCyc pathways | ID | Archaeal MetaCyc pathways |
|---|---|---|---|
| **a1** | pentose phosphate pathway (non-oxidative branch) | **b1** | incomplete reductive TCA cycle |
| **a2** | L-isoleucine biosynthesis II | **b2** | L-methionine biosynthesis III |
| **a3** | L-isoleucine biosynthesis IV | **b3** | methanogenesis from $H_2$ and $CO_2$ |
| **a4** | superpathway of pyrimidine nucleobases salvage | **b4** | coenzyme B biosynthesis |
| **a5** | adenosine ribonucleotides *de novo* biosynthesis | **b5** | L-isoleucine biosynthesis IV |
| **a7** | L-isoleucine biosynthesis I | **b6** | guanosine ribonucleotides de novo biosynthesis |
| **a8** | L-valine biosynthesis | **b7** | coenzyme A biosynthesis I (prokaryotic) |
| **a9** | pyruvate fermentation to isobutanol | **b8** | L-isoleucine biosynthesis II |
| | | **b9** | L-isoleucine biosynthesis I (from threonine) |
| | | **b10** | pyruvate fermentation to isobutanol (engineered) |

**Figure 2.6 Relationship between predicted MetaCyc pathways and rumen microbiota.** The relationship was analysed using the Spearman's rank correlation. Each round node represents a microbial taxon, the colour represents the kingdom it belongs to and the size of each node

represents the relative abundance. Each square node represents a MetaCyc pathway predicted from bacterial or archaeal sequences. The edge colour indicates the correlation relationship, with red indicates positive correlation and blue indicates negative correlation. Only these correlations with $|r|>0.3$ and $P_{adj}<0.05$ were presented.




| ID | genus | ID | genus | ID | genus |
|---|---|---|---|---|---|
| **bac4** | uncultured *Clostridiales* | **bac26** | uncultured *Saccharimonas* | **bac45** | [Eubacterium] *cellulosolvens* group |
| **bac6** | *Lachnospiraceae* NK3A20 | **bac28** | *Ruminococcaceae* UCG-014 | **bac46** | uncultured *Bacteroidales* |
| **bac7** | *Ruminococcus* 2 | **bac30** | *Ruminococcaceae* CAG-352 | **bac49** | *Blautia* |
| **bac9** | *Prevotellaceae* UCG-001 | **bac31** | *Mogibacterium* | **bac52** | uncultured *Rickettsiales* |
| **bac12** | [Ruminococcus] *gauvreauii* group | **bac33** | [Eubacterium] *ruminantium* group | **bac53** | *Prevotellaceae* UCG-003 |
| **bac14** | [Eubacterium] *coprostanoligenes* group | **bac35** | *Ruminococcaceae* UCG-010 | **bac61** | [Eubacterium] *nodatum* group |
| **bac15** | *Kandleria* | **bac38** | uncultured *Bacteroidales* | **bac63** | *Ruminococcaceae* V9D2013 group |
| **bac17** | uncultured *Ruminococcaceae* | **bac40** | *Marvinbryantia* | **bac68** | *Shuttleworthia* |
| **bac18** | *Acetitomaculum* | **bac41** | *Olsenella* | **bac69** | *Schwartzia* |
| **bac27** | *Fibrobacter* | **bac42** | uncultured *Lachnospiraceae* | **pro7** | undetected protozoa |

**Figure 2.7 Relationship between ADG, VFA and rumen microbiota.** RFI, ADG, VFA profiles and microbial taxonomic datasets from 32 samples (16 in drylot and 16 under grazing) were included in the analysis using the Spearman's rank correlation. Each node represents a microbial taxon or ADG and VFA, the colour represents the phylum it belongs to and the size of each taxa node represents the relative abundance. The edge colour indicates the correlation relationship, red indicates positive correlation and blue indicates negative correlation. Only these correlations with $|r|>0.3$ and $P_{adj}<0.05$ were presented.

# Chapter 3. General Discussion

Nowadays, more and more rumen microbiota studies of ruminants under the grazing system have been published. Previous studies of rumen microbiota in ruminants under grazing system either used different groups of animals in drylot and under grazing (Mohammed et al., 2014; Zhou et al., 2017; O'Callaghan et al., 2018), or applied same group of animals but the diet compositions were different in drylot and under grazing (Pitta et al., 2010; Noel et al., 2017; Belanche et al., 2019). However, none of these studies involved the feed efficiency of animals during the investigation of rumen microbiota. The present study in Chapter 2 was the first study to explore the patterns of rumen microbiota changes in heifers with divergent RFI facing the shift of feeding systems. The novel part of this study was the breeding strategy to generate a group of heifers with a distinct genetic background of feed efficiency (Figure 2.1). Specifically, low RFI cows were used to cross with low RFI bulls, medium RFI cows were bred with medium RFI bulls, and high RFI cows were crossed with high RFI bulls. Since the RFI is a moderate heritable trait in beef cattle (Berry and Crowley, 2013), such breeding strategy could produce offspring with different RFI. Since the RFI is repeatable trait when beef cattle are fed with similar forage diets (Coyle et al., 2016), in addition to the genetic background of feed efficiency, the diets of barley silage fed in drylot and forage oat grazed on pasture had similar energy levels (Table 2.1), which may provide a repeatable RFI in the present study. Therefore, we speculated that the RFI difference would consistently exist with these 16 heifers in drylot and under grazing, but future research on validation is needed.

The results from the present study revealed that heifers with H-RFI have less stable rumen microbiota. The relative abundance of two predominant phyla *Firmicutes* and

*Bacteroidetes* were significantly increased and decreased respectively only in H-RFI heifers when the feeding system changed from drylot to grazing. In addition to the microbiota compositions, only heifers with H-RFI had significant increases in 7 out of 10 major MetaCyc pathways predicted with bacterial sequences when the feeding system changed from drylot to grazing. Together with the correlation analysis of the rumen microbial taxa and predicted MetaCyc pathways, these results indicated that inefficient heifers had diverse rumen microbial communities than efficient heifers. This also suggested that L-RFI heifers have certain functions to maintain their efficient rumen microbiota (such as *Coprococcus* 1 genus) to keep their efficient microbial fermentation.

Although the present study has its advantages and novel aspects compared with previous studies, there were several limitations of the research conducted for this thesis. The six pairs of fungi primers (shown in Table 2.3) tested for the present thesis were not successful, so the amplicon sequencing of fungi group was excluded in the present study. This exclusion of the fungi limited our understanding of the eukaryotic group under grazing systems. More fungi primers should be tested in the future to fill this knowledge gap. Another limitation came from the database for 16S and 18S rRNA gene sequencing. In the present study, the group of unassigned archaea and protozoa actually had correlations with some or all predicted MetaCyc pathways. However, due to the limitation of the database, it was not easy to know which archaea and protozoa played important roles in the predicted MetaCyc pathways. In addition, the functions are only predicted based on amplicon sequences, future studies on metagenomics and/or metabolomics are needed. For the future work of the current study, validations of the feed efficiency related bacteria abundance using qPCR are necessary because the limitations of the database for taxonomic analysis may provide inaccurate information. An additional limitation

was that the RFI values of heifers under the grazing system were not tested. Although based on a few limited previous studies of RFI reranking and RFI repeatability, we speculate that the RFI differences in the present study would exist under different feeding system based on the advantages of experimental design, it would be more accurate if the RFI could be validated under grazing system in the future. Another limitation and future direction for the present study was that the 100% forage oat pasture in this thesis may not totally reflect the real grazing conditions with different vegetations on pasture. In reality, the differences in eating behavior between each individual cattle may also play an important role in feed efficiency and rumen microbiota.

In conclusion, the present study provided fundamental knowledge to understand the rumen microbiota of cattle under different feeding systems and support the development of potential methods to manipulate rumen microbiota to improve the feed efficiency of cattle. For example, beef producers may apply targeted grazing management strategy for cattle on pasture with low feed efficiency to manipulate their rumen microbiota and thus improve the feed efficiency of the whole herd.

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