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Changes in ruminal bacterial communities of beef cattle during high concentrated diet transition and experimental induced subacute acidosis

by

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ABSTRACT

Rapid dietary transition is a common practice in the nutritional management of feedlot cattle, which may lead to ruminal acidosis in some animals. Ruminal acidosis is a prevenient digestive disorder in livestock industry which significantly impacts animal health and production. To date, the understanding of the changes in the ruminal microbial community during diet transition or acidosis is very limited. In this study, we have investigated: 1) the epithelial tissue associated bacterial diversity changes in the rumen of beef cattle during dietary transition to high grain diets; and 2) variation of digesta and epithelium attached bacterial communities in the rumen of steers differing in susceptibility to subacute ruminal acidosis using culture-independent molecular based methods. PCR- denaturing gradient gel electrophoresis and quantitative real time PCR analysis of 24 beef heifers adapted from a diet containing 97% hay to the diet containing 8% hay over 29 days revealed that the epimural bacterial diversity from rapid grain adaptation heifers changed in response to the dietary transition. Similar analysis of bacterial profiles of rumen samples from acidosis-resistant and acidosis-susceptible steers showed that the diversity and density of digesta and epithelial attached bacterial communities are different between AS and AR animals.

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LIST OF ABBREVIATIONS

AR	Acidosis resistant
AS	Acidosis susceptible
ANOSIM	Analysis of similarity
CP	Crude protein
DGGE	Denaturing gradient gel electrophoresis
DMI	Dry matter intake
GC	Gas chromatography
MDS	Multi-dimensional scaling
NDF	Neutral detergent fiber
OM	Organic matter
PBS	Phosphate-buffered saline
PCA	Principle components analysis
PCR	Polymerase chain reaction
qRT-PCR	Quantitative real time polymerase chain reaction
SARA	Sub-acute ruminal acidosis
SCFA	Short-chain fatty acids, refers to both the dissociate and undissociated states
VFA	Volatile fatty acids

Chapter Ⅰ. Literature Review

1.0 Introduction

In Canada, there are about 13 million cows of which 34.3% is beef cattle, 10.8% is dairy cattle and 54.9% is calves (Statistics Canada 2010; Canada's Beef Industry Fast Facts, 2010), all of which significantly contributes to Canada's economy. For example, beef production contributed $23 billion to Canada's economy in 2009 (Canfax, Statistics Canada, 2009). In past decades, the population of both dairy cows and beef cattle have increased by 0.6% year-over-year due to the consistent increase in demand of meat and milk products. To improve the meat and milk production of cattle, the nutritional density of diets has been increased by feeding more concentrates and less forage (Plaizier et al., 2007). However, feeding highly concentrate diet can significantly affect rumen microbial ecosystem and ruminal fermentation, leading to reduced rumen buffer and higher concentration of fermented acids (Kleen et al., 2003; Stone, 2004; Rustomo et al., 2006a, b, c). This then leads to the depression of rumen pH and an increased occurrence of subacute ruminal acidosis (SARA) (Owens et al., 1998; Nagaraja and Titgemeyer, 2007). If the rumen pH continues to decrease, SARA may lead to acute acidosis which may, in the worst case, cause the cattle death (Britton et al., 1986; Goad et al., 1998). During acidosis, cows can experience diarrhea, weight loss, reduced milk production, and increased susceptibility to other metabolic disorders (Vasconcelos and Galyean, 2008).

It is known that rumen microbial symbiosis is vital for the uptake of nutrients by the cow. The microbes ferment plant materials to carbohydrates,

ammonia, and microbial proteins that are used by the host for energy metabolism (Annison and Bryden, 1998). In the meantime, the host supplies a niche for these microbes to colonize and grow. Therefore, the changes in rumen environment due to the feeding of high concentrate diet can alter the diversity and density of the microbes in the rumen, which may subsequently change the rumen functions. This chapter aims to summarize the current understanding of rumen microbial ecology in cattle and their relationship with SARA. Among rumen microorganisms, bacteria are the most investigated population and have significant effects on the animal's performance. Therefore, special emphasis will be given to the ecology of rumen bacterial communities and their adaptation to different diets as well as their association with SARA. In addition, the knowledge about ruminal epimural bacteria and recent applications of molecular based techniques to identify rumen microbes will also be summarized in this chapter.

1.1 Rumen fermentation and rumen microbes

Ruminants have four stomach compartments: rumen, reticulum, omasum and abomasum. The rumen is the first and largest compartment and it holds the feed which it is being digested by the symbiotic microbes. The feed is softened further in the reticulum and transformed into small wads of ingesta. Compared to the rumen, the reticulum has a more selective function; its role is to move the ingesta to the omasum or have it regurgitated. The third compartment, the omasum, serves as a 'filter' and it filters the large pieces of digesta back into the reticulum. The cud is then pressed and broken down further in the real stomach or

abomasum, where the protein is digested. The cells of the abomasum wall produce enzymes and hydrochloric acid which hydrolyse proteins in the feed. Hydrolysis breaks proteins into smaller sub-units such as dipeptides, having them ready for further digestion and absorption in the small intestine.

The rumen is the major fermentation vat when compared with the other three compartments. Besides being an anaerobic environment, the rumen also has to maintain an optimal temperature, a desirable pH, and an environment rich in sodium and with the adequate moisture level, aided by water and saliva (Caldwell and Hudson, 1974) because all of these are critical factors that impact the growth of rumen microbes and their activities. It is known that the rumen microbial community is comprised of bacteria, fungi, protozoa and archea. Among them, bacteria have the largest population, up to $10^{11\text{-}12}$ cells/g of rumen content (Russell, 2002; Li et al., 2009), and preferentially digest structural carbohydrates, non-structural carbohydrates, and protein. Protozoa are present in more than 10^6 cells/ml of rumen fluid (Sato et al., 2010), and derive their nutrients through phagocytosis of other microbes; they degrade and digest feed carbohydrates, especially starch, sugars, and proteins (Russell, 2002). Ruminal fungi counts reach only 1.8×10^4~10^5 zoospores/ml of rumen fluid (Khejornsart and Wanapat, 2010); nevertheless they occupy an important niche in the rumen because they hydrolyse the ester linkages between lignin and hemicellulose or cellulose, and help break down the digesta particles (Lowe et al., 1987; Srinivasan et al., 2001). Rumen archaea, present in approximately 1.8×10^6~10^7 cells/ml of rumen fluid

(Khejornsart and Wanapat, 2010), are mostly autotrophic methanogens and produce methane through anaerobic respiration (Kebreab et al., 2006).

Due to the fact that the bacteria population is the largest, and also because of their role in the production of volatile fatty acids (VFA), the following sections will mainly focus on rumen bacteria.

1.1.0 Ruminal bacteria

Based on their localization in the rumen, the bacterial community can be classified into three groups (Sadet et al., 2007): inhabiting in the rumen fluid; adhering to the feed particles; and attached to the rumen epithelium wall (defined as epimural bacteria) (Cheng et al., 1979; Cheng and Costerton, 1986).

1.1.1.1 Ruminal bacteria inhabiting the rumen digesta

To date, most of the researches have focused on the bacteria associated with rumen digesta, including those inhabiting in the rumen fluid and adhering to the feed particles. Early studies using culture based and microscopy methods showed that these bacteria were rods, cocci, or ovals, under the light microscopy (Bryant, 1959) with most of them being Gram positive and Gram negative bacteria (Stewart and Bryant, 1988). Using the amount of G + C in double-stranded chromosomal DNA as a criterion for differentiating bacteria, the G + C content of ruminal bacteria only ranges from 30 to 54% (Hobson, 1965). This was reported to be caused by the narrow temperature range of the rumen. Using the direct counting method, Krause and Russell (1996) estimated that the density of ruminal bacteria can reach up to 10^{10} cells per gram of ruminal content. In recent years,

ruminal bacteria have been re-evaluated with newer, more objective, and genetically valid methods of classification, like culture-independent molecular identification methods. Using a culture-independent based 16S rDNA clone library analysis, the major bacteria in the rumen content is low G+C Gram-positive bacteria that were over 50% of the total number following by the orders of Cytophaga-Flexibacter-Bacteroides, Proteobacteria and Spirochaetes, respectively (Tajima et al., 1999; Edward et al., 2004). Recent study using small subunit (SSU) rRNA sequence analysis further revealed that there are up to 722 phylotypes in the bovine rumen (Brulc et al., 2009). In addition, the rumen digesta associated bacteria have been also grouped based on their fermentation pathways. For example, based on their fermentation substrates, the rumen bacteria have been categorized into different groups, such as amylolytic, maltose-fermenting, glucose-fermenting, and lactic acid-fermenting bacteria.

1.1.1.2 Functions of rumen digesta associated bacteria

The digestion of the cellulose or fibre by rumen bacteria was not well understood until 1940s. Elsden (1945) performed the first study that identified that rumen bacteria digested the plant fibre and converted it to VFAs, including acetate, propionate and butyrate, the essential carbohydrates for host nutrition uptake. A following study by Gray et al. (1951) revealed that more VFAs such as valerate, isobutyrate and isovalerate were also present in the rumen. The VFAs are produced by the relevant bacteria (Table 1.1) via hydrolysing monosaccharides or disaccharides in the feed component. These products then

undergo glycolysis or other biochemical pathways, like lipid metabolism, to yield energy for their own growth. In addition, rumen bacteria have been reported to be able to digest not only cellulose but also other carbohydrates and nitrogen-containing compounds. For example, Hobson et al. (1981) identified and isolated *Streptococcus bovis*, *Lactobacillus* sp, *and Selenomonas rumitanium,* those are involved in starch digestion, and utilize amylase to carry out degradation; their end of product is lactate (Figure 1.1). When lactate is produced, it can serve as substrate for lactate utilizing bacteria such as *Anaerovibrio lipolytica*, *Fusobacterium necrophorum*, *Megasphaera elsdenii*, *Peptostreptococcus asaccharolyticus*, *S. ruminantium* ssp. *lactilytica*, *Propionibacterium acnes*, and *Veillonella parvula* who also grow in the rumen.

Table 1.1 Characteristics of predominant ruminal bacteria (*adapted from Ressull, 2002*)

Species	Products	Primary Niches
Fibrobacter succinogenes	S, F, A	CU
Ruminococcus albus	A, F, E	CU
Ruminococcus flavefaciens	S, F, A	CU
Butyrivibro fibrisolvens	B, F, L, A	CU, HCU, ST, PC, SU
Ruminobacter amylophilus	S, F, A	ST
Selenomonas ruminantium	L, A, P, B, H_2	SU, ST, L
Prevotella sp.	S, F, A, P	ST, HCU, PC, β-GL, PT
Succinomonas amylolytica	S, A, P	ST
Succinivibrio dextrinosolvens	S, F, A, L	MD
Streptococcus bovis	L, F, A, E	ST, SU
Eubacterium ruminantium	L, F, A, B	MD, SU
Megasphaera elsdenii	P, B, A, Br	L, MD, AA
Lachnospira multiparus	L, F, A	PC, SU
Anaerovibrio lipolytica	S, P, A	GY, L
Peptostereptococcus anaerobius	Br, A	PEP, AA
Clostridium aminophilum	A, B	AA, PEP
Clostridium sticklandii	A, B, P, Br	PEP, AA
Wolinella succinogenes	S	MAL, FUM

A, acetate; B, butyrate; P, propionate; F, formate; L, lactate; E, ethanol; S, succinate; Br, branched chain VFA; CU, cellulose; HCU, hemicelluloses; ST, starch; SU, sugars; MD, maltodextrins; AA, amino acids; GY, glycerol; PT, protein; PEP, peptides; PC, pectin; MAL, malate; FUM, fumarate; β-GL, β-glucans

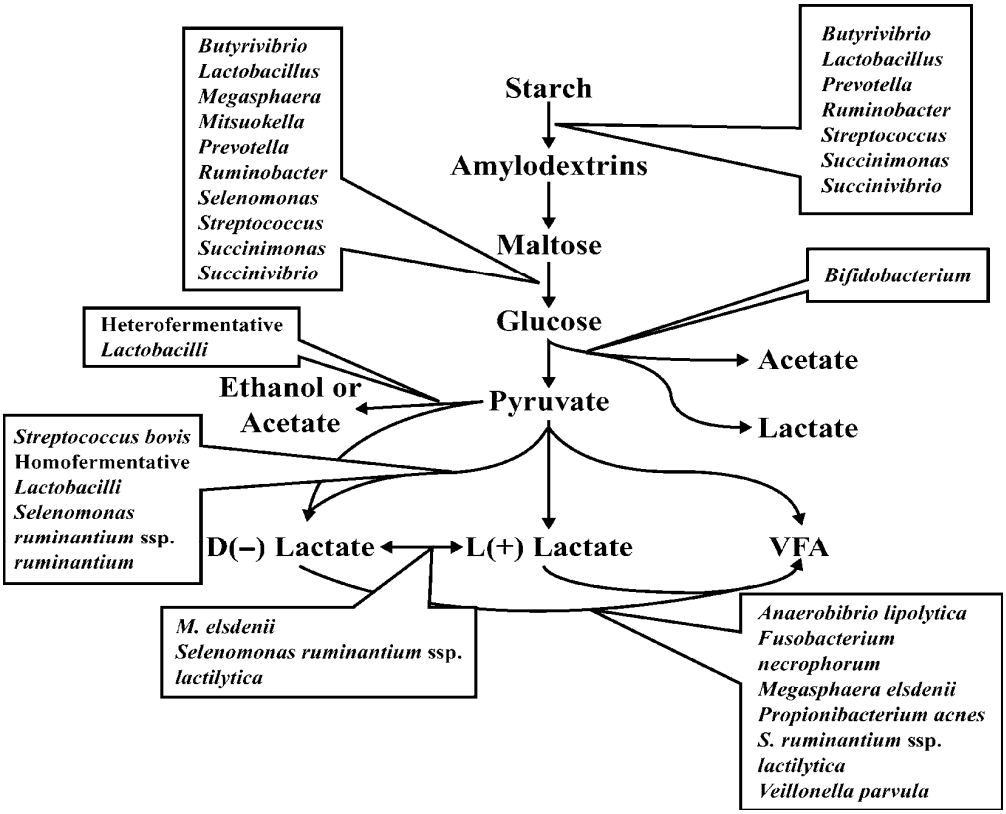

Figure 1.1 Different fermenting bacteria involved in starch fermentation to lactic acid and VFA in the rumen of grain-fed cattle **(*adapted from Nagaraja and Titgemeyer 2007*)**

Amino acids can also serve as substrates for ATP formation and bacterial growth in the rumen. Only certain rumen bacteria (e.g., *Prevotella* species) can ferment amino acids, but the ATP yield is low (Rychlik et al., 2002; Walker et al., 2005). They need to ferment more than 20 amino acids to gain enough energy to polymerize a single amino acid into protein (Russell, 2002). During microbial protein production, vitamin B acts as a coenzyme. The specialized bacteria in the rumen synthesize all eight vitamin B compounds, as well as vitamin K, inside their cells (Strobel, 1992; Nagaraja et al., 1997). Several studies have confirmed that vitamin B_{12} is an important growth factor for some ruminal microorganisms (Tanner and Wolfe, 1988; Strobel, 1992). Other microorganisms can synthesize it in pathways that produce propionate (Chen and Wolin, 1981), finally supplying these vitamins to the protozoa when the rumen bacteria are passed to the abomasum.

1.1.1.3 Factors that impact on the ecology of the rumen digesta associated bacteria

Many factors have been reported to have an effect on the rumen microbial community, among which are: changes in diet (Tajima et al., 2001), age of the animal, antibiotic usage (Kleen et al., 2003), health of the host animal (Kleen et al., 2003; Rustomo et al., 2006a), geographical location and season (Bryant, 1959; Hungate, 1966) and feeding regimen (Rustomo et al., 2006c). Amongst these factors, the effects of diet on the changes of rumen microbial community have been the most studied.

Commonly, the diets used to feed beef cattle contain the following components: hay/forage (grass or legume) and grain (corn, barley, wheat, oats and sorghum). To achieve a high level of productivity, one strategy is to feed animals with highly fermentable (high grain) diets, to increase VFAs production in the rumen (Penner et al., 2010), thereby increasing the total metabolizable energy for the animal. When the diet is switched from the hay based diet to the grain based diet, the quantity of the fibrolytic bacterium, *F. succinogenes* falls 20-fold on the third day and 57-fold on day 28 after the switching (Tajima et al., 2001). Once the number of this bacterium is low, large amounts of fibre or cellulose accumulate in the rumen resulting in reduced rate of utilization of the pentoses (Strobel, 1993).

Rumen pH is another factor that can also impact the bacterial growth (Russell and Dombrowski, 1980; Hoover, 1986; Grant and Mertens, 1992; Allen et al., 2006), especially for the ruminal cellulolytic bacteria which are known to cease growth when pH value drops below 6.0 (Russell and Dombrowski, 1980; Shi and Weimer, 1992; Weimer, 1993). When bacteria are exposed to a low rumen pH, their ability to bind cellulose, or catabolism of hydrolytic products, is reduced or inhibited, and they are unable to survive. This could in turn lead to changes in the population of sugar and cellodextrin-fermenting bacteria that could utilize products of enzymatic hydrolysis of fiber (Nagaraja and Titgemeyer, 2007). Supporting this statement, Brown et al. (2006) reported that, when the ruminal pH dropped to 5.8, the population of *R. flavefaciens* (cellulolytic) and *B. fibrosolvens* declined rapidly, while the population of *S. bovis* increased 100-fold within the first 24 h. Based on above results, they suggested that when the pH is reduced, the

populations of bacteria rather than the bacterial diversity are changed. A recent study showed that bacterial diversity remains quite stable at different ruminal pH (Palmonari et al., 2010).

There are limited studies that investigate the effects of geographical location, and season on rumen microbial community. No difference was detected on counts of bacteria from rumen contents of deer feeding in their natural habitat that could be related to location, sex, or age of the animals (Pearson, 1969). In contrast to the previous finding, the enhanced digestibility of poor-quality feeds, reduced rate of feed passage within the digestive tract, and increased recycling of nitrogen observed in North American buffalo when compared to Asian water buffalo, suggested that geographic location might have an impact on the rumen microbiota (McAllister, 2009). Research by Orpin et al. (1985) indicated that rumen bacterial concentrations in the rumen of Svalbard reindeer could be affected by the change of season. During the summer months, the estimated rumen bacterial concentration was 2.1×10^{10}/g, while in the winter season it decreased by more than 20% (Orpin et al., 1985).

In conclusion, many evidences have shown that various factors, including those discussed above, can alter or disrupt the normal bacterial diversity and density in the rumen. Therefore, to improve animal production by altering the rumen function, it is essential to first understand what species there are, what they are doing, and how they can respond to various factors.

1.1.2.1 The bacteria attached to the ruminal epithelium (epimural bacteria)

The internal surface of the rumen is covered with small flattened nipple or fingerlike projections-papillae of the ruminal epithelium surface. The important function of this extended surface area is to facilitate the absorption of fermentation products, especially VFAs. An early study using microscopy showed the bacterial population attached to this surface (Cheng et al., 1979). However, the understanding of the ecology and the function of such population is very limited.

The existence of the bacteria associated with the rumen epithelial surface of sheep was demonstrated by Bauchop et al. (1975) using scanning electron microscopy. They found that most of the bacteria were on the dorsal, caudal, and lateral surfaces of the rumen wall, and that the densest populations were on the top of the dorsal rumen and on the bottom of the caudodorsal blind sac. Mueller et al. (1984) identified the diversity of the epimural community succession in young lambs and the species *Lactobacilluis ruminus, Clostridiurm ramosurm, Butyrivibrio fibrisolvens, Ruminococcus albus, Streptococcus* sp., *Bacteroides* sp., *Succinivibrio dextrinosolvens, Acidaminococcus* sp., *Streptococcus bovis,* and *Ruminococcus flavefaciens* were present at different ages. The first study on bovine epithelial wall associated bacteria was carried out by Tamate et al. in 1971, and it revealed similar colonization patterns in the rumen of cattle to those of sheep. Following these studies and still using cultured based methods and microscopy, McCowan et al. (1978) and Cheng et al. (1979 and 1980) provided further knowledge when they found that the bacteria attached to the rumen wall of cattle were taxonomically distinct from those in the rumen fluid and/or rumen

solid particles. They reported that the bacteria attached to the rumen wall of cattle included Micrococcus, Staphylococcus, Streptococcus, Corynebacterium, Lactobacillus, Fusobacterium, Propionibacterium and other unidentified anaerobic species.

Recently developed molecular based techniques, such as PCR-DGGE (Sadet et al., 2007; 2010) and 16S rRNA sequence analysis (Mitsumori et al., 2002; Cho et al., 2006; Pei et al., 2010) (See the following section for details), have been applied to identify the microbial diversity associated with rumen epithelial tissue. These studies have confirmed that the profiles of the microbes attached to the ruminal tissue are different from those found in the ruminal digesta.

Although many factors have been found to affect the bacterial diversity and bacterial population associated with digesta, very little is known with respect to the factors that can affect the bacteria attached to the epithelial tissue wall. Recent research on ovine epimural bacterial diversity under different diets, by Sadet et al. (2007 and 2010), indicated that bacterial diversity tended to be different between high concentrate and high forage diet; while sampling site did not appear to have an effect. Up to date, it is not known whether diet has an impact on epimural bacteria of the bovine rumen.

Although bacterial diversity and density under different diets have been compared, the population of bovine epimural bacteria has not yet been accurately defined. Early study showed that the counts of epimural bacteria in hay-fed sheep range from 4.4×10^7 to 2.2×10^8 per g of wet tissue weight (Wallace et al. 1979). However the count was 1.4 ~1.8 x 10^7 CFU/cm^2 of tissue surface of the epimural

community on rumen epithelial tissue in young lambs (Mueller et al., 1984). In cattle, it is estimated that approximately 1 to 2% of the total bacterial population is in the rumen (Russell et al., 2002), while the density of such bacteria has not been reported.

1.1.2.2 The predicted function of epimural bacteria

It is believed that the epimural bacteria are involved in oxygen scavenging (Cheng et al., 1979), hydrolysis of urea entering the rumen across the wall (Fay et al., 1979; Wallace et al., 1979), and tissue recycling (McCowan et al., 1978). Moreover, Mitsumori et al. (2002) detected Nitrosomonas from the rumen epithelium and suggested the possibility that the bacterium oxidizes ammonia and methane on the rumen surface. These functions are distinct from those of the bacteria in the rumen content, suggesting that the epimural bacteria may play a different role during rumen fermentation. Due to the intimate contact between animal tissue and epimural bacteria, they also play some role in host-microbial interactions since they also have barrier functions, such as biofilm formation (Macfarlane and Dillon, 2007).

1.2 Rumen subacute acidosis

1.2.1 The identification of acidosis

Following the medical definition of acidosis by Stedman (1982): acidosis is a decrease in the alkali (base excess) in body fluids relative to the acid (hydrogen ion) content. The biological definition is “biochemical and physiological stresses

caused by rapid production and absorption of ruminal organic acids and endotoxins" (Britton and Stock, 1986). Ruminal acidosis is a common "digestive disorder" in dairy cows and beef cattle. Two types of acidosis have been defined and are widely accepted by researchers and industry: acute and subacute acidosis. Rumen epithelium damage and barrier function failure are the phenomena observed when the acidosis occurs (Nagaraja and Titgemeyer, 2007; Khafipour et al., 2009a; Penner et al., 2010). Acute acidosis, defined as a rumen pH between 4.0 and 5.0 with lactic acid accumulation, is less common but more severe. Subacute ruminal acidosis (SARA) is more common in feedlot cattle (Huntington, 1988; Cooper and Klopfenstein, 1996; Owens et al., 1998). SARA can cause epithelial parakeratosis (Kleen et al., 2003), rumenitis (Enemark, 2008) and greater histamine absorption from the rumen, due to high rumen histamine concentrations and increased permeability of the epithelial membrane. The consequent increase in plasma histamine concentrations can lead to bronchial constriction and cardiovascular disorders (Plaizier et al., 2008).

The engorgement of large amounts of starch or other rapidly fermented carbohydrates has been considered to be the cause of acidosis (Britton and Stock, 1987; Owens et al. 1998). High concentrate diet (Gill et al., 2000), excess feed intake or starch-rich supplement (Owens et al., 1998) can cause an overload of starch or carbohydrate in ruminants. These substrates can all influence the salivary secretion, limiting the rumen buffering ability, which can imbalance the pH when the animals consume high grain diets. Feeding high grain diet is a common practice in the dairy and beef industries, since it can increase VFAs

production which supplies higher energy for milk or meat production compared to those fed high forage diet. However, feeding highly fermentable diets may increase the severity of acidosis in dairy cattle (Oba et al., 2010). When cattle are fed a high concentrate diet or are rapidly switched to grain diet, the incidence of acidosis increases compared to hay based diets (Gill et al., 2000). To prevent ruminal acidosis during the production period, Penner et al. (2007 and 2010) recommended that when high fermentability diets are going to be used, the transition must be gradual, going first from low to moderate and then to high grain.

A pH decrease has been commonly used as an indicator for ruminal acidosis and pH of 5.8 is widely accepted as the threshold of subacute ruminal acidosis (Beauchemin et al., 2001; Ghorbani et al., 2002; Koenig et al., 2002). When rumen pH is lower than 6.0, the cellulolytic ruminal bacteria cannot survive (Russell and Wilson, 1996), causing a decrease in fibre digestion and feed efficiency. Since values of pH change constantly, recent studies have applied parameters such as duration (h/d) and area (pH × min) below the pH threshold of 5.8, to define acidosis or SARA (Oba and Allen, 2000; 2003) together with the pH value. Some researchers have used other thresholds to define SARA. For example, Khafipour et al. (2009b) used duration of rumen pH below 5.6 for above 180 min/d as the threshold for SARA. The pH below the norm (<5.6) already has significant impact not only on microbial activity, but also on rumen function and animal productivity and health (Nagaraja and Titgemeyer, 2007).

1.2.2 SARA and VFAs

Lactate accumulation has been observed during acute acidosis (Krause and Oetzel, 2006; Stone, 2004), while during SARA the depression in ruminal pH is largely caused by the increase of SCFA concentration without a concomitant increase in lactate concentration (Oetzel et al., 1999). Normally, lactate is present in the digestive tract at low concentrations, but it can accumulate when the carbohydrate supply is increased abruptly (i.e., following grain engorgement or during adaptation to high-concentrate diets) (Owens et al., 1998). This was first described by Mackie et al. (1984) in an *in vivo* study. In their study, the ruminal metabolism of lactic acid was investigated under normal feeding conditions in four sheep each adapted to one of the following diets: high-concentrate, intermediate, high-roughage containing 65, 43 or 10% maize meal and molasses respectively, or Lucerne hay. The turnover of ruminal lactate was found to increase 10- to 40-fold immediately after feeding. They also indicated that approximately 8, 6.5, 5 and 2.5% of the total VFA were formed through lactate on the high-concentrate, intermediate, high-roughage and Lucerne hay diets, respectively. They suggested that diet can not only have an impact on lactic acid concentration, but also on the concentration of total VFA.

Comparing acidity in the rumen, lactic acid is about 10 times stronger than VFAs (pKa 3.9 vs. 4.9) (Nagaraja and Titgemeyer, 2007), meaning that the higher the accumulation of lactic acid in the rumen, the more the enrichment of protons which increase the probability of acidosis. Thus, the lactate and VFA utilization and absorption are critical to prevent their accumulation in the rumen, leading to a

reduced probability of acidosis. Recently, sheep with a greater capacity for apical uptake of SCFA were found, and they were less susceptible to SARA (Penner et al., 2009b), thus suggesting that the enhanced transepithelial transport of SCFAs may balance the rumen pH. The advantage of rapid absorption may be associated with a shift in microbial populations towards lactic acid production, which will further reduce ruminal pH.

1.2.3 Effects of SARA on microbial profiles

Bacterial changes associated with SARA primarily include shifts in the populations of starch- and soluble sugar-fermenting bacteria (amylolytic, maltose-, and glucose-fermenting bacteria), and lactic acid producing bacteria (Owens et al., 1998). A significant increase in the population of ruminal lactobacilli is a common feature of both acute and subacute acidosis (Slyter, 1976; Nagaraja and Miller, 1989; Goad et al., 1998). Since Lactobacilli can grow under low pH, they are considered to have an important role on the pH decrease (Owens et al., 1998; Asanuma and Hino, 2002; Nagaraja and Titgemeyer, 2007) because of their capacity to be acid-tolerant and extrude H^+ at acidic pH (Booth, 1985). *In vitro* studies showed that only these bacteria survived when the pH dropped to near 5.0 or below for a sustained period while most other microbes (methanogenic, cellulolytic bacteria, protozoa then lactic acid utilisers) were not able to survive under such conditions (Slyter, 1976; Nagaraja and Miller, 1989; Goad et al., 1998). In addition, Lactobacilli can change their fermentation pathways from

fermenting glucose to lactate or biosynthesis of VFA (Asanuma and Hino, 1997; Russell and Hino, 1985).

Besides the Lactobacilli, some other bacterial species have also been reported to be associated with SARA. *Streptococcus bovis*, found by Wojcicchowicz and Ziolccki (1984), can also produce lactic acid and grow very fast. When conditions are favourable, populations of this species can double comparing to those of *E.coli in vivo* (Russell and Robinson, 1984). In addition, the quorum sensing signal system of this species can monitor its population density when pH decreases. Asanuma et al. (2004) demonstrated that this species has the *luxS* gene that encodes for an autoinducer-2, an interspecies quorum sensing system. Although its transcription was not directly related to cell density in pure culture, it is believed that the autoinducer-2 activity may act as a signal for adjusting cell physiology and metabolism in response to ruminal conditions (Asanuma et al., 2004). In addition, *S. bovis* is considered to be the major etiologic agent of acute acidosis. The intervention strategies for prevention of SARA or acidosis, such as antibiotics and vaccines, are often targeted at controlling the growth of this species in the rumen (Nagaraja and Miller, 1989; Gill et al., 2000).

In addition, rumen bacteria *Anaerovibrio lipolytica*, *Fusobacterium necrophorum*, *Megasphaera elsdenii*, *Peptostreptococcus asaccharolyticus*, *Selenomonas ruminantium* ssp. *lactilytica*, *Propionibacterium acnes*, and *Veillonella parvula* are lactic acid utilizing bacteria (Nagaraja and Titgemeyer, 2007). Not all of them are found to have increased population in SARA except *M.*

elsdenii and *S. ruminantium* ssp. *lactilytica* (Goad et al., 1998). *Selenomonas ruminantium*is is less active than *M. elsdenii* as lactate utilizers, because the lactic acid dehydrogenase is suppressed by glucose in *S. ruminantium* (Asanuma and Hino, 2005), but not in *M. elsdenii* (Hino and Kuroda, 1993). *Fusobacterium necrophorum* has garnered considerable attention because of its importance as the primary cause of liver abscesses in cattle (Nagaraja and Chengappa, 1998). Although ruminal acidity may kill *Fusobacterium* in ruminal contents, some ruminal wall associated *Fusobacterium* can survive under acidity (Narayanan et al., 1997) and can be translocated to the liver to cause liver problems (Tadepallia et al., 2009) when acidosis occurs.

In summary, during SARA, the diversity and density of rumen microbes can be changed. To date, there are more than 400 species of rumen microbes demonstrated. The interactions among different groups of bacteria and how these change in response to highly fermentable diets, as well as their associations with SARA are not clear.

1.2.4 SARA and animal health

During acidosis, bacteria are reported to produce toxins including ethanol, methanol, histamine, tyramine, and endotoxins which can result in host tissue damage or disease (Irwin et al., 1979; Aschenbach et al., 1998; Bruewer et al., 2003). For example, histamine produced by *Allisonella histaminiformans* (Garner et al., 2002) has long been suspected to be related to the onset of laminitis (Nocek, 1997). Biological amines are reported to attribute the initiation of the immune

response and disruption of barrier function (Nagaraja et al., 2005; Plaizier et al., 2008). Ruminal lipopolysaccharide (LPS), the endotoxin produced by gram negative bacteria (Andersen et al., 1994; Gozho et al., 2005) can cause a systemic immune or inflammatory response (Nagaraja and Titgemeyer, 2007; Khafipour et al., 2009a) during their translocation into the portal blood stream. However, a recent study by Khafipour et al. (2009a) showed that LPS can cause inflammation depending on diets. In this study, no inflammatory response was observed under both grain- and alfalfa pellet-induced SARA; only in grain-fed animals.

Incidence of SARA can also have other detrimental health effects. Rapid adaptation to a high concentrate diet, which decreases ruminal pH, can cause rumen thiamanase, which breaks down thiamin to levels where polioencaphalomalacia, a disorder of the forebrain, can occur (Brent, 1976). Also, SARA may affect gelatinoproteinases, causing an elongation of collagen fibre, ultimately making the third phalanx in the hoof more mobile (Cook et al., 2004). This makes cows more susceptible to claw horn lesions and laminitis.

1.2.5 SARA Prevention strategies

Management and feed additives are the major SARA prevention strategies. Management strategies include variation in feed intake, dietary roughage amount and source, dietary grain amount and processing, and step-up regimen (Allen et al., 2006; Nagaraja and Titgemeyer, 2007; Khafipour et al., 2009b). Some management has already been established and applied in the present dairy farms. Gradual transition from diets with low to moderate fermentability to those with

high fermentability has been proposed as one of the improved management approaches to minimize the risk for digestive disorders, especially ruminal acidosis (Bevans et al., 2005; Penner et al., 2007; Steele et al., 2009a). Feed intake patterns of cattle managed in a feedlot setting need further study, as this would provide a baseline for understanding the variability in intake that can trigger acidosis (Galyean and Eng, 1998; Schwartzkopf-Genswein et al., 2003).

Buffers (bicarbonate and sesquicarbonate), ionophores, and antibiotics are three common feed additives that have been applied to prevent acidosis. Feeding ionophores can reduce lactate production *in vitro* and *in vivo* (Newbold and Wallace, 1988; Bauer et al., 1992; Syntex, 1994), either through inhibition of lactate-producing bacteria or reduction of the meal size. The antibiotic tetronasin has been reported to control the *S. bovis* population, which may prevent acidosis, (Owens et al., 1998). Although antibiotics can maintain the productivity and feed efficiency, the use of chemicals and antibiotics in modern livestock agriculture is strictly limited by the concern of developing antibiotic resistant bacterial strains from livestock species. Therefore the probiotic approach, the use of live microbes as inocula, may have potential to manipulate the rumen ecosystem. One successful instance of the microbial inocula procedure involved using *Synergistes jonesii* to protect cattle feeding on *Leucaena leucocephala* from mimosine poisoning (Klieve et al., 2002), and demonstrates that it may be a practical solution in the future.

1.3 Rumen bacterial identification using molecular techniques

Earlier knowledge of rumen microbial diversity and ecology was largely based on classical anaerobic culture techniques and phenotypic characterization of cultural isolates, as well as light and electron microscopic examination. However, only 10-15% of rumen microbes have been reported to be culturable (Kobayashi et al., 2000). Recently developed molecular based methods, predominantly based on the analysis of 16S rRNA genes, have allowed the identification of the unkown and unculturable bacterial species in the rumen. Such approaches can help to describe the bacterial composition independent of isolating, maintaining, and propagating bacteria under laboratory conditions. These techniques include in situ hybridisation (Stahl et al., 1988; Lin et al., 1994; Forster et al., 1997), restriction fragment length polymorphism (RFLP) analysis (Wood et al., 1998), competitive polymerase chain reaction (cPCR) (Reilly and Attwood, 1998; Kobayashi et al., 2000; Koike and Kobayashi, 2001), denaturing gradient gel electrophoresis (DGGE) (Kocherginskaya et al., 2001; Guan et al., 2003, 2008; Li et al., 2009), analysis of 16S rRNA libraries generated by PCR amplification (Whitford et al., 1998; Edward et al., 2004; Tajima et al., 1999; 2000; 2001a; b), and recently developed next generation sequencing technology (Schuster, 2008).

1.3.1 PCR-Denaturing gradient gel electrophoresis (DGGE)

PCR-DGGE is a molecular fingerprinting method that separates the same length of polymerase chain reaction (PCR)-generated DNA fragments with different sequences on a polyacrylamide gel with denaturing gradients. Differing

sequences of DNA will migrate at different locations on the gel with a single band representing a different bacterial population in the community (Muyzer et al., 1993). The similarity of generated profiles can be assessed to investigate microbial structural differences (Muyzer et al., 1993; Edward et al., 2005). This method has been used widely to analyze the ruminal bacterial structure changes in digesta or fluid associated population. Larue et al. (2005) compared the bacterial PCR-DGGE profiles in the rumen content of sheep under two different diets (orchard grass hay diet and hay mixed with corn diet), and revealed that dietary factors influence bacterial community structure. In addition, they indicated PCR-DGGE was useful for detection of gross differences in bacterial diversity attributable to exogenous (dietary) and endogenous (host derived) parameters, which were supported by Karnati et al. (2007) by detecting altered protozoan PCR-DGGE profiles under different diets. Furthermore, Li et al. (2009) presented the effect of location and time of sample collection on the assessment of bacterial diversity in the rumen using PCR-DGGE analysis and it successfully showed that the distribution of detectable bacteria in the rumen was relatively stable among different locations within the rumen over time, and that the microbial taxonomy varied by host animals to a greater extent than by sampling location or sampling time. In addition, a recent study by Hernandez-Sanabria et al. (2010) revealed the potentials to link the PCR-DGGE patterns to host phenotypes. Therefore, the PCR-DGGE banding patterns can be considered to be representative of the dominant bacterial groups and applied to screen changes of dominant species in

the microflora of rumen and to link them to phenotypic characteristics of the host, specifically to feed efficiency.

To date, not many studies have been performed on the diversity changes of bacteria attached to the rumen epithelial wall of cattle.

1.3.2 Sequencing analysis of 16S rRNA gene libraries

Although PCR-DGGE can directly compare the predominant microbial profiles from a population of animals, the bands need to be cloned and sequenced for taxonomic identification. Therefore, the direct sequencing analysis of 16S rRNA gene clone libraries provides direct taxonomy identification. Many studies have applied this method to study rumen microbial community at taxonomic levels (Whitford et al., 1998; Tajima et al., 1999; Edward et al., 2004). For example, up to 20 novel Gram-positive bacteria and six previously uncharacterized groups of Gram-negative bacteria were identified in the rumen fluid from mature Holstein dairy cows (Whitford et al., 1998). A high degree of genetic diversity was detected between bacterial populations attached to the plant particles and rumen fluid in the rumen of matured Holstein dairy cows (Tajima et al. 1999). In the library from the rumen fluid, the sequences were indentified as: low G+C Gram-positive bacteria (52.4%), Cytophaga-Flexibacter-Bacteroides (CFB) (38.1%), Proteobacteria (4.7%), Spirochaetes (2.4%) and unknown species (2.4%), while the vast majority of sequences from the rumen solids were found to be related to low G+C Gram-positive bacteria (71.4%) and the remaining sequences were placed within the CFB (26.2%) and Spirochaetes (2.4%) phyla.

This method was also used to investigate phylogenetic changes of ruminal bacteria in the rumen content under different diets, and all these studies demonstrated that dietary factors influence bacterial community structure (Tajima et al., 2000; 2001a; Sadet et al., 2010). Moreover, this method was also used to investigate other microbial communities in the rumen. Tajima et al. (2001b) investigated the sequences from archaeal 16S rRNA libraries from the rumen and suggested the existence of a novel group of archaea which were associated with known methanogens. A recent study by Sadet et al. (2010) has characterized the epimural bacterial community using this method and found that the sequences from a concentrate-rich diet were different from those obtained from a forage diet. This suggests the composition of the bacterial epimural community may be affected by diets.

1.3.3 Quantitative real-time PCR (qRT-PCR)

qRT-PCR is a method that can measure the copy numbers of DNA in real time. Compared to direct cell counts and culture based measurement, the culture-independent qRT-PCR analysis allows the direct measurement of the copy number of a targeted microbial gene, including those from unculturable or unidentified species. It can also be applied to detected group-specific or species specific rumen bacteria by measuring the abundance of each targeted taxon using taxon-specific and eubacterial domain specific primers.

A recent study by Li et al. (2009) showed that copy numbers of the 16S rRNA gene of total bacteria in the rumen content of dairy cows are around

$5.2\sim9.5\times10^9$ copy number/g rumen digesta using a universal bacteria primer. This study also used group-specific primers to estimate the copy number of eight species including *Fibrobacter succinogenes, Ruminococcus albus, Ruminococcus flavefaciens, Butyrivibrio fibrisolvens, Eubacterium ruminantium, Prevotella bryantii, Selenomonas ruminantium* and *Streptococcus bovis* in the same rumen content samples, indicating that this method is a powerful tool to quantify ruminal bacteria. qRT-PCR can be more sensitive when detecting species of low abundance compared to other molecular based techniques. Stevenson and co-worker (2007) quantified three *Prevotella* species and ten non-*Prevotella* species in the bovine rumen and found that *Prevetella* is the predominant genus in the rumen digesta. Their data suggest that the aggregate abundance of the most intensively studied ruminal bacterial species is relatively low and a large fraction of the uncultured population represents a single bacterial genus.

Despite its advantages, qRT-PCR, like all molecular techniques, is subject to certain artifacts. To date, the qRT-PCR analysis of bacterial populations is based on targeting the 16S rRNA gene, and the bacterial population obtained from measurement of the copy numbers of the 16S rRNA gene may be overestimated. The 16S rRNA gene has been reported to have multiple heterogeneous copies within a genome (Case et al., 2007; Crosby and Criddle, 2003; Dahllöf et al., 2000). Case et al. (2007) have shown that 460 copies of 16S rRNA gene were recovered from 111 bacterial genomes, giving an average of 4.2 copies per genome. The recent studies showed that other housekeeping genes such as *ropB* can be used to replace 16S rRNA gene (Case et al., 2007; Küpfer et al., 2006;

Santos and Ochman, 2004; Weimer et al., 2010). Future studies seeking other target genes are necessary to improve the precision of this method for measuring the bacterial population in the rumen.

1.3.4 Metagenomics

Recently developed next generation sequencing technology has led to high resolution when generating sequences of the microbial community from an environment, and can be used to predict the ecology and function of the microbial community. Metagenomics has applied this technology to study the total DNA information of a microbiome. To date, this method has been widely applied to study human gut microbiomes and their association with human health related problems such as obesity (Turnbaugh et al., 2006; 2009), metabolic disease (Manichanh et al., 2006; Zhang et al., 2009), and diabetes (Larsen et al., 2010).

Metagenomics has just started to be applied to study the rumen microbiome. A recent study by Brulc et al. (2009) compared 3,617 sequences of 16S rRNA genes from rumen fluid and solid associated samples from three 5-yr old Angus Simmental Cross steers, and revealed that even though three animals were fed the same diet, the community structure, predicted phylotype, and metabolic potentials in the rumen were markedly different with respect to nutrient utilization. Hess et al. (2011) generated 268 gigabases of metagenomic DNA from microbes attached to plant fiber in cow rumen, identifying 27,755 putative carbohydrate-active genes and 54 candidate proteins for enzymatic activity against cellulosic substrates. These data sets provide a substantially expanded catalogue of genes participating in the deconstruction of cellulosic biomass. In

addition, Khafipour and co-worker (2009) have applied pyrosequencing to compare the phylogenetic changes of the rumen microbiome during experimentally induced SARA and found a decline in gram-negative Bacteroidetes in cattle. However, the proportion of Bacteroidetes was greater in alfalfa pellet-induced SARA than that in mild or severe grain-induced SARA (35.4% versus 26.0% and 16.6%, respectively). *Streptococcus bovis* and *Escherichia coli* were dominant in severe grain-induced SARA cattle, whereas *Megasphaera elsdenii* and *Prevotella albensis* were dominant in mild grain-induced SARA and alfalfa pellet-induced SARA cattle, respectively. Future studies to apply this technique to study rumen microbiome changes under various factors such as diet, antibiotic use and stress may lead to better management of cows and cattle production by improving their rumen functions.

1.4 Summary

Although many studies have been performed to study rumen microbes, the diversity of rumen bacteria has not been well identified due to the existence of uncultivated and/or unknown species. These species may play very important roles in rumen functions. Many factors can impact the ecology of rumen bacteria associated with the digesta (rumen fluid and solid particles). However, how these factors impact the epimural bacteria is unknown. There is currently a paucity of knowledge on the ecology of rumen epimural bacteria and how this community responds to changes in diet and feeding management.

SARA is a common health related problem for ruminants when they are fed with high concentrate diets. Although species such as *Lactobacillus sp.* and *Streptococcus bovis* have been found to be associated with pH decrease, the bacterial diversity and density of rumen microbial communities, including digesta associated and epithelial attached populations, during SARA has not been well studied.

Our hypothesis is that the diversity and density of epimural bacteria in the rumen of beef cattle can be impacted by diet transition or subacute acidosis. The objectives of further studies are: 1) to evaluate the diversity and density of the epimural bacteria during dietary transition from forage based diet to high grain based diet; 2) evaluate the diversity and density of the epimural bacteria as well as rumen digesta associated bacteria in the rumen of acidosis resistant and acidosis susceptible steers; 3) investigate the potential relationships between the ecology of bacteria and fermentative characteristics during diet transition and experimental induced SARA.

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Chapter Ⅱ. The Epithelial Tissue Associated Bacterial Diversity Changes in the Rumen of Beef Cattle during Dietary Transition to High Grain Diets[1]

2.0 Introduction

The ruminal bacteria digest complex and simple carbohydrates in the rumen and produce nutrients such as volatile fatty acids (VFA), microbial protein, and vitamins for the host. The rumen bacteria have been classified into three groups based upon their location of colonization within the rumen: those associated with liquid, attached to solid particles, and attached to the ruminal epithelium (defined as epimural bacteria) (Cheng and Costerton, 1986). To date, most studies have focused on the bacteria attached to solid particles and associated with liquid (Hungate, 1966; Stewart et al., 1997; Sadet et al., 2007; Li et al., 2009). These studies have revealed that the bacterial diversity in ruminal contents is highly responsive to the changes in diet, age, and antibiotic use with the health status of the host, geographical location, and season further contributing to variation in diversity (Stewart et al., 1997).

While only accounting for approximately 1 to 2% of the total bacterial population in the rumen (Russell et al., 2002), it has been suggested that epimural bacteria carry-out essential roles in oxygen scavenging (Cheng et al., 1979a), urea hydrolysis (Fay et al., 1979; Wallace et al., 1979), and tissue recycling

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(McCowan et al., 1978). As epimural bacteria are directly attached to the epithelial lining, they may also be involved in host-microbial interactions and they may have a role in barrier function for rumen tissue which is exposed to various fermented products. The epimural bacteria were reported to be taxonomically distinct from those in the rumen fluid or attached to the solid bacteria in dairy cattle (Cho et al., 2006; McCowan et al., 1978) and sheep (Cheng et al., 1979b) using culture based techniques. Recent studies using culture-independent techniques have also confirmed that the epimural bacterial community is distinctly different from the liquid and particle associated materials (Mitsumori et al., 2002; Cho et al., 2006; Sadet et al., 2007; 2010). However, the ecology of the epimural bacteria and whether diet affects the diversity of this population in the rumen of beef cattle has not been studied.

Rapid dietary transition from a high-forage diet to a high-grain diet is common practice in the nutritional management of feedlot cattle. It is known that changing the proportion of forage and concentrate in diets affects ruminal fermentation characteristics such as volatile fatty acid (VFA) and ruminal pH (Schwartzkopf-Genswein et al., 2003) and the known effects of changes in ruminal pH and VFA concentrations on microbial activity have been documented (Nagaraja and Titgemeyer, 2007; Russell, 2002). For example, low pH (< 6.0) has negative effects on fibrolytic bacteria in the rumen and the population of amylolytic bacteria decreases as pH continues to decline (Martin et al., 2002; Nagaraja and Titgemeyer, 2007). In the current study, we hypothesized that diet affects the diversity and population of the ruminal epimural bacteria. Therefore

we evaluated the diversity and density of rumen epimural bacteria from beef heifers (n = 18) while transitioned from a high-forage to a high-grain diet in comparison to heifers (n = 6) fed the high-forage diet throughout the study. The diversity and density of rumen epimural bacteria were investigated using PCR-denaturing gradient gel electrophoresis (PCR-DGGE) and quantitative real time PCR (qRT-PCR) analysis. In addition, correlation analysis was used to evaluate the association between the diets and/or fermentation characteristics including the molar proportion of VFA and ruminal pH and the epimural bacteria diversity and population.

2.1 Materials and methods

2.1.1 Animals and sampling. Twenty-four ruminally cannulated beef heifers (about eight months old, weighing 244 kg to 369 kg) were cared for in the Laired McElroy environmental and metabolic centre at the University of Alberta. Animal care and use were followed the guidelines of the Canadian Council on Animal Care (2009). The animal experiment protocol was pre-approved by the University of Alberta Animal Care and Use Committee for Livestock (Protocol number OBA077).

Heifers were randomly assigned to either the control (CON; n = 6) or a rapid grain transition treatment (RGA; n = 18) through a 29-day experiment period. Heifers in the RGA group were initially fed a diet containing 97% hay (d 1 to 4), and transitioned to a final diet containing 8% hay using the following intermediate diets; 60%-hay (d 5 to 8), 40%-hay (d 9 to 12), 25%-hay (d 13 to 16), 15%-hay (d 17 to 20), and 8%-hay (d 21 to 29). Heifers assigned to the CON

group were fed the 97%-hay diet throughout the experiment. Detailed descriptions of the dietary ingredients, chemical composition, and rapid transition protocol are presented in Table 2.1.

Ruminal papillae were biopsied when heifers were fed 97%-hay at 14:00 pm on the d 3 (3rd d of a 4-d feeding period), 25%-hay on d 15 (3rd d of a 4-d feeding period), and 8%-hay diets on d 26 (6th d of a 9-d feeding period), respectively. The excised ruminal papillae (approximately 500 mg) were washed with sterile 0.01 M phosphate-buffered saline (PBS) buffer (pH 6.8) immediately. The papillae were scraped to remove attached feed particles and rinsed three times to remove the non-adherent bacteria. Cleaned tissues were then transferred into RNA-later solution (Invitrogen, Carlsbad, CA, USA) and stored at -20°C until further molecular analysis.

Rumen digesta were collected at 8:00 am, 10:00 am, 12:00 noon and 14:00 pm respectively on d 2 (2nd d of a 4-d 97%-hay feeding period), d 14 (2nd d of 4-d 25%-hay feeding period), and d 25 (5th d of 9-d 8%-hay feeding period). Fifty ml of digesta sample were directly collected using a 50 ml sterile Falcon tube and were immediately placed on dry ice after collection and then stored at -80°C until further molecular analysis. The digesta samples (approximately 200 ml) were immediately strained through a perforated screen (Petex, pore size = 355 µm; Sefar Canada Inc., Scarborough, Ontario, Canada). Each 10 ml of strained rumen fluid was acidified with 2 ml of metaphosphoric acid and stored at -20°C until analysis for VFA including acetate, propionate, isobutyrate, butyrate, isovalerate, valerate, and total VFA concentration measurement. To determine VFA

concentrations, acidified fluid samples were centrifuged at 13,000 × *g* for 20 min, and supernatant was transferred into a gas chromatography (GC, model 3400, Varian) analysis using 170°C auto sampler (model 8200; Varian, Walnut Creek, CA) into a Stabilwax®- DA column (30 m, 0.53 mm i.d., 0.5 µm DF) and measured by 190°C detector. Peak integration was performed using galaxie software (Varian) (Li et al., 2009).

2.1.2 Rumen pH measurements. The ruminal pH measurement system (DASCOR, Inc, Escondido, CA) was used in this study to measure ruminal pH for the whole testing period according to the method described by Penner et al. (2006). The pH meter was inserted into the rumen through cannula on d 1 and was removed every 4 days for standardization and to download the data for each diet treatment period. This process was repeated until d 29. Ruminal pH (minimum, mean, and maximum pH) was obtained by average the pH data for each minute and ruminal pH characteristics under pH threshold of 5.5 including duration (total time when pH lower than threshold per day, h/d) and area (pH value multiple the time when pH lower than threshold per day, pH × min/d) values were calculated for each heifer per day. Since the absolute pH values change consistently in the rumen, the duration (h/d) and area (pH × min/d) values have been widely used as indicators of ruminal acidosis (Ghorbani et al., 2002). In this study, they were used to study the relationship between bacterial diversity and density changes with pH related features during the diet transition. The pH 5.5 threshold was used

because it is a typical threshold for beef cattle for they are fed more fermentable diets.

2.1.3 DNA extraction. Rumen tissue samples were thawed and washed using fresh sterile 0.01 M PBS buffer (pH 6.8) three times to ensure the removal of non-adherent bacteria and the residual of RNA later solution. In brief, the tissue was transferred onto a sterile plastic patri dish (90 mm X 15 mm) containing ~20 ml PBS buffer and was incubated for 3 min at room temperature with light shaking on the bench every 1 min. The buffer was then removed and the same amount of buffer was added to repeat the wash step for three times. Total DNA was extracted using a bead-beating method (Walter et al., 2001). Briefly, the tissue sample (100 to 250 mg) was transferred to a 2-ml micro-centrifuge tube containing Zirconium beads (0.3 g, diameter 0.1 mm) and washed with 1 ml of TN150 buffer (10 mM Tris-HCl [pH 8.0], 150 mM NaCl) twice by vortex and centrifugation at 14,600 × *g* for 5 min at 4°C. Then the pellet was resuspended in 1 ml of TN150 followed by a physical disruption in a Mini Bead-Beater^{TM}-8 (BioSpec Product, Bartlesville, OK, USA) at 4,800 rpm for 3 min. The tube was immediately placed on ice and incubated for a couple of minutes. The beat-beating process was then repeated to ensure the maximum recovery of bacterial DNA. Phenol and chloroform-isoamyl alcohol (24:1) were used to remove protein and DNA was precipitated with 70% cold ethanol and dissolved in 30µl of nuclease-free water. The amount and quality of DNA were measured based on

absorbance at 260 and 280 nm using a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA).

2.1.4 Design of a reference marker for PCR-DGGE analysis. In this study, an internal reference lane containing 47 amplicons from 16S rRNA full length sequences with known taxonomy identity was used for all PCR-DGGE analysis. According to the principals of PCR-DGGE, the DNA fragments consisting of the same sequences migrate to the same location of the DGGE gel, indicating that if the bands migrate to the same location of the reference sequences, they are likely to have high identify with the reference sequence to the different species at genus level based on 93- 96% of similarity (Ben-Dov et al., 2006). Based on many studies using the reference system using the know sequence and the confirmed identity for the DGGE bands (Guan et al., 2003; Konishi et al., 2009; Zhou et al., 2010), we decided to use a reference system containing the distinct taxonomy identification at V2-V3 region for DGGE analysis to predict the taxonomy of DGGE bands at genus level. To generate this reference lane, 1026 full-length 16S rDNA sequences were selected from a previous study on sequencing analysis of epimural bacterial community (Li et al., unpublished data; NCBI accession numbers GU303006- GU304593). Sequences were aligned from position 300 to 600 bases of V2-V3 region 16S rRNA gene using ClustalX program (http://www.molecularevolution.org; Thompson et al., 1997). Forty-seven unique sequences were determined based on the alignment score and taxonomy identification (Table 2.2). The plasmid DNA extracted from colonies containing

each of these 47 full length 16S rDNA sequences was used as a template to amplify the DNA fragment (~200 bp) using HDA1-GC and HDA2 primers and outlined program (Walter et al., 2000). Then the fourth-seven amplicons were mixed to be reference marker and loaded in one lane of DGGE gel, while the individual amplicon was loaded in the separate lanes of the same gel with reference marker in order to determine the migration position of each band in the reference marker. The DGGE (30 to 55% gradients with a 6% acrylamide) was run at 130 V, 60 °C for 4 h using Bio-Rad Dcode Universal Mutation Detection System (Hercules, CA, USA). After electrophoresis, gels were stained with 0.1% ethidium bromide for 20 min and detained with milliQ water for 30 min. The gels were then photographed with FluorChem SP imaging system (Alpha Innotech, San Leandro, CA, USA). These 47 sequences were confirmed to have distinct migration location on the gel under above DGGE condition and were the mixture of them were used as a reference maker for this study.

2.1.5 PCR-DGGE analysis. Total DNA (10 ng/μl) extracted from individual ruminal tissue was used as a template to generate the amplicon for PCR-DGGE analysis using nested PCR. The nested PCR was performed by amplifying the 1.5 kb product targeting the full length 16S rRNA gene with a universal bacterial primer pair 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT; Lane, 1991). The nested PCR conditions were: an initial denaturation for 5 min at 94°C; 30 cycles of 94°C for 30 s, 58°C for 45 s, and 72°C for 90 s; and a final elongation for 7 min at 72°C. This PCR

product was then diluted 10 times as a template to amplify a ~200 bp DNA fragment using HDA1-GC and HDA-2 primers (Walter et al., 2000). The PCR and DGGE analysis was performed using the same conditions described above for the reference marker.

The obtained PCR-DGGE profiles were analyzed using the BioNumerics software package (version 6.0; Applied Maths, Austin, TX). To be able to predict the identity of the PCR-DGGE bands using the reference maker and to compare the PCR-DGGE profiles among different gels, it is critical to define the optimal parameters such as optimization and tolerance for similarity coefficient settings for all given fingerprint types obtained within an experiment. In this study, optimization of 0.8% and the tolerance position of 0.88% were obtained based on the calculation function for these parameters from all gels and all lanes from the software and were applied for comparison of all the DGGE profiles for this particular experiment. Similarity matrices were generated using the Dice similarity coefficient (D_{sc}) and the dendrogram was obtained using the unweighted pairwise grouping method with mathematical averages (UPGMA) clustering algorithm (Fromin et al., 2002; Nicol et al., 2003). Similarity between bacterial PCR-DGGE profiles was obtained in percentage. Multi-dimensional scaling (MDS) and principal components analysis (PCA) were also performed using MDS and PCA analysis modules supplied with the BioNumerics software package.

To identify which PCR-DGGE bands were affected by the diet, a best-fit Gaussian curve for each band from all DGGE patterns was calculated. All the

assigned bands were then exported with the normalized relative position. After the band matching procedure, a binary matrix where all the bands were allocated into the define numbers of categories was created for the 24 heifers. Three dietary conditions: 97% hay, 25% hay and 8% hay were used to define the presence or absence of particular bands on each variable using the PROC CATMOD model as developed in house (Hernandez-Sanabria et al., 2010). In this model, the effect of all variables on the prevalence of each band was determined based on the transformation of the cell probabilities (response function). Afterwards, the FREQ Procedure of SAS (version 9.2; SAS Institute, Cary, NC) was used to estimate the frequency of the bands in all animals and results were plotted.

2.1.6 Estimation of total epimural bacteria population using qRT-PCR. The qRT-PCR analysis was performed to estimate the total rumen epimural bacterial population by measuring the copy numbers of 16S rRNA gene using the primer pair: U2 (Forward: 5'-ACTCCTACGGGAGGCA G-3'; Reverse: 5'-GACTACCAGGGTATCTAATCC-3') (Stevenson and Weimer, 2007) with StepOnePlus^{TM} Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using the SYBR green chemistry. The total volume of each reaction solution contained 10µl Fast SYBR® Green Master Mix (Applied Biosystems), 0.5 µl of each primer (20 pmol μl^{-1}), 8 µl of nuclease-free water and 1 µl of DNA template (10 ng μl^{-1}). The standard curve was constructed using plasmid DNA containing insert of 16S rRNA gene of *Butyrivibrio hungatei* (ATCC BAA456) with the serial dilution of the initial concentration of 9.1×10^{10}molecule μl^{-1}. The

range of the copy numbers in the standard curve was from 9.1×10^2 to 9.1×10^6 molecule μl^{-1}. Each standard dilution and sample was assayed in triplicates. Amplification was carried out using the following program: 95°C for 10 min for initial denaturation and then 40 cycles of 95°C for 20 s followed by annealing/extension for 1 min at 62°C.

Standard curves were plotted in StepOnePlus software version 2.0. The copy numbers of total 16S rRNA gene in the samples were determined by relating the C_T values to standard curves. The calculation of the copy number for the 16S rRNA gene in 0.5g of tissue was performed using the formula from the study of Li *et al*. (2009). The corresponding RT-PCR efficiency ranged between 86 and 100% in this study.

2.1.7 Statistical analysis. This experiment was analyzed as a randomized complete block design. The PROC MIXED procedure of SAS (version 9.2; SAS Institute, Cary, NC) was used to analyze the VFA concentration and total rumen epimural bacterial population. In the statistical model, period (diet), and treatment were analyzed as fixed effects with heifer as random effect and with all potential 2- and 3-way interactions. Interactions having $P > 0.05$ were removed from the model and the data were reanalyzed using reduced models. Least squares means were compared using the Bonferroni mean separation method, and significance was declared at $P = 0.05$.

Principal component analysis (PCA) was performed using the composite data set of the normalized location and intensity of each DGGE band, ruminal pH

variables (mean, minimum, and maximum pH or the duration or area below a threshold) and the molar proportion of VFA. All these data were also used to evaluate the relationships and to investigate the contribution of these variables to the variation of the data using PRINCOMP procedure in SAS. For each dietary treatment (period), the mean value of each measured molar proportion VFA or total VFA concentration from four time points was used for analysis. The rumen variables included all the ruminal pH characteristics (mean, minimum, and maximum pH or the duration or area below a threshold) and VFA molar proportions (acetate, propionate, isobutyrate, butyrate, isovalerate, valerate, and total VFA) as PCA ordinations. This procedure standardizes the variables to a mean of zero and a standard deviation of one. The correlation matrix was used to generate principal component eigenvalues and associated the loadings (SAS Institute. 1998). Correlation among total epimural bacterial population, ruminal pH and the molar proportion of VFA were analyzed using the PROC CORR and REG procedures of SAS.

2.2 Results

2.2.1 Rumen fermentation parameters. Interactions between period and treatment were significant for all data except for the area under the pH threshold 5.5 (pH × min/d), molar proportion of isobutyrate, and the concentration of total VFA. There were no differences for any measured variables including ruminal pH, and the concentration of total and individual VFA over the 3 periods for CON group and for RGA group when fed the 97%-hay diet (period 1, P1; Table 2.3).

When RGA heifers were fed the 25%-hay diet, mean ruminal pH decreased from 6.75 to 6.12 (P = 0.001), the duration below pH 5.5 increased from 0 to 356 min/d, and the area (pH × min/d) increased from 0 to 156 relative to 97% hay fed heifers. For VFA profiles, the molar proportions of propionate, butyrate, valerate, and isovalerate increased, while no differences were detected for total VFA concentration for heifers fed the 25%-hay diet compared to those fed the 97%-hay diet (Table 2.3). When heifers were fed the 8%-hay diet, ruminal pH and the VFA profile did not differ except for the molar proportion of valerate (P = 0.03) compared with those fed the 25%-hay diet. However, when RGA heifers were fed the 8% hay diet they had lower mean ruminal pH and molar proportion of acetate but higher molar proportions of propionate, butyrate, valerate, and isovalerate compared with those fed the 97%-hay diet (Table 2.3). Among individual heifers, mean ruminal pH change was strongly dependant on the host. Our data showed that the mean ruminal pH changes of 18 RGA cattle were found to follow three patterns: the mean ruminal pH continually decreased as the proportion of hay decreased (6 heifers), ruminal pH decreased only when the diet was transitioned from the 97% hay to 25% hay, and then increased during the transition to the diet containing 8% hay, (7 heifers) and, ruminal pH decreased when the diet transitioned from 97% hay to 25% hay, with little change in ruminal pH (5 heifers) (Figure 2.1).

2.2.2 PCR-DGGE profiling of ruminal epimural bacteria. The PCR-DGGE profiles of epimural bacteria from 24 heifers under different diets were generated

and compared (Figure. 2.2). The PCR-DGGE profiles of the 6 heifers in the CON group did not change among three periods (P = 0.58) when the similarity was compared using D_{sc}. For heifers on the RGA treatment, the bacterial PCR-DGGE profiles were clustered by diet (period; Figure 2.2; 2.3A). Similarity analysis showed that the bacterial profiles were more similar (Average D_{sc} = 81.22%) within the same diet than among different diets (Average D_{sc} = 69.32%).

To further verify the bacterial PCR-DDGE profiles changes in response to diet, MDS and PCA, two alternative grouping methods, were used to produce two or three-dimensional plots for relatedness of the bacterial diversity among all animals. The MDS analysis showed that there were six groups from all the PCR-DGGE profiles under three diets in total. The PCR-DGGE profiles from heifers fed 97% hay (symbols with green color), 25% hay (symbols with dark blue) diets were grouped closely based on the diet (Figure. 2.3B), while the PCR-DGGE profiles from heifer fed 8% hay diet were scattered into several groups (symbols with purple, red and yellow colar) and mixed with the outliners from other two diets. Principal component analysis (PCA) of PCR-DGGE profiles across diets also revealed the trend that the PCR-DGGE profiles were grouped by diets (data not shown).

2.2.3 Assessment of PCR-DGGE bands. In total, 88 bands were detected from all PCR-DGGE profiles. When the PCR-DGGE bands were subjected to multivariate statistical analysis, the frequency of presence for most of the PCR-DGGE bands changed in response to diet (Table 2.4). For instance, with the

concentration of hay decreasing, the frequency of presence of bands 23, 39, 53, 42, 44, 51, 52, 57, 59, 60, 64, 78 and 84 reduced, while it increased for bands 4, 9, 15, 20, 30, 48, 72, 82 and 83. Comparing the bacterial community profiles for the high-forage diet (97% hay) to the high-grain diet (8% hay), bands 79, 85, 86, 87 and 88 were only detected in samples under the 97% hay diet, whereas bands 2, 14, 25 and 81 were only present under the 8% hay diet. When the PCR-DGGE bands were compared under three diet conditions, bands 2, 14, 25, 79 and 85 were also detected when RGA heifers were fed the 25% hay diet while band 81 was only detected in RGA heifers when fed the 8% hay diet. Among all detected PCR-DGGE bands, 44 of them migrated with the exact location of those in the reference marker lane. Based on the phylotypes of these 44 bands, the predominant bacterial taxa belonged to the phyla Firmicutes, Proteobacteria, Bacteroidetes as well as unknown bacteria were identified for both CON and the RGA samples (Figure 2.4). The phyla of Spirochaetes, Tenericutes, Actinobacteria and Lentisphaerae were also found in some heifers.

2.2.4 Comparison of the total population of rumen epimural bacteria. The total epimural bacterial population was estimated using the total copy number of bacterial 16S rRNA genes. It did not change over time ($P = 0.78$) for CON heifers, but was different between CON and RGA cattle ($P = 0.05$) (Table 2.3). For RGA heifers, the total epimural bacterial numbers dramatically increased when the heifers were fed the 25% hay diet compared to those fed the 97% hay diet ($P = 0.01$) and significantly decreased when the dietary hay content decreased from 25

to 8% ($P < 0.01$). When comparing 97% hay diet (Period 1) and 8% hay diet (Period 3), the total epimural bacteria population was not different ($P = 0.36$). When each individual heifer in RGA group was compared, the changes of total epimural bacterial population under three diets did not follow a consistent pattern (Figure 2.5).

2.2.5 Correlations among total population of rumen epimural bacteria, rumen pH and VFA profiles. To identify whether epimural bacteria are associated with rumen fermentation parameters, the relationships among the total epimural bacteria population, ruminal pH and proportion of individual VFA were evaluated. Significant positive correlations ($P < 0.05$) were detected only between total population of epimural bacteria and duration (min/d) that ruminal pH was below 5.5 (Table 2.5). There was no correlation between the total epimural bacterial population and the molar proportion of VFA.

2.2.6 Correlations among epimural bacterial PCR-DGGE profiles, ruminal pH and molar proportion of VFA. To identify the correlations between rumen epimural bacterial diversity (PCR-DGGE bands) and fermentation characteristics (pH and VFA), the association among all variables were investigated using PCA analysis. Three significant principal components were extracted, describing 60% of the total variance. Two significant principal components were extracted describing 39.1% (PC1) and 16.9% (PC2) of the variation. In the first principal component (PC1), isovalerate, isobutyrate, acetate, and bands 1, 13 (*Treponema*

sp.), 19, 21 (Proteobacteria), 27, 28, 34 (Rumen bacterium), 38 (Uncultured rumen bacterium), and 40 had the highest contributions and they were orthogonal to VFAs trait. Rumen pH, minimum of pH, bands 3 (Uncultured *Mycoplasma* sp.), 10 (Clostridiales), 22, 24, 28, 35, 37 (Rumen bacterium) and 61 (*Desulfobulbus* sp.) which were described in the second principal component (PC2).

2.3 Discussion

Diet is one of the major factors influencing the structure and function of microbial community in the rumen contents (Tajima et al., 2000; Kocherginskaya et al., 2001; Zhou et al., 2010). The nature of feed materials, and the physicochemical changes induced by their fermentation are known to favor the development of certain microbial ecotypes in the ruminal solid and liquid phases (Martin et al., 2002). Meanwhile, the diversity of the ruminal epimural microflora has been reported to be stable relative to those in ruminal contents through 8 wk (Sadet et al., 2007) and under different dietary regimens (Sadet et al., 2007; 2010). In this study, we hypothesized that dietary transition from a high forage diet to a high grain diet, can affect the diversity of epimural bacteria in beef cattle. The PCR-DGGE profiles and total bacterial population of CON animals throughout this study indicates that the epimural bacteria composition was not altered by time at both the structural and density levels which confirm the findings of a previous study with a static diet (Li et al., 2009). The observed changes in the PCR-DGGE profiles and total epimural bacterial population from rumen of RGA animals in

response to the decreased forage proportion of diet show that dietary changes can induce marked changes in the diversity and density of the epimural bacteria.

On the phylum level of bacteria detected in this study, phyla Firmicutes, Proteobacteria, Bacteroidetes and unknown bacteria were predominant in the epimural bacterial community (Fig. 2). This finding confirmed that the gram-positive bacteria (Firmicutes) are relatively common in tissue adherent populations from previous studies (Cheng et al., 1979a; Mitsumori et al., 2002; Tajima et al., 1999). When our study was compared to a study of sheep by Sadet et al. (2010), phyla Firmicutes, Bacteroidetes, Proteobacteria, Spirochaetes have been detected from both sheep and heifers suggesting they are the predominant bacteria in epimural bacterial community in ruminants. Lower proportion of Phyla Firmicutes and Bacteroidetes (55% in heifer vs 86% in sheep) and higher proportion of unknown bacteria in our study can be the results of the low resolution of the PCR-DGGE analysis and the identified numbers of bands. Further study is necessary to sequence and identify all PCR-DGGE bands may improve the taxonomy identification of the bovine epimural bacterial community. Despite above limitation, the proportion of bacteria belonged to phyla Firmicutes, Bacteroidetes and Proteobacteria significantly changed under three diets (P = 0.006 for 97% hay, P = 0.02 for 25% hay, and P = 0.005 for 8% hay, respectively, Figure 2.4), revealing the evidence that diet influencing on the ecology of the epimural bacterial community.

Contrary to previous findings (Sadet et al., 2007; 2010), we observed that dietary transition to the high grain diets affected the diversity of the epimural

bacterial community. The discrepancy between our results and past studies may due to several reasons. Firstly, in the current study we evaluated changes in the bacterial diversity as individual heifers were transitioned from a high-forage diet to a high-grain diet. With this design we were able to utilize individual RGA heifers as their own control, after demonstrating that there was no time effect for the CON group. This differs from previous studies (McCowan et al., 1980; Sadet et al., 2007; 2010) where dietary effects were compared using two groups of animals. The use of two groups of animals does not account for variation in the epimural bacterial composition and density without proper controls and thus, the diet effect can be partially masked by host effect.

Secondly, different components of diets between previous studies and this study may have altered the epimural bacterial response. Wheat was used as the concentrated diet for the sheep study (Sadet et al., 2007; 2010) while barley was the grain component in our study. These cereal grains differ in the rate of ruminal degradation. Meanwhile, the forage component was also different. The previous studies used 20% alfalfa hay (Sadet et al., 2007) and 30% alfalfa hay (Sadet et al., 2010) in the diets, while the forage component in our study was 25% and 8% alfalfa or grass hay. This suggests that the variation in type of wheat and proportion of forage in the diet may also contribute to the difference of detected ruminal epimural bacterial changes.

Thirdly may be the most importantly, there may be host genetic effect between beef heifers and lambs when comparing our results to those of Sadet et al. (2007). Because the epimural bacteria inhabit the host tissues, we can speculate

that host may have a role in regulating microbial diversity and density, and its response to the rumen environmental changes as a result of consuming different dietary components. The host genetics have played a role in gut microbial community adaptation and evolution (Ley et al., 2008). However, the understanding of the variation of rumen bacterial diversity and density among different ruminant species is very limited. Varel and Dehority reported population difference of ruminal cellulolytic bacteria and protozoa from bison, cattle-bison hybrids and cattle (Varel et al., 1989). A recent study by Fuente *et al.* compared population of ciliated protozoa in the rumen of different domestic ruminant species including cattle, sheep, goats and reindeer using a culture based method, showing the divergence of the diversity and population among these species (Fuente et al., 2006). Our study showed that the total bacterial population of epimural bacteria was ranged from 7.3×10^9 to 2.0×10^{10} per g of wet tissue for hay-fed heifers, which were higher than that of hay-fed sheep ranging from 4.4×10^7 to 2.2×10^8 per g of wet tissue weight as reported by Wallace *et al.* (1979). All these suggest that we cannot ignore the host effect when we compare the studies on epimural bacterial community. Future studies to investigate how the host genetics can control ruminal epimural bacteria changes are warranted to better explain the different findings between sheep and cattle studies.

This is the first study to report the density of epimural bacteria can be impacted by diet using culture-independent methods. McCowan et al. (1978, 1980) used classic microscopy techniques to enumerate the epimural bacterial population in the rumen of Hereford bulls, showing a high density of the bacteria

attached to the ruminal wall. The previous studies from young lambs (Rieu et al., 1990) showed that the epimural bacterial population was up to 10^5-10^6 cells cm^{-2} in the rumen of 2-day old lamb, and this value increased very rapidly with age. It reached 10^8-10^9 cells cm^{-2} at day 21 after birth. DNA-based qRT-PCR analysis from our study further confirmed that such population can be as high as 7.3×10^9 to 2.0×10^{10} cells per g of wet tissue. Culture-independent techniques are more sensitive to detect the species with low abundances as well as unculturable bacteria. However, it is important to be aware of the limitations for estimating the total bacterial population based on the copy numbers of the 16S rRNA gene. It has been known that microbial genomes can display multiple numbers of operons of this gene with bacterial genome having up to 15 operons (Acinas et al., 2004). Acinas *et al.* suggested a correction factor ~2.5 for single operon for estimation of the population of bacteria and archaea (Acinas et al., 2004). A recent study is Case et al. (2007) evaluated the copy number of the 16S rRNA gene in 111 bacterial genomes, and 460 copies of this gene were recovered (giving an average of 4.2 copies per genome). In addition, other studies (Klappenbach et al., 2007) have shown the copy numbers of 16R rRNA gene vary depending on the growth of the bacteria: fast colony formers (~5.5 copies per genome) and slow colony former (~1.4 copies per genome). Therefore, the detected population based on 16S rRNA gene copy numbers may be overestimated due to the multiple heterogeneous copies within a genome (Crosby and Criddle, 2003; Case et al., 2007; Dahllöf et al., 2000). To date, there is no data to show the average copy number of this gene per genome in rumen. To address this limitation, recent

studies have been conducted indicating that other housekeeping genes such as *rop*B or *gyr*B and/or some functional genes can be used instead of 16S rRNA gene (Santos and Ochman, 2004; Küpfer et al., 2006; Powell et al., 2006; Case et al., 2007; Weimer et al., 2010). However, our attempt to estimate the bacterial population by measuring copy numbers of the *rop*B gene showed that this gene was not suitable for rumen bacteria since the detected population were 1000 fold lower than the known population (data not shown). Further studies are needed to identify more suitable genes to replace 16S rRNA gene to detect the total bacteria in the rumen.

Multivariate statistical analysis of individual PCR-DGGE band further confirmed that diet can impact on the presence and or absence of particular bacterial phylotypes. For example, bands 2, 14, and 25 (*Ruminobacter* sp.) were only present in the ruminal epithelial samples collected from heifers fed the 25% and 8% hay diets, suggesting that this genus attach to the epithelial tissue and their detectable population can be impacted by the diet. Another example is band 79 (Rumen bacterium YS2-like). It was not detected under 8% hay diet and it was associated with total bacterial population under 97% hay diet, suggesting that the diet not only can change the presence or absence of particular species, but also may impact on the their interactions with the community. Bands 29 and 78, the phylotypes with the identify with uncultured rumen bacterium clones, were associated with molar proportion of acetate, butyrate (band 29), valerate (25% hay, band 29 and band 78), isobutyrate (97% hay, band 78), rumen pH (8% hay, Band 19) and duration (min, pH < 5.5) (8% hay, band 78), suggesting that diet may

impact the association between particular bacterial species including unknown bacteria with rumen fermentation parameters.

Through the data analysis of the whole study, the variation among individuals in all measured parameters was noticeable. For example, bacterial phylotypes belonged to Tenericutes and Lentisphaerae were only detected in 4 of 18 heifers. These suggest that ruminal epimural bacterial diversity and its response to diets can vary depending on host animals. Taking heifers 170, 172 and 360 as examples (Figure 2.6), the percentage of bacterial phylotype belonged to Firmicutes was highest in heifers 170 and 172 fed with the 97% hay , while it was highest under 25% hay fed condition for heifer 360. Moreover, heifer 360 had a significant pH increase from period 2 (25% hay diet) to period 3 (8% hay diet), such pH change may directly associated detected difference of bacterial diversity at phylum level comparing to other animals. Although there was a significant increase of the estimated total epimural bacterial population detected in the rumen of most animals when fed with the 25% hay diet, the patterns of the bacterial population changes in each animal were different (Figure 2.5). These differences can be also associated with variation in pH response among individuals. For example, cattle 178 and 360 had highest numbers of the total bacterial population while they had lowest mean pH under 25% hay diet. The observation of different pH change pattern in response to the high grain diet from each animal suggests that the variation of the individual bacterial diversity may have a role in different ways of responses at population level to diet or pH. This

strongly suggests that the individual variation needs to be taken into account when studying the association between ruminal microbial diversity and host phenotypes.

In conclusion, this study showed that the diet transition from forage to high grain based diet significantly altered the diversity of epimural bacteria in the rumen of beef heifer. These changes may be explained by their association with the concentration of VFA and ruminal pH characteristics in the rumen. In addition, the ruminal epimural bacterial diversity and its response to diets can be variety depending on host animals. Furthermore, the different correlation between particular species of epimural bacteria and fermentation characteristics might indicate a variety of different types of relationships with the host. Deeply, it supplies some preliminary knowledge of the potential roles of epimural bacteria.

Table 2.1 Ingredient and nutrient composition in each diet during 29 d adaptation period

	Forage-to-concentrate ratio					
	97:3	60:40	40:60	25:75	15:85	8:92
Days fed[1]	Baseline d1 to 4	d 5 to 8	d9 to 12	d13 to 16	d16 to 20	d21 to 29
Ingredient composition, dry matter basis						
Grass hay	97.0	60.0	40.0	25.0	15.0	8.0
Barley grain	0	37.0	57.0	72.0	82.0	89.0
Vitamin and mineral supplement	2.5	2.5	2.5	2.5	2.5	2.5
Limestone	0.5	0.5	0.5	0.5	0.5	0.5
Chemical composition						
Dry matter	92.3	92.2	92.2	92.2	92.1	92.1
Organic matter, %DM	92.5	94.1	94.8	95.4	95.8	96.1
Crude protein , %DM	13.7	13.5	13.4	13.4	13.3	13.3
Neutral detergent fibre, %DM	57.3	43.5	36.6	31.4	28.0	25.6
Starch, %DM	3.1	20.7	29.6	36.2	40.6	43.7

[1]Depicts the feeding days for heifers on the rapid adaptation protocol. Control heifers received the 97% forage diet throughout the study.

[2]Each kg of supplement contained 14% Ca, 7% P, 3.1% Mg, 11% Na, 0.5% S, 30 mg Se, 50 mg Co, 2000 mg Cu, 100 mg I, 4000 mg Mn, 6000 mg Zn, 500 KIU vitamin A, 50 KIU vitamin D, and 2000 IU vitamin E.

Table 2.2 Identification of reference marker used in the all bacterial profiles

No.	Clone ID	Taxonomy identification	Access No.
1	406RT6-G12	Uncultured *Mycoplasma* sp. (90%)	AB089057.1
2	406RT1-G07	*Treponema refringens* (92%)	AF426101.1
3	206RT5-D08	Prevotella (100%)	AB239482.1
4	206RT2-A09	Clostridiales (97%)	DQ394637.1
5	406RT3-E12	*Treponema bryantii* (96%)	M57737.1
6	206RT1-D07	*Treponema bryantii* (98%)	M57737.1
7	406RT1-G01	Succinivibrionaceae (95%)	AB185751.1
8	406RT5-D01	*Comamonadaceae bacterium* MPsc (93%)	AY651926.1
9	406RT2-F02	Clostridiales (97%)	EU381508.1
10	206RT1-A03	Proteobacteria (82%)	EU835464.1
11	206RT1-C06	*Ruminobacter amylophilus* strain H18 (97%)	NR_026450.1
12	206RT2-B09	Uncultured rumen bacterium clone TWBRB53 (98%)	FJ799156.1
13	206RT1-A06	*Incertae Sedis XV* (98%)	EU381431.1
14	206RT5-G02	*Ruminococcus flavefaciens* strain AR72 (90%)	AF104841.1
15	406RT1-F10	Ruminococcaceae (100%)	EU381687.1
16	406RT3-A06	Rumen bacterium (95%)	AB239489.1
17	206RT2-F11	Rumen bacterium R-9 gene (93%)	AB239482.1
18	206RT3-A06	Uncultured rumen bacterium clone P5_G03 (96%)	EU381963.1
19	406RT3-F10	*Desulfobulbus* sp. (94%)	AY005036.1
20	406RT1-C02	Uncultured rumen bacterium clone T33H60F43 (88%)	AB270115.1

21	406RT4-C06	*Anaerovibrio lipolytica* (98%)	AB034191.1
22	206RT1-B02	Butyrivibrio (94%)	EU381489.1
23	206RT1-D02	*Mitsuokella jalaludinii* strain M9 (94%)	NR_028840.1
24	206RT2-A08	*Eggerthella sinensis* strain HKU14 (93%)	AY321958.1
25	206RT4-G01	Clostridiales (98%)	AB185741.1
26	406RT4-D04	*Lachnospiraceae bacterium* DJF_VP52 (90%)	EU728778.1
27	406RT2-C03	*Campylobacter fetus* strain 03-427 (96%)	AY621303.1
28	406RT3-A04	Alpha-proteobacteria (97%)	AY297796.1
29	206RT6-A08	*Desulfonosporus* sp. AAN04 gene (96%)	AB436739.1
30	206RT1-E07	*Desulfobulbus* sp. oral clone CH031 (94%)	AY005036.1
31	206RT1-A02	*Deferribacteres* sp. oral clone JV001 (98%)	AY349370.1
32	206RT1-D04	Bacteroidales (100%)	EF436307.1
33	206RT1-F08	Unidentified rumen bacterium JW16 (95%)	AF018445.1
34	406RT4-B05	*Succiniclasticum ruminis* strain DSM 9236 (93%)	NR_026205.1
35	406RT3-F08	*Desulfitobacterium hafniense* DCB-2 (96%)	CP001336.1
36	406RT3-A01	*Desulfobulbus* sp. oral clone CH031 (94%)	AY005036.1
37	406RT2-H02	Clostridiales (100%)	AB185814.1
38	206RT6-G05	*Eubacterium* sp. C2 (90%)	AF044945.1
39	406RT4-A10	Neisseriaceae (100%)	AY551997.1
40	406RT1-A02	Mogibacterium (96%)	AB034014.1
41	406RT2-D09	Victivallis (99%)	FJ028789.1
42	406RT1-C01	Rumen bacterium YS2 (91%)	AF544207.1
43	406RT1-A11	Uncultured bacterium clone CHIMP1_aaj40e05 (94%)	EU462343.1

44	406RT3-F09	Porphyromonadaceae (94%)	EF686526.1
45	406RT4-C04	Uncultured rumen bacterium clone YRC60 (93%)	EU259436.1
46	406RT3-E11	*Atopobium parvulum* DSM (93%)	CP001721.1
47	206RT5-A07	Rumen bacterium YS2 (92%)	AF544207.1

Table 2.3 Analysis of ruminal fermentation parameters and total epimural bacterial population in the inter- and intra-treatment

	CON(N=6)			RGA(N=18)					
	P1 (97%HAY)	P2 (97%HAY)	P3 (97%HAY)	P1 (97%HAY)	P2 (25%HAY)	P3 (8%HAY)	T	P	T*P
Rumen pH									
Mean	6.73±0.05^{a}	6.73±0.04^{a}	6.74±0.05^{a}	6.75±0.02^{a}	6.12±0.08^{b}	6.12±0.07^{b}	<0.001	<0.001	<0.001
Minimum	6.48±0.07^{a}	6.46±0.06^{a}	6.44±0.09^{a}	6.49±0.04^{a}	5.42±0.09^{b}	5.55±0.08^{b}	<0.001	<0.001	<0.001
Maximum	6.96±0.04^{a}	6.98±0.03^{a}	7.01±0.04^{a}	6.99±0.02^{a}	6.76±0.06^{b}	6.71±0.05^{b}	0.002	0.141	0.022
Rumen pH under 5.5									
Duration, min/d	0^{b}	0^{b}	0^{b}	0^{b}	357±71.6^{a}	346±47.8^{a}	<0.001	0.007	0.007
Area, pH x min/d	0	0	0	0	157±52.9	97.1±16.7	0.011	0.147	0.147
VFAs									
Acetate, %	70.1±0.92^{a}	69.8±0.86^{a}	69.6±0.76^{a}	69.6±0.76^{a}	57.7±1.80^{b}	54.6±1.44^{b}	<0.001	<0.001	<0.001
Propionate, %	17.3±0.58^{b}	18.1±0.22^{b}	17.7±0.16^{b}	17.3±0.24^{b}	22.4±1.43^{a}	24.0±2.35^{a}	0.034	0.170	0.013
Isobutyrate, %	0.95±0.06	0.74±0.04	0.93±0.13	0.91±0.03	1.00±0.10	1.12±0.10	0.130	0.358	0.376
Butyrate, %	9.11±0.44^{b}	9.35±0.66^{b}	8.93±0.51^{b}	9.61±0.75^{b}	15.2±1.45^{a}	14.8±1.13^{a}	<0.001	0.097	<0.001
Isovalerate, %	1.07±0.07^{b}	0.74±0.07^{b}	0.95±0.1^{b}	1.07±0.05^{b}	1.93±0.29^{a}	1.71±0.22^{a}	0.004	0.538	0.007
Valerate, %	1.11±0.09^{b}	1.04±0.07^{b}	1.19±0.07^{b}	1.22±0.11^{b}	1.49±0.18^{b}	3.19±0.40^{a}	0.002	0.005	0.013
Total VFAs, mM	84.3±9.05	81.2±5.12	90.4±7.09	82.0±4.61	95.6±5.67	89. 8±7.09	0.544	0.591	0.056
Total epimural bacterial population (copy number/g)	(1.54±0.55) ×$10^{10\ ab}$	(1.63±0.55) ×$10^{10\ ab}$	(1.09±0.29) ×$10^{10\ ab}$	(1.06±0.13) ×$10^{10\ b}$	(2.00±0.38) ×$10^{10\ a}$	(0.73±0.19) ×$10^{10\ b}$	0.599	0.042	0.045

T: treatment (CON and RGA); P: periods (P1, P2 and P3); T*P: interaction between treatment and period. Analysis within treatment: a, b Within a column, means with different letters are significantly different. For control treatment, within each period, there is no significant difference. The model was used in this analysis: Y= T + P + T*P + e. The unit of total bacteria population is copy number per gram of tissue.

Table 2.4 Identification of PCR-DGGE bands and associations with different rumen parameters

DGGE band	Predicted Taxonomy identification of Genus level (similarity)	Predicted Taxonomy identification of Phylum level	Presence (+) or absence (-) in the rumen Probable associations with different parameters		
			(97%hay fed diet)	(25%hay fed diet)	(8%hay fed diet)
1	UD	UD	--	+	--
2	UD	UD	--	+	+ Acetate, Total VFAs, isovalerate, isobutyrate,
3	Uncultured *Mycoplasma* sp. (90%)	Tenericutes[81%]	+ pH	+ Minimum, butyrate	+
4	*Treponema* sp. (92%)	Spirochaetes[100%]	+	+	+
7	Prevotella (100%)	Bacteroidetes[100%]	+	+	+ Propionate
10	Clostridiales (97%)	Firmicutes[98%]	+	+ Valerate	+ pH, min[2], max[3], propionate, butyrate
11	*Treponema* sp. (96%)	Spirochaetes[100%]	+ Total VFAs	+	+ Propionate
13	*Treponema* sp. (98%)	Spirochaetes[100%]	+ Propionate	+	+ TEBP[4]
14	UD	UD	--	+	+ pH, min, time[5], area[6]
15	Succinivibrionac	Proteobacteria[10	+	+	+

	eae (95%)	0%]			
20	Clostridiales (97%)	Firmicutes[99%]	+	+	+
21	Proteobacteria (82%)	Proteobacteria[82%]	+	+ Total VFAs, isobutyrate	+ Time
25	*Ruminobacter* sp. (97%)	Proteobacteria[100%]	--	+ Butyrate, valerate	+
29	Uncultured rumen bacterium clone TWBRB53 (98%)	Firmicutes[100%]	+	+ Acetate, butyrate, valerate	+ pH, min, time, max
30	*Incertae Sedis XV* (98%)	Firmicutes[99%]	+	+	+
32	*Ruminococcus* sp. (90%)	Firmicutes[100%]	+ Isobutyrate, Isovalerate	+ Butyrate	+
33	Ruminococcaceae (100%)	Firmicutes[100%]	+	+	+ Min
34	Rumen bacterium (95%)	Firmicutes[100%]	+	+	+ Valerate
37	Rumen bacterium R-9 gene (93%)	Bacteroidetes[100%]	+ Isovalerate	+ Valerate	+
38	Uncultured rumen bacterium clone P5_G03 (96%)	Bacteroidetes[100%]	+	+ Propionate	+
39	*Desulfobulbus* sp. (94%)	Proteobacteria[100%]	+	+	+
41	Uncultured rumen bacterium clone T33H60F43 (88%)	Firmicutes[98%]	+	+	+ Total VFAs
42	*Anaerovibrio* sp. (98%)	Firmicutes[100%]	+	+ Total VFAs, isobutyrate	+ Propionate, butyrate
43	Butyrivibrio	Firmicutes[100%]	+	+	+

	(94%)			Min	
44	*Mitsuokella* sp. (94%)	Firmicutes[100%]	+ Isovalerate,	+	+
45	*Eggerthella* sp. (93%)	Actinobacteria[100%]	+	+	+
46	Clostridiales (98%)	Firmicutes[99%]	+	+	+
48	*Lachnospiraceae* sp. (90%)	Firmicutes[100%]	+	+	+ Acetate
50	*Campylobacter* sp. (96%)	Proteobacteria[100%]	+	+	+ pH, Max
51	Alphaproteobacteria (97%)	Proteobacteria[97%]	+ Min, propionate	+	+ TEBP, propionate, total VFAs, butyrate
52	*Desulfonosporus* sp. (96%)	Firmicutes[99%]	+	+	+
53	*Desulfobulbus* sp. (94%)	Proteobacteria[100%]	+ Isovalerate	+	+
54	*Deferribacteres* sp. (98%)	Firmicutes[98%]	+ Isovalerate	+ TEBP, min, area, total VFAs	+
56	Bacteroidales (100%)	Bacteroidetes[100%]	+ TEBP	+	+ Area
58	Unidentified rumen bacterium JW16 (95%)	Bacteroidetes[97%]	+	+	+
59	*Succiniclasticum* sp. (93%)	Firmicutes[100%]	+ TEBP, isobutyrate	+ Propionate	+
60	*Desulfitobacterium* sp. (96%)	Firmicutes[100%]	+	+ TEBP, isobytyrate	+
61	*Desulfobulbus* sp. (94%)	Proteobacteria[100%]	+	+	+
62	Clostridiales	Firmicutes[100%]	+	+	+

	(100%)			Isovalerate, isobutyrate,	
65	*Eubacterium* sp. C2 (90%)	Firmicutes[100%]	+	+	+
69	Mogibacterium (96%)	Firmicutes[100%]	+	+	+
70	Victivallis (99%)	Lentisphaerae[100%]	+ Butyrate	+ Isobutyrate	+
72	Rumen bacterium YS2 (91%)	Proteobacteria[68%]	+	+ TEBP, Isovalerate,	+
74	Uncultured bacterium clone CHIMP1_aaj40e05 (94%)	Firmicutes[58%]	+ TEBP, total VFAs	+	+
75	Porphyromonadaceae (94%)	Bacteroidetes[100%]	+	+	+
78	Uncultured rumen bacterium clone YRC60 (93%)	Bacteroidetes[100%]	+ Isobutyrate	+ Valerate	+ Time
79	Rumen bacterium YS2 (92%)	Proteobacteria[52%]	+ TEBP	+	--
80	UD	UD	+	--	+
81	UD	UD	--	--	+ Valerate
85	UD	UD	+	+ Butyrate	--
86	UD	UD	+ Propionate	--	--
87	UD	UD	+	--	--
88	UD	UD	+	--	--

+ Presence of the bacterium**;** -- Absence of the bacterium

[1]UD, undefined identity; [2]min: of mean rumen pH; [3]Max: Maximum of mean rumen pH; [4]TEBP: Total epimural bacteria population

[5]The min/d that ruminal pH was < 5.5.

[6]The area (pH × min/d) that ruminal pH was < 5.5.

Table 2.5 Correlation among total epimural bacteria population, rumen pH and VFAs production

	TEBP[1]	
	R-value	*P-value*
Mean	-0.20	0.09
Minimum	-0.15	0.10
Maximum	-0.25	0.05
Duration[2]	0.35	0.02
Area[3]	0.43	0.001
Acetate	0.05	0.73
Propionate	-0.07	0.63
Isobutyrate	-0.05	0.71
Butyrate	-0.007	0.96
Isovalerate	0.25	0.07
Valerate	-0.12	0.40
Total VFAs	0.08	0.56

[1]Total epimural bacterial population.

[2]The min/d that ruminal pH was < 5.5.

[3]The area (pH × min/d) that ruminal pH was < 5.5.

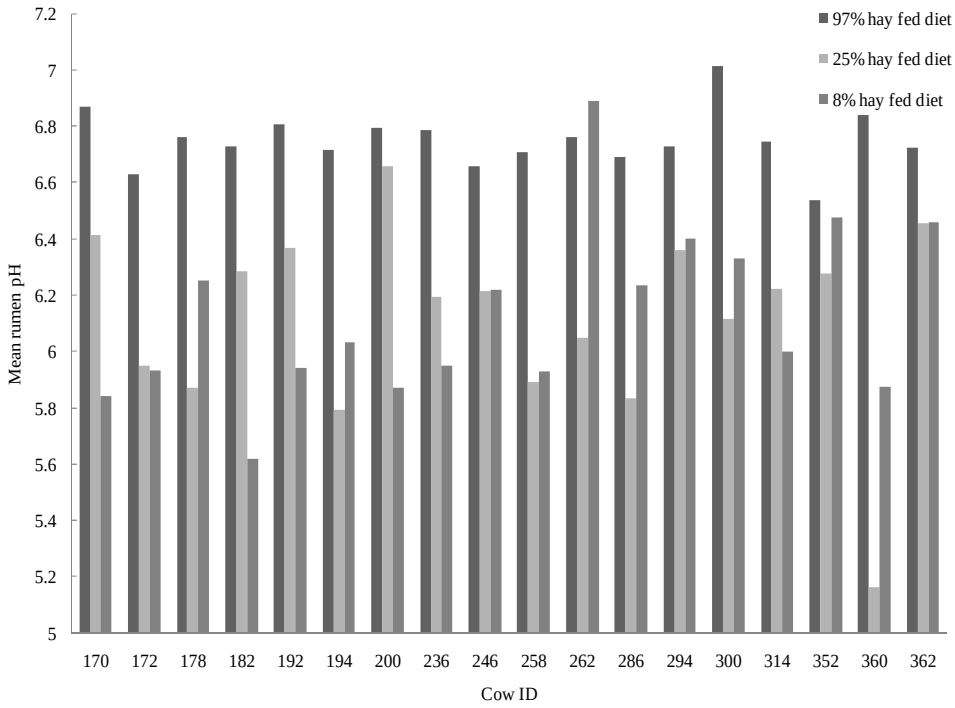

Figure 2.1 Mean rumen pH measured from each of 18 RGA beef heifers under 97%-, 25%- and 8%-hay diet, respectively.

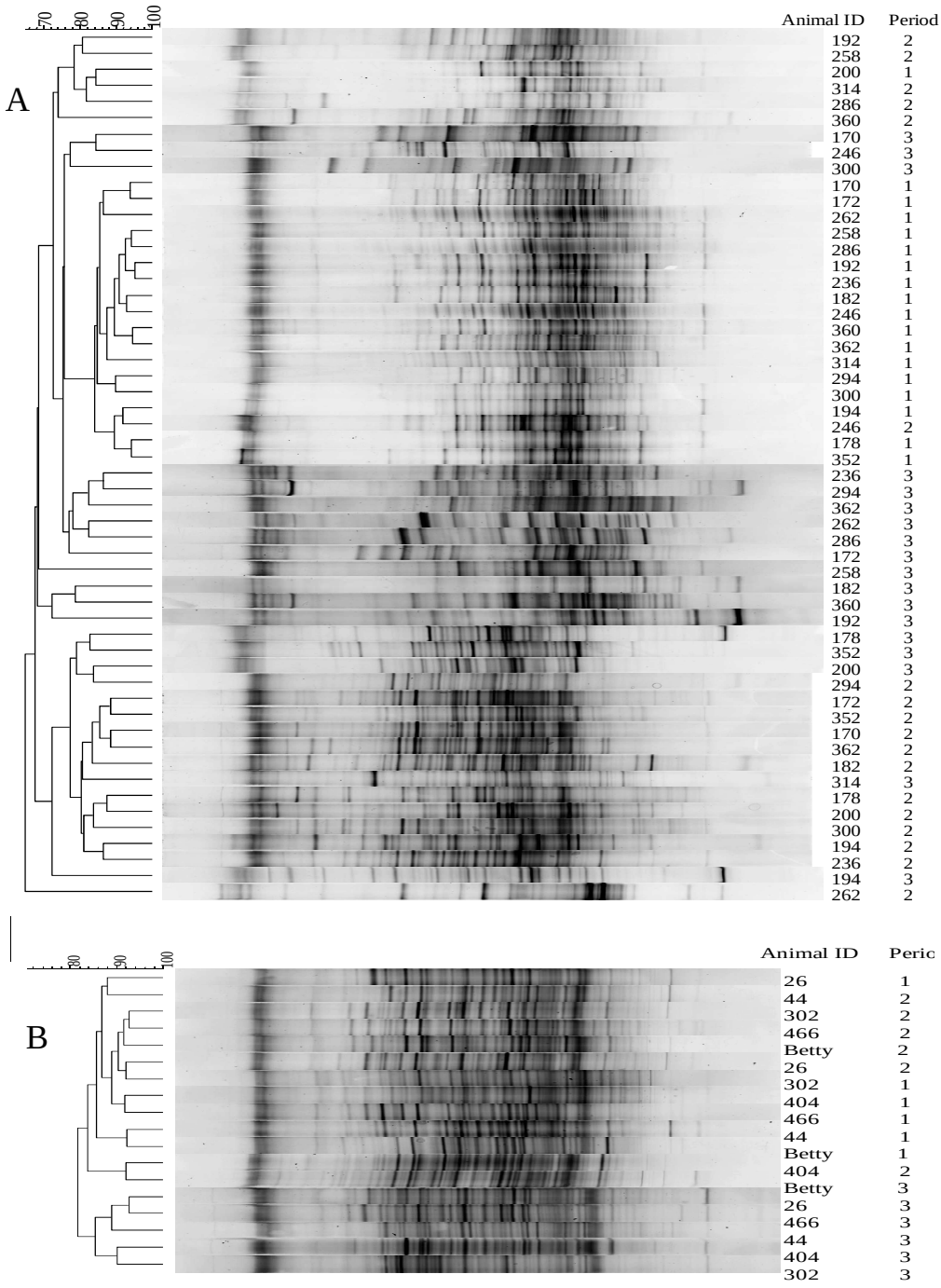

Figure 2.2 The cluster of the PCR-DGGE profiles of epimural bacterial profiles from (A) the 18 RGA cattle fed three diets: 97% hay (P1), 25% hay (P2), 8% hay (P3) respectively and (B) control cattle fed 97% hay diet.

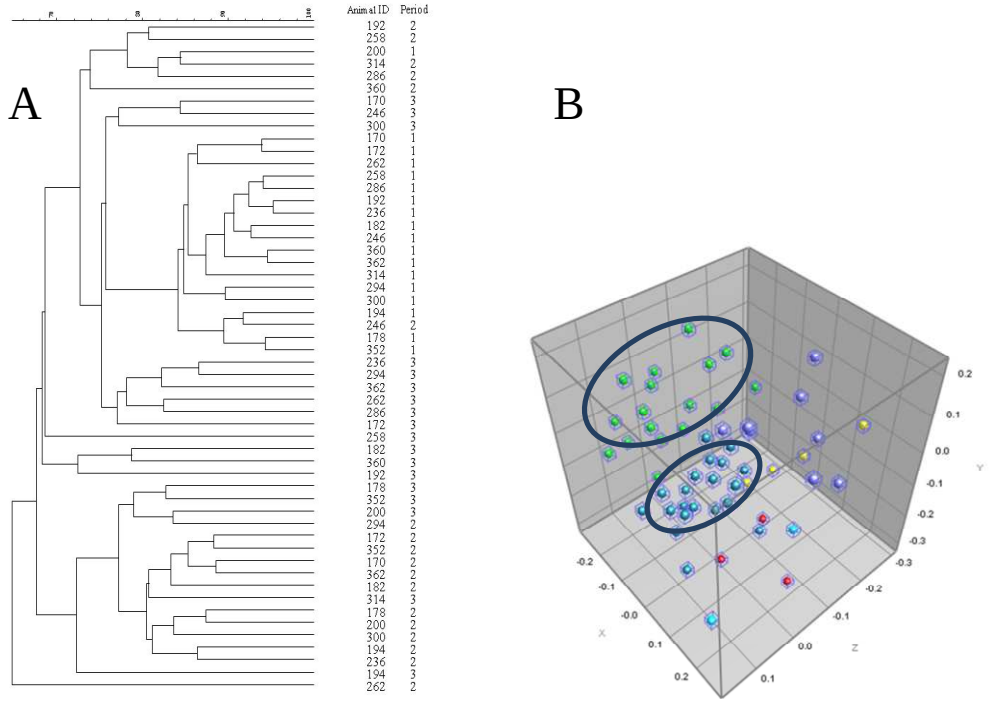

Figure 2.3 Analysis of epimural bacterial profiles using PCR-DGGE analysis. (A) The epimural bacterial profile detected from the 18 RGA cattle fed three diets: 97% hay (P1), 25% hay (P2), 8% hay (P3) respectively. The cluster of the PCR-DGGE profiles was generated with the Bionumerics software package using UPGMA method as described in the text. Optimization of 0.8% and the tolerance position of 0.88% were applied to comparison of DGGE profiles among the gels. (B). MDS plot of PCR-DGGE profiles shown in (A). Different groups were indicated with different color: Dark blue (n=20): 97%-hay fed cattle (14), 8%-hay fed cattle (5) and 25%-hay fed cattle (1); Light green (n=17): 25%-hay fed cattle (11) plus 8%-hay fed cattle (6); Purple (n=8): 25%-hay fed cattle (4), 97%-hay fed cattle (3) and 8%-hay fed cattle (1); Yellow (n=4): 8%-hay fed cattle (3) plus 97%-hay fed cattle (1); Red (n=3): 8%-hay fed cattle (3); Light blue (n=2): 8%-hay fed cattle (2).

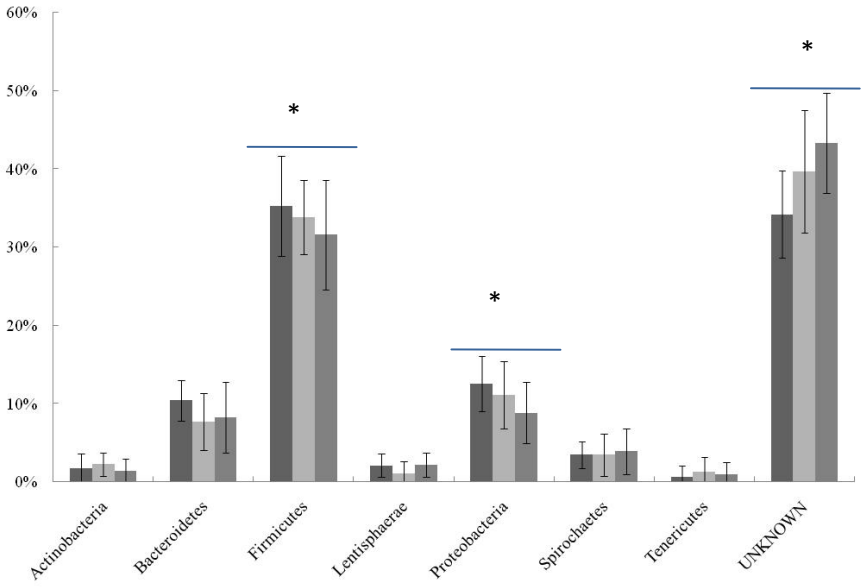

Figure 2.4 Comparison of distribution of epimural bacteria at phylum level from 18 RGA heifers fewer than 97%-, 25%- and 8%-hay diet, respectively based on the sequence information from the reference marker.

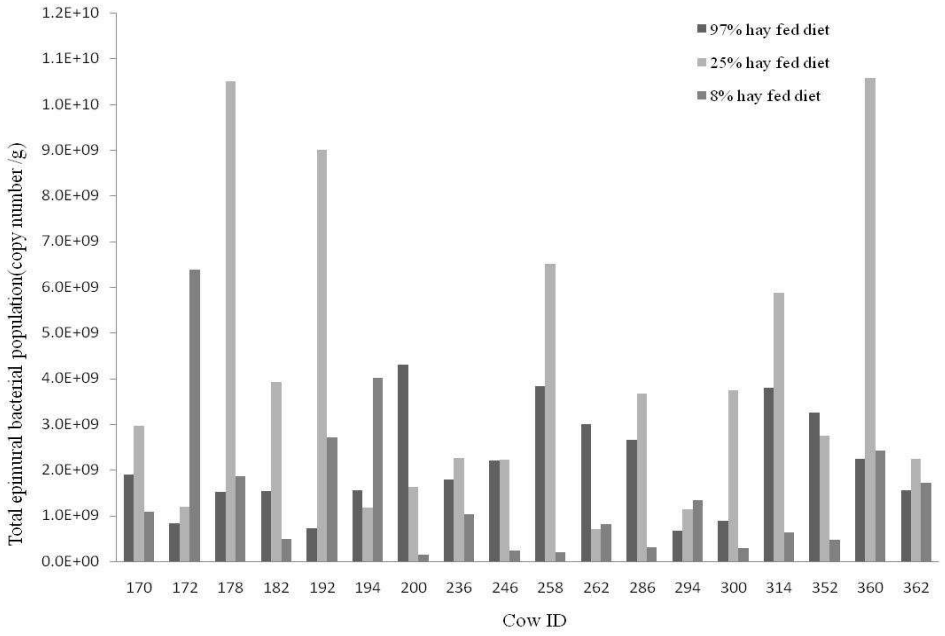

Figure 2.5 Changes of estimated total population of epimural bacteria measured by total copy numbers of 16S rRNA genes from each individual of 18 RGA heifers in response to 97%-, 25%- and 8%-hay diet, respectively.

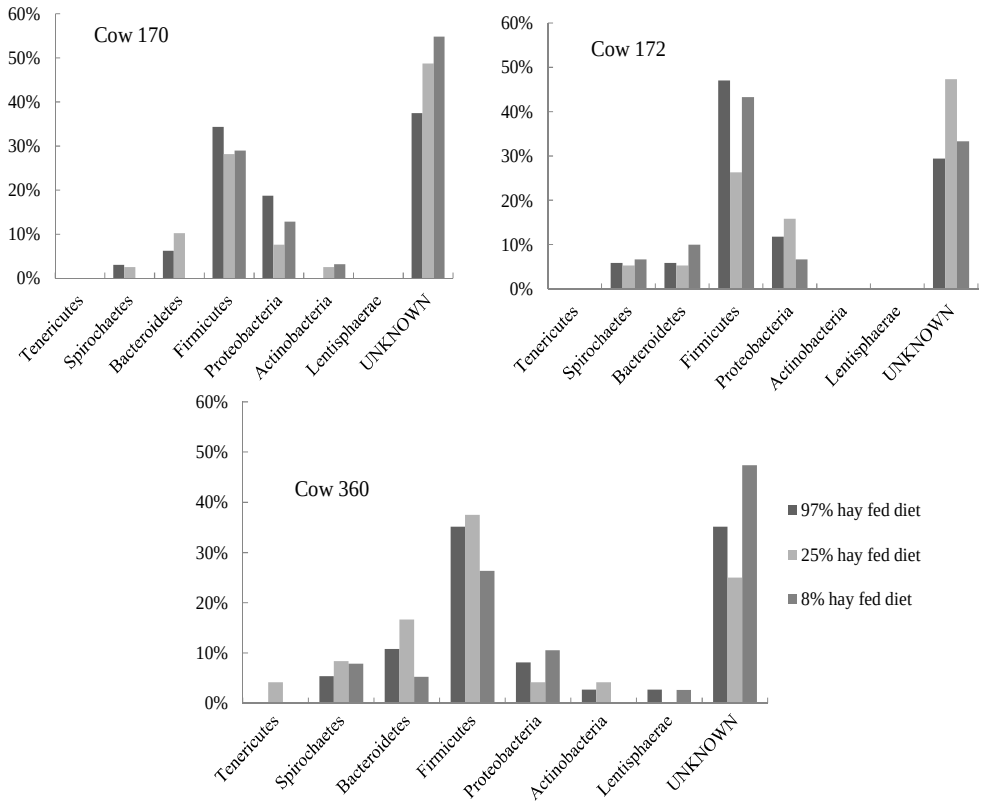

Figure 2.6 Variation of the distribution changes of epimural bacteria at phylum level of heifer 170, 172 and 360 in response to 97%, 25%and 8% hay diet, respectively based on the sequence information from the reference marker.

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Chapter Ⅲ. Variation of Digesta and Epithelial Attached Bacterial Community and Expression of TLRs in the Rumen of Steers Differing in Susceptibility to Subacute Ruminal Acidosis

3.0 Introduction

Subacute ruminal acidosis (SARA) is considered as one of the most common digestive disorders in the ruminant industry, and it can lead to significant reductions in animal performance (Britton and Stock, 1987; Stock and Britton, 1993). The majority of previous research examining ruminal acidosis has focused on dietary factors, including ration particle size distribution (Kononoff et al., 2003; Kononoff and Heinrichs, 2003a; b) and diet fermentability (Krause et al., 2002; Krause and Combs, 2003). A variation on susceptibility of individual ruminants to SARA has been discussed, but few studies have investigated the relationship between ruminal bacteria and such variation. Recently, the ruminal microbial and fermentative changes have been proposed to be associated on experimentally-induced SARA (Goad et al., 1998), and the microbial changes associated with ruminal acidosis have been observed from experimentally-induced acidosis in cattle and sheep (Dunlop, 1972; Huber, 1976; Nagaraja et al., 1985; Goad et al., 1998). Because ruminal acidosis represents varying degrees of acidity in the rumen, differences in ruminal pH dynamics could be related to different ruminal bacterial communities (Khafipour et al., 2009a; Palmonari et al., 2010). In addition, previous studies have revealed that changes in some bacteria in population or diversity were associated with ruminal acute acidosis (Dunlop, 1972; Slyter, 1976; Nagaraja and Titgemeyer, 2007).

To date, most studies on microbial changes associated with acute acidosis and SARA have been focused only on those bacteria inhabiting the rumen digesta. Recent work from our group (Chen et al., 2011) revealed that bacterial communities attached to rumen epithelia (epimural bacteria) can be impacted during diet transition, and that their population was associated with severity of SARA characterized by the area (pH value under 5.8 multiplied by the duration that pH is lower than 5.8, pH × min/d). We hypothesized that the diversity and density of epimural bacteria are different between acidosis susceptible (AS) and acidosis resistant (AR) animals, similar to the digesta associated bacteria. Recent studies using culture-independent techniques have revealed that the epimural bacterial community is distinctly different from those associated with rumen digesta at taxonomic level in dairy and beef cattle (Cho et al., 2006; Sadet et al., 2010; Li et al., 2011), and sheep (Cheng et al., 1979b; Sadet et al., 2007; 2010). As epimural bacteria are directly attached to the epithelial lining, their end products indirectly influence rumen tissue barrier function (Wallace et al., 1979; Penner et al., 2010; 2011) and they may be involved in alteration of volatile fatty acid absorption or expression of host genes.

Toll like receptors (TLRs) are essential for innate immune response induction, and among the 10 bovine TLRs, TLR4 recognizes ligands secreted by gram-negative bacteria (Takeuchi et al., 1999) while TLR2 recognizes the molecular patterns from gram-positive bacteria (Yoshimura et al., 1999). However, the changes in epimural bacterial community during SARA and weather these changes are associated with expressions of these TLR have not

been studied. Therefore, the objectives of this study were to investigate the diversity and density of ruminal digesta and epimural bacterial communities in the rumen of AR and AS steers and host TLR 2 and TLR4 expressions.

3.1 Materials and methods

3.1.1 Animal and sampling: Seventeen, ruminally cannulated steers (~ 1 year old, with an average body weight of 413±45 kg) were cared in the Laired McElroy environmental and metabolic centre at the University of Alberta. The animal study was approved by Animal Care and Use Committee, University of Alberta (Guan 019). The diet used in this experiment was a high grain based diet (85% grain in total) containing 56.7% dry-rolled barley grain, 28.3% dry-rolled oats grain, 10.0% sun-cured alfalfa pellet and 5.0% mineral vitamin mixture (feedlot 32, Nutrena^{TM} , Camrose, AB, Canada) with monensin (440mg/kg). All nutrient components of the diet are presented in Table 3.1. All steers were fed this diet more than 58 days.

Among these, six steers were selected based on acidosis index, which was calculated by dividing the area that ruminal pH below 5.8 by DMI and an indicator of the severity of ruminal acidosis normalized for DMI (Schlau et al., 2011). The three steers having the highest acidosis index value were categorized as acidosis-susceptible animals (AS) group and the other three with lowest acidosis index value were categorized as acidosis-resistant (AR) animals. Before ruminal tissue and content were sampled, six steers were force-fed 60% of expected daily DMI within 30 min in order to induce a subacute acidosis. Rumen pH data and ruminal papilla samples were collected analyzed as described by

Schlau et al. (2011). Briefly, rumen pH was measured continuously by an indwelling system as described previously (Penner et al., 2006) over the 6-h postprandial sampling period. At 0, 2, 4, and 6 h after feeding, ruminal papillae were biopsied, approximately 500 mg from the main ventral sac of the rumen. The samples were washed with 0.01 M phosphate-buffered saline (PBS) buffer (pH 6.8) immediately after collection. The samples of clean tissue were then transferred to the RNA-later solution (Invitrogen, Carlsbad, CA, USA) and stored at -20°C until analysis. At the same time, rumen digesta samples were collected from areas adjacent to the location of the biopsies. The digesta samples were collected in 50 ml sterile falcon tubes and stored in dry ice immediately. After collection, the samples were transferred and kept at -80°C until further analysis.

3.1.2 DNA extraction and PCR-denaturing gradient gel electrophoresis (DGGE): Total DNA from digesta and papillae samples was extracted following the bead-beating method protocol reported previously reported by our group (Guan et al., 2008; Li et al., 2009) with physical disruptions performed twice for rumen epithelium samples using a Mini Bead-BeaterTM-8 (BioSpec Product, Bartlesville, OK, USA). After extraction, total DNA was diluted to10 ng/µl and then 1 µl of diluted DNA was used as a template to generate the amplicon using nested PCR. The nested PCR was performed by amplifying a ~1.5 kb product targeting the full length 16S rRNA gene with a universal bacterial primer pair 27F -1492R (Table 3.2). The nested PCR was performed using the following program: an initial denaturation for 5 min at 94°C; 30 cycles of 94°C for 30 s, 58°C for 45 s,

and 72°C for 90 s; and a final elongation for 7 min at 72°C. This PCR product was then diluted 10 times as a template to amplify a ~200 bp DNA fragment using HDA1-GC and HDA-2 primers (Table 3.2) following the program outlined by Walter et al. (2000).

The amplicons (~200 bp) were then subjected to DGGE analysis using a 30 to 55% gradient with a 6% acrylamide gel. In this study, two internal reference lanes containing the known sequences were used for all PCR-DGGE analysis. The DGGE run conditions were 130 V, 60 °C for 4 h using Bio-Rad Dcode Universal Mutation Detection System (Hercules, CA, USA). After electrophoresis, gels were stained with 0.1% ethidium bromide for 20 min and detained with milliQ water for 30 min. The gels were then photographed with FluorChem SP imaging system (Alpha Innotech, San Leandro, CA, USA).

3.1.3 DGGE profiles analysis: PCR-DGGE profiles were analyzed using the BioNumerics software package version 6.0 (Applied Maths, Austin, TX). In this study, optimization of 0.5 and the tolerance position of 1% were applied for comparison of all the DGGE profiles. These two parameters were calculated by the software and were used to optimise similarity coefficient settings for all given profiles among different gels. In addition, the PCR-DGGE profiles were analyzed using multi-dimensional scaling (MDS) procedure supplied with the BioNumerics 6.0 software package. In MDS plots, points separated farer represent samples that are more different in bacterial species composition (Clarke and Gorley, 2001; Clarke and Warwick, 2001). Analysis of similarity (ANOSIM) was performed

using global R statistics. The overall significance level of the samples statistic was set at $P < 0.05$.

3.1.4 Quantitative real time PCR analysis: The copy numbers of 16S rRNA gene was measured to estimate the total bacterial population using qRT-PCR analysis outlined by Li et al. (2009) using the primers shown in Table 3.2. The plasmid DNAs containing the 16S rRNA gene of *Butyrivibrio hungatei* were used to generate standard curves for each species. The standard curve was constructed using with the initial concentration of 8.5×10^{10} molecular/µl for total bacteria. Each standard dilution and sample was assayed in triplicates. The corresponding RT-PCR efficiency ranged between 86 and 100% in this study. The final copy numbers of total bacteria or each species per gram rumen samples were calculated by the function that total DNA amount divided 50 ng (template used for real-time PCR), the folds obtained were multiplied quantity mean exported from RT-PCR and subsequently divided weigh of samples used in DNA extraction.

3.1.5 RNA extraction and gene expression: Total RNA was extracted from around 0.1 g of rumen biopsy samples. Rumen papillaes were homogenized with beads (CK 14 Precellys® lysine) and TRIzol Reagent (Invitrogen, CA, USA) by Precellys® 24 homogenizer (Bertin technologies, Montigny, France) with two cycles of 30s at 5500rpm with 10s stop between each cycle. Extracted total RNA was first reverse transcript to synthesize first strand and then second strand cDNAs, following cDNA purification and subsequently in vitro transcript to

synthesize aRNA using Ambion Amino Ally Message Amp Ⅱ aRNA kit (#1753) (Life Technologies™/Applied Biosystems™, Austin, TX). After aRNA purification, the amount and quality of aRNA were measured based on absorbance at 260 nm using a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA).

The expression of TLR2 and TLR4 in the rumen epithelium was evaluated using qRT-PCR analysis using the primer pair as shown in Table 3.2. The qRT-PCR was performed with StepOnePlus^{TM} Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using the SYBR green chemistry. Beta-actin gene was used as a reference gene. The reaction was carried out using the following program: 95°C for 5s for initial denaturation and then 40 cycles of 95°C for 10s followed by annealing/extension for 30s at 60°Cwith a final melting curve stage (from 95°C 15 s - 60°C 1 min - 95°C 15 s, with fluorescence collection at 0.1°C intervals). The data for ΔCT and ΔΔCT values was calculated by the function: $\Delta CT = CT_{sample} - CT_{\beta\text{-actin}}$; $\Delta\Delta CT = \Delta CT_{AR} - \Delta CT_{AS.}$ And the fold changes between AS and AR group was obtained using $2^{-\Delta\Delta CT}$.

3.1.6 Statistical analysis: Data were analyzed using the Proc Mixed procedure of SAS (version 9.2; SAS Institute, Cary, NC). In the statistical model (repeated measured model), group (AS and AR) and hour (time relative to feeding) were analyzed as fixed effects with steers as random effect and with an interaction between group and hour. The element of design structure was subject (steer) and the unstructured type of variance and co-variance structure was used in the

procedure by generalized least square approach. Differences between groups were considered significant when P <0.05. When the F-test was significant, the Bonferroni mean separation test was used to determine whether means differed.

Correlation among total bacterial population detected from the rumen digesta and tissue samples, ruminal pH and VFA variables including the molar proportion of acetate, propionate, isobutyrate, butyrate, isovalerate, valerate, and concentration of total VFA were analyzed using the PROC CORR procedures of SAS.

3.2 Results

3.2.1 PCR-DGGE analysis of bacteria associated with digesta and epithelial tissue in the rumen of AR and AS animals. When bacterial PCR-DGGE profiles were compared between AS and AR group, bacterial profiles of rumen digesta samples from AR group was 69.8±1.54% similar to those from AS group while the epimural bacterial profiles from AR group was 73.9±0.91% similar to those from AS group (Figure 3.1). MSD analysis of PCR-DGGE profiles revealed a clear separation of PCR-DGGE profiles between AS and AR groups from both digesta and epithelial tissue samples (Figure 3.2). To further confirm the above observation of differences in bacterial community profiles between AR and AS group, pairwise comparison were performed by ANOSIM using Global R statistics. No differences in bacterial profiles were observed at each time point after feeding (P > 0.05). While the bacterial community in rumen digesta (P =0.001) and attached to the epithelial tissue (P=0.002) were significant different between AR and AS animals (Table 3.3).

3.2.2 Analysis of the density of bacteria in the rumen of AR and AS steers. The time point after feeding (hour) and interaction between group and hour had no effect (P>0.05) on total copy number of 16S rRNA genes of both bacterial communities (Table 3.4). The total copy number of 16S rRNA genes in the rumen digesta of AS steers was 10-fold higher than that of AR steers, while it was not different between AR and AS epimural communities.

3.2.3 Expression of TLR2 and TLR4. The expressions of TLRs were evaluated based on ΔCT, the relative amplification of the targeted genes to the house keeping gene. Therefore, the lower value of ΔCT indicates the earlier amplification and higher level of gene expression. The value of ΔCT of TLR4 in the rumen epithelium of AR group was ranged from 2.0~2.7 while it was from 11.4~12.4 in the AS group, which they were significant different (P <0.001) (Figure 3.3). The fold change of TLR4 expression for AR group was 741.7±62.0 higher than that of AS group. In addition, the value of ΔCT of TLR2 in the rumen epithelium of AR group (8.6~9.0) was significantly lower than (12.4~13.5) that for AS group (P<0.01), the expression of TLR for AR group was 22.4±2.1folds higher than that of AS group. Moreover, TLR4 expression in the rumen epithelium significantly changed at 0, 2, 4, and 6 h after feed, within each group (P=0.02) while TLR2 expression didn't change comparing four different time point (P=0.59).

3.2.4 Correlation between bacterial density, fermentation variables and TLR expression. The copy number of total 16S rRNA genes of epimural bacteria was positively correlated with ruminal pH (r = 0.59, P = 0.04) and negatively correlated with total VFA concentration (r = -0.59, P = 0.05) for AR steers, but no such relationship was found for AS animals (Table 3.5). Furthermore, the copy number of total 16S rRNA genes of content bacteria was positively correlated with molar proportion of butyrate (r=0.74, P=0.006) and negatively correlated with molar valerate (r = -0.58, *P* =0.04) for AR animals, while it was negatively correlated with molar butyrate (r=-0.73, *P*=0.007) and positively correlated with molar valerate (r = 0.53, *P* =0.05) for AS animals. Overall the expression data, compared between two groups, the expression of TLR4 was significantly positively related (P<0.01, Table 3.5) to the copy number of total 16S rRNA genes of epimural bacteria in AR group. However, there was no association found between the expression of TLR2 and density of epimural bacteria community between two groups.

3.3 Discussion

Rumen acidosis is a common health related problem in ruminant livestock industry. Recent studies have revealed that the onset of ruminal acidosis can induce changes in the microbial population including decreases in the number of gram negative bacteria and increases in the number of gram positive bacteria (Nagaraja and Titgemeyer, 2007). The detected difference of rumen digesta PCR-DGGE profiles between AS and AR group is in agreement with previous data that reported a bacterial composition of diversity changed in different severity of

SARA (Khafipour et al., 2009b), supporting that the bacterial diversity in the rumen digesta may have a role in SARA development. Our observed significant higher total VFA concentrations in the rumen of AS animals at 2, 4 and 6h compared to that of AR steers (Schlau et al., unpublished data) suggest that AS animals may have higher microbial fermentation and lower absorption activities than AR steers when they were fed with the same diet. These activities may be due to the variation of bacterial density. Higher copy numbers of total 16S rRNA gene in the digesta for AS group suggest that increased microbial fermentation in rumen of these animals may be related to decreased rumen pH during high concentrated diet induced acidosis. This confirmed the finding by Goal et al. (1998) that induction of ruminal acidosis resulted in increased total viable anaerobic bacteria. The rumen pH of AS steers was lower than that of AR group which indicated the excessive VFA production exceeds the ability of the ruminal papillae absorption leading the accumulation of protons in the rumen. Significant relationships between density of total bacteria in the rumen digesta and VFA such as the positive correlation with butyrate in the AR suggested that population and activities of butyrate producers are higher in rumen of AR steers. Future study to investigate this population and its functions including butyrate kinase and butyryl-CoA/acetate CoA transferase will provide better understandings of the roles of butyrate producers (Macfarlane and Gibson, 1997; Miller and Wolin, 1979; Duncan et al., 2002) in acidosis resistance. The observed higher expression of sodium hydrogen exchanger, isoform 3, which imports Na^+ to the epithelial cell and exports H^+ to the rumen (Schlau et al., unpublished data), supporting the

speculation that higher rumen pH for AR steers might be attributed to faster rate of VFA absorption.

Besides the changes of bacteria diversity and density in the rumen digesta, this study is the first to report the diversity and density difference of epimural bacteria community between AR and AS steers. Although the function of these epimural bacteria is not well known, the fact that bacteria intimately associated with the host's epithelial tissues is more likely to indicate epimural bacteria could have an influence on the surface protection or the assistance in absorption (Freter, 1969; 1970). No significant difference in density of epimural bacteria between AR and AS steers suggests that the diversity difference rather than density changes are associated with different susceptibility of steers to SARA. Our recent study revealed that the epimural bacterial diversity was changed during the high grain diet adaptation and the diversity variation was associated with difference in pH changes for host animal (Chen et al., 2011). These further pinpoint the importance of the epimural bacteria in SARA development.

The observed difference in TLR expression has further supported the importance of the epimural bacteria. TLRs may homeostatically function in the gut epithelium (Medzhitov et al., 2009) and the stimulation of TLR expressions by commensal intestinal flora is critical for protecting against intestinal epithelial injury (Rakoff-Nahoum et al., 2004). The identified higher expression of TLRs 2 & 4 in the rumen papillae of AR steers than those in AS steers indicating that host immune responses may be higher in the rumen of AR steers to protect rumen epithelium from damage and reducing the barrier function of the rumen by SARA.

Moreover, TLR4 has been identified as a major contributor to autoimmune functions which may replace or impair the epithelium barrier failure (Testro and Visvanathan, 2009). Therefore, higher VFA absorption could occur in the rumen epithelium in AR steers, and better host maintenance functions due to the epimural bacteria community which may prevent the VFA accumulation which leads to the resistance to SARA, comparing to AS steers. In addition, the relationship between the copy number of total 16S rRNA genes of epimural bacteria with ruminal pH and total VFA concentration (positively correlated with ruminal pH and negatively correlated with total VFA concentration) and expression of TLR4, which is the first time to be investigated, suggests that the epimural bacterial population may be related to maintain the higher pH and lower acidity on the rumen surface of AR animals and stimulate appropriate activation of the immune system (Sartor, 2000; Farrell and LaMont, 2002) which is thought to be prevent to damage to the epithelial tissues. Future study to investigate rumen epithelial functions (e.g. VFA absorption or transport) and response (e.g. inflammation) associated with epimural bacteria will provide clues on the mechanisms on how host function can be impacted by these bacteria and their roles in acidosis tolerance.

In conclusion, this study showed the differences of bacterial diversity and density in the rumen between AR steers and AS steers. The different bacterial diversity in both rumen digesta and epimural community in AS animals suggest that particular structure of the ruminal microbial community may be related to the higher density of the bacteria when the high concentrate diet was fed. This is the

first attempt to compare the epimural bacteria diversity and density and their association with host TLRs 2 and 4 expressions between AR and AS steers. Higher expression of host innate immunity in AR animals indicates that host gene expressions were altered by changes in epimural bacterial diversity and density which mitigate the severity of acidosis in AR animals. This warrants future study of host genetic markers which regulate the microbial-host interaction with respect to tolerance to SARA.

Table 3.1 Ingredient composition and nutrient composition of diet fed to AR and AS steers

Ingredient composition, % DM	
Dry-rolled barley grain	56.7
Dry-rolled oats grain	28.3
Alfalfa pellet	10.0
Premix of mineral , vitamin and monensin[1]	5.0
Nutrient composition, % DM	
DM	92.2
OM	93.5
Starch	38.6
NDF	28.7
CP	17.6

[1]Contained 1.60% Na, 0.49% P, 6.00% K, 6.0% Ca, 0.20% S, 0.65% Mg, 1220mg/kg Zn, 242 mg/kg Cu, 11mg/kg Mn, 242mg/kg Co, 13mg/kg I, 220mg/kg Fe, 90000 IU/Kg Vitamin A, 13333 IU/Kg Vitamin D, and 400.0 IU/kg Vitamin E.

Table 3.2 The primers used for detection of rumen bacteria in this study

Target bacterium	Primers (5'→ 3')	Annealing temp (°C)	Product size (bp)	Reference
27F-1492R	F: AGAGTTTGATCMTGGCTCAG R: TACGGYTACCTTGTTACGACTT	58	1465	Yang et al., 2010
HDA	F:ACTCCTACGGGAGGCAGCAG T R:GTATTACCGCGGCTGCTGGC AC	54	200	Walter et al., 2000
U2	F: ACTCCTACGGGAGGCAG R: GACTACCAGGGTATCTAATCC	62	468	Stevenson and Weimer, 2007
TLR2	F: CTGTGTGCGTCTTCCTCAGA R: TCAGGGAGCAGAGTAACCAGA	60	228	This study
TLR4	F: GGTTTCCACAAAAGCCGTAA R: AGGACGATGAAGATGATGCC	60	137	This study

Table 3.3 ANOSIM of bacteria profiles between AR and AS steers

		Bacterial profiles of rumen digesta		The epimural bacterial profiles	
	The time point after feeding	***R* statistic**	***P*-value**	***R* statistic**	***P*-value**
	0h	0.370	0.191	-0.037	0.496
AR *vs*	**2h**	0.259	0.285	0.111	0.189
AS	**4h**	-0.074	0.798	0.519	0.202
	6h	0.185	0.295	0.333	0.301
Overall: AR *vs* AS		0.367	0.001	0.412	0.002

Table 3.4 The diversity and density of bacteria in the rumen

	AR				AS				*P*-value		
	0H	**2H**	**4H**	**6H**	**0H**	**2H**	**4H**	**6H**	**G**	**H**	**G* H**
Similarity(%, content vs tissue)	65.5.±2.2 [b]	61.4±2.0 [b]	71.4±1.3 [a]	69.4±2.4 [a]	67.6 ±1.6 [a]	67.9±0.8 [a]	67.3±1.1 [a]	69.3±1.5 [a]	0.36	0.02	0.02
Total bacterial population in the rumen content (copy number /g)	(2.46 ±1.98) $\times 10^9$	(3.50 ±2.58) $\times 10^9$	(3.92 ±2.35) $\times 10^9$	(2.04 ±1.14) $\times 10^9$	(5.10 ±1.22) $\times 10^{10}$	(3.00 ±0.92) $\times 10^{10}$	(2.71 ±0.78) $\times 10^{10}$	(3.09 ±0.94) $\times 10^{10}$	<0.001	0.38	0.31
Total epimural bacterial population (copy number /g)	(2.19 ±0.29) $\times 10^{10}$	(8.24 ±4.36) $\times 10^9$	(1.00 ±0.53) $\times 10^{10}$	(1.67 ±0.69) $\times 10^{10}$	(1.55 ±0.74) $\times 10^{10}$	(2.19 ±1.17) $\times 10^{10}$	(3.37 ±1.47) $\times 10^{10}$	(2.69 ±1.70) $\times 10^{10}$	0.17	0.89	0.52

G: Group (AR and AS); H: the time point during the 6-h postprandial sampling period (At 0, 2, 4, and 6 h after feeding). [a, b] Within a column, means with different letters are significantly different.

Table 3.5 Correlation between density of bacteria community and fermentation variables

	Total bacterial population					
	Content	Tissue	Content		Tissue	
			AR	AS	AR	AS
acetate	-0.48*	0.19	0.35	0.16	0.19	0.43
propionate	0.69**	-0.05	-0.59*	0.02	-0.05	-0.26
isobutyrate	0.22	0.45	-0.003	0.61*	0.45	-0.25
butyrate	-0.82**	-0.33	0.74**	-0.73**	-0.33	-0.41
isovalerate	-0.20	0.14	0.49	0.45	0.14	-0.31
valerate	0.51*	0.47	-0.58*	0.53*	0.47	-0.15
Total VFAs	0.23	-0.59*	0.15	-0.37	-0.59*	0.45
pH	-0.23	0.59*	-0.40	0.30	0.59*	-0.26
ΔCT of TLR4		0.32			-0.71**	0.32
ΔCT of TLR2		0.33			-0.26	0.07

* ***P*<0.05** * * ***P*<0.01**

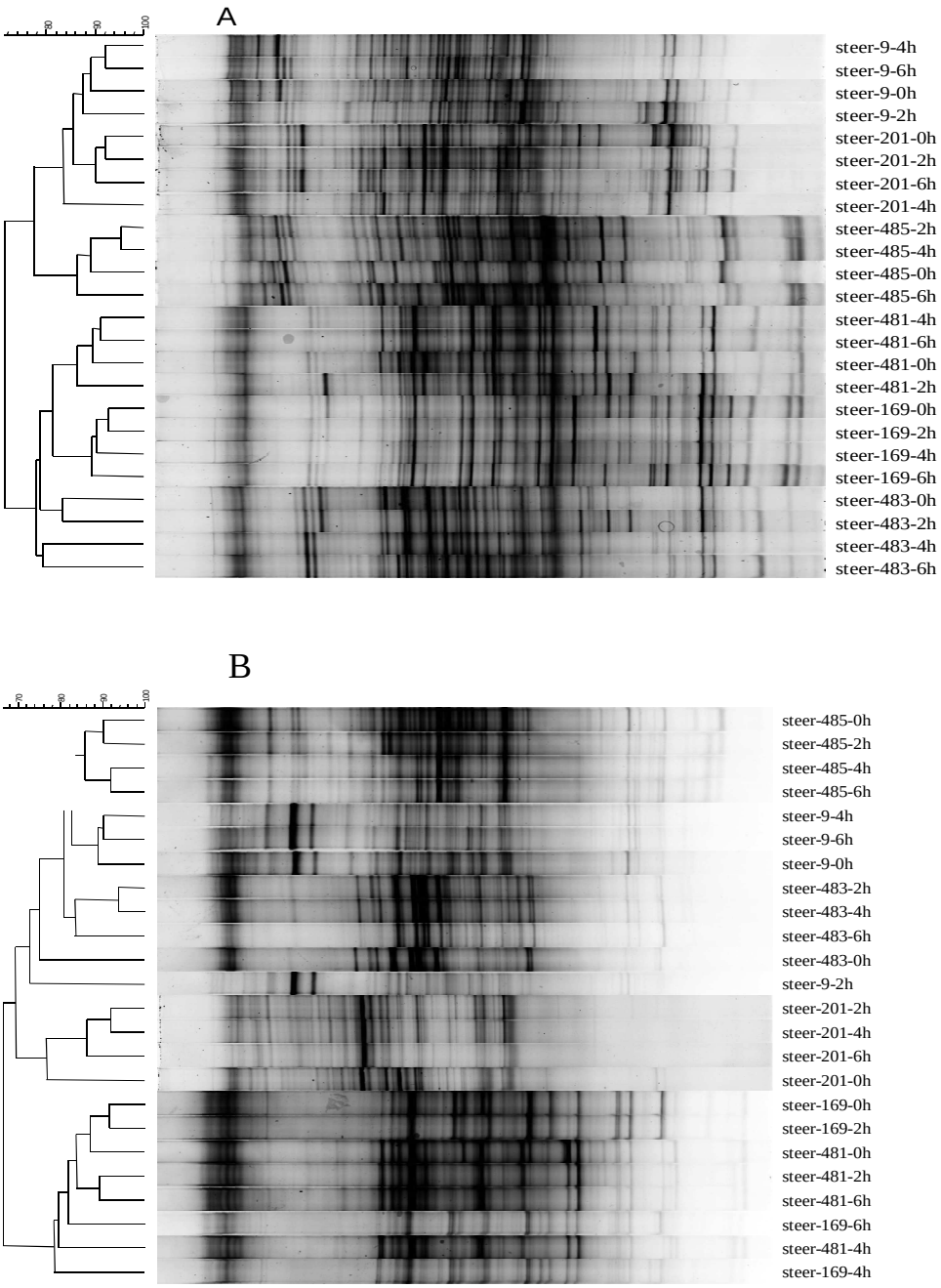

Figure 3.1 The cluster of the PCR-DGGE profiles of bacterial profiles of AR and AS steers from (A) rumen tissue samples (B) rumen digesta samples collected at the four different time point after feeding.

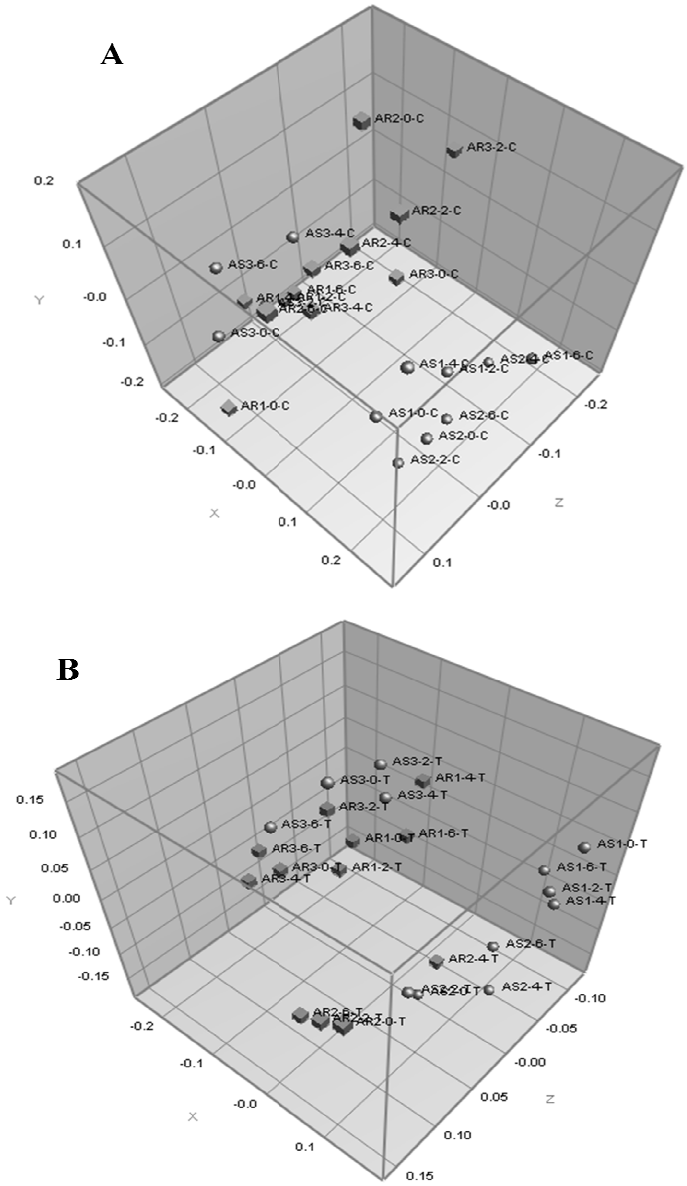

Figure 3.2 MDS analysis' plots of bacteria PCR-DGGE profiles generated from (A) rumen content samples (B) rumen epithelium tissue samples. Different groups were indicated with different shapes: ball (n=12): acidosis-resistant steers; cube (n=12): acidosis-susceptible steers.

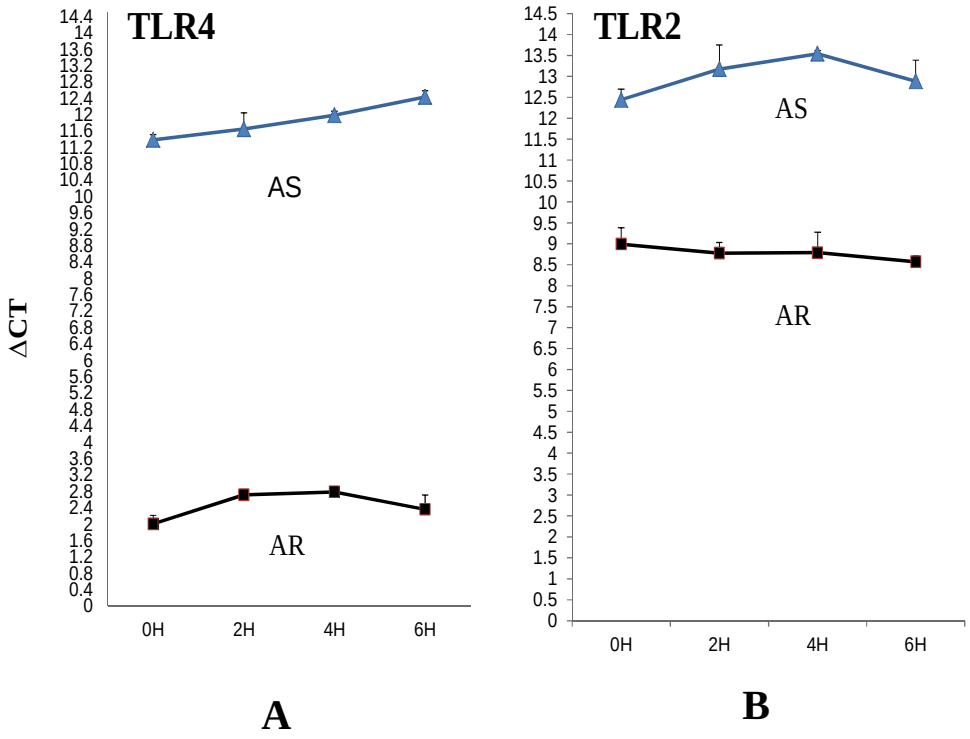

Figure 3.3 TLR4 expression (A) and TLR2 expression (B) in the rumen epithelium between AS and AR steers at each time point after feeding. ΔCT: the relative amplification of the targeted genes to the house keeping gene. The lower value of ΔCT indicates the earlier amplification and higher level of gene expression.

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Chapter Ⅳ. General Discussion

Subacute ruminal acidosis (SARA) is a common digestive disorder in beef and dairy cattle, which happens during a sudden diet transition or with an overload of starch fermentation in the rumen. When SARA occurs, the ruminal pH significantly decreases due to the accumulation of fermented acids, such as lactic acid or VFAs, which are produced by rumen microbes and supply the energy resources for the host development. It has been reported that the bacteria associated with rumen digesta is altered during dietary transition and experimental SARA induced (Goad et al., 1998; Nagaraja and Titgemeyer, 2007). However, there is very limited understanding on the diversity and density of ruminal epithelial wall attached bacteria and their changes during diet transition and experimentally induced SARA, in the rumen of beef cattle. Two studies were performed in this project, in which, the effect of diet on the diversity and density of epimural bacteria, and in rumen bacterial variations including digesta and ruminal epimural bacterial communities, between SARA resistant (AR) and susceptible (AS) animals were observed.

Our study 1 was the first to monitor the epimural bacterial diversity changes from the same host animal through a dietary transition from a 97% forage based diet to an 8% forage based diet including a transition period with a 25% forage based diet. This study was the first to report that diet can change the diversity of epimural bacterial community, similar as the digesta associated community. Although feeding the high concentrate diet (85% grain) reduced rumen pH, not all animals developed SARA. In Study 2, we identified bacterial diversity and

density differences in the rumen between AR and AS animals using a trial that fed beef steer with high concentrate diet. It has been reported, that rumen microbial diversity and population change during experimentally induced SARA (Goad et al., 1998; Khafipour et al., 2009). However, the microbial profiles between AR and AS animals had not been compared and there was no data on epimural bacterial community. Our study showed that the diversity of rumen digesta and epimural communities were different between AR and AS animals, and that the total copy number of bacteria in rumen digesta was much higher in the rumen of AS steers compared to that of AR steers. These results suggest that the diversity of bacterial community in the rumen may be associated with the host response to SARA, and that this could be achieved by regulating density of total bacteria. Interestingly, in study 2, the copy number of total 16S rRNA genes of digesta associated bacteria was positively correlated with the molar proportion of butyrate in AR animals, while it was negatively correlated in AS animals. Higher butyrate proportions were identified in the rumen of the AR group (Schlau et al., unpublished data). During ruminal acidosis, the rumen epithelium can be damaged due to the long duration of low rumen pH, what majorly leads to epithelial barrier function failure in the rumen (Penner et al., 2010). We proposed that higher butyrate and a positive relationship with rumen bacteria in AR steers could activate certain growth factors, such as IGF-1 and EGF (Gálfi et al., 1981, 1993; Neogrady et al., 1989a, b; Baldwin, 1999) of the rumen epithelial tissue. These growth factors may impair the damage of rumen epithelium preventing inhibition of VFAs absorption. As butyrate induces the release of hormones and

growth factors, these factors bind to respective cellular membrane receptors (Baldwin, 1999) leading to the induction of cellular responses in the rumen of AR steers. Future studies that further investigate these factors will supply fundamental understanding and explanations for the observed differences on the above relationships.

To date, many studies have pointed out the influence of the host on rumen microbial diversity (Guan et al., 2008; Li et al., 2009). In our case, a significant individual variation was also observed on ruminal pH changes (Chapter 2, Figure 2.5), bacterial profiles changes (Chapter 2, Figure2.2 and Figure4.1), and total bacterial population changes among the 18 steers under the three diets (Study 1) (Chapter 2, Figure2.3). These results strongly suggested that the individual variation needs to be taken into account when studying the association between ruminal microbial diversity and SARA. In addition to the differences among the animals with regard to their susceptibility to SARA, there were significant differences in bacterial diversity detected between AR and AS steers. However, within each group, individual variation of diversity and density was also observed. For example, steer 485 seems to be an outlier (Figure3.1) from the AS group, and is more similar to the AR group at diversity level. The observation of individual variation suggests, again, that the host has an effect on ruminal bacterial ecology in rumen. So it has been considered that there are three factors, host genetics, environment (including diet) and microbial interactions that can impact on microbial diversity. Future researches on understanding the roles of rumen

microbial community in SARA susceptibility and resistance need to include all these factors.

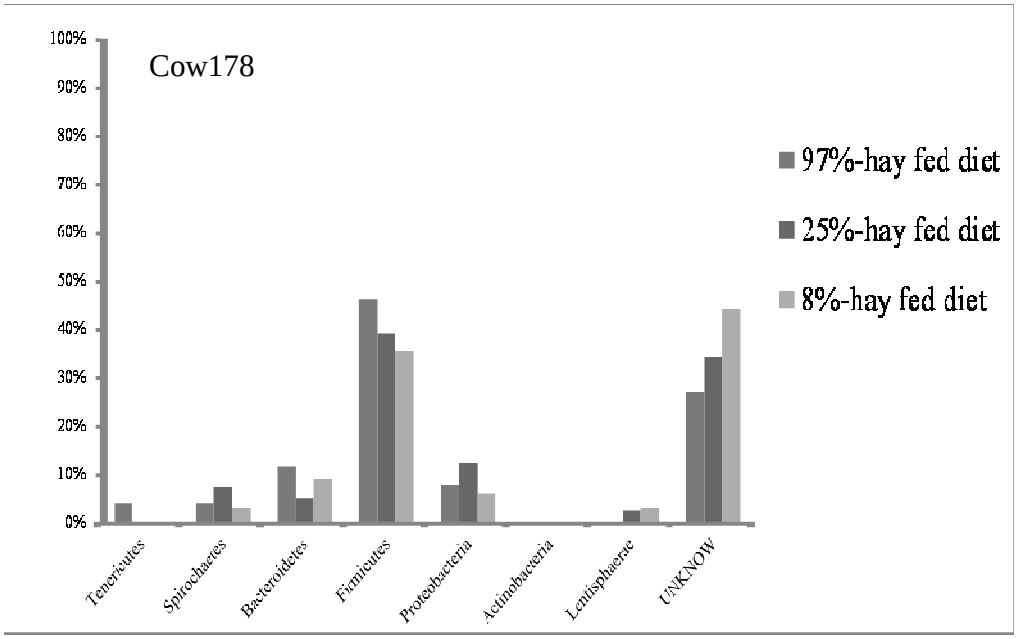
The linkage of the diversity and density of microbial communities in rumen, including contents and epimural bacteria to host function has not been well studied. Host immune response was considered to be one of the linkages investigated in this project. We speculated epithelial surface structure including receptors and cell proliferation may determine the diversity and density of the epimual bacteria. In the study, we only investigated expression of 2 TLRs. The expression of TLR2 and TLR4 were 22 and 742 folds higher in AR animals than in AS animals, suggesting that the host immune cell responses were more highly activated by TLRs in AR steers. However, there is no study to date on the regulatory mechanisms of the expression of these genes. Are the detected expression differences between AR and AS animals due to genetic variation of these genes? Or, are they due to some other post transcriptional and translational regulation? In the bovine genome, there are 10 TLRs, which can recognize the molecular patterns from microbes. Future studies to investigate the expression of other TLRs in AS and AR animals may elucidate whether the by-products of ruminal epimural or digesta associated bacteria have an impact on rumen epithelial immune functions. Also, future studies that investigate cytokines, chemokines and other immune related functions will aid a better understanding of the host function in AR and AS animals. In addition, based on the variation in susceptibility we observed on the steers of this study, genotyping would seem to be a good way to determine gene variation among the two groups. For example,

single nucleotide polymorphisms (SNPs) genotyping could be performed, and then designate them as genetic markers to investigate microbe-host interaction with respect to tolerance to SARA.

In this thesis, there were some intrinsic limitations that this research was faced with. First, the low resolution of PCR-DGGE led to the identification of bands only at phylum or genus level, 59% of the bands are still not identified, and only 88 PCR-DDGE bands were identified while rumen can contain up to about 400 species (Brulc et al., 2009). Future studies using next generation sequencing technology will supply better identification of the ruminal epimural bacterial communities and their alteration under different diets at species or strain level. Second, the functions of the identified bacterial community were not directly measured. Currently our research group is working on the application of metagenomics to these samples, and it is expected that this will help to understand whether and how the functions of epimural bacteria change during dietary transition. This understanding could lead to improved management practices of dairy and beef cattle in order to improve their rumen functions. A microbial model that could be used to investigate changes in rumen Gram-negative and Gram-positive bacteria populations and such relationship to host gene expression during SARA is currently lacking. Approaches currently being attempted by our group to identify Gram-negative and Gram-positive epimural bacteria and their by-products which stimulated the host gene expression in the rumen of AR and AS animals, may supply potential model to investigate their roles in SARA. Third and last, we only collected rumen tissue from one location of the rumen. In future

studies it would be desirable to utilize sampling from multiple locations within the rumen; this would provide direct evidence of the linkage between rumen epimural bacteria and host function changes during dietary transitions and SARA.

Therefore, our overall contribution to the understanding of the ecology of epimural bacteria under different conditions constitutes importantly fundamental information, which can be applied to diet management and future regulation of rumen acidosis disease in the livestock industry.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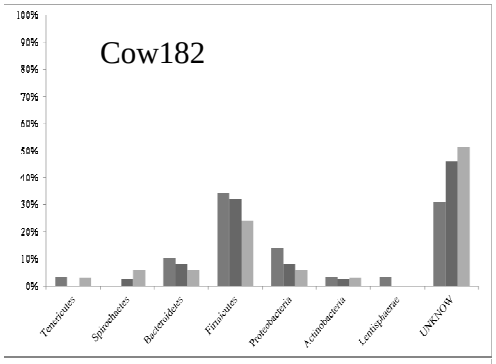
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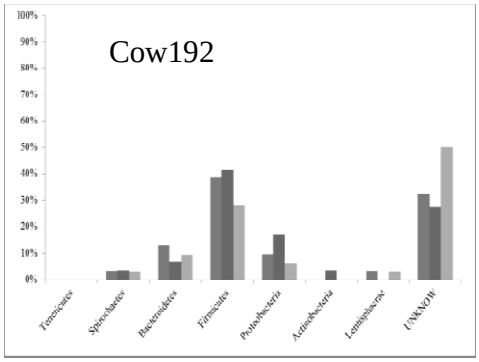
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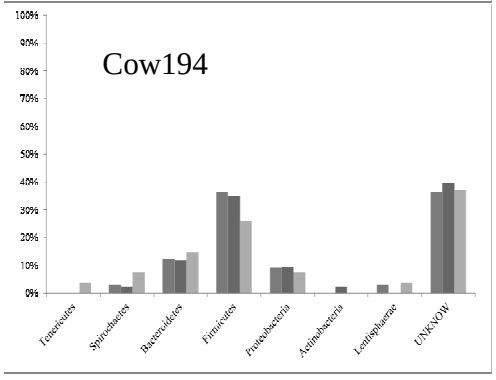
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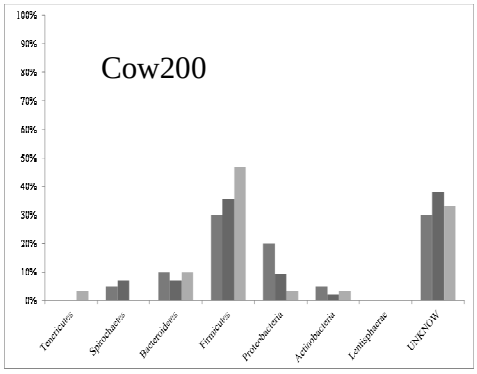
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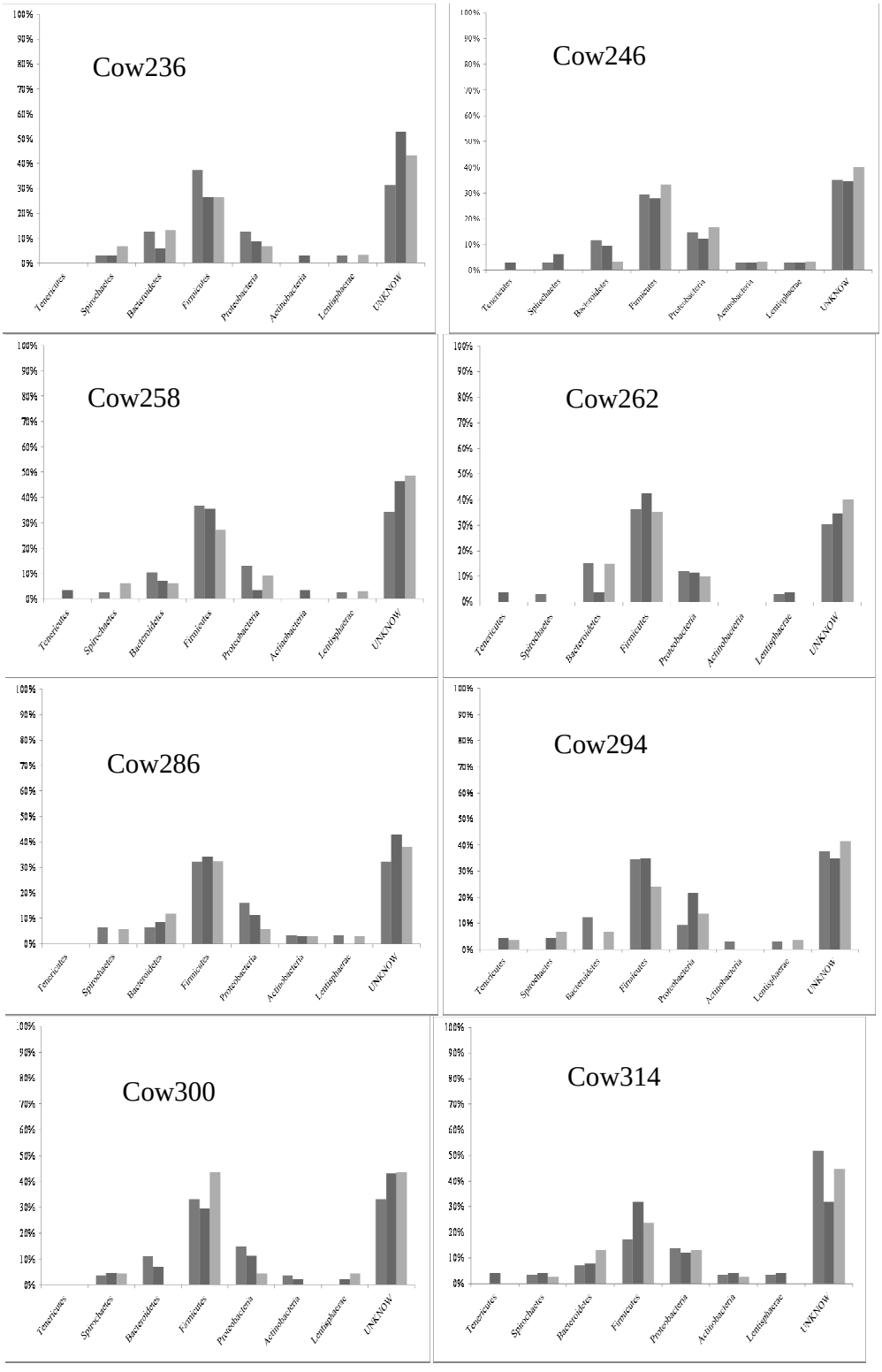
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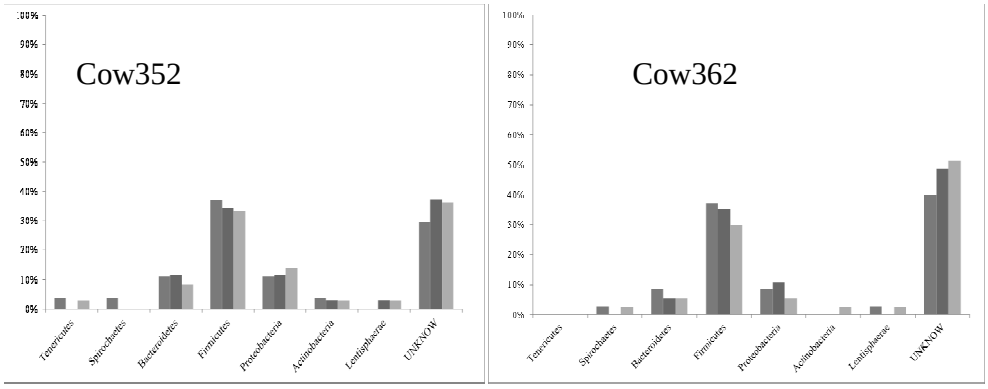

Figure 4.1 Variation of the distribution changes of epimural bacteria at phylum level of the other 15 heifers in response to 97%, 25%and 8% hay diet, respectively based on the sequence information from the reference marker.

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