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Associations between Rumen Bacteria and Feed Efficiency in Beef Cattle

By

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Dedicated to my family.

Gracias Papá, Mamá, Peri y Lups por su apoyo.

ABSTRACT

Feed efficiency affects profitability and sustainability in beef production systems. Since ruminal microbes play essential roles in feed digestion and conversion, the overall objective of this project was to investigate the association between ruminal bacteria and feed efficiency of beef cattle. PCR-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) was applied to investigate ruminal microbial communities. A statistical model was developed to link the PCR-DGGE band patterns to host phenotypic measurements such as feed efficiency and rumen fermentation variables. Therefore, we determined how the numbers and species of ruminal bacteria present varied dependently as well as independently from diet and their associations with feed efficiency. Likewise, the impact of these interactions on the metabolic capacity and feed efficiency of the host was assessed. Study 1 demonstrated that particular bacteria in the rumen contributed to differences in feed efficiency when host was fed low energy diet. Study 2 evaluated whether structure of bacterial populations remained stable in spite of the dietary changes; we also determined how specific bacterial groups could impact the feed efficiency under low and high energy density diets. Populations of three bacterial species (*Succinivibrio* sp., *Eubacterium* sp., and *Robinsoniella* sp.) were identified to be correlated with feed efficiency measurements; their predicted metabolic mechanisms influencing feed efficiency were proposed (propionate synthesis, formate production and cross-feeding interaction with methanogens). Furthermore, host factors were elucidated in Study 3. Frequency

analysis of bacterial PCR–DGGE bands showed that *Prevotella* sp. was abundant in Angus rumen liquid and contents while *Clostridium* sp. was present in contents and tissue from Charolais steers. Rumen tissue from Hybrid animals presented high frequency of *Prevotella* sp. but no species were particularly abundant in liquid, suggesting a strong association between host and the colonization of rumen bacteria. This is the first study linking rumen microbial diversity and microbial metabolites to host feed efficiency traits and their implications on individual variations in Residual Feed Intake of beef cattle.

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TABLE OF CONTENTS

EXAMINING COMMITTEE……II

DEDICATED TO MY FAMILY…….....III

ABSTRACT ……...IV

ACKNOWLEDGEMENTS……...VI

TABLE OF CONTENTS……VIII

LIST OF TABLES……XV

LIST OF FIGURES……..XIX

LIST OF ABBREVIATIONS…….XXVII

CHAPTER 1. LITERATURE REVIEW……1

1.1. FEED INTAKE AND UTILIZATION EFFICIENCY…….2

1.1.1. Feed efficiency measurements……...3

1.1.1.1. *Gross efficiency*..……....3

1.1.1.2. *Maintenance efficiency*……4

1.1.1.3. *Partial efficiency of growth*…….4

1.1.1.4. *Cow/calf efficiency*……..4

1.1.2. Residual Feed Intake…….5

1.1.2.1. *Association with physiological factors*……6

1.1.2.2. *Effects of variations in diet*..……..8

1.2. EVOLUTIONARY ADAPTATIONS OF RUMINANTS……9

1.2.1. Digestive adaptations……..10

1.2.2. Host–microbe co-evolution……11

1.3. HOW DOES THE RUMEN WORK?.....14

1.3.1. Rumen anatomy and functions……14

1.3.1.1. *Rumen*……14

1.3.1.2. *Reticulum*……...15

1.3.1.3. *Omasum*……15

1.3.1.4. *Abomasum*……15

1.3.2. Rumen environment and fermentation……16

1.3.2.1. *Rate of fibre digestion*……18

1.3.2.2. *Production of microbial protein*……18

1.3.2.3. *Ammonia accumulation*……19

1.3.2.4. *Methane production*……...19

1.3.2.5. *Lactate production*……19

1.3.3. Fermentation of carbohydrates……20

1.3.3.1. *Pentose metabolism*……...21

1.3.3.2. *Pyruvate metabolism*……21

1.3.3.3. *VFA synthesis*……21

1.3.3.3.1. *Acetate and butyrate*……...22

1.3.3.3.2. *Propionate*……..22

1.3.3.3.3. *Other VFA*……..23

1.3.3.4. *Metabolism of Nitrogen*……23

1.3.3.4.1. *Microbial protein*……...24

1.3.3.5. *Production of Methane*……..25

1.4. MICROBIAL ECOSYSTEM IN THE RUMEN……..27

1.4.1. Bacteria……27

1.4.2. Archaea……29

1.4.3. Protozoa……...31

1.4.4. Fungi……33

1.4.5. Bacteriophages……34

1.4.6. Interactions among different microbial populations……35

1.5. HISTORICAL PERSPECTIVES OF RUMINAL MICROBIAL ECOLOGY……..39

1.6. METHODOLOGICAL ADVANCES……...42

1.6.1. rRNA/rDNA – based methods……42

1.6.1.1. *FISH*……..43

1.6.1.2. *Quantitative Real-Time PCR*……44

1.6.2. Community fingerprinting techniques……45

1.6.2.1. *RFLP and T-RFLP*……45

1.6.2.2. *DGGE / TGGE*……..46

1.6.3. Sequence-based approaches……48

1.6.3.1. *Clone libraries*……...49

1.6.3.2. *Metagenomics*……50

1.7. SUMMARY……...53

1.8. LITERATURE CITED……..54

CHAPTER 2. LINKAGE OF PARTICULAR BACTERIAL PCR-DGGE PATTERNS TO BOVINE RUMINAL FERMENTATION PARAMETERS AND FEED EFFICIENCY TRAITS. ……...**90**

2.1. INTRODUCTION……90

2.2. MATERIALS AND METHODS……..92

2.2.1. Animals and sampling……92

2.2.2. DNA Extraction……...93

2.2.3. PCR-DGGE analysis……...94

2.2.4. Similarity analysis of PCR-DGGE profiles……95

2.2.5. Cloning and sequencing analysis of DGGE bands……..95

2.2.6. Multivariate Statistical Analysis……..96

2.2.7. Analysis of fermentation parameters……...98

2.2.7.1. *VFA and NH_3-N*……98

2.3. RESULTS. ……98

2.3.1. PCR-DGGE analysis of detectable bacteria in rumen samples……98

2.3.2. Analysis of fermentation profiles……..100

2.3.3. Correlations among PCR-DGGE profiles, fermentation characteristics and host feed efficiency traits……..101

2.4. DISCUSSION……...102

2.5. CONCLUSIONS……...109

2.6. LITERATURE CITED……128

CHAPTER 3. IMPACT OF FEED EFFICIENCY AND DIET ON THE ADAPTIVE VARIATIONS IN THE BACTERIAL COMMUNITY IN THE RUMEN FLUID OF CATTLE IDENTIFIED BY PCR-DGGE AND QUANTITATIVE REAL TIME PCR ANALYSIS……135

3.1. INTRODUCTION…….....135

3.2. MATERIALS AND METHODS……...137

3.2.1. Animals and sampling……...137

3.2.2. DNA Extraction and PCR DGGE analysis……138

3.2.3. Analysis of fermentation profiles: VFA and NH_3-N…….139

3.2.4. Quantitative real-time PCR……140

3.2.5. Statistical Analysis…….143

3.3. RESULTS . ……..145

3.3.1. PCR-DGGE profiling of rumen bacterial dynamics between diets……145

3.3.2. Characterization of fermentation metabolites in response to diet and RFI classification……146

3.3.3. Interactions between PCR-DGGE profiles, fermentation characteristics and host feed efficiency under different diets……147

3.3.4. Comparison of specific and total bacterial populations…….150

3.4. DISCUSSION……...150

3.5. CONCLUSIONS……...157

3.6. LITERATURE CITED……183

CHAPTER 4. POTENTIAL ASSOCIATIONS BETWEEN RUMINAL BACTERIAL PCR–DGGE PROFILES AND HOST BREEDS IN BEEF CATTLE……193

4.1. INTRODUCTION……..193

4.2. MATERIALS AND METHODS……195

4.2.1. Animals and sampling……...195

4.2.2. DNA Extraction and PCR DGGE analysis……196

4.2.3. Analysis of ruminal fermentation products……...197

4.2.4. Statistical Analysis……198

4.3. RESULTS. ……..199

4.3.1. Evaluation of PCR–DGGE profiles among rumen fractions……199

4.3.2. Assessment of fermentation profiles and feed efficiency variables among breeds……200

4.3.3. Linkage of PCR–DGGE profiles, ruminal fermentation and feed efficiency measures with diverging breeds……201

4.4. DISCUSSION……...204

4.5. CONCLUSIONS……...209

4.6. LITERATURE CITED……225

CHAPTER 5. GENERAL DISCUSSION……...232

5.1. DEVELOPMENT OF A STATISTICAL ANALYSIS TO LINK THE PCR-DGGE BACTERIAL PROFILES TO PHENOTYPIC PARAMETERS……..232

5.2. IDENTIFICATION OF BACTERIAL SPECIES ASSOCIATED TO RESIDUAL FEED INTAKE (RFI) IN THE RUMINAL LIQUID……..238

5.3. ASSESSMENT OF THE RELATIONSHIPS AMONG THE THREE BACTERIAL POPULATIONS INHABITING THE RUMEN AND HOST'S RESIDUAL FEED INTAKE (RFI)…….241

5.4. EVALUATION OF THE IMPACT OF HOST GENETIC BACKGROUND AND RESIDUAL FEED INTAKE (RFI) ON MICROBIAL POPULATIONS IN THE RUMEN……...243

5.5. LITERATURE CITED……245

CHAPTER 6. GENERAL CONCLUSIONS…….249

LIST OF TABLES

Table 1.1. Factors impacting Residual Feed Intake (RFI)…….....8

Table 1.2. Interactions among rumen bacteria and diverse ruminal microorganisms……..38

Table 1.3. Recent perspectives on the use of qRT-PCR to screen bacterial populations…….45

Table 1.4. Novel applications of T-RFLP (1) and RFLP (2) to monitor ruminal bacteria……...46

Table 1.5. Contributions of PCR-DGGE technique to the understanding of the rumen bacterial ecosystem…….48

Table 1.6. Current trends in library-based methods applied to rumen microbial ecology……...50

Table 2.1. Sequence identification of the PCR-DGGE bands…….111

Table 2.2. Fermentation and feed efficiency measurements in steers differing RFI and fed low energy diet (n = 58)……..115

Table 2.3. Correlation (r) of fermentation measurements in the rumen of steers differing RFI with indicators of feed efficiency (RFI, DMI, ADG and FCR, n = 58), ***p<0.0001, **p<0.05……116

Table 3.1. Primers used for detection of particular functional bacterial in Study 2……158

Table 3.2. Variations in Residual Feed Intake classification of steers under two different diets. For illustration purposes, only the steers classified as L- or H-RFI in LE diet were included……...159

Table 3.3A. Taxonomical identification of RFI-associated PCR – DGGE bands within particular diets (n = 19)……160

Table 3.3B. Taxonomical identification of diet-associated PCR – DGGE bands within particular RFI groups (n = 19)……..162

Table 3.4. Rumen metabolites and feed efficiency traits in steers differing RFI and diet (n = 19)……...164

Table 3.5A. Differences in particular bacterial species between Low and High Energy density diets within RFI group (n = 19)……..165

Table 3.5B. Differences in particular bacterial species population between L– or H – RFI group within Low and High Energy density diets (n = 19)……...166

Table 3.6. Changes by diet in classification assigned to ruminal metabolites (n = 19). * Diet 1: LE diet, Diet 2: HE diet, RFI: Residual Feed Intake, DMI: Dry Matter Intake, ADG: Average Daily Gain, FCR: Feed Conversion Ratio, Acet: acetate, Prop: propionate, But: butyrate, Isobut: isobutyrate, Val: valerate, Isoval: isovalerate, Total: total VFA, A: P ratio: acetate to

propionate ratio, St: Br ratio: straight to branched – chain VFA, NH3 – N: Ammonia – N……...167

Table 3.7A. Correlation (r) of fermentation metabolites in the rumen of Low RFI steers with indicators of feed efficiency (n = 13) *** $P < 0.0001$, **$P < 0.05$……...168

Table 3.7B. Correlation (r) of fermentation metabolites in the rumen of High RFI steers with indicators of feed efficiency (n = 6) *** $P < 0.0001$, ** $P < 0.05$……..169

Table 3.8. Correlation (r) of fermentation metabolites in the rumen of steers differing RFI under LE with indicators of feed efficiency (RFI, DMI and FCR) and bacterial population (n = 19), *** $P < 0.0001$, ** $P < 0.05$, *trend……...170

Table 3.9. Correlation (r) of fermentation metabolites in the rumen of steers differing RFI under high energy diet with indicators of feed efficiency (RFI, DMI and FCR) and bacterial population (n = 19), *** $P < 0.0001$, ** $P < 0.05$, *trend……..171

Table 3.10A. Correlation (r) of fermentation metabolites and feed efficiency indicators in the rumen of Low RFI steers with functional bacterial populations (n = 13), ***$P < 0.0001$, **$P < 0.05$, * $P < 0.1$……...172

Table 3.10B. Correlation (r) of fermentation metabolites and feed efficiency indicators in the rumen of High RFI steers with functional bacterial populations (n = 6), ***$P < 0.0001$, **$P < 0.05$, * $P < 0.1$…….173

Table 4.1. Feed Efficiency and Ruminal Metabolic Measurements in steers differing breed and Residual Feed Intake (n = 48)……..211

Table 4.2. Feed Efficiency Measurements in steers from diverging sire breed and differing Residual Feed Intake (only H-RFI and L-RFI individuals, n = 27)……212

Table 4.3A. Correlation (r) of fermentation parameters in the rumen of Angus steers differing RFI with indicators of feed efficiency (RFI, DMI, ADG and FCR). n = 5, ***$P < 0.0001$, **$P < 0.05$, *trend…….213

Table 4.3B. Correlation (r) of fermentation parameters in the rumen of Charolais steers differing RFI with indicators of feed efficiency (RFI, DMI, ADG and FCR), n = 19. *** $P < 0.0001$, ** $P < 0.05$, *trend……214

Table 4.3C. Correlation (r) of fermentation parameters in the rumen of Hybrid steers differing RFI with indicators of feed efficiency (RFI, DMI, ADG and FCR), n =24. *** $P < 0.0001$, ** $P < 0.05$, *trend……..215

LIST OF FIGURES

Figure 1.1. Most Probable Number (MPN) procedure to estimate ruminal bacteria……...41

Figure 1.2. Prospective and current opportunities for increased understanding and targeted manipulation of ruminal metabolism (Adapted from Zoetendal et al., 2008)……..52

Figure 2.1. PCR-DGGE profiles generated from ruminal fluid DNA from fifty-eight steers fed with low energy diet using primers HDA1-GC and HDA2 (22 to 55% DGGE). H and L represent the steers with high RFI (H-RFI > 0.5, inefficient), M-RFI (-0.5 < RFI < 0.5) and low-RFI (L-RFI < -0.5, efficient), respectively. RFI, residual feed intake, a parameter to measure feed efficiency in cattle (Basarab et al., 2003). The comparison of the PCR-DGGE profiles was generated with the BioNumerics software package using UPGMA (unweighted pair-group) method as described in the text……117

Figure 2.2A. Principal component analysis of fermentation and feed efficiency measurements. Scatter plot of the first two principal component scores (symbol equals RFI class: low, L; medium, M; high, H)……119

Figure 2.2B. Principal component analysis of fermentation and feed efficiency measurements. Loading plot describing the relationship among measured

variables. ADG, average daily gain; DMI, daily dry matter intake; FCR, feed conversion ratio; RFI, residual feed intake; A:P, acetate to propionate ratio; ST:BR VFA, straight chain to branched chain VFA ratio……...120

Figure 2.3. Frequency of PCR-DGGE bands in animals categorized on the basis of (A) Dry Matter Intake (DMI), and (B) Average Daily Gain (ADG) using PROC CATMOD analysis. The x-axis represents 85 identified bands and the order of the bands reflects the migration locations on the PCR-DGGE gel from top to the bottom. The arrows indicate the frequency of bands detected in the tested the population of each trait……..121

Figure 2.3. Frequency of PCR-DGGE bands in animals categorized on the basis of (C) Feed Conversion Ratio (FCR, F: G), and (D) Residual Feed Intake (RFI using PROC CATMOD analysis. The x-axis represents 85 identified bands and the order of the bands reflects the migration locations on the PCR-DGGE gel from top to the bottom. The arrows indicate the frequency of bands detected in the tested the population of each trait…….122

Figure 2.3. Frequency of PCR-DGGE bands in animals categorized on the basis of (E) Butyrate, and (F) Straight to Branched-chain VFA using PROC CATMOD analysis. The x-axis represents 85 identified bands and the order of the bands reflects the migration locations on the PCR-DGGE gel from top to the bottom. The arrows indicate the frequency of bands detected in the tested the population of each trait……123

Figure 2.3. Frequency of PCR-DGGE bands in animals categorized on the basis of (G) Isobutyrate, and (H) Isovalerate using PROC CATMOD analysis. The x-axis represents 85 identified bands and the order of the bands reflects the migration locations on the PCR-DGGE gel from top to the bottom. The arrows indicate the frequency of bands detected in the tested the population of each trait……..124

Figure 2.4. Frequency of PCR-DGGE bands in animals categorized on the basis of (A) Acetate, and (B) Propionate using PROC CATMOD analysis. The x axis represents 85 identified bands and the order of the bands reflects the migration locations on the PCR-DGGE gel from top to the bottom. The arrows indicate the frequency of bands detected in the tested the population of each trait……..125

Figure 2.4. Frequency of PCR-DGGE bands in animals categorized on the basis of (C) Valerate, and (D) Total VFA concentration using PROC CATMOD analysis. The x axis represents 85 identified bands and the order of the bands reflects the migration locations on the PCR-DGGE gel from top to the bottom. The arrows indicate the frequency of bands detected in the tested the population of each trait……126

Figure 2.4. Frequency of PCR-DGGE bands in animals categorized on the basis of (E) Acetate to Propionate ratio, and (F) NH_3-N using PROC CATMOD analysis. The x axis represents 85 identified bands and the order of the bands reflects the migration locations on the PCR-DGGE gel from top to the

bottom. The arrows indicate the frequency of bands detected in the tested the population of each trait……127

Figure 3.1. PCR-DGGE profiles generated from ruminal fluid DNA from fifty-five steers fed with low and high energy density diet, using primers HDA1-GC and HDA2 (22 to 55% DGGE). H, M and L represent the steers with high RFI (H-RFI, mean plus 0.5 SD, inefficient), medium RFI (M-RFI, -0.5 SD < mean RFI < 0.5 SD) and low-RFI (L-RFI, < mean minus 0.5 SD, efficient), respectively. RFI, residual feed intake, is a parameter to measure feed efficiency in cattle (Basarab et al., 2003). The comparison of the PCR-DGGE profiles was generated with the BioNumerics software package using UPGMA (unweighted pair-group) method as described in the text; the first column indicates the ID of the steer, the second column corresponds to the diet and the third column represents the RFI classification when steer was either under LE or HE……..174

Figure 3.2. PCR-DGGE profiles generated from ruminal fluid DNA from fifty-eight steers fed with high energy density diet using primers HDA1-GC and HDA2 (22 to 55% DGGE). H and L represent the steers with high RFI (H-RFI > 0.5, inefficient), M-RFI (-0.5 < RFI < 0.5) and low-RFI (L-RFI < -0.5, efficient), respectively. RFI, residual feed intake, a parameter to measure feed efficiency in cattle (Basarab et al., 2003). The comparison of the PCR-DGGE profiles was generated with the BioNumerics software package using UPGMA (unweighted pair-group) method as described in the text……176

Figure 3.3. Comparison of PCR–DGGE bacterial profiles for different RFI groups, from nineteen steers (H–RFI, n = 6; M–RFI, n = 4 and L– FI, n = 13) fed low and high energy density feedlot diets. (A) Individuals grouped in the H–RFI group throughout diets, (B) steers belonging to the M–RFI category and (C) animals in L–RFI classification under both feed trials. Dice similarity coefficient is indicated for each clustering analysis…….177

Figure 3.4. Comparison of PCR–DGGE bacterial profiles for different RFI groups from nineteen steers (H–RFI, n = 6; M–RFI, n = 4 and L–RFI, n = 13) fed low and high energy density feedlot diets. (A) Individuals with L-RFI in LE diet that switched to H-RFI in HE, and (B) steer that switched from H-RFI in LE to L-RFI in HE. Dice similarity coefficient is indicated for each clustering analysis……178

Figure 3.5. Specific bacterial species correlated to LE and HE diet and to L– and H–RFI groups. Asterisks indicate the number of species significantly correlated to each group; taxonomy is indicated in the squares corresponding to each quadrant……...179

Figure 3.6A. Frequency of PCR-DGGE bands in animals categorized on the basis of Dry Matter Intake (DMI), Average Daily Gain (ADG), Feed Conversion Ratio (FCR, F:G), Acetate, Isobutyrate, and NH_3–N, under Low Energy diet (A) using PROC CATMOD analysis. The x-axis represents 85 identified

bands and the symbols plotted reflect the frequency of the bands detected in the tested the population of each trait……..180

Figure 3.6B. Frequency of PCR-DGGE bands in animals categorized on the basis of Dry Matter Intake (DMI), Average Daily Gain (ADG), Feed Conversion Ratio (FCR, F:G), Acetate, Isobutyrate, and NH_3–N, under High Energy diet (B) using PROC CATMOD analysis. The x-axis represents 85 identified bands and the symbols plotted reflect the frequency of the bands detected in the tested the population of each trait……..181

Figure 3.7. Proposed cross-feeding interactions among three bacterial groups with similar functions (*Succinivibrio* sp., *Eubacterium* sp., and *Robinsoniella* sp.) and their potential associations with RFI……..182

Figure 4.1A. PCR-DGGE profiles generated from ruminal contents DNA from forty-eight steers fed high energy density diet using primers HDA1-GC and HDA2 (22 to 55% DGGE). H and L represent the steers with high RFI (H-RFI > 0.5, inefficient), M-RFI (-0.5 < RFI < 0.5) and low-RFI (L-RFI < -0.5, efficient), respectively. RFI, residual feed intake, a parameter to measure feed efficiency in cattle (Basarab et al., 2003). The comparison of the PCR-DGGE profiles was generated with the BioNumerics software package using UPGMA (unweighted pair-group) method as described in the text……216

Figure 4.1B. PCR-DGGE profiles generated from ruminal tissue DNA from forty-eight steers fed high energy density diet using primers HDA1-GC and

HDA2 (22 to 55% DGGE). H and L represent the steers with high RFI (H-RFI > 0.5, inefficient), M-RFI (-0.5 < RFI < 0.5) and low-RFI (L-RFI < -0.5, efficient), respectively. RFI, residual feed intake, a parameter to measure feed efficiency in cattle (Basarab et al., 2003). The comparison of the PCR-DGGE profiles was generated with the BioNumerics software package using UPGMA (unweighted pair-group) method as described in the text……218

Figure 4.2. Multidimensional Scaling Analysis of the PCR-DGGE profiles generated from ruminal fluid, digesta and tissue DNA from forty-eight steers fed with low and high energy density diet, using primers HDA1-GC and HDA2 (22 to 55% DGGE). Colours represent a particular rumen fraction: blue, rumen liquid; green, rumen contents and pink, rumen tissue. The comparison of the PCR-DGGE profiles was generated with the BioNumerics software package using UPGMA (unweighted pair-group) method as described in the text; comparison was optimised using the default settings of the software……..220

Figure 4.3. Specific bacterial species correlated to particular rumen fractions. Asterisks indicate the number of species significantly different within each group; taxonomy of the species with high frequency among breeds (>90%) is indicated in the squares corresponding to each quadrant…….221

Figure 4.4A. Frequency of PCR-DGGE bands in animals categorized on the basis of Low Dry Matter Intake (L–DMI), High Average Daily Gain (H–ADG),

and Low Feed Conversion Ratio (L–FCR, F:G), in rumen liquid using PROC CATMOD analysis. The x-axis represents 85 identified bands and the symbols plotted reflect the frequency of the bands detected in the tested the population of each trait. * $P < 0.05$……..222

Figure 4.4B. Frequency of PCR-DGGE bands in animals categorized on the basis of Low Dry Matter Intake (L–DMI), High Average Daily Gain (H–ADG), and Low Feed Conversion Ratio (L–FCR, F:G), in rumen digesta using PROC CATMOD analysis. The x-axis represents 85 identified bands and the symbols plotted reflect the frequency of the bands detected in the tested the population of each trait. * $P < 0.05$……..223

Figure 4.4C. Frequency of PCR-DGGE bands in animals categorized on the basis of Low Dry Matter Intake (L–DMI), High Average Daily Gain (H–ADG), and Low Feed Conversion Ratio (L–FCR, F:G), in rumen tissue using PROC CATMOD analysis. The x-axis represents 85 identified bands and the symbols plotted reflect the frequency of the bands detected in the tested the population of each trait. * $P < 0.05$……..224

LIST OF ABBREVIATIONS

ADG	Average Daily Gain
ANG	Angus breed
A: P ratio	Acetate to Propionate ratio
CCAC	Canadian Council on Animal Care
CH_4	Methane
CHAR	Charolais breed
CP	Crude Protein
DGGE	Denaturing Gradient Gel Electrophoresis
DMI	Dry Matter Intake
DNA	Deoxyribonucleic acid
D_{sc}	Dice Similarity Coefficient
FCR	Feed Conversion Ratio
HE	High Energy density feedlot diet
H-RFI	High Residual Feed Intake
HYB	Hybrid breed
LE	Low Energy density feedlot diet
L-RFI	Low Residual Feed Intake
MDS	Multi-dimensional Scaling Analysis
ME	Metabolisable Energy
M-RFI	Medium Residual Feed Intake
MWT	Mid-Weight on Test (for RFI)
NFE	Net Feed Efficiency

NH_3-N	Ammonia-N
PC	Principal Component
PCA	Principal Components Analysis
PCR	Polymerase Chain Reaction
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
PEG	Partial Efficiency of Growth
RFI	Residual Feed Intake
RNA	Ribonucleic acid
SARA	Sub-acute ruminal acidosis
SCFA	Short-Chain fatty acids
SD	Standard Deviation
St: Br VFA ratio	Straight- to Branched-chain Volatile Fatty Acids
UPGMA	Unweighted pair group with mathematical averages
VFA	Volatile Fatty Acids

Chapter 1. Literature Review.

Throughout history, agriculture has been permanently proactive in the incorporation of novel technologies. The outcome has been an ever increasing efficiency of food production. But, in spite of the "Green Revolution" success, additional challenges have emerged. As soon as the economic status of the population improves, and immediately after the basic nutritional needs are met, the demand for meat starts escalating at a much higher rate. By 2025, the world population will have increased by 25% (UN, 2004); even assuming that the per capita meat consumption remains constant, a 25% increase in production will be required.

The competition with the bioethanol industry as well as with the production of edible crops for the use of agricultural land has promoted the development of approaches to increase the productive efficiency with the current arable land area. The production of 1 kg of meat can require between 3 and 10 kg of grain (Tilman et al., 2002); thus, enhancing feed efficiency in livestock has become a key goal for the beef industry to remain sustainable and competitive. Improving feed efficiency in cattle would also have a very positive economic impact, as feed costs account for approximately 65% of total variable costs (Arthur et al., 2001) and even a 5% improvement in feed efficiency in growing beef cattle have a greater economic impact than a 5% improvement in average daily gain (ADG) (Gibb et al., 1999). It has been calculated that it costs $38 USD less to feed an efficient bull compared to an inefficient one for a 150-day period. Based on the number of cattle in Alberta, improvements in efficiency may

translate into savings of more that $100 million in finishing feed costs (Crews, 2005).

Microbial fermentation of the ingested feed plays a major role in the metabolism of cattle, contributing to the nutrition, production and energetic metabolism of the host (Leedle et al., 1982). By increasing feed efficiency, reductions in nutrient excretion (Herd et al., 2003) and methane emissions (Hegarty et al., 2007), can be achieved, thereby promoting environmental sustainability. Since economic efficiency of farming systems rely on improved feed efficiency, research is warranted.

1.1. FEED INTAKE AND UTILIZATION EFFICIENCY**.**

Feed use and feed efficiency are important for the survival and selective advantage of an animal (Van Soest, 1982). More importantly, feed consumption drives profitability and sustainability in beef production systems. Efforts to address the need for increased energetic efficiency in beef production have resulted in the identification of factors that affect their energy intake and usage. Maintenance energy requirements vary with physiological state (Archer et al., 1999), body weight (Nkrumah et al., 2007), body composition (Basarab et al., 2003), organ mass (Castro Bulle et al., 2006), feeding behaviour (Kelly et al., 2010), fat and protein synthesis and turnover (Richardson et al., 2004b), and digestive efficiency (Swanson et al., 2008).

The efficiency of nutrient utilisation and absorption in ruminants is mainly determined by a balanced rumen fermentation, which is ultimately controlled by

the ruminal microbiota (Russell et al., 1981; McSweeney et al., 1994). Since ruminal microbes play essential role in feed conversion, their diversity and activities are also associated with the host feed efficiency (Guan et al., 2008).

1.1.1. Feed efficiency measurements.

Biological efficiency in animal systems has been defined as the capacity to convert inputs (feed) into marketable product (beef, output) within the prevailing production conditions (Nkrumah et al., 2007). As the success of the production systems relies on the feed inputs and production outputs, examination of these factors has to be performed in large groups of animals to gain accuracy and produce statistically significant results (Archer et. al., 1999). Diverse indices describe feed efficiency of cattle and each reflects different biological and mathematical aspects of efficiency (Mujibi et al., 2010). Some indices for feed efficiency are summarized below:

1.1.1.1. *Gross efficiency.*

Gross efficiency represents the gain: feed ratio (G: F) and it is the most used index of efficiency together with its inverse, feed conversion ratio (FCR, F: G). For beef production systems, outputs are measured as weight gain so feed conversion ratio represents the ratio of feed intake to weight gain measured over a defined period of time (Archer et al., 1999; Nkrumah et al., 2007). Feed conversion ratio of each animal can be computed as the ratio of daily dry matter intake (DMI) to ADG (Nkrumah et al., 2004) and reflects the increased proportion

of energy being utilised for production compared with maintenance (Archer et al., 1999).

1.1.1.2. *Maintenance efficiency.*

Maintenance efficiency can be defined as the ratio of body weight to DMI when body weight does not change (Archer et al., 1999). To obtain a true measure of maintenance requirements it is necessary to hold animals at a constant live weight, which is a process that can take up to 2 years in beef cattle and can be performed only in small number of animals (Archer et al., 1999).

1.1.1.3. *Partial efficiency of growth.*

Partial efficiency of growth (PEG) is the ratio of ADG to the difference between average daily DMI and expected DMI for maintenance (Arthur et al., 2001; Nkrumah et al., 2004), and it is an index to estimate of the efficiencies of energy retention and energy loss (Veerkamp et al., 1995). Maintenance requirements can be calculated from feeding tables (NRC) or from metabolic studies, but both calculation methods have biases associated with their estimations (Archer et al., 1999).

1.1.1.4. *Cow/calf efficiency.*

The efficiency of the calf/cow unit has been used to examine the total feed intake of the cow and her progeny over an entire production cycle from weaning of one calf to weaning of the next calf (Archer et al., 1999). The efficiency of the cow/calf unit is expressed in terms of kg calf weaned per kg of feed consumed

(Jenkins et al., 1994). This method of expressing efficiency is likely to be correlated to the "true" production efficiency of the beef enterprise in both biological and economical terms (Archer et al., 1999).

1.1.2. Residual Feed Intake.

Most measures of feed efficiency take into account live weight and growth rate, and are generally expressed as a ratio relative to feed intake. Residual Feed Intake (RFI), also known as Net Feed Efficiency (NFE; Koch et al., 1963), has been shown to have great potential as an index of feed efficiency for beef cattle (Archer et al., 1999). It has been proposed as a measure of efficiency that is independent of the size of the mature animal and of production (Mujibi et al., 2010). Residual feed intake has been defined as the difference between an animal's actual DMI and its expected DMI based on its body weight (BW) and growth rate over a period of time (Nkrumah et al., 2004, 2006). Genotypic residual feed intake is moderately heritable, and due to its lack of phenotypic correlation with production traits (Kennedy et al., 1993; Archer et al., 1999), animals selected for improved RFI do not show alterations in their productive measurements; thereby physical characteristics remain uniform among cattle.

RFI is usually calculated by linear regression of DMI (dry matter intake) on ADG (average daily gain) and MWT (metabolic mid weight), with the residual being the RFI (the difference between the predicted intake and actual DMI). RFI calculation is obtained using a linear regression model so that it has a normal distribution with mean zero and variance equal to the variance for the group tested

for feed intake at the same time. This implies that approximately half of the group will have values below zero (more efficient) and the other half will have values above zero (less efficient). Animals can be grouped according to RFI, using standard deviation as cut-off point, after the above calculation; high RFI is represented on the right extreme of the normal curve (less efficient) while low RFI is on the left extreme portion of the normal curve (more efficient) such that efficient animals have a negative RFI value, and inefficient animals have a positive RFI value. Thus, efficient animals consume less feed than expected based on their growth and maintenance requirements (Moore et al., 2008).

1.1.2.1. *Association with physiological factors.*

Understanding the physiological mechanisms responsible for the reported variations in feed efficiency among individual animals might aid in developing alternative, non-genetic, methods to manipulate cattle metabolism and improve feed efficiency (Archer et al., 1999). Improving feed efficiency could potentially ameliorate profits for beef cattle enterprises as well as reduce the dietary energy losses and environmental impact of methane emissions from livestock, contributing to address the feed-related challenges faced by this agricultural industry.

There are multiple levels at which regulation of metabolic processes influence feed efficiency, including intake, digestion, absorption and post absorptive utilisation of nutrients (Mader et al., 2009). Several predictive measurements have explained 0.52 of the phenotypic variation in RFI (Richardson et al., 2004a). Researchers have proposed that differences in

digestion contribute to approximately 10% of the overall variation in RFI (Richardson et al., 2004a). Differences in lean and fat tissue deposition patterns explain less than 5% of the variations in RFI observed in cattle, while 2% corresponds to feeding patterns (Herd et al., 2009). Differences in heat increment due to feeding have been reported to contribute nearly 9% (Herd et al., 2004), and general activity contributes another 9% (Richardson et al., 1999). The remaining 37% is considered to be accountable for differences in estimates of protein turnover, tissue metabolism and other factors not yet measured (Richardson et al., 2004b, Herd et al., 2009).

The potential roles of different whole-animal and metabolic factors influencing RFI are addressed in Table 1.1.

Table 1.1. Factors impacting Residual Feed Intake (RFI).

Factor	Impact RFI when/by:	Reference
Nutrient digestion/absorption	Dry matter intake differences	Basarab et al., 2003
	Differences in digestibility of feed	Swanson et al., 2008
	Increased energy requirements for maintenance	Richardson et al. 1996
Protein turnover	Variations in genetic composition	Herd et al., 2004
	Metabolic differences in protein synthesis and degradation rates	Richardson et al., 2004
	Muscle turnover increases energy requirements for maintenance	Castro-Bulle et al., 2007
	Stress response increases tissue breakdown	Herd et al., 2009
Fat metabolism	Variations in fat weight proportion	Mader et al., 2009
	Increased body lipogenesis	Kelly et al., 2010
	Increased fat deposition in carcass	Schenkel et al., 2004
Energetic metabolism	Methane production	Hegarty et al., 2007
	Heat production	Nkrumah et al., 2006

1.1.2.2. *Effects of variations in diet.*

Residual feed intake has been demonstrated to be influenced by environmental factors as well (Mujibi et al., 2010). Apart from season, diet has

been identified as a source of variation in RFI. The proportion of grain in the diet, grain source and processing method influence the digestion of starch (Mader et al., 2009) and the feeding behaviour (Durunna et al., 2011b), ultimately affecting feed efficiency. Moreover, feeding a high concentrate total mixed ration (TMR) could potentially be associated with less feeding sessions (Friggens et al., 1998).

The impaired ability of the animal to rapidly adjust to changing feedstuffs may cause a switch from one RFI category to another (from low RFI to high RFI, for example) (Durunna et al., 2011a). For instance, changing abruptly from feeding a low energy diet to a high energy diet alters ruminal pH and populations of rumen bacteria (Chen et al., 2011) and reduces intake (Calsamiglia et al., 2008). Guan et al. (2008) demonstrated that rumen microbiota plays an important role in animal performance. Because individual animals perform differently on various diets (Russell et al., 1992), different rumen microbial populations also influence the diet-associated RFI re-ranking. However, data on how specific bacterial populations might be linked to variations in feed efficiency measurements such as DMI, RFI and FCR is lacking.

1.2. EVOLUTIONARY ADAPTATIONS OF RUMINANTS.

Ruminants evolved approximately 50 million years ago and initially they were small, forest-dwelling species (Janis, 1976) that ingested parts of the vegetation rich in intracellular carbohydrates (Perez-Barbería et al., 2001). Today there are nearly 200 extant ruminant species distributed in 6 families (Hackmann et al., 2010), which have common attributes such as a preference for grass intake, a large body weight (up to 800 kg) and that they belong to the Bovidae family

(Hackmann et al., 2010). Ruminant species display varied feeding behaviours across species (Perez-Barbería et al., 2004), so they have been classified according to these preferences as 1) browsers, if they prefer to eat fruits, shoots or leaves; 2) grazers, if they prefer grasses; or 3) intermediate feeders, which rely on browsing or grazing depending on the seasonal availability of feedstuffs (Hofmann, 1989; Clauss et al., 2010). Among the domestic species, the majority are grazers except goats and reindeer (Hackmann et al., 2010). Sheep have been classified as grazers but many investigators consider them as intermediate feeders (Perez-Barbería et al., 2004).

1.2.1. Digestive adaptations.

In the Late Oligocene, when forests decreased, grasses evolved as the dominant form of vegetation (Janis et al., 2000) and grazing species proliferated. Thus, ruminants evolved adaptations to effectively consume and digest fibrous plant material (Weimer et al., 2009). The main site of cellulosic conversion in ruminants is a pre-gastric fermentation chamber, the rumen, whose functions are beyond than only acting as a storage device. Unlike other herbivores, ruminants subsist on the structural parts of the plant such as the stem and the leaves (Van Soest, 1982) which contain cellulose, a polysaccharide that is incorporated into the plant cell wall. Ruminants *per se* are not able to produce enzymes to break down cellulose, but through evolution they became associated with cellulase-producing microorganisms (Van Soest, 1982) that enabled them to make use of the nutrients from the cellulose itself and from other digestible materials attached to the plant cell walls (Janis, 1976).

The most obvious characteristic of ruminants is the rumination process, which consists of the regurgitation, rechewing and reswallowing of their foregut digesta. Foregut fermenting mammals (with a primary fermentation chamber proximal to the small intestine) also share two enzymes: stomach lysozyme and pancreatic ribonuclease, which are adaptations to this mode of digestion (Janis, 1976). Additionally, ruminants maximise digestion of forages through several physiological mechanisms. The omasum evolved from a simple "floodgate" in primitive ruminants to a more defined and separated compartment in grazing ruminants; the well-developed laminae and the complex motor activity supported absorption, fibre fermentation and digesta flow, providing improved capacity of processing large amounts of fibre (Hackmann et al., 2010). A sorting mechanism in the forestomach of ruminants (the reticulo-omasal orifice) retains large particles in the reticulo-rumen to be further digested (Clauss et al., 2007) but pushes out those already broken down (Lechner et al., 2010). This selective mechanism represents an effective strategy to increase the efficiency of chewing in ruminants (Fritz et al., 2009). Nevertheless, the selective advantage of the ruminant sorting mechanism comes at a price and ruminants must rely on gravity and keep their forestomach in a vertical plane to fully perform the above functions (Clauss et al., 2010). Also, energetic losses due to methane production represent another cost associated with ruminant digestive physiology adaptations (Van Soest, 1982).

1.2.2. Host–microbe co-evolution.

Probably the best-documented case of the crucial role of host-microbe co-evolution comes from the analysis of gut microbiota (Fraune et al., 2010). Comparative sequencing studies of faecal samples from a wide variety of mammals revealed that bacterial communities co-diversified together with their hosts (Ley et al., 2008). The microbial community inhabiting the ruminant gastrointestinal tract is represented by all groups of microbes (bacteria, archaea, ciliate protozoa, anaerobic fungi and bacteriophages) and is characterised by its high density, diversity and complex interactions (Mackie, 2002). Although more than 80% of the identified bacterial phylotypes belong to only two phyla, the Firmicutes and Bacteroidetes, the genera and species diversity are large (Ley et al., 2008). The microbial community in the gastrointestinal tract of the ruminant has co-evolved with the host into a symbiotic relationship which is mainly involved in the digestion and fermentation of plant polymers (Fraune, et al., 2010).

During the evolution of ruminants, the abundance of carbohydrates in plant cell walls (cellulose and hemicellulose) was the trigger for the development of a cooperative model between the host and the resident microbes (Mackie, 2002). As the carbohydrates in plant cell walls were indigestible by most ruminants, a microbial partner aids to hydrolyse and ferment the substrates and the generated end-products of the fermentation, so the microbial cells can be utilised by the host animal (Van Soest, 1982). Nonetheless, a disadvantage of this relationship is the breakdown of dietary protein by ruminal microorganisms before enzymatic digestion by the host. However, rumen microbiota compensates for the protein it utilizes from the diet through the production of microbial protein

(Dehority, 2004). The microorganisms which the ruminants have developed symbiotic relationships with also synthesise and provide the host with essential amino acids (Baldwin et al., 1982). In ruminants, dietary protein is fermented to ammonia-N (NH_3-N), which can be used as a protein source by the bacteria or absorbed through the rumen wall and sent to the liver (Russell et al., 1981). Ammonia-N is converted to urea, which can be returned to the rumen, either through the saliva or by simple diffusion from the blood through the rumen wall (Firkins et al., 2007). Urea is utilised by bacteria for growth and reproduction, but the rumination process carries large numbers of bacteria to the abomasum, where they are digested by proteases. The ingested nitrogen thus finally becomes available to the animal as microbial protein (Firkins et al., 2008). Urea from metabolic sources is re-circulated into the cycle rather than excreted in the urine (Firkins et al., 2007). The nitrogen cycle could be considered a strategy to conserve water in ruminants (Janis, 1976) and also supplements the host with amino acids to fulfil the requirements when imbalances in the diet occur (Van Soest, 1982). The rumen microbiota are also responsible for the synthesis of vitamins, metabolism of toxic and non-digestive compounds, stimulation of the immune system and maintenance of intestinal mucosal integrity (Chaucheyras et al., 2008).

Ruminants are a much diversified group that has proved to be a very successful example of thriving co-evolution strategies. Studies of the ecology and evolution of domestic ruminants will provide additional insights and may

potentially support current management strategies or aid in proposing more holistic alternatives for animal production.

1.3. HOW DOES THE RUMEN WORK?

1.3.1. Rumen anatomy and functions.

Ruminants have a different gastrointestinal tract than that of monogastric animals, with modifications that make it an ideal habitat for its symbiotic microorganisms. Feedstuffs ingested by ruminants consist mainly of fibre and complex polysaccharides which cannot be broken down by mammalian digestive enzymes (Dehority, 2004). Consequently, ruminants have developed digestive mechanisms involving microbial fermentation of feed prior to the exposition to the digestive enzymes (McDonald et al., 1995). The ruminant stomach has four compartments: rumen, reticulum, omasum and abomasum, allowing the animal to extract energy from fibrous feedstuffs, which otherwise would not be available to the animal.

1.3.1.1. *Rumen.*

It is the main compartment of the ruminant stomach. The interior of this organ is divided into sacs; due to special structures in the epithelium (ruminal pillars), contractions are carried out to mix digesta, to inoculate the feed with microorganisms and to further transfer fermentation products to the surface in order to be absorbed (Russell et al., 2001).

1.3.1.2. *Reticulum.*

It is a small pouch-like organ, distinctive of the rumen, with epithelial surface resembling a honeycomb (Russell, 2002). The role of the reticulum is transferring feed particles to the oesophagus and to the mouth during the rumination process.

1.3.1.3. *Omasum.*

It is the third compartment of the ruminant stomach. Its epithelium is organised in longitudinal laminae that act as a filter. Through muscular contractions of the reticulo-rumen, bacteria and small particles can either pass through the reticulo-omasal orifice to the omasum or to flow to the abomasum, while larger feed particles remain in the rumen to be further reduced (Church et al., 1979).

1.3.1.4. *Abomasum.*

It is known as the gastric stomach but, its pH is not as low as in the stomach of non-ruminant species (Russell, 2002). In the abomasum, digesta and microbes undergo the first phases of protein digestion and then they are transferred to the small intestine where additional enzymatic breakdown is carried out.

When ingested feed is chewed, plant material is broken down to be mixed with the saliva and forms a bolus that is swallowed into the reticulo-rumen. Through this process, particle density is increased and salivation induces a buffering action in the rumen pH (Church et al., 1979). Rumination starts after an

inactive period upon the first meal, when the animal has collected enough feedstuff. Rumination is the process of regurgitating ingesta from the reticulo-rumen into the mouth, where the bolus is masticated again and mixed with saliva for 30 to 60 seconds and then re-swallowed (Beauchemin, 1991). This mechanism reduces the particle size of the feedstuffs, exposing the cell walls for the microbial digestion and sorting particles according to their density (Dehority, 2004). The duration of rumination can vary from periods of 15 to 30 minutes, up to 2 to 6 hours (Saras-Johansson, 2011). During the regurgitation, the chewing of the digesta produces some fermentation gases that are directly eructated (Beauchemin, 1991).

1.3.2. Rumen environment and fermentation.

The rumen is a very suitable environment for the growth of microorganisms (Dehority, 2004). Water and saliva create a moist and buffered environment with constant supply of nutrients from the ingested feedstuffs. This is accompanied by a continuous removal of end-products, such as volatile fatty acids that are absorbed rapidly through the rumen walls, or fermentation gasses which are expelled or consumed by the microbiota (Russell et al., 2001). Contractions of reticulo-rumen keep the food in motion so microbial growth is favoured (Shimada, 1984).

Rumen fluid has a high buffering capacity due to its content of sodium, bicarbonate and phosphate but these levels might vary with the diet (Russell, 2002). Cattle fed forage have higher potassium content than those fed grain

(Russell, 2002). The overall ion concentration after feeding creates an osmotic pressure of approximately 400 mOsmol per litre of rumen fluid (Dehority, 2004) but then decreases to a hypotonic equilibrium with respect to plasma. Rumen pH is variable during the fermentation process; fluctuations can occur after feeding, due to feedstuff type and frequency of feeding (Hristov et al., 2001). The normal pH range is between 5.5 and 7.0 but outer limits lie between 4.5 and 7.5 (Dehority, 2004). The pH homeostasis in the rumen determines the biodiversity of the ruminal ecosystem and the nutritional value of the end products (Aschenbach et al., 2011); deviations in this measure might cause health disturbances (Penner et al., 2007). The main gasses in the rumen are CO_2 (65%) and CH_4 (27%); both are end-products of the microbial fermentation (Dehority, 2004). Carbon dioxide is recycled from the saliva, amino acids and other organic acids (Van Soest, 1982); H_2 is used by methanogens to reduce CO_2 to CH_4 (Hungate et al., 1970).

In the rumen, digestion and fermentation of feedstuffs are carried out by the microbial population. Some end-products of these processes are volatile fatty acids (VFA) such as acetic, propionic and butyric, carbon dioxide, methane and ammonia. Energy (as ATP) is conserved during fermentation by either substrate-linked or by electron transport-linked phosphorylation (Nagaraja et al., 1997) and used for the synthesis of cellular components, microbial growth and functions of the microbial cells.

The main site of VFA absorption is the reticulo-rumen; then rumen microorganisms and partially fermented digesta flow to the omasum, where there is some absorption of VFA and water, and later into the abomasum and small

intestine (Church et al., 1979). The microbial fermentation is performed by a mixed population of bacteria and ciliate protozoa, together with a number of anaerobic fungi (Newbold, 2003). The interactions between microbial species in the rumen are complex, representing some of the best examples of microbial symbiosis (Baldwin et al., 1983).

Ruminant animals have two metabolic systems that differ in their nutrient requirements, microbial metabolism in the rumen and mammalian metabolism in the tissues (Nagaraja et al., 1997). Optimising ruminant productivity involves meeting the requirements for both metabolic systems. In ruminants, fermentation of ingested feedstuffs is helpful to break down substrates that cannot be digested by the animal enzymes. Microbial fermentation in the rumen produces heat, which added to that produced by the animal, contributes to the ability of the animals to survive cold weather (Forbes et al., 1993). However, the fermentation of proteins, amino acids and sugars is an inefficient process and generates losses (Dehority, 2004), which may influence the energetic efficiency of the host (Russell et al., 1981). These losses can be detected at the following levels:

1.3.2.1. *Rate of fibre digestion.*

Fibre (along with starch and sugars) is essential for producing VFA; however, when its digestibility is low, feed intake and VFA production decrease, affecting microbial growth and host absorption (Russell et al., 2001).

1.3.2.2. *Production of microbial protein.*

Microbial protein efficiecy depends on the amount of energy used for maintenance and energy losses (Kelly et al., 1993) and accounts for nearly 90% of the amino acids reaching the small intestine (Russell, 2002).

1.3.2.3. *Ammonia accumulation.*

Protein fermentation in the rumen removes amino acids from the animal, since excessive NH_3-N must be converted to urea and thus the animal expends energy (as ATP) to compensate for wasteful amino acid degradation (Russell, 2002).

1.3.2.4. *Methane production.*

As much as 11% of the energy from feed is wasted as methane (Russell, 2002), reducing energy availability for the animal's functions (Nagaraja et al., 1997).

1.3.2.5. *Lactate production.*

Lactic acid can be produced from some rumen bacterial species and isotrichid protozoa (Firkins et al., 2008) and bring undesirable effects such as reduced ruminal pH, decreased feed intake, inhibition of microbial protein synthesis and subacute ruminal acidosis (Aschenbach et al., 2011).

A fundamental understanding of microbial ecology and its relationships to ruminant physiology is essential for later manipulation of ruminal microflora and a subsequent improvement in animal production (McSweeney et al., 1994).

Principles such as niche occupancy and interactions among populations (Weimer, 1998) have to be taken into account when evaluating the ruminal microflora and host interactions.

1.3.3. Fermentation of carbohydrates.

Structural polysaccharides such as cellulose, hemicellulose and pectin are the main carbohydrates in forage-based diets, constituting around 15-70% of most ruminant diets (Russell et al., 1981). They might not be completely physically accessible, so their fermentation rate in the rumen is variable. There is evidence that cellulose digestion is limited not by the population or activity of the cellulolytic microbes, but rather by the amount of cellulose available for microbial degradation (Weimer, 1998). Non-structural polysaccharides such as starch and fructosans are the most important sources in grain-based diets (Baldwin et al., 1983). Starch constitutes more than 80% (on a DM basis) of the total material of the grains (Nagaraja et al., 1997). Because of their high fermentability, non-structural carbohydrates are increased in the diet to meet the energy requirements of highly productive animals.

The first step in fermentation of dietary carbohydrates is usually hydrolysis (Baldwin et al., 1983). After feeding, colonization of forage appears to be carried out by microorganisms that have enzymes degrading the available side chains of polysaccharides but not the more resistant main chains, especially cellulose (Brulc et al., 2009). Following, a secondary group of colonizing microorganisms (Edwards et al., 2008) degrades the chains of cellulose and xylan

(Brulc et al., 2009). In the final stage, small saccharide molecules are fermented inside microbial cells (Russell et al., 1981).

Through the fermentative processes, some of the energy is conserved as ATP (France et al., 1993) which is used to maintain cell functions by rumen microbes. In order to produce ATP there are several pathways carried out in the cell.

1.3.3.1. *Pentose metabolism.*

Baldwin et al. (1983) have pointed out that feedstuffs contain mainly hexoses, but hemicellulose contains pentoses, which can be metabolised either in the pentose cycle or by the phosphoketolase. The ATP yield from the pentose cycle is greater; around of 75% of the xylan is fermented by this pathway (Russell, 2002).

1.3.3.2. *Pyruvate metabolism.*

Pyruvate and $NADH_2$ from hexose and pentose fermentation can be metabolised to several products including formate, acetate, propionate, butyrate, lactate, succinate, methanol, ethanol, CO_2 and H_2 (Theodorou et al., 1993). To continue with the digestive processes, NAD has to be converted into NADH during glycolysis (Church et al., 1977); pyruvate carboxylase can be converted into acetyl CoA and then to acetate (Baldwin et al., 1983).

1.3.3.3. *VFA synthesis.*

In ruminants, VFA (mainly acetate, propionate and butyrate) are the major sources of energy. As their proportions can be influenced by the diet, increased production of propionate, which is the most important precursor of glucose synthesis, will lead to increased energy retention from the fermentative process and therefore will enhance the distribution of the nutrients in the tissues (Weimer, 1998).

1.3.3.3.1. ***Acetate and butyrate.***

Acetyl CoA from pyruvate metabolism can be converted to either acetate or butyrate (Bergman, 1990). This is achieved through two main pathways, either by using a butyrate kinase or by using a butyryl CoA acetyl CoA transferase (Diez-Gonzalez et al., 1999). France et al. (1993) mentioned that acetate and butyrate are used mainly as energy sources being the former the principal substrate for lipogenesis. ∅rskov (1977) indicates that the balance between the supply of glucogenic propionate relative to that of the non-glucogenic acetate and butyrate influences the efficiency with which the VFA are used.

1.3.3.3.2. ***Propionate.***

Propionate can be synthesized through two different pathways. In the first, ruminal bacteria use succinate and oxaloacetate as intermediates to produce propionate (Baldwin et al., 1983). In the second, the reductive pathway, lactate (or pyruvate from glucose) is converted to acrylyl CoA ester that is subsequently dehydrated and reduced (Bergman, 1990).

1.3.3.3.3. ***Other VFA.***

Valerate is formed by condensation of acetate and propionate (Bergman, 1990). Longer VFA are formed by the condensation of acetyl CoA (Baldwin et al., 1983); from this process, the ATP yield is decreased because the free energy of the CoA ester is used to form carbon-carbon bonds rather than ATP (Bergman, 1990). Branched-chain VFA (isobutyrate and isovalerate), originate from the branched-chain amino acids valine, leucine and isoleucine (Pátek, 2007).

1.3.3.4. *Metabolism of Nitrogen.*

The digestion of the N in ruminants has two stages: fermentation of the protein and non-protein nitrogen (NPN) in the reticulo-rumen by the microbial enzymes; and the partition of the proteins and peptides by the digestive enzymes present in the abomasum and duodenum (Nolan et al., 1993). Dietary protein is divided into rumen-degradable protein (RDP), composed of nonprotein and true protein N (Bach et al., 2005), and undegraded protein (RUP). True protein is degraded to peptides and amino acids and deaminated into NH_3-N or incorporated into microbial protein (Russell et al., 1981). Non-protein N includes N present in nucleic acids, ammonia, amino acids, and peptides and is further used for microbial growth (Baldwin et al., 1983).

Protein partition in the rumen begins with bacteria attachment to feed particles, followed by the action of bacterial proteases and protozoal phagocytosis (Russell et al., 2001), resulting either in the release of peptides or in the formation of protozoal protein. The peptides obtained can be used to synthesise tissue

proteins, enzymes, hormones and other metabolites for the host, used directly for microbial protein synthesis or being deaminated to VFA, CO_2, or NH_3-N among other soluble N-material (Bach et al., 2005). Ammonia-N is the major source of N for ruminal bacteria and is absorbed through the wall of the reticulum and rumen, converted to urea and excreted in the urine (Church et al., 1979).

Amino acids can also be substrates for ATP formation, obtaining a lower yield than from carbohydrates. Only specialized bacteria (Russell, 1993) can utilize amino acids as their only energy source but at the expense of low ATP yield. Other ruminal bacteria (e.g. *Megasphaera elsdenii* and *Prevotella* sp.) can ferment amino acids but the rate of ammonia and ATP production is slow (Russell, 2002).

1.3.3.4.1. ***Microbial protein.***

The rumen is a complex ecosystem consisting of numerous types of microbial species that degrade feed N for their own benefit, or which uptake N either from other microorganisms or from recycled N (Bach et al., 2005). These metabolic pathways provide most of the amino acid requirements for the host (Bach et al., 2005). Since not all the NH_3-N produced in the rumen is incorporated into microbial protein (Firkins et al., 2008), some of the protein in the diet may be replaced by NPN.

Ruminal microorganisms are responsible for providing the major part of the energy requirements of the host animal by transforming dietary carbohydrates to VFA (Theodoru et al., 1993). In order to more completely extract energy from

the feedstuffs, it is essential that they grow and proliferate; thus, large amounts of NH_3-N and amino acids are needed (Baldwin et al., 1983). Bacteria that degrade structural carbohydrates have low maintenance requirements, grow slowly, and use NH_3-N as their main N source; whereas microorganisms that degrade non-structural carbohydrates (amylolytic) have higher maintenance requirements, grow rapidly, and use NH_3-N, peptides, and amino acids, as N sources (Russell et al., 1992). The N for these species is obtained by breakdown of the nitrogen fraction of the feed. The production of the microbial protein of the rumen is the least expensive source of amino acids for the host animal so this process needs to be as efficient as possible (Nolan, 1993). Further, microbial protein yield has a major impact on the nitrogen economy of the host since it determines the amino acid mixture available for protein synthesis at tissue level (McDonald et al., 1995). Ammonia-N may be used for microbial protein synthesis or it may be absorbed across the rumen wall, resynthesised into urea and excreted or recycled in the gut (Firkins et al., 2008).

1.3.3.5. *Production of Methane.*

Methane is a major end-product of ruminal fermentation. It is produced by a group of rumen *Archaea* known collectively as methanogens, which belong to the phylum *Euryarcheota* (Hook et al., 2010). Cattle can produce as much as 17 litres of methane (CH_4) each hour (Russell, 2003), wasting between 2 and 12% of their gross energy intake (Johnson et al., 1995). This is a great environmental concern, since cattle account for nearly 70% of the total methane produced by livestock each year (Johnson et al., 1995).

The production of methane in the rumen implies the removal of H_2 and the reduction of CO_2 (Weimer, 1998) but also, the production of acetate, H_2, CO_2, formate and other products including long chain VFA has been discussed (Baldwin et al., 1983). In the rumen, formate can also be used as a substrate by methanogens (McDonald et al., 1995), but as formate users grow slowly, they may be washed out from the rumen. Other compounds can also be converted to methane, like methanol from pectin breakdown and methyl-amines and triethylamines (Hungate, 1970). Ruminal methanogens keep a low partial pressure of H_2. Under these conditions, H_2 production provides an alternative oxidation pathway (Sharp et al., 1998). Disposal of the metabolic hydrogen (from NADH or $FADH_2$), via the propionate production or in the saturation of long-chain VFA within the rumen, can be considered more efficient than the removal of excessive H_2 as methane (Beever, 1993). Reduction of fumarate to succinate, which will result in propionate formation, is actually more thermodynamically favourable than methanogenesis within the range of H_2 partial pressures seen in the rumen (Ellis et al., 2008).

Energy losses from methane in feedlot cattle vary from 3.5–6.5% (Hook et al., 2010) based on geographical location and feed characteristics such as quality, intake, composition, and processing (Johnson et al., 1995). It is possible to decrease methane production by adding antimicrobial drugs to the diet (Van Nevel et al., 1995) but with rather inconsistent effects (Odongo et al., 2007; Hook et al., 2009). Feed additives such as the ionophores may alter the acetate to propionate ratio and reduce methane production by increasing feed efficiency.

Monensin, the most utilized ionophore, alters the ratio of microbial species within the rumen (Beever, 1993).

1.4. MICROBIAL ECOSYSTEM IN THE RUMEN.

The ruminal microbial community is characterised by its high population density, broad diversity and complexity of interactions. The rumen contains large numbers of bacteria (as high as 10^{11} viable cells per ml, encompassing nearly 200 species), archaea, ciliate protozoa (10^{4}-10^{6} per ml, distributed in 25 genera), anaerobic rumen fungi (with a zoospore population density of 10^{3}-10^{5} per ml, divided into five genera) and bacteriophages (10^{7}-10^{9} particles per ml) (Klieve et al., 1993; Hespell et al., 1997; Mackie et al., 2000).

1.4.1. Bacteria.

Bacterial density is the highest in the rumen and direct counts can be as high as 10^{11} cells per gram of ruminal contents (Mackie et al., 2000). Even though bacterial mass has not been precisely quantified, some authors have proposed values in the range of 14 to 18 mg bacterial dry weight per ml of rumen fluid (7 to 9 mg bacterial protein per ml) (Russell, 2002). Russell (2002) points out that the bacteria are approximately 10% N (62.5% crude protein) but only 80% of the crude protein is true protein, the remainder is nucleic acids. Ruminal bacteria range in size from approximately 0.3 to 5 μm (Czerkawski, 1986).

In the rumen, three different subpopulations of bacteria can be distinguished: (i) a planktonic population composed of bacteria free in the rumen

fluid; (ii) a population attached to feed particles (loosely attached or firmly adhered, McAllister et al., 1994); and (iii) a population attached to the rumen epithelium, named epimural (Mead et al., 1981; Cheng et al, 1981). An additional group is attached to the surface of protozoa and fungi sporangia (Miron et al., 2001). The population of free-floating, planktonic bacteria is composed by bacteria detached from feed particles as well as those that consume soluble feed components from the ruminal fluid (McAllister et al., 1994). Despite its minor role in digesting insoluble feed particles, this subpopulation is fundamental to initiate the digestion of ingested feed particles (Leedle et al., 1982). Bacterial population associated with feed particles account for up to 75% of the total population (Koike et al., 2003) and play the most important role in feed digestion (McAllister et al., 1994). The population attached to the rumen epithelium represents less than 1% (Czerkawski, 1986) and contributes to the hydrolysis of systemic urea diffusing from the blood across the rumen wall (Wallace et al., 1979) and to the tissue recycling and oxygen scavenging processes. Due to their close contact with the ruminal tissue, the host might have a strong influence on their structure (Larue et al., 2005). Since the intermediate metabolism occurs in the rumen epithelium (Žitňan et al., 1993), it is likely that the epimural community is more closely related to the metabolic activity of the host than the bacteria from the rumen contents (Wallace et al., 1979).

The complex bacterial ecosystem of the rumen is responsible for the breakdown of feed components, enabling ruminants to derive about 70% of their metabolic energy from the ruminal fermentation of feedstuffs (Bergman, 1990).

Previous research has demonstrated that rumen bacteria are highly responsive to alterations in diet (Bevans et al., 2005; Sun et al., 2009), age (Fonty et al., 1987, 2007), and health of the host animal (Wanapat et al., 2009). Even further, ruminal bacteria are also affected by variations in geographical location (Sundset et al., 2007), season (Orpin et al., 1985; Crater et al., 2007), photoperiod (McEwan et al., 2005), stress level (Uyeno et al., 2010) and environment temperature (Romero-Pérez et al., 2011). Similarly, the microbial species and activities have also been shown to be influenced by feed intake levels (Crater et al., 2007) and frequency of feeding (Kocherginskaya et al., 2001; Pulido et al., 2009), demonstrating that it is possible to modify the composition of ruminal bacteria *in vivo* by manipulating diet.

1.4.2. Archaea.

Archaea (formerly Archaeobacteria) are a group of prokaryotes that constitute a phylogenetically separate domain of life (Vetriani, 2001). Although Archaea resemble Bacteria morphologically, they differ in their cell wall and membrane composition. Bacteria contain diacyl glycerol diesters, whereas Archaea are composed by isoprenoid glycerol diethers or diglycerol tetraethers (Woese et al., 1990). From a genetic standpoint, Archaea have a unique combination of genes because some of the archaeal genes, including those encoding major metabolic pathway enzymes, are similar to those of Bacteria; others, such as those for RNA polymerase subunits, are more similar to eukaryal genes. Besides, there are also Archaea-specific genes (McLain, 2005).

Three general categories represent the patterns of archaeal adaptations to extreme environments: thermophilic, methanogenic and halophilic (Vetriani, 2001). The majority of Archaea present in the rumen belong to the methanogenic group. Methanogens are responsible for regulating the overall fermentation in the rumen by removing H_2 during methane production, encouraging the activity of H_2-producing species and altering their metabolism towards higher-yielding pathways (Leahy et al., 2010). Methane is the final product of breaking organic materials down into the simple methanogenic substrates and thus, methanogens complete the last step in the digestion of organic matter (McLain, 2005). These ruminal microorganisms utilize the CO_2 and H_2 produced by the protozoa, fungi and bacteria from the catabolism of hexoses to produce CH_4 and generate ATP (Ferry et al., 2010). These processes benefit the donors by providing an electron sink for reducing equivalents to minimize the partial pressure of H_2 (Wolin et al., 1997; Russell, 2002).

Because they are dependent on other organisms for provision of substrate and the establishment of reducing conditions, methanogens are found in a symbiotic association with ruminal bacteria (Wolin et al., 1997), protozoa (Lange et al., 2005) and fungi (Miron et al., 2001). Ruminal methanogenic populations identified with molecular techniques indicate the presence of species from the classes *Methanobacteria* and *Methanomicrobium*, and a large, uncultured group of rumen archaea referred to as rumen cluster C (Leahy et al., 2010). Sequences from *Methanobrevibacter* accounted for 61.6% of rumen archaea, with sequences associated with *M. gottschalkii* (33.6%) and *M. ruminantium* (27.3%) being

prominent (Janssen et al., 2008). The *Methanomicrobiales* are a group primarily with free-living lifestyle (Mackie et al., 2000) while the order *Methanobacteriales* can exist either associated with ruminal ciliates or free living (Sharp et al., 1998) but showing a preferred association with ruminal protozoa. Because CH_4 production is affected by the composition of the feedstuffs consumed, the methanogen population is also likely to be impacted.

When ruminal CH_4 is eructated, the CO_2 and the reducing equivalents comprising each molecule are also expelled from the ruminal environment resulting in a loss of energy (Leahy et al., 2010). Feed energy lost as CH_4 may vary from 2 to 12% and can be affected by a several factors including DMI, feed processing, carbohydrate type, dietary fat, among other factors (Johnson et al., 1995). When DMI increases, feed energy lost as CH_4 decreases (Johnson et al., 1995; Benchaar et al., 2001) and this effect is more pronounced in digestible (concentrate-based) diets than in forage-based diets (Hook et al., 2010). Therefore, limited intake of a highly digestible feed will result in decreased CH_4 losses (Johnson et al., 1995). Forage processing, for instance grinding (Hook et al., 2010) and pelleting (Benchaar et al., 2001) has also been reported to decrease CH_4 production. Other strategies to reduce methanogenesis are defaunation and vaccines (Hook et al., 2010). These mitigation methods target the methanogen population of the rumen, but with varying degrees of efficacy.

1.4.3. Protozoa.

The other important population in the rumen is the rumen protozoa. They are large microorganisms of about 20 to 200 μm of size. Protozoa numbers in the

rumen do not exceed 10^7 cells per ml (more often they are 10^5–10^6 per millilitre of rumen liquid) but they can account for as much as half of the biomass in the rumen (Czerkawski, 1986). When protozoa numbers increase, bacterial mass declines and vice versa (Russell, 2002). Protozoa are associated with the feed particles to access substrate and in order to extend their stay in the rumen (Dehority, 2004). The majority of the protozoa in the rumen are ciliate species, although some flagellates have been reported (Dehority, 2004). Rumen ciliates are believed to have been originated from free-living (anaerobic) ciliates that were introduced into the ruminant digestive tract after it already harboured anaerobic prokaryotes (Mackie et al., 2000). These free-living ancestors adapted and evolved, resulting in a group of specialised organisms (Finlay, 2001). Anaerobic ciliates contain distinctive modified mitochondria (hydrogenosomes), which ferment pyruvate produced by glycolysis into acetate; the H_2 resulting from this process is used for energy conservation and ATP synthesis via substrate level phosphorylation (Müller, 1988; Embley et al., 1997). These H_2 fermentative processes in protozoa have resulted in symbiosis with methanogens, which partially contribute to the methane emissions from ruminants (Mackie et al., 2000). Most rumen ciliates have bacteria living either inside (endosymbionts) (Finlay et al, 1994) or adhered to their external surfaces (exosymbionts), which can be attached or detached based on H_2 chemotaxis (Firkins et al., 2007).

Recent research using molecular-dependent techniques revealed a taxon-specific association between protozoa and methanogens in the rumen and in an *in vitro* system (Sharp et al., 1998). *Methanobacteriaceae* were the most abundant

population in the rumen comprising 89.3% of total archaea and 99.2% in the protozoal fraction. After 48 h in the fermentor, a loss of protozoa in the system was concurrent with a 54% reduction of the archaea. These studies indicated the specificity of *Methanobacteriaceae* as symbionts of protozoa (Mackie et al., 2000). Rumen protozoa forage bacteria and microscopic fragments of grass and plant material (Dehority, 2004). Some protozoa are capable of degrading cellulose and other structural carbohydrates (Russell et al., 1981), while others are involved in the degradation of dietary and microbial proteins in the rumen. Due to the ability of protozoa to ingest particulate material suspended in the rumen, protozoa are more active in degrading insoluble than soluble proteins (Jouany, 1996). Besides contributing to VFA production, protozoa engulf starch granules, preventing rapid bacterial degradation, excessive lactate production (entodinomorphs are important lactolytic microbes) and a drop in pH; thus, preventing these detrimental activities in the rumen (Russell et al., 1981, 2002).

1.4.4. Fungi.

The anaerobic fungi in the rumen are a group of zoosporic fungi occupying a unique niche in the digestive tract of ruminants (Theodorou et al., 1996). Since anaerobic fungi are often found to colonise and degrade plant material with a high phenolic content, such as lignified sclerenchyma or vascular tissues (Borneman et al. 1991, 1992), ruminal fungi have been reported to play a fundamental role as the initial colonisers of plant fibre in the rumen (Joblin et al., 2002; Lee et al., 2000). France et al. (1990) have suggested that bacteria and fungi have a mutualistic relationship; they both live in two different niches in the rumen,

whereby bacteria degrade plant particles by surface erosion while fungi penetrate and degrade by invasive colonisation. These modes of attack are mutually beneficial to both microorganisms, allowing the survival in the rumen of two fibrolytic populations consuming the same substrates. To facilitate colonisation, rumen fungi produce a range of plant cell wall-degrading enzymes. These enzymes are mainly present on the fungal surface, on the rhizoids and rhizomycellia, and they are secreted into the cell (Lowe et al., 1987).

It has been estimated that fungi constitutes approximately the 6% of the total biomass (Russell, 2002) in the rumen. Rumen fungi are difficult to quantify because their complex lifecycle covers the feed particles. The lifecycle of anaerobic fungi in the rumen consists of two stages where the motile zoospores in the rumen fluid move with the fungal thalli, which colonise and degrade solid digesta (Davies et al. 1993; Nielsen et al. 1995). Lifecycles of 24-32 h have been described *in vitro*, although under *in vivo* conditions zoosporogenesis can occur even at 8 h after encystment (France et al. 1990; Theodorou et al. 1996). Anaerobic fungi are important in the digestive tract ecosystem, particularly where animals feed on highly fibrous diets.

1.4.5. Bacteriophages

A large and diverse population of bacteriophages is known to be present in the rumens of sheep and cattle (Klieve et al., 1993), and they could play a major role in the population dynamics of ruminal bacteria due to their lytic activity (Hespell et al., 1997). Lysis of bacteria in the rumen and subsequent breakdown

of the protoplasm results in the reduction of the efficiency of feed conversion in ruminants (Klieve et al., 1993).

1.4.6. Interactions among different microbial populations.

Recently published studies have described the molecular diversity of rumen bacteria based on culture-independent techniques (Welkie et al., 2010) and suggest that the numerically prevalent species, even under different diet conditions, are made up of bacteria belonging to the Firmicutes phylum (Tajima et al., 2001). However, some authors have reported that there is significantly different bacterial diversity among individuals (Brulc et al., 2009, Li et al., 2009).

More than 200 ruminal bacterial species have been isolated and phylogenetically and physiologically characterized (Kong et al., 2010b). A group of them has the ability to hydrolyse cellulose: species belonging to the cellulolytic group include *Fibrobacter succinogenes*, *Ruminococcus albus*, and *flavefaciens* (Dehority, 2004). In the rumen, interactions among a consortium of fibrolytic bacteria are crucial to digest plant cell walls (McAllister et al., 1996; Shinkai et al., 2010). In addition to cellulose, two other forage polysaccharides occur in significant amounts: hemicellulose and pectin (Dehority, 2004). It appears that *Butyrivibrio fibrisolvens*, *Prevotella ruminicola*, *Ruminoccocus flavefaciens* and *Ruminococcus albus* are the predominant ruminal species digesting hemicellulose in the rumen (Russell et al., 1981); *Eubacterium* spp. also seem to contribute to the process (Dehority, 2004). *Lachnospira multiparus* is one of the main pectinolytic species.

Research has indicated that fibre degradation is further accelerated by interactions among fibrolytic and non-fibrolytic species (Koike et al., 2003), although the role of uncultured species may also might be as important as that of characterised species (Stiverson et al., 2011). Several species of ruminal bacteria attach to starch granules due to their high amylase activity (McAllister et al., 1994). The affinity of amylolytic bacteria for starch granules may differ among bacterial species; for instance, *B. fibrisolvens* digests isolated starch granules (Cotta, 1988) whereas *Streptococcus bovis* attaches to both starch granules and to the protein matrix in grains (McAllister et al., 1990). Other starch-fermenting species identified are *Selenomonas ruminantium*, *Succinivibrio dextrinosolvens*, *Succinomonas amylolytica* and *Ruminobacter amylophilus* (Stevenson et al., 2007).

Lactate-utilising bacteria might be of considerable importance, since they supply NH_3-N and branched-chain VFA for the growth of cellulolytic bacteria (Russell et al., 1981). *Megasphaera elsdenii* and *Veionella alcalescens* might be important fermentors of lactate in the rumen during periods of adaptation to a different diet, contributing to decreased pH (Dehority, 2004). Anaerobic *Lactobacillus* sp. and *Bifidobacterium* sp. have been also identified in young calves (Vlková et al., 2006).

Protein breakdown in the rumen is mostly carried out by rumen bacteria (Brock et al., 1982). Many proteolytic bacteria have been isolated in the rumen and their occurrence influenced by the diet of the animal (Attwood et al., 1996). On forage and concentrate diets fed to ruminants, *Butyrivibrio fibrisolvens*,

Ruminobacter amylophilus, *Selenomonas ruminantium*, *Streptococcus bovis*, and *Prevotella ruminicola* are usually isolated (Russell et al. 1981). Also, the amino acid and peptide-fermenting species like *Clostridium aminophilum*, *Clostridium sticklandii* and *Peptostreptococcus anaerobius* may be important in ruminal ammonia production (Eschlenauer et al., 2002). Other bacteria with proteolytic activity are *Streptococcus* and *Eubacterium* spp. (Dehority, 2004) and high degrading activity has been identified in *Eubacterium*-like species (Attwood et al., 1995).

Bacteria that use CO_2 to produce acetate form a phylogenetically diverse group designated "homoacetogens" (Diekert et al., 1994). These bacteria grow with H_2 or other suitable electron donors (sugars or formate) and CO_2 as electron acceptor; they are also capable of using methoxylated compounds and other organic substrates (Henderson al., 2010). Several homoacetogens, including *Acetitomaculum ruminis* (Greening et al., 1989), *Eubacterium limosum* (Joblin, 1999), *Blautia schinkii*, and *Blautia producta* (Fonty et al., 2007), have been isolated from ruminants. Although they may play an important role in the disposal of H_2, they might be outcompeted by methanogens, because they have lower affinity for H_2 (Lopez et al., 1999). However, other researchers propose that they could be important in the H_2 removal in the low-methane emission forestomach of wallaby (Gagen et al., 2010).

Interactions among rumen bacteria can be positive or negative and they are summarised in table 1.2.

Table 1.2. Interactions among rumen bacteria and diverse ruminal microorganisms.

	Bacteria	Protozoa	Fungi	Methanogens
Bacteria	• Synergism (i.e. cellulose and starch digestion). • Cross feeding (branched-chain VFA, vitamins and amino acids). • Inhibition • Redundancy of functions	• Predation • Parasitism (bacteria provides amino acids; protozoa are unable to grow without bacteria).	• Mutualism (bacteria provides B vitamins and amino acids). • Synergism (cellulose and hemicellulose digestion) • Inhibition (bacterial fermentation products affect fungal cellulolysis)	• Competition (homoacetogens) • Cross feeding (CO_2 + H_2 from cellulose digestion).
Protozoa		• Predation (antagonism and cannibalism among populations). • Competition for nutrients.	• Predation (ingestion of rhizoids and sporangia as a carbon source).	• Mutualism (methanogens are endosymbionts or exosymbionts).
Fungi			• Synergism? (Not determined yet).	• Synergism (cellulose digestion).
Bacteriophages	• Affinity	• Affinity? (Undetermined)	• Not determined	• Not determined

1.5. HISTORICAL PERSPECTIVES OF RUMINAL MICROBIAL ECOLOGY.

The rumen ecosystem contains a complex conglomerate of bacteria, archaea, protozoa and fungi. Historically, cultivation-based techniques have enabled the identification of a number of species present in the rumen (Edwards et al., 2008). Methods to enumerate these populations can make a valuable addition to the measurements of rumen fermentation products carried out by ruminant nutritionists (Dehority, 2004). However, the rumen microbial population is complex and their growth requirements are highly variable. Additionally, over 70% of the rumen bacteria are associated with particulate matter (Koike et al., 2003) within the rumen and thus difficult to recover and culture. The counts obtained might vary not only with media and methods used but also between laboratories apparently using the same methods. For these reasons it is unlikely that viable counts made using culture techniques will manage to account for more than 20% of the ruminal bacteria (Dargie et al., 2005), so they could only be considered as a comparative tool rather than absolute measurements.

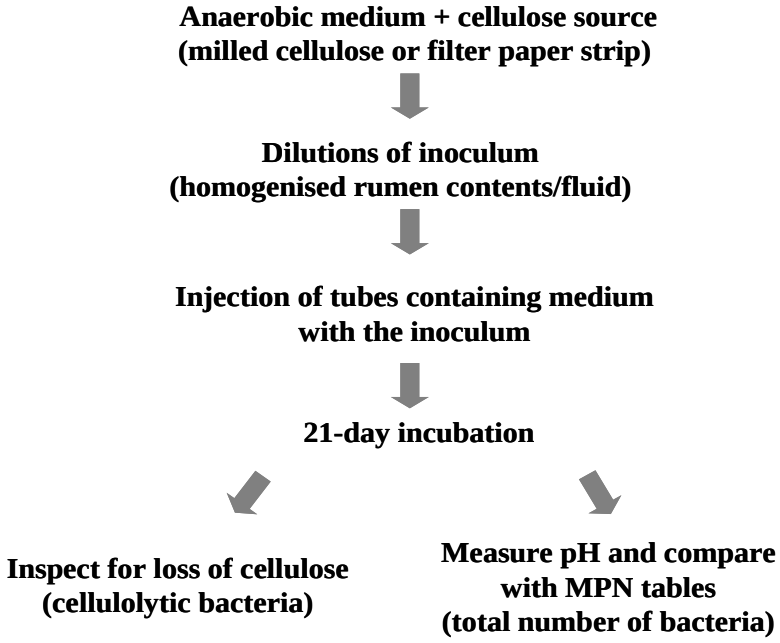
Dilution methods such as Most Probable Number (MPN) have been used to quantify total bacteria in ruminal contents (Neumann et al., 2008) and specific bacterial species in faeces (Fegan et al., 2004) and it is advantageous if bacteria cannot be cultivated or grow poorly in agar (McSweeney et al., 2005). If isolation and identification of species are not required, the MPN method can estimate the number of viable microbial cells, is simpler and less time-consuming than other culturing methods such as roll tubes. Cellulolytic bacteria can also be estimated as described by Dehority et al. (1989) (Figure 1.1). The number of viable cells is

calculated from the MPN tables that use statistical methods to combine data from different dilution levels (McSweeney et al., 2005). Cellulose digestion in MPN tubes is generally determined by visual loss of cellulose in the tube (Wedekind et al., 1988). Final pH appears to be a suitable criterion for estimating bacterial growth in MPN tubes (Dehority et al., 1989).

The roll tube method used for enumerating total numbers of anaerobic bacteria in ruminal contents was first described by Hungate (Bryant, 1972). This method is time consuming, requires training and skill (Holdeman et al., 1972) and involves counting colonies in the roll tube, further isolation and picking colonies to be grown in liquid medium (Dehority, 2004).

The introduction of molecular techniques opened new perspectives to understand microbial diversity, confirming that previous culturing methods had been highly selective (Edwards et al., 2004) and failed to reveal the "real" diversity (Yu et al., 2006). However, culturing is still important to provide quality material for further application of molecular techniques (Dähllof, 2002), so novel isolation approaches, mimicking the environmental conditions from which the samples are collected in conjunction with molecular-based strategies are clearly the way forward.

Figure 1.1. Most Probable Number (MPN) procedure to estimate ruminal bacteria.



1.6. METHODOLOGICAL ADVANCES.

The study of microbial diversity and community analysis in the rumen has risen since the advent of DNA sequencing, which in turn has revolutionised our understanding of ruminant nutrition (Kobayashi, 2006). Examination of the microbial diversity at a deep level (groups, species and strains) is essential to improve our understanding of the relationships between structure–function among ruminal communities and to analyse the interactions among microbes, their environment and the host. At present, the bacterial ribosomal RNA operon, encompassing the 16S rRNA gene, is the most frequently used molecular marker due to: (1) its highly conserved sequence (Zoetendal et al., 2008b), (2) the discriminatory potential of the hypervariable regions of the gene (V regions) (Justé et al., 2008), which contain the signatures of phylogenetic groups and even species, and (3) the availability of sequences in public databases such as GenBank (Benson et al., 2004), which enable an accurate description of the microbial populations in a community (Chakravorty et al., 2007). Therefore, molecular techniques based on 16S rDNA can be useful tools to gain insight on phylogenetic and functional relationships among rumen microbiota.

1.6.1. rRNA/rDNA – based methods.

Fingerprinting of the 16S rRNA gene has been successfully applied to monitor community shifts (Zoetendal et al., 2004), and to compare different communities upon amplification of variable regions of the rDNA (Kobayashi,

2006). The heterogeneity of the 16S rRNA gene is one of the disadvantages when using it as a target for amplification (Nübel et al., 1996), since, on average, 2.2 copy numbers per organism have been detected in the regions V2-V3 of the bacterial 16S rDNA (Schmalenberger et al., 2001). Therefore, these techniques require extraction of high-quality nucleic acids prior to the amplification of RNA/rDNA, and final analysis of PCR products (Deng et al., 2008). Nevertheless, the use of 16S rRNA gene is widely spread and online resources such as BLAST in GenBank (Madden et al. 1996) (http://www.ncbi.nlm.nih.gov/Blast) and Ribosomal Database Project (RDP) (http://www.cme.msu.edu) (Maidak et al. 2001) aid in the identification of novel species obtained with this technique (Deng et al., 2008).

1.6.1.1. *FISH.*

Fluorescence in **S**itu **H**ybridisation (FISH) targets rRNA molecules using fluorescently labelled probes that penetrate fixed cell membranes and hybridise to intracellular rRNA (Amann et al., 2001). After removing unbound probes, labelled cells can be detected by microscopy or flow cytometry (Amann et al., 1995). Due to the limited number of reported probes for FISH, its use in rumen microbial ecology is still limited (Zoetendal et al. 2004). However, it has allowed detecting *Oscillospira* spp. populations in different ruminants (Mackie et al., 2003), identified specific niches in fibrolytic bacteria (Shinkai et al., 2007) and revealed abundance of sulphur-reducing bacteria in liquid and solid fractions of rumen contents (Kong et al., 2010a). The full potential of this technique coupled with additional culture-independent tools is yet to be explored, but it may provide

key information on the functional role of microbial populations within complex ecosystems (Lee et al. 1999; Nielssen et al., 2010), even possibly in the rumen.

1.6.1.2. *Quantitative Real-Time PCR.*

Real-time (quantitative) PCR was developed to quantify DNA rapidly in a single process (Higuchi et al. 1993). This technique employs a DNA-binding dye (SYBR Green biochemistry, for example), which binds to the minor groove of double-stranded DNA and increasing its fluorescence as more amplicons are produced in the reaction (Denman et al., 2005). The cycle at which fluorescence is detectable above the background during the exponential phase, is termed the cycle threshold (Ct) and the values reported at this stage are then used for quantification (Denman et al., 2005). The analysis of the dissociation curve is performed on completion of the amplification cycles to reveal the purity of the amplicon produced for each reaction. Universal probes have been used to estimate the total gene copy of 16S rRNA from all bacterial populations present in a particular sample (Deng et al., 2008). Furthermore, from the total bacterial 16S rRNA gene copy number, the proportion of a taxon-specific SSU rRNA (determined with specific probes) can be verified (Li et al., 2009) to evaluate variations in bacterial population due to location in the rumen and time of sampling (Li et al., 2009). Because this is one of the most commonly applied techniques, it has led to major findings in ruminal bacterial ecology (Table 1.3).

Table 1.3. Recent perspectives on the use of qRT-PCR to screen bacterial populations.

Innovation	Reference
Adaptive dynamics in populations during diet transition	Fernando et al., 2010
Detection of novel homoacetogenic bacteria	Henderson et al., 2010
Discovery of synergistic fibrolytic consortia	Shinkai et al., 2010
Monitoring and isolation of uncultured bacterial strains	Koike et al., 2010
Enumeration of the epimural bacterial community	Chen et al., 2011

1.6.2. Community fingerprinting techniques.

In order to study population structure and dynamics, genetic fingerprinting techniques are valuable tools (Zoetendal et al., 2004). Following the PCR amplification of 16S rRNA genes, these methods are used to monitor the amplified DNA. The amplicons generated from extracted nucleic acids by PCR assays, using bacteria-specific primers, are separated according to their intrinsic properties, and the resulting pattern is reflective of the community diversity (Talbot et al., 2008).

1.6.2.1. *RFLP and T-RFLP.*

Restriction fragment length polymorphism (RFLP) and terminal restriction fragment length polymorphism (T-RFLP) are techniques that allow differentiating organisms based on the patterns derived from enzymatic cleavage of the DNA (Acinas et al., 1997). In T-RFLP, one or both primers are fluorescent-labelled;

detection of the terminal restriction fragment fluorochrome is performed by separating the fragments on an automated sequencer (Liu et al., 1997). Both methods have been widely applied to examine the microbial diversity in the gut of mammalian species; results of these techniques applied to study rumen bacteria are described in Table 1.4.

Table 1.4. Novel applications of T-RFLP (1) and RFLP (2) to monitor ruminal bacteria.

Innovation	Reference
Composition of rumen microbiome during SARA[(1)]	Khafipour et al., 2009
Comparison of the microbial populations in GI tract [(1)]	Frey et al., 2009
Adaptation of faecal bacteria to adverse environment [(1)]	Romero-Pérez., 2011
Report of ammonia-hyperproducing (HAP) bacteria [(2)]	Attwood et al., 1998
Diet-dependent diversity of *Streptococcus bovis* [(2)]	Jarvis et al., 2001

1.6.2.2. *DGGE / TGGE*.

Denaturing Gradient Gel Electrophoresis (DGGE) and Temperature Gradient Gel Electrophoresis (TGGE) are methods that examine microbial diversity based upon electrophoresis of PCR-amplified 16S rDNA fragments (Muyzer et al., 1997). Initially, partial regions of the 16S rDNA (V1–V9) are amplified with either universal or specific primers to generate amplicons of identical size (Yu et al., 2004). A GC-rich sequence (40 bp) is attached to the 5’

end of the forward primer to prevent DNA dissociation under denaturing conditions (Muyzer et al., 1993). Separation is achieved by the decreased electrophoretic mobility of a partially melted double stranded DNA molecule in a polyacrylamide gel containing a linear gradient of denaturant (urea and formamide) or a linear temperature gradient (Muyzer, 1999). Because molecules with diverse sequences may have different melting behaviour, they will stop migrating at different positions in the gel (Muyzer et al., 1998), resulting in distinctive band profiles, even if PCR products appeared of approximately equal size (McCracken et al., 2001). Further, DNA bands in DGGE profiles can be visualized upon staining (Deng et al., 2008).

Among the molecular-based techniques, PCR-DGGE analysis is reproducible, rapid and inexpensive; and because separation is dependent on the sequence of the PCR product, it is more selective than other approaches (Moeseneder et al., 1999) when the communities of large number of samples are compared. This method can determine microbes that constitute up to 1% of the total bacterial community, so it is capable to detect the most dominant bacteria (Zoetendal et al., 2004) (Table 1.5). Therefore, PCR-DGGE analysis allows comparing several samples at a time, it is a better estimator of biodiversity (Pedros-Alio, 2006) and enables us to monitor temporal and/or diet induced changes in the rumen microbial population when large numbers of animals are used (Nicolaisen et al., 2002). Additionally, this procedure allows direct identification of the presence and relative abundance of different species (Deng et al., 2008) as well as re-amplification of excised bands or hybridisation analysis

with specific probes (McCracken et al. 2001; Temmerman et al. 2003). However, the analyses of re-amplified bands needs careful interpretation, as the separation of amplicons by DGGE may not be as accurate as desired (Nikolausz et al., 2005) due to the multiple copies of the 16S rRNA gene and one band may contain more than one sequence. Additionally, the small fragment size of the PCR product might not contain enough information to render a precise taxonomic classification of the DNA bands in the profile (Justé et al., 2008).

Table 1.5. Contributions of PCR-DGGE technique to the understanding of the rumen bacterial ecosystem.

Innovation	Reference
Bacterial changes in response to photoperiod	McEwan et al., 2005
Identification of main starch-utilising bacteria	Klieve et al., 2007
Characterisation of the dynamics of forage colonisation	Edwards et al., 2007
Correlation of microbial ecology to feed efficiency	Guan et al., 2008
Detection of host specificity for bacterial populations	Shi et al., 2008
Development of rumen ecosystem in young cattle	Belanche et al., 2010
Screening of epimural community	Sadet-Borgeteau et al., 2010
Analysis of acetogen populations in the rumen	Gagen et al., 2010
Determination of the role of bacteria in biohydrogenation	Huws et al., 2011

1.6.3. Sequence-based approaches.

The bacterial 16S rRNA molecule has a length of approximately 1500 nucleotides and thus contains sufficient information for reliable characterization of bacterial taxa at species and/or strain level (Zoetendal et al., 2008a). Molecular methodologies relying on 16S rRNA gene sequences are suitable to describe the active members of a population (McSweeney et al., 2009). Sequence-driven analysis relies on the conserved DNA regions to design hybridization probes or PCR primers for screening bacterial clones that are expected to contain nucleotide sequences of interest (Deng et al., 2008). Most phylogenetic information from the GI tract has been gathered by sequencing of cloned 16S rRNA gene amplicons.

1.6.3.1. *Clone libraries.*

The composition of microbial communities in the rumen has been also determined by constructing 16S rRNA clone libraries, followed by phylogenetic identification of the clones. Ribosomal RNA sequences can be obtained either by reverse transcriptase (RT) PCR from rRNA or from regular PCR from rRNA genes (Zoetendal et al., 2008a). These PCR products are then cloned into plasmid vectors, with each clone containing one copy of the rRNA gene of interest (Talbot et al., 2008). For economical and practical reasons, sequences of 16S rRNA genes are determined by creating rRNA gene clone libraries rather than cDNA libraries from rRNA (Zoetendal et al., 2004). After generating the clone library, the sequences of the cloned amplicons are determined and compared to sequences available in the DNA databases followed by phylogenetic analysis (Cole et al., 2003; Ludwig et al., 2004). However, to monitor changes in the community over time, this approach might be expensive, time-consuming (Talbot et al., 2008) and

biases due to the PCR and vector cloning might occur (Deng et al., 2008). Table 1.6 shows recent outcomes from this methodology.

Table 1.6. Current trends in library-based methods applied to rumen microbial ecology.

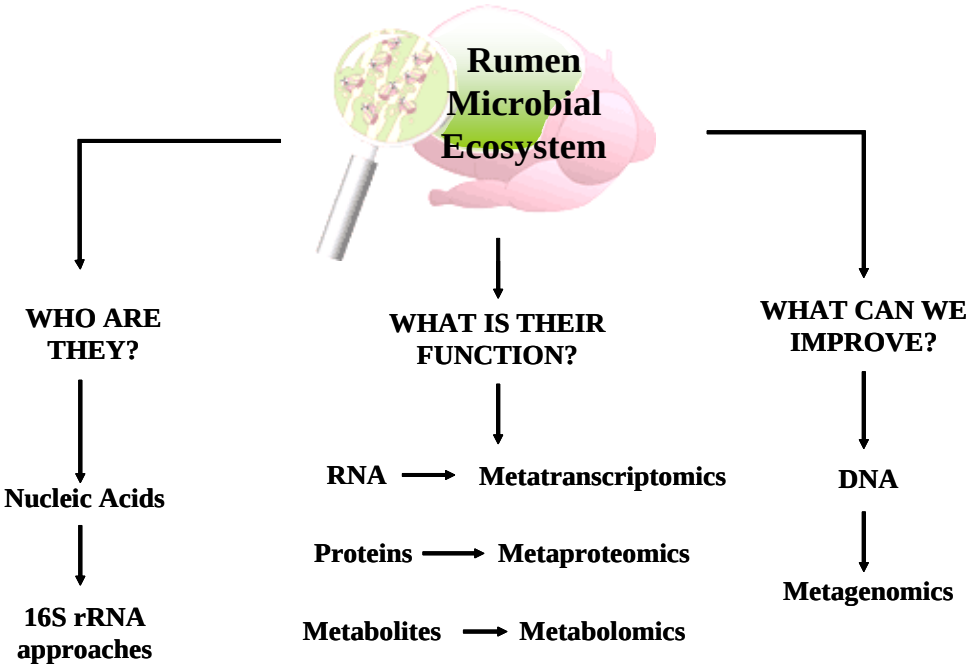
Innovation	Reference
Diet-dependent shifts in bacterial diversity	Tajima et al., 2001
Analysis of fibre-associated bacterial community	Koike et al., 2003
Influence of geographic separation on bacterial diversity	Sundset et al., 2007
Individual variation in microbial diversity	Durso et al., 2010
Genetic diversity of rumen *Prevotella* spp.	Bekele et al., 2010
Effect of heat stress on rumen microbial composition	Uyeno et al., 2010
Impact of Sub-Acute Acidosis (SARA) on bacterial density	Hook et al., 2011
Dietary associations of particular rumen bacterial species	Bekele et al., 2011

1.6.3.2. *Metagenomics.*

Metagenomics refers to the analysis of combined genomes from an ecosystem, aiming to assess the phylogenetic, physical and functional properties of the microbial communities present (Handelsman, 2004). To achieve these objectives, DNA is extracted from the microbial community and cloned in a suitable host using a vector, such as fosmids or bacterial artificial chromosome vectors (Zoetendal et al., 2008b). This results in a metagenomic library that can be used for sequence- or function-driven analysis. Sequence-driven analyses

contribute to obtain an overview of the diversity of the microbial ecosystem (Gabor et al., 2004), while function-driven analyses aid in screening libraries for novel enzymes and functional interactions (Ferrer et al., 2005). Metagenomics techniques have great potential to identify genes encoding plant-degrading enzymes, thus contributing to increase the understanding of the mechanisms mediating digestion in the ruminants (Singh et al., 2008; Brulc et al., 2009). Moreover, the functional analysis of these genes might uncover strategies for improving fibre digestion in the rumen (Attwood et al., 2008; Hess et al., 2011) that could further be applied to manipulate pathways associated with bioreactor processes for biofuels production (Lissens et al., 2004). However, because not all the detected genes in metagenomic libraries are functionally relevant, metagenomics should be considered a supplementary tool to screen functions (Booijink et al., 2007). Additional ‘‘omics’’ approaches targeting RNA, proteins and metabolites, might be more efficient for gaining insight into the activity, functions and interactions of the microbes in the rumen ecosystem (Figure 1.2).

Figure 1.2. Prospective and current opportunities for increased understanding and targeted manipulation of ruminal metabolism (Adapted from Zoetendal et al., 2008).



1.7. SUMMARY

One of the main issues in the current beef industry is how to optimize the utilization of feed resources to maximize efficiency of feed utilization, because feed efficiency ultimately affects profitability and sustainability of the industry. In fact, it has been considered that feed costs account for nearly 75% of the total input costs (Moore et al., 2009). Moreover, an improvement in feed efficiency has been proposed to bring reductions in nutrient excretion (Herd et al., 2003) and in methane emissions (Hegarty et al., 2007). Previous studies have employed culture-independent techniques to report differences in bacterial diversity and have aided to establish the concept of microbiome, to describe the interaction among the microbes, their genomes and the environment (Brulc et al., 2009). Work performed by Guan et al., (2008) using these techniques have found a correlation between bacterial diversity and animal performance, specifically with Residual Feed Intake, a desirable measure of feed efficiency (Nkrumah et al., 2006). However, the paucity of data has not allowed establishing the link between the bacterial population and the host productive performance. Further, reports on the associations between specific bacterial populations and feed efficiency measurements were nonexistent. Therefore, information on how phenotypic variation of the host and diet affect the bacterial diversity in the rumen and whether there were associations with sire breed are missing. With these facts in mind, it was hypothesised that ruminal microflora structure and the fermentation variables may be associated with feed efficiency of the host.

Therefore, three studies were designed to fulfil the overall objective of this thesis; to investigate the association between the microbial diversity, the microbial metabolites produced in the rumen, and the feed efficiency trait. The specific objectives are 1) to investigate the association between the bacterial profiles in the rumen fluid and feed efficiency measurements under low energy diet; 2) to explore the associations between the above profiles and rumen fermentation and feed efficiency variables under different diets and for different RFI rankings; 3) to investigate the associations among the bacterial profiles, fermentation measurements, RFI, and sire breeds. The long term goal of this study is to elucidate the biological functions of rumen microbes, and to reveal how the genetic variation of the host influences the microbial population. This information may be applied to identify genetic markers for economic important traits such as Residual Feed Intake and other fed efficiency traits.

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Chapter 2. Linkage of Particular Bacterial PCR-DGGE patterns to Bovine Ruminal Fermentation Parameters and Feed Efficiency Traits. [1]

2.1. INTRODUCTION.

A fundamental understanding of microbial ecology and relationships to ruminant physiology is essential for successful manipulation of ruminal microflora and subsequent improvement in animal production since rumen microflora play important roles in the nutrient and energy uptake of the host (McSweeney et al., 1994). Hence, principles such as niche occupancy, selective pressure, adaptation, and interactions among populations (Weimer, 1998) as well as the kinetics of substrate utilisation (Kelly et al., 1993) have to be taken into account when evaluating the ruminal microflora and host interactions. Bacterial density in the rumen is high, with direct counts as high as 10 billion cells per gram of ruminal contents (Russell et al., 2001; Kobayashi, 2006). Due to the limited understanding of the complex nature of the microbial component and activities in the rumen, the mechanisms of host–microbial, microbial–microbial interactions and whether such interactions impact on host biology have not been well established.

Many recent studies have employed molecular based culture-independent techniques to investigate bacterial profiles (Tajima et al., 2001; Mackie et al., 2003; Edwards et al., 2005). PCR-denaturing gradient gel electrophoresis (PCR-

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DGGE) analysis has been applied to assess ruminal microbial diversity based upon PCR-amplified 16S rRNA fragments to study community interactions (Sadet et al., 2007), monitor populations shifts (Mao et al., 2007) and screen clone libraries (Deng et al., 2008). The PCR-DGGE banding patterns are considered to be representative of the dominant bacterial groups (Muyzer et al., 1993) and can be applied to screen changes of dominant species in the microflora for large numbers of environmental samples. A new terminology of "microbiome" has been applied to study the rumen microbial community and further confirmed the complexity of this environment (Brulc et al., 2009). However, many questions remain unanswered. For example, how does the microbiome change in large numbers of animals in response to host, diet, environment, health and other factors?, which is more important to the host: whole microbiome? Or core microbiome? Functional particular microbiome? Therefore, defining the ruminal microbiome to study its functions and interactions with the host has been an immense challenge. The selection of the rumen microbiome with particular functions after screening by culture-independent methods such as PCR-DGGE, therefore, is essential for the high throughput sequence analysis.

Feed efficiency is one of the most critical factors that impact on the feed utilization by cattle. I hypothesized that particular bacterial populations in the rumen are associated with fermentation metabolites which can also influence on host feed efficiency. A recent study suggested that the bacterial structure may be associated with cattle's residual feed intake (Guan et al., 2008); however, the few number of animals used in this study did not provide a direct linkage between

particular microbial population and host feed efficiency traits. The rumen microbial community changes in response to the feeding time (Li et al., 2009). Since previous studies have shown that the pre-feeding concentration of volatile fatty acids (VFA) had less variation by diet (Peters et al., 1990) or by feeding cycles (Welkie et al., 2010) and due to the limited access to rumen fluid sampling from the examined commercial population in this study, we centred on the characterization of the pre-feeding dynamics in the ruminal bacterial composition and in the fermentation metabolites in 58 steers, to test our hypothesis. Therefore, I focused on investigating the associations between rumen bacteria and host feed efficiency traits using PCR-DGGE analysis, aiming to identify the functional rumen microflora. The traits evaluated were daily dry matter intake (DMI), average daily gain (ADG), feed conversion ratio (FCR) (Feed: Gain), and residual feed intake (RFI), to measure the feed efficiency of cattle (Archer et al., 1999; Arthur et al., 2001; Nkrumah et al., 2006). Furthermore, a multivariate statistical analysis was developed to correlate bacterial PCR-DGGE profiles to fermentation measurements such as VFA and ammonia-nitrogen (NH_3-N) in the rumen and to feed efficiency traits including DMI, FCR, ADG, and RFI.

2.2. MATERIALS AND METHODS.

2.2.1. Animals and sampling.

Fifty-eight 10-month-old, Hereford × Aberdeen Angus steers were raised following the guidelines of the Canadian Council on Animal Care (CCAC) under feedlot conditions at the Kinsella Research Station, University of Alberta, on a

finishing diet as described by Nkrumah et al. (2006). The animal protocol was approved by Animal Care and Use Committee, University of Alberta (CCAC, 1993). Feeding intake data were collected using the GrowSafe automated feeding system (GrowSafe Systems Ltd., Airdrie, Alberta, Canada). Diet consisted of a total mixed finishing ration composed by approximately 74% oats, 20% hay, 6% feedlot supplement (32% CP beef supplement containing Rumensin (400 mg/kg), and 1.5% canola oil (ME 2.6 Mcal/kg). Steers were ranked and allocated to high RFI (H-RFI, inefficient, mean plus 0.5 SD, n=20), medium RFI (M-RFI, -0.5 SD < mean RFI < 0.5 SD, n=16), and low RFI (L-RFI, efficient, mean < –0.5 SD, n=22) groups, based on calculated RFI values as described by Nkrumah et al. (29). Similarly, DMI, ADG, and FCR were obtained following the procedures outlined by Basarab et al. (2003) and Nkrumah et al. (2006).

Rumen samples were collected before feeding on the same day within one week of completion of RFI measurement. For each animal, fifty to one hundred ml of rumen fluid including feed particles were obtained by inserting a flexible plastic tube into the rumen and transferred into a sterile 200 ml container, immediately frozen in dry ice and stored at –80 °C until further analysis.

2.2.2. DNA Extraction.

Total DNA was extracted from rumen samples using physical disruption with the bead beating method (Guan et al., 2008). Briefly, rumen samples were thawed, were visually inspected for saliva contamination, manually homogenized and centrifuged at 14,600 × g for 5 minutes at 4°C. The pellet was washed twice

and re-suspended in 1 ml of TN150 (10 mM Tris-HCl [pH 8.0], 150 mM NaCl) buffer and transferred to a 2-ml micro centrifuge tube containing 0.3 g of Zirconium beads (diameter 0.1 mm). The cells were lysed in a BioSpec Mini Bead-Beater-8 at 4,800 rpm for 3 min following by phenol-chloroform-isoamyl ethanol (25:24:1) extractions. DNA was precipitated with cold ethanol and re-suspended in 30 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]). The amount and quality of DNA were measured using a Nanodrop ND 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

2.2.3. PCR-DGGE analysis.

PCR amplifications of the V2-V3 region (~200 bp) of the 16S rRNA gene of bacteria were performed with universal bacterial primers HDA1-GC and HDA-2 using the program outlined by Walter et al. (2000). All PCR products were purified with a QIAquick PCR Purification Kit (Qiagen; Carlsbad, CA, USA) according to the manufacturer's instructions and the final concentration was measured using Nanodrop ND 1000 spectrophotometer (NanoDrop Technologies) before subjecting to DGGE analysis.

DGGE was run on 1X TAE buffer (40 mM Tris-base, 20 mM glacial acetic acid, 1 mM EDTA) with a 6% polyacrylamide gel with 22 to 55% linear denaturing gradient, using Bio-Rad DCode Universal Mutation Detection System (Hercules, CA, USA) and 1294 ng of purified PCR product from each sample. The gel was run at 130 V for 4 h, stained with 0.1% (vol/vol) ethidium bromide after electrophoresis, and photographed using FluorChem SP imaging system

(Alpha Innotech, San Leandro, CA, USA). In order to normalise for differences among different gels, a PCR product from one animal and a ladder containing purified PCR products from all animals were loaded as reference lanes on each gel.

2.2.4. Similarity analysis of PCR-DGGE profiles.

PCR-DGGE patterns were analysed using BioNumerics software version 5.1 (Applied Maths, Austin, TX, USA), herein hierarchical cluster comparisons were carried out to group similar profiles and to generate a binary matrix of band classes. All the images were normalised using the internal control samples described above and the comparison among whole profiles was performed using the Dice similarity coefficient (D_{sc}). The dendrogram was generated using the unweighted pair group with mathematical averages (UPGMA) method at 1% position tolerance. Furthermore, the arithmetic average of the D_{sc} (%) values was calculated. To assign categories to the fingerprint patterns, the D_{sc} was specified at 80% and an application ("script") from BioNumerics software package was run. Accordingly, relationships between fingerprint patterns and metabolites, as well as between fingerprint patterns and RFI were assessed.

2.2.5. Cloning and sequencing analysis of DGGE bands.

The separated bands were excised aseptically from the gel and transferred to diffusion buffer (0.5 M ammonium acetate; 10 mM magnesium acetate; 1 mM EDTA, pH 8.0; 0.1% SDS). DNA fragments were extracted using QIAEX II Gel Extraction Kit (QIAGEN Sciences, MD) following the manufacturer's

instructions for polyacrylamide gel extraction. Further, the extracted products were reamplified using the same primer pair and same amplification conditions as mentioned above. The fresh PCR products were then cloned into the TOP10 vector (TOPO TA Cloning kit, Invitrogen, Carlsbad, CA, USA) using chemical transformation. Colonies were selected on S-Gal medium (Sigma, St. Louis, MO, USA), randomly picked and from three replicates with insertions, plasmid DNA was extracted using Millipore Plasmid Extraction Kit (Millipore, Billerica, MA, USA). Sequence reaction was performed in 10 µl total volume, containing 0.5 µl of BigDye, 3.2 pmol of M13 Forward (CGC CAG GGT TTT CCC AGT CAC GAC) primer, 2.0 µl of 5x sequencing buffer, 20 ng of plasmid DNA as the template with ABI 3730 sequencing system using ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). All sequences were subjected to BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine the closest known taxon and were aligned using the ClustalW program (http://www.ebi.ac.uk/Tools/clustalw2). The sequence composition of each band was compared using the RDP Classifier online tool (http://rdp.cme.msu.edu/) (Wang et al., 2007).

2.2.6. Multivariate Statistical Analysis.

Principal Components Analysis (PCA) was used to observe the structure of the data and to identify the response variables accounting for the largest source of variation. Thirteen variables (Table 2.1.) were included in the analysis (acetate, propionate, butyrate, isobutyrate, valerate, isovalerate, total VFA, acetate: propionate ratio, branched VFA: straight VFA ratio, ammonia, daily dry matter

intake, average daily gain and feed conversion ratio). PCA was performed using PRINCOMP procedure in SAS (SAS System version 9.1, SAS Institute, Cary, NC, USA). This procedure standardises the variables to a mean of zero and a standard deviation of one. The loadings (eigen vectors) in each principal component were retained when the loadings were greater than the absolute average eigen value for that component.

Each DGGE pattern from an individual steer was assigned by BioNumerics software to generate a calculated best-fit Gaussian curve for each band. All the assigned bands from each animal were then exported with the normalised relative position. According to above PCR-DGGE profile similarity analysis, 1% tolerance was used to rectify the shifts among all the bands from all profiles. A binary matrix where all the bands were allocated into 85 new categories was created for 58 animals. Once clustering tendency was observed, a categorical model in SAS based on maximum likelihood was fit to analyse the interaction of the phenotypic traits with the bands. All variables were categorised in High (H) and Low (L) using PROC MEANS in SAS and taking 0.5 standard deviation as the cut-off point; then they were used to define the presence of particular bands on each variable: in PROC CATMOD, the effect of all variables on the prevalence of every band was determined based on the transformation of the cell probabilities (response function). This model analysed a data matrix containing either the averaged Gaussian position of the band or zero, indicating class. Afterwards, two-way contingency tables of cross classifications containing

the frequencies of the bands per category (High/Low) were obtained using PROC FREQ and results plotted.

2.2.7. Analysis of fermentation parameters.

2.2.7.1. *VFA and NH_3-N.*

Rumen fluid was centrifuged and supernatant was subjected to VFA profiling using Gas Chromatography (GC) analysis with a Perkin-Elmer Gas Chromatographer (Waltham, MA, USA) following standard procedures (Hristov et al., 2001). An enzymatic assay was carried out to measure NH_3-N using a commercial kit (R-Biopharm Roche Inc., South Marshall, MI, USA), following the manufacturer's instructions, based on spectrophotometer readings at a wavelength of 340 nm (SpectraMax 190, Molecular Devices, Sunnyvale, CA, USA).

Differences of VFA composition and ammonia were compared using the simple covariance mixed model of SAS; to find the interactions between the metabolites, statistical correlations were carried out using the CORR procedure in SAS and P values recorded. Significance was assumed at the $P < 0.05$.

2.3. RESULTS.

2.3.1. PCR-DGGE analysis of detectable bacteria in rumen samples.

The predominant bacteria in the rumen of 58 steers were initially compared using PCR-DGGE analysis. The PCR-DGGE profiles showed that each animal harboured an individual bacterial flora, evidenced by the presence of

complex band patterns (Figure 2.1) with average Dice similarity coefficient (D_{sc}) of all PCR-DGGE profiles of 75.5%. When RFI values were included into the similarity analysis, no significant trend was observed. The RFI was chosen to be correlated with DGGE profiles because it has been described as the most desirable measure for feed efficiency (Arthur et al., 2001).

Based on the positions of each band from the PCR-DGGE band patterns from all animals, 85 band categories were identified using BioNumerics Software. To characterize the taxonomy of the bands, all of them were purified, cloned and sequenced, and seventy-four bands were identified (Table 2.1.). The following criteria were used to determine the taxonomy of each band: a 96% or higher match between the clone sequence and the GenBank data was considered to represent at the species level (with accession number, Table 2.1.), and 90 to 95% identity at the genus level, given by the RDP Classifier online tool. When accession number is not provided, the percentage corresponds to the identity with the genus as matched by the RDP classifier. From the sequences obtained from the 74 PCR-DGGE bands, thirty-three of them corresponded to strains from the following known species: *Prevotella* sp., *Clostridum* sp., *Ruminococcus* sp., *Succinivibrio* sp., *Butyrivibrio* sp., *Robinsoniella* sp., *Eubacterium* sp., *Moryella* sp., *Coprococus* sp., *Bifidobacterium* sp., *Pelotomaculum* sp., *Succiniclasticum* sp., *Ruminobacter* sp., *Anaerophaga* sp., *Succinomonas* sp., *Selenomonas* sp., and *Lactobacillus* sp. Six sequences were identified only at genus level (band 1 and band 2, *Prevotella* sp.; band 24, *Clostridium* sp.; band 34 and band 56, *Ruminoccocus* sp.; band 63, *Eubacterium* sp.). Fifteen sequences matched

uncultured clones from the following species: *Prevotella* sp., *Succinivibrio* sp., and *Roseburia* sp. Four sequences were identified only at family level (band 39, *Prevotellaceae*; band 14 and band 28 *Lachnospiraceae*; band 15, uncultured *Lachnospiraceae).*

2.3.2. Analysis of fermentation profiles.

To investigate the associations between bacteria diversity and its functions in the rumen, we measured VFA and Ammonia-nitrogen (NH_3-N) concentration of the rumen samples. To minimise the influence of the sampling method on the VFA concentrations, due to the dilution of the rumen fluid by the saliva and the time elapsed since last meal, the proportion of each VFA to the total VFA concentration was obtained and used as the dependent variable for the metabolite analysis. The VFA profiles detected in the rumen samples were consistent with those previously reported (Sharp et al., 1982; France et al., 1993). The proportion of isovalerate was significantly higher in the H-RFI animals (P = 0.03), while the straight chain VFA to branched chain VFA ratio was significant lower (P = 0.03) in the same group of animals (Table 2.2.). Butyrate tended to be higher in the rumen of H-RFI steers (P = 0.10). DMI and FCR were significantly different between L-RFI and H-RFI animals, similar as previously reported (Nkrumah et al., 2006). In addition, significant statistical correlations were observed among all VFA, between butyrate and DMI, and between isovalerate and RFI as well as between DMI and isovalerate (Table 2.2.). On the other hand, the concentration of NH_3-N was not significantly different among different groups of animals (Tables 2.2. and 2.3.).

2.3.3. Correlations among PCR-DGGE profiles, fermentation characteristics and host feed efficiency traits.

To identify the correlations among rumen microbial structure, fermentation measurement and host feed efficiency, the association among all variables showing in Table 2.2. was investigated using Principal Components Analysis (PCA). Three significant principal components were extracted, describing 70% of the total variance (Figure 2.2A). In the first principal component (PC1), Acetate: Propionate ratio (A: P), isovalerate, isobutyrate and acetate had the highest contributions and they were orthogonal to DMI and butyrate, which were described in the second principal component (PC2). Also, RFI, FCR (Feed: Gain) and Straight: Branched-chain VFA were in PC3, independent from those variables in PC1 and PC2. The only variable associated with RFI was FCR as they had similar loadings in PC3. When PC1 was plotted with PC2, five variables including butyrate, isovalerate, DMI, FCR and RFI were in the same quadrant (Figure 2.2B).

A maximum likelihood approach was used to reveal the direct linkage between a specific group of bands and particular fermentation/feed efficiency estimates since the above PCA analysis failed to include all the bands as variables as well as to show the direct linkage among different variables. Using the CATMOD procedure in SAS, the effect of fermentation and feed efficiency traits on the prevalence of every band was analysed based on 85 bands determined from all animals. After two-dimensional tables were created, all bands were significantly different among each other by their locations on the DGGE gel (*P*

<0.0001). Based on the above analysis, the frequency plots of the bands showed that there were eight bands associated with DMI: 6 to high DMI and 2 to low DMI (Figure 2.3A); ten bands were correlated to ADG: 8 with high ADG and 2 with low ADG (Figure 2.3B); thirteen bands were associated with FCR: 5 to high FCR and 8 to low FCR (Figure 2.3C); eight bands exclusive to the RFI: 6 with L-RFI and 2 with H-RFI (Figure 2.3D). Additionally, particular PCR-DGGE bands were also found to be linked to fermentation measures. Nine bands with isovalerate: 6 with high and 3 with low isovalerate respectively (Figure 2.3G); ten with Straight: Branch chain VFA ratio: 1 with high and 9 with low (Figure 2.3F), while these two traits were significant different between L-RFI and H-RFI animals (Table 2.2.). Ten bands were correlated to butyrate; 5 with high and low butyrate, respectively (Figure 2.3E); and four bands were associated with isobutyrate (Figure 2.3G), while these two traits were tentatively associated with RFI (Table 2.2.). Similarly, associations between PCR-DGGE bands and other eleven variables were observed (Figures 2.4 A-F). Some bands were commonly identified from more than one trait. For example, band 15 (Uncultured *Lachnospiraceae* bacterium) was associated with low FCR, low propionate, low butyrate, low isobutyrate, low valerate, low isovalerate, low total VFA, L-A:P ratio and low Straight: Branch chain VFA ratio, and band 20 (*Succinivibrio dextrinosolvens*) was related to low FCR, low acetate, low butyrate, low valerate, low isovalerate and low A:P ratio.

2.4. DISCUSSION.

Ruminant animals derive about 70% of the metabolic energy from microbial fermentation of feed particles (Bergman, 1990). Microbial populations degrade feed components and generate end-products such as short chain VFA, carbon dioxide, methane and ammonia. Metabolic energy is used in the synthesis of cellular components needed for the microbial growth and other functions (Nagaraja et al., 1997) whereas the VFA are mainly absorbed and used as the main energy source by the host. To date, the relationship among the rumen microbial community, the microbial fermentation profiles and RFI is not well studied.

It was speculated that the differences in some bacterial profiles (PCR-DGGE bands) may be related to the probable associations observed between the fermentation measurements and feed efficiency. In this study, attempts to link the bacterial community structure with phenotypic traits were accomplished by correlating PCR-DGGE profiles to VFA concentrations and to various feed efficiency traits. VFA concentrations in the rumen represent the balance between microbial production and host epithelial transport and absorption. I considered the concentration of VFA can be interpreted as one of the indicators of microbial-microbial interactions as well as host-microbial interactions. A direct method to categorize the whole PCR-DGGE patterns did not allow the separation of the animals with different RFI, suggesting that not all the dominant bacteria are relevant for this trait. From the multivariate statistical analysis, the frequency of the bands in 58 steers revealed that all individuals have a “core” bacterial structure (bands 1-12, bands 37-75) (Figures 2.3. and 2.4.), which was not

correlated to any variable examined and it was present in all traits. Since all the animals shared the majority of the bands, the results agree with those observed in the PCR-DGGE dendrogram and in the pattern analysis, suggesting that some bacterial species in the rumen may be the key players influencing the feed conversion in the rumen. Three sequenced bands associated with low straight-chain to branched-chain VFA ratio (Band 11, *Prevotella* sp.), high butyrate, low FCR, low straight-chain to branched-chain VFA ratio (band 25, *Prevotella oulora*.), and low ammonia-N (band 31, *Butyrivibrio fibrisolvens*) were identified. As a predominant population in the rumen (Stevenson et al., 2007), it is not surprising that *Prevotella* sp. is associated with fermentation profiles. However, *Prevotella oulora* (band 25) was also associated with the same trait and FCR, one of the feed efficiency traits, suggesting that more than one bacterial species are associated with such low straight-chain to branched-chain VFA ratio; this is also supported by the fact that *Clostridium* sp. (band 24) is associated with low straight-chain to branched-chain VFA and FCR.

Our study has also identified potential associations of novel species with specific functions; for instance, band 21, which was associated with low FCR and low propionate, corresponds to an anaerobic propionate-oxidizing bacterium (Imachi et al., 2002), *Pelotomaculum thermopropionicum.* Similarly, band 76, which was related with low acetate, low butyrate and low A: P ratio was identified to be *Moryella indoligenes*, an anaerobic bacteria that has acetate and butyrate as major metabolic end-products (Carlier et al., 2007). In addition, the band representing *Butyrivibrio fibrisolvens* is associated with ammonia-N but not

with butyrate, also revealing the limitations of studying the rumen microbial community at taxonomy level; further functional studies such as investigation of the enzymes involved in the amino acid metabolism need to be done.

The observation of significant correlation between butyrate, isovalerate and DMI, and between isovalerate and RFI in this study supplied more preliminary data to support our hypothesis of the associations between rumen microbial diversity, fermentation profiles and host feed efficiency. The energetic metabolism has been reported to be significantly different in beef cattle with different RFI (Nkrumah et al., 2006), suggesting that the ruminal microbial fermentation plays important roles in this trait. The possible association between butyrate and DMI under low energy diet found in this study suggests that the microbial fermentation generated substrates involved in the energetic metabolism of the host that may be associated with the differences in RFI, since DMI is a fundamental element in deriving RFI, and represents the extreme end of the feed conversion axis. Although higher concentration of butyrate has been found to be associated with L-RFI animals (Guan et al., 2008), in our study contrasting observations were recorded and lower butyrate was associated with L-RFI animals. The diet difference in the present experiment might explain the above difference, since animals tested were under a low-energy density feedlot diet, whereas Guan et al. results are based in steers fed high-energy feedlot density diet. Previous studies showed that butyrate is mainly used as energy source for the host (France et al., 1993) and that butyrate increased significantly when animals were fed high-energy diet compared to low-energy diet (Russell et al., 1981; France et

al., 1993), suggesting that this and different butyrate metabolic pathways may contribute to RFI under different diets. Therefore, the effect of the diet needs to be considered when the interaction between the ruminal microflora and host RFI is investigated. Future studies on the relationships between the differences in butyrate concentration in the same steers under the high energy diet may supply extensive evidence which could assist in associating feed efficiency traits with this VFA.

Branched-chain VFAs are derived from branched-chain amino acids such as leucine, valine, and isoleucine, and the variations in this ratio indicate altered branched-chain amino acid catabolism (Pátek, 2007). Therefore, the ratio of branched-chain to straight-chain volatile fatty acids was considered as an indicator of amino acid fermentation in this study. I hypothesized that the higher Straight-chain to Branched-chain VFA ratio as well as the higher concentration of isovalerate and isobutyrate in the L-RFI animals suggest that more efficient N flow may also be associated with improved feed efficiency when low energy density diet was fed. Further studies on the association between microbial crude protein and RFI will supply better understanding of the relationship between microbial fermentation profiles and host feed efficiency.

Although possible associations between some VFA and feed efficiency traits we shown, data are limited and can be biased, because the VFA measured in this study were collected from a single time point and feed efficiency traits were recorded for a longer period. Recent studies have shown that the rumen fermentation was highly associated with the time after feeding (Li et al., 2009),

and the total VFA concentration reached highest level at 9 h after the morning feeding, but did not differ at other sampling times (Soto-Navarro et al., 2000). Despite the total VFA concentrations remaining above pre-feeding levels throughout post-feeding, pre-feeding VFA concentrations were similar between diets (Peters et al., 1990) as well as individual cow and within each feeding cycle (Welkie et al., 2010). Welkie et al. (2010) reported that VFA concentrations first increased due to microbial fermentation, reaching a maximum value 6 h post-feeding and then declining approximately to the initial values. Similarly, a previous study by Bevans et al. (2005) has shown that VFA concentrations were lowest before feeding, highest at 8 h after feeding and intermediate 18 h after feeding. Hence, the VFA measured before feeding may be applied as the baseline measurement, avoiding overestimation of the rumen bacterial community and can be representative for the variations of rumen microbial diversity for each individual animal. In addition, Sun et al. (2009) found that VFA concentrations peaked at 12 days after feeding goats with a diet similar to the one used in our study, but returned to lower levels afterwards, indicating that longer periods of adaptation can lead to a more stable rumen environment and fermentation characteristics. Such a trend was supported by Hristov et al. (2001), who detected a decline in VFA concentrations after 15 d of feeding a high barley diet. Thus, a sample taken after 90 days of feeding can be considered stable enough to represent the fermentation profiles. Furthermore, there is the inconvenience of cannulating 58 steers to obtain samples and while the variations in the VFA concentration among animals increase with the oral sampling, a larger sample size

leads to increased precision in estimating these variables. Lodge-Ivey et al. (2009) conducted a study to determine if sampling rumen contents via a ruminal cannula or oral lavage tube would yield similar PCR-DGGE profiles of the bacterial community and fermentation metabolites. When samples were grouped according to band pattern similarity, groups were most stable according to individual animal and species rather than sampling method. These data indicate that rumen samples collected via oral lavage or rumen cannula yield similar results.

Therefore, the VFA data measured pre-feeding is valid to indicate the potential associations between the rumen microbial community and fermentation profiles of the individual and its feed efficiency traits, which is a new concept that links the microbial molecular ecology to animal production. However, in order to provide a comprehensive biological relationship between VFA and feed efficiency traits, it is necessary to collect rumen samples at multiple time points during the period for recording intake variables.

In addition, the observed correlation amongst the butyrate and isovalerate concentration and the feed efficiency traits might also be associated with microbial interactions such as bacteria-archaea interactions. Another recent study revealed that the methanogenic community in animals with L-RFI was more diverse than that in animals with H-RFI (Zhou et al., 2009), indicating that interactions between different microbial groups may also impact fermentation and feed efficiency parameters. To achieve a realistic estimate of total microbial growth as well as relative numbers of individual species within the rumen, a quantitative understanding of microbial relationships is essential.

Compared to DMI, ADG and FCR traits, RFI has been recently considered as a more desirable measurement for feed efficiency (Crews, 2005). Many factors such as host genetics, diet, environment, management as well as the genetic-environmental interactions may directly or indirectly influence RFI. Our method to associate RFI to the identified PCR-DGGE bands (bacterial species) provides a better insight of the complexity of this trait, supporting our speculation that rumen fermentation may play a key role in this trait.

2.5. CONCLUSIONS.

This study identified probable associations between ruminal ecology and activities with cattle's feed efficiency by defining a statistical method to link the PCR-DGGE profile, microbial fermentation parameters and RFI. This is the first attempt to categorise bacterial PCR-DGGE band patterns in the rumen and to link them to phenotypic characteristics of the host, specifically to feed efficiency. From the multiple variable analysis, the bands representing specific groups of bacteria may be associated with measurable phenotypic parameters: six bacterial phylotypes were related exclusively to the H-RFI individuals whereas three phylotypes were exclusive of L-RFI steers. To identify the functions of ruminal microbes, it has been believed that a "consortium" may play more important roles than single species. This study supplies a way to identify such "consortium", which can be applied in screening the microbial community from large numbers of animals; further, the sequencing analysis of all the bands supplies information of the consortium. However, due to the limitations of the existing database, the results obtained from any sequencing analysis (including the output of the

recently developed pyrosequencing technology) usually indicate many "unculturable" and "unidentified" ruminal bacteria. The statistical analysis to identify the "specific bacterial PCR-DGGE profiles" will supply the functions of such "unculturable" and "unidentified" ruminal species. This work also supplies a potential method to identify functional "rare" (non-core) species of gut microbes contributing to host biology which will supply fundamental knowledge to understand the microbial-host interactions, and can also be extended to study functional microbes in various environmental microbial communities.

Table 2.1. Sequence identification of the PCR-DGGE bands.

PCR-DGGE Band Category	Taxonomy (GenBank Accesion No.)	Identity (%)	Trait Associated
1	*Prevotella* sp. (AF218619)	95%	
2	*Prevotella* sp. (AF218619)	97%	L-Acet, L-Val, L-A:P ratio
3	*Prevotella maculosa* strain W1609 (EF534315)	94%	
4	- *		
5	Uncultured *Succinivibrio* sp. clone EMP_B23 (EU794184)	100%	
6	*Prevotella* sp. BP1-56 (AB501155)	95%	
7	*Lactobacillus* sp. DI71 (AB290831)	100%	
8	-		
9	*Blautia* sp. BM-C2-0 (GQ456220)	97%	L-A:P
10	*Clostridium symbiosum* strain 69 (EF025909)	98%	L-Prop, L-But
11	*Prevotella oulorum* strain WPH 179 (NR_029147)	94%	L-St:Br VFA ratio
12	*Prevotella denticola* clone WWP_SS6_P23 (GU409439)	97%	
13	*Prevotella ruminicola* isolate L16 (AY699286)	93%	L-RFI, L-DMI, L-ADG, L-FCR, L-Acet, L-Prop, L-But, L-Isobut, L-Val, L-Isoval, L-Total VFA, L-A:P ratio, L-St:Br ratio, L-Ammonia
14	*Lachnospiraceae genomosp*. C1 (AY278618)	99%	L-Ammonia
15	Uncultured *Lachnospiraceae* bacterium	99%	L-FCR, L-Prop, L-But, L-Isobut, L-Val, L-Isoval, L-Total VFA, L-A:P, L-St:Br ratio
16	*Ruminococcus gauvreaui* strain CCRI 16110 (EF529620)	100%	L-RFI, L-DMI, L-ADG, L-FCR, L-Acet, L-Prop, L-But, L-Isobut, L-A:P ratio, L-St:Br ratio, L-

17	*Prevotella aff. ruminicola* Tc2-24 (AJ009933)	97%	Ammonia L-Acet, L-Prop, L-But, L-Total VFA, L-A:P ratio, L-St:Br ratio
18	*Prevotella ruminicola* (AB219152)	99%	L-DMI, H-ADG, L-FCR, L-Prop, L-But, L-Isobut, L-Val, L-Isoval, L-Total VFA, L-A:P, L-St:Br ratio, H-Ammonia
19	-		
20	*Succinivibrio dextrinosolvens* strain 0554 (NR_026476)	98%	L-FCR, L-Acet, L-But, L-Val, L-Isoval, L-A:P
21	*Pelotomaculum thermopropionicum* SI (AP009389)	93%	H-ADG, L-FCR, L-Prop, L-But, L-Val, L-Isoval, L-Total VFA, L-A:P, L-St:Br ratio
22	Uncultured *Succinivibrio* sp. clone EMP_B23 (EU794184)	100%	L-FCR, L-Acet, L-Prop, L-But, L-Val, L-Isoval, L-A:P
23	-		
24	*Clostridium populeti* strain 743A (NR_026103)	100%	L-FCR, L-Prop, L-Val, L-St:Br ratio
25	*Prevotella oulora* (L16472.2)	94%	High Butyrate, Low FCR, Low St: Br VFA
26	-		
27	Uncultured *Prevotella* sp. clone T0505 (GU458954)	93%	L-DMI, L-ADG, L-Prop, L-But, L-Val, L-St:Br ratio
28	Lachnospiraceae		

29	-		
30	*Prevotella maculosa* strain GEJ21 (GU561342)	97%	L-ADG, L-A:P ratio
31	*Butyrivibrio fibrisolvens*, strain Mz3 (AM039822)	98%	Low Ammonia-N
32	*Prevotella ruminicola* (AB219152)	94%	
33	*Photobacterium* sp. M2 (EU046607)	87%	
34	*Ruminococcus gauvreaui* strain CCRI 16110	100%	L-ADG, L-A:P ratio
35	Uncultured *Prevotella* sp. clone JD9 (FJ268952)	96%	
36	*Butyrivibrio fibrisolvens* strain H15 (EU887842)	98%	
37	Uncultured *Prevotella* sp. clone Sew1-325 (FJ219872)	95%	
38	*Clostridium indolis* (AF028351)	97%	
39	Prevotellaceae		
40	*Vibrio* sp. WH134 (FJ847833)	86%	
41	*Ruminococcus* sp. ZS2-15 (FJ889653)	90%	
42	*Prevotella* sp. 152R-1a (DQ278861)	97%	
43	Uncultured *Roseburia* sp. clone M2-35 (EU530245)	100%	
44	Uncultured *Prevotella* sp. clone JD9 (FJ268952)	96%	
45	-		
46	Uncultured *Prevotella* sp. clone JD9 (FJ268952	96%	
47	*Hespellia porcina* strain PC80 (NR_025206)	98%	
48	*Lactobacillus* sp. DI71 (AB290831)	100%	
49	Uncultured *Prevotella* sp. clone Gull85-50 (FJ220908)	98%	
50	*Prevotella ruminicola* strain TC2-3 (AF218617)	97%	
51	*Robinsoniella peoriensis* strain HGUE-09/9434 (GU322806)	98%	
52	*Succiniclasticum ruminis* strain DSM 9236 (NR_026205)	97%	
53	*Ruminobacter amylophilus* strain H 18 (NR_026450)	99%	
54	*Succinivibrio dextrinosolvens* strain 0554 (NR_026476)	96%	
55	Uncultured *Succinivibrio* sp. clone EMP_J46 (EU794280)	89%	
56	Ruminococcaceae		
57	*Eubacterium xylanophilum* (L34628)	98%	
58	*Moryella indoligenes* strain AIP 220.04 (DQ377947)	99%	
59	Uncultured *Succinivibrio* sp. clone EMP_V30 (EU794288)	100%	
60	*Anaerophaga thermohalophila* strain Fru22 (NR_028963)	88%	
61	*Eubacterium rangiferina* (EU124830)	97%	
62	*Robinsoniella peoriensis* strain HGUE-09/9434 (GU322806)	98%	
63	*Eubacterium rectale* ATCC 33656 (CP001107)	98%	
64	*Butyrivibrio fibrisolvens* strain H15 (EU887842)	94%	
65	*Robinsoniella peoriensis* strain HGUE-09/9434 (GU322806)	98%	
66	Uncultured *Succinivibrio* sp. clone EMP_B23 (EU794184)	97%	
67	*Succinivibrio dextrinosolvens* strain 0554 (NR_026476)	96%	
68	*Succinivibrio dextrinosolvens* strain 0554 (NR_026476)	97%	
69	Uncultured *Prevotella* sp. clone 3083 (FJ976203)	93%	
70	*Succinivibrio dextrinosolvens* strain 0554 (NR_026476)	95%	
71	*Coprococcus eutactus* strain ATCC 27759 (EF031543)	99%	

72	*Clostridium indolis* (AF028351)	97%	
73	Uncultured *Succinivibrio* sp. clone EMP_V30 (EU794288)	100%	
74	Uncultured *Succinivibrio* sp. clone EMP_V30 (EU794288)	100%	L-But
75	-		
76	*Moryella indoligenes* strain AIP 220.04 (DQ377947)	98%	L-Acet, L-But, L-A:P ratio
77	*Succinivibrio dextrinosolvens* strain 0554 (NR_026476)	98%	L-Acet, L-Prop, L-But, L-Isoval, L-A:P ratio
78	-		
79	*Succinimonas amylolytica* strain DSM 2873 (NR_026475)	94%	L-Isoval
80	*Ruminococcus bromii* strain YE282 (DQ882649)	93%	L-ADG, L-Acet, L-But, L-Isobut, L-Isoval, L-Total VFA, L-A:P ratio, L-St:Br ratio L-Ammonia
81	-	96%	L-RFI, L-DMI, L-ADG, L-FCR, L-Acet, L-Prop, L-But, L-Isobut, L-Isoval, L-A:P ratio
82	-		
83	*Selenomonas ruminantium* strain: S211 (AB198441)	96%	L-Isoval, L-St:Br ratio, L-Ammonia
84	*Bifidobacterium ruminantium* strain KCTC 3425 (GU361831)	100%	L-Acet, L-Ammonia
85	-		

* '-' represents the bands that could not be successfully cloned and sequenced.

Table 2.2. Fermentation and feed efficiency measurements in steers differing RFI and fed low energy diet (n = 58).

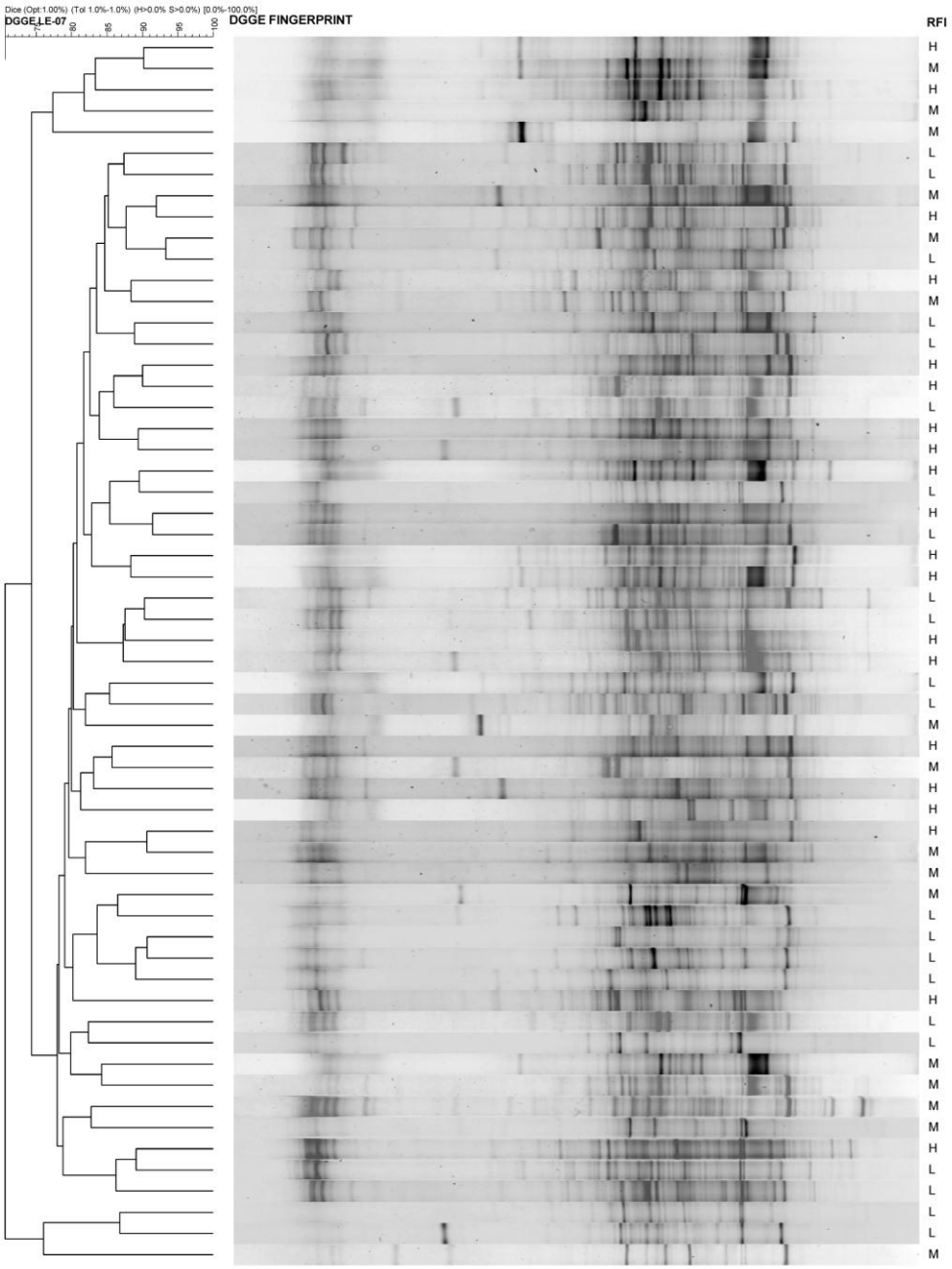
Variable	High RFI Mean ± SEM (n = 20)	Low RFI Mean ± SEM (n = 22)	*P* value
Acetate (%) *	54.58 ± 1.23	54.92 ± 1.18	0.84
Propionate (%) *	31.45 ± 1.22	33.41 ± 1.16	0.25
Butyrate (%) *	9.51 ± 0.76	7.26 ± 0.73	0.10
Isobutyrate (%) *	9.53 ± 0.06	8.29 ± 0.05	0.11
Valerate (%) *	1.00 ± 0.07	1.04 ± 0.07	0.67
Isovalerate (%) *	2.37 ± 0.15	1.92 ± 0.14	0.03
Total VFA (mM)	58.55 ± 5.49	64.17 ± 5.24	0.75
Acetate : Propionate ratio	1.87 ± 0.13	1.69 ± 0.12	0.32
Straight VFA : Branched VFA ratio	30.95 ± 2.27	38.06 ± 2.17	0.03
Ammonia (mM)	0.096 ± 0.01	0.11 ± 0.01	0.43
Dry Matter Intake (kg DM)	8.65 ± 0.14	6.94 ± 0.13	<0.0001
Average Daily Gain (kg)	1.26 ± 0.04	1.22 ± 0.04	0.39
Feed Conversion ratio (Feed: Gain)	6.95 ± 0.16	5.76 ± 0.15	<0.0001

* Values are given as a proportion of the total VFA concentration.

Table 2.3. Correlation (r) of fermentation measurements in the rumen of steers differing RFI with indicators of feed efficiency (RFI, DMI, ADG and FCR, n = 58), ***p<0.0001, **p<0.05

Variable	Propionate	Butyrate	Isobutyrate	Valerate	Isovalerate	Total VFA	Straight VFA : Branched VFA ratio	Acetate : propionate ratio	NH_3-N	RFI	DMI	ADG	FCR (F:G)
Acetate	-0.714***	-0.460**	0.635***	-0.537**	NS	-0.555***	NS	0.847***	NS	NS	NS	NS	NS
Propionate		-0.466**	-0.377**	NS	-0.701***	0.564***	0.667***	-0.892***	NS	NS	NS	NS	NS
Butyrate			NS	0.347**	0.609***	NS	-0.377**	NS	NS	NS	0.415**	NS	NS
Isobutyrate				NS	0.259***	-0.689***	-0.631***	0.701***	NS	NS	NS	NS	NS
Isovalerate						-0.329**	-0.883***	0.431**	NS	0.325**	0.351**	NS	NS
Total VFA							0.529**	-0.487***	-0.309**	NS	NS	NS	NS
Straight VFA : Branched VFA ratio								-0.554***	NS	-0.317**	NS	NS	NS
RFI											0.836***	NS	0.732***
DMI												0.433**	0.539**
ADG													-0.513**

Figure 2.1. PCR-DGGE profiles generated from ruminal fluid DNA from fifty-eight steers fed with low energy diet using primers HDA1-GC and HDA2 (22 to 55% DGGE). H and L represent the steers with high RFI (H-RFI > 0.5, inefficient), M-RFI (-0.5 < RFI < 0.5) and low-RFI (L-RFI < -0.5, efficient), respectively. RFI, residual feed intake, a parameter to measure feed efficiency in cattle (2). The comparison of the PCR-DGGE profiles was generated with the BioNumerics software package using UPGMA (unweighted pair-group) method as described in the text.


Dice (Opt:1.00%) (Tol 1.0%-1.0%) (H>0.0% S>0.0%) [0.0%-100.0%]
DGGE-LE-07
75
80
85
90
95
100
DGGE FINGERPRINT
RFI
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M
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Figure 2.2A. Principal component analysis of fermentation and feed efficiency measurements. Scatter plot of the first two principal component scores (symbol equals RFI class: low, L; medium, M; high, H).

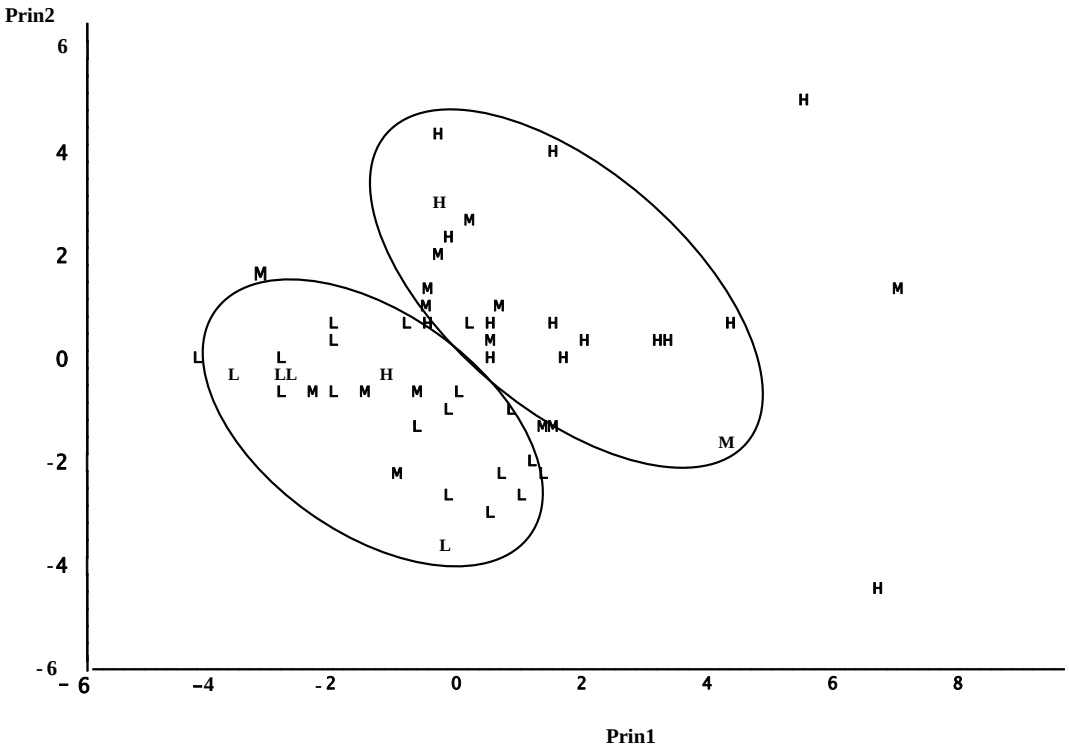


Figure 2.2B. Principal component analysis of fermentation and feed efficiency measurements. Loading plot describing the relationship among measured variables. ADG, average daily gain; DMI, daily dry matter intake; FCR, feed conversion ratio; RFI, residual feed intake; A:P, acetate to propionate ratio; ST:BR VFA, straight chain to branched chain VFA ratio.

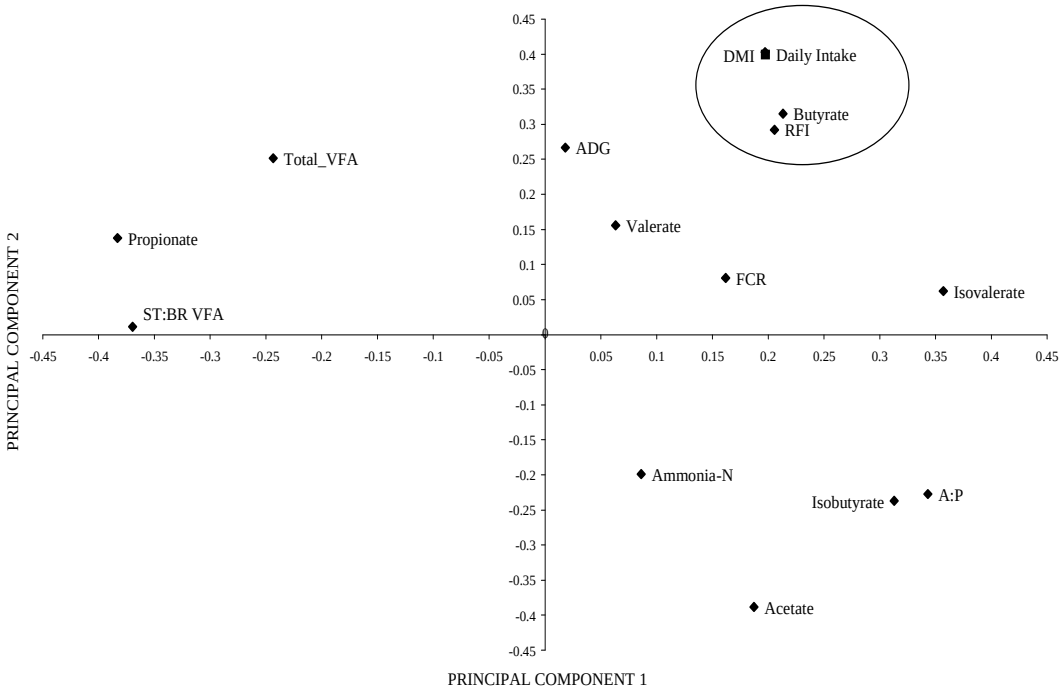


Figure 2.3. Frequency of PCR-DGGE bands in animals categorized on the basis of (A) Dry Matter Intake (DMI), and (B) Average Daily Gain (ADG) using PROC CATMOD analysis. The x-axis represents 85 identified bands and the order of the bands reflects the migration locations on the PCR-DGGE gel from top to the bottom. The arrows indicate the frequency of bands detected in the tested the population of each trait.

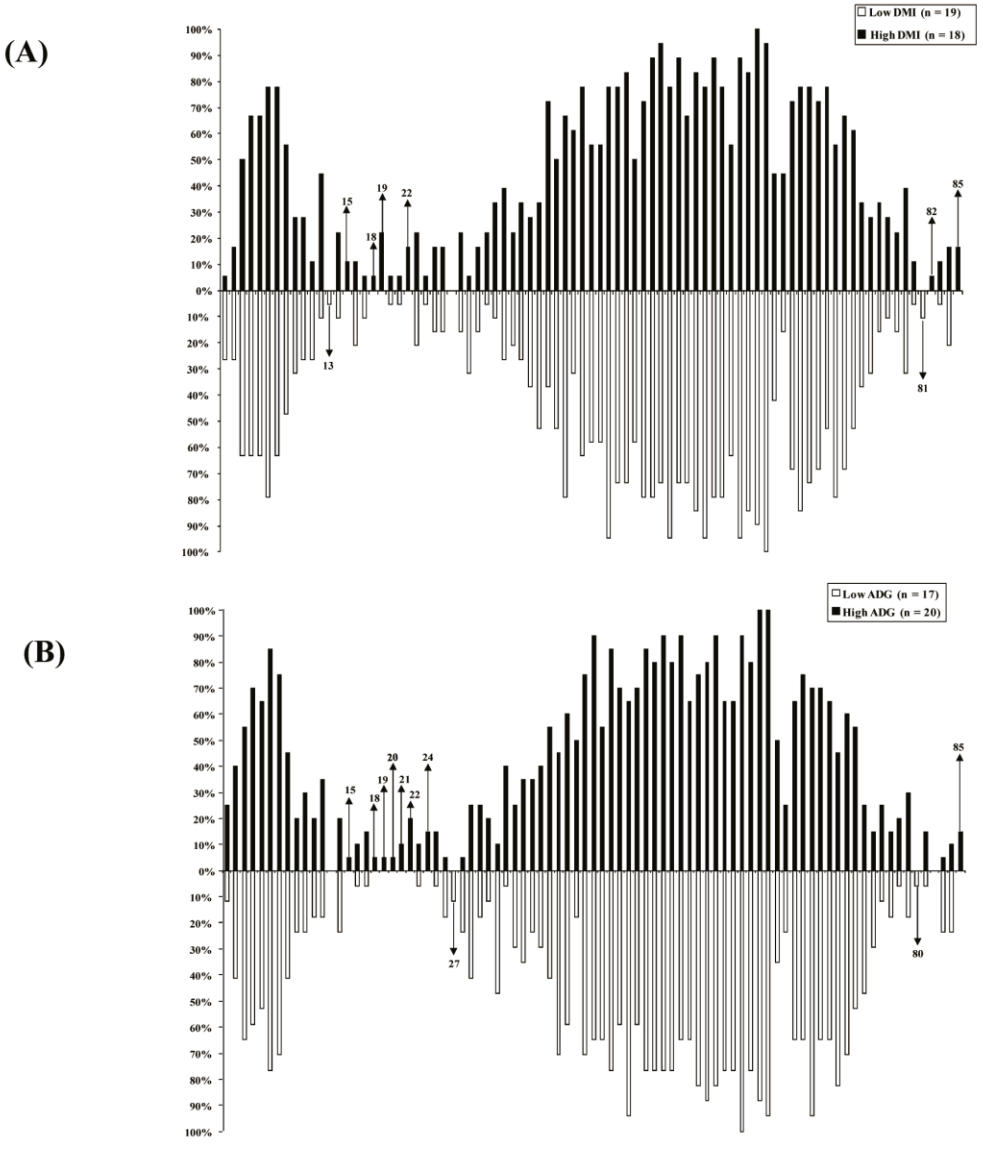


Figure 2.3. Frequency of PCR-DGGE bands in animals categorized on the basis of (C) Feed Conversion Ratio (FCR, F: G), and (D) Residual Feed Intake (RFI) using PROC CATMOD analysis. The x-axis represents 85 identified bands and the order of the bands reflects the migration locations on the PCR-DGGE gel from top to the bottom. The arrows indicate the frequency of bands detected in the tested the population of each trait.

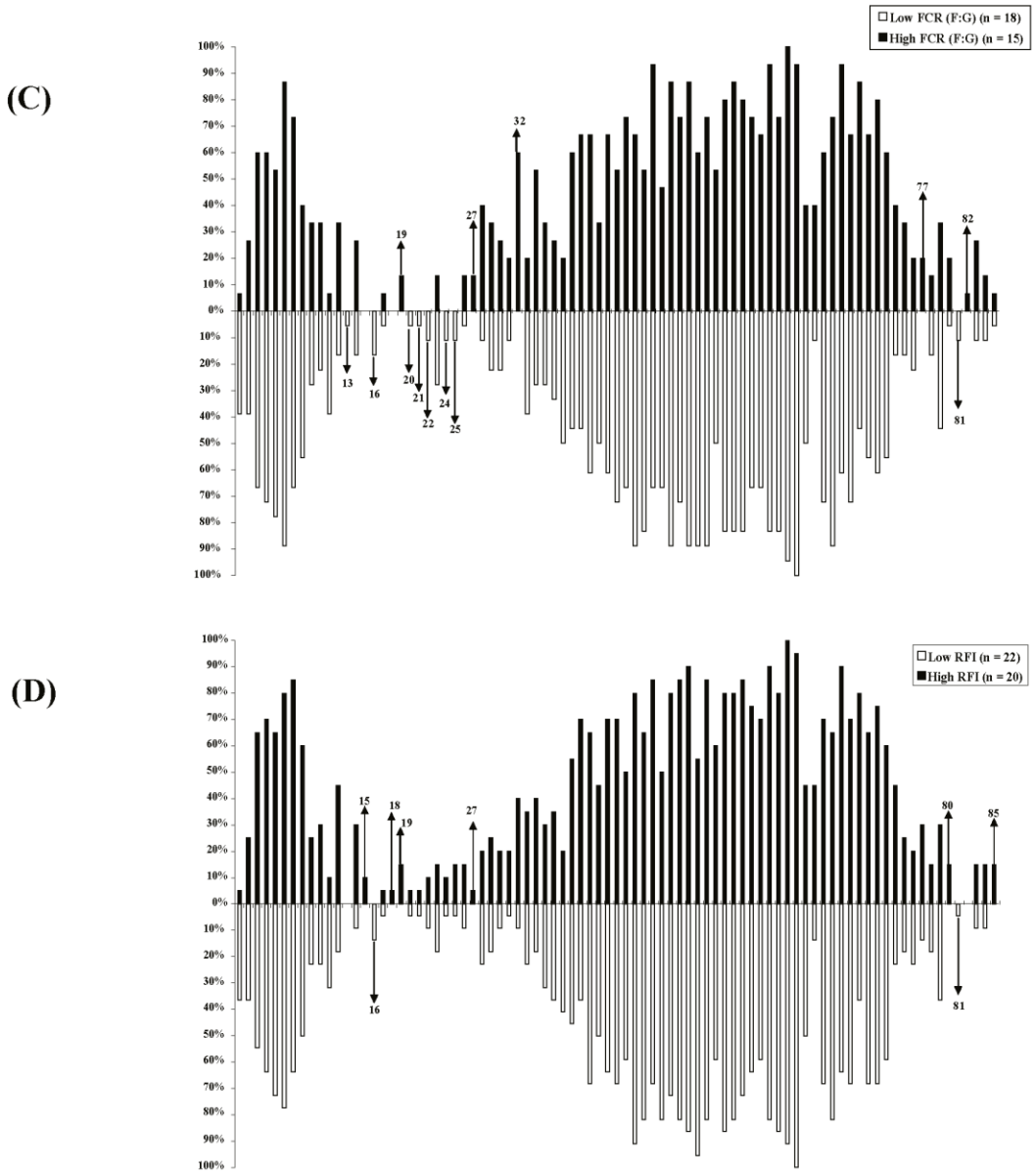


Figure 2.3. Frequency of PCR-DGGE bands in animals categorized on the basis of (E) Butyrate, and (F) Straight to Branched-chain VFA using PROC CATMOD analysis. The x-axis represents 85 identified bands and the order of the bands reflects the migration locations on the PCR-DGGE gel from top to the bottom. The arrows indicate the frequency of bands detected in the tested the population of each trait.

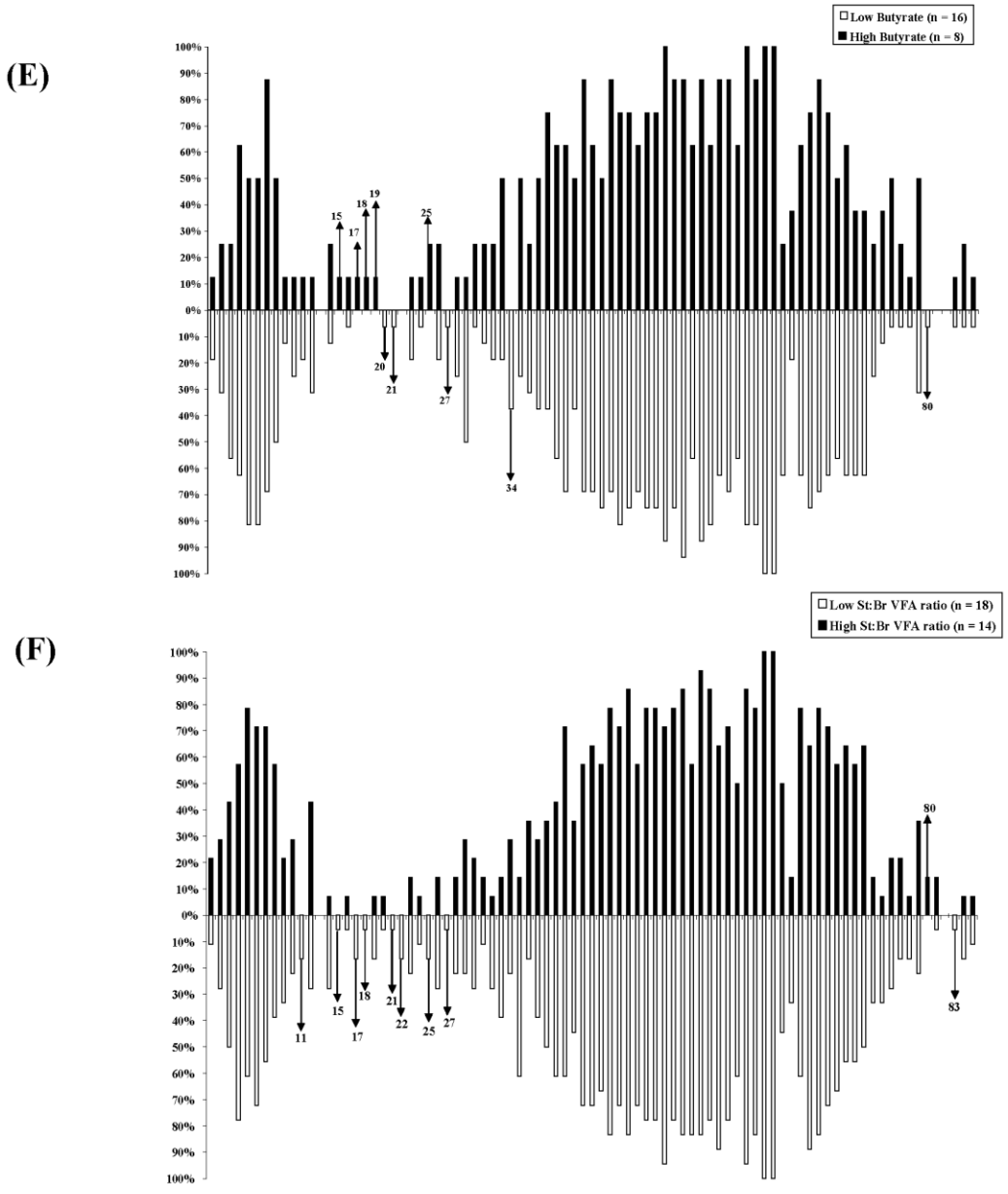
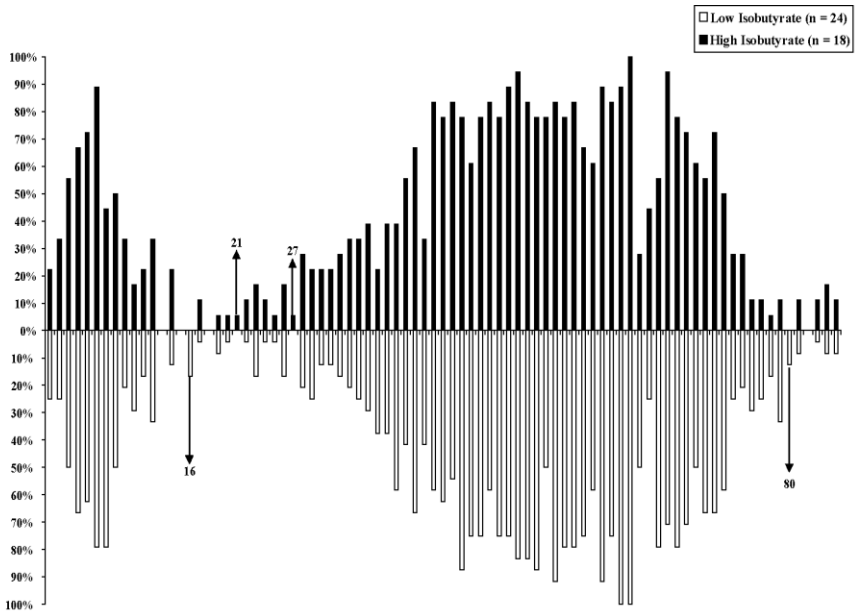


Figure 2.3. Frequency of PCR-DGGE bands in animals categorized on the basis of (G) Isobutyrate, and (H) Isovalerate using PROC CATMOD analysis. The x-axis represents 85 identified bands and the order of the bands reflects the migration locations on the PCR-DGGE gel from top to the bottom. The arrows indicate the frequency of bands detected in the tested the population of each trait.

(G)



(H)

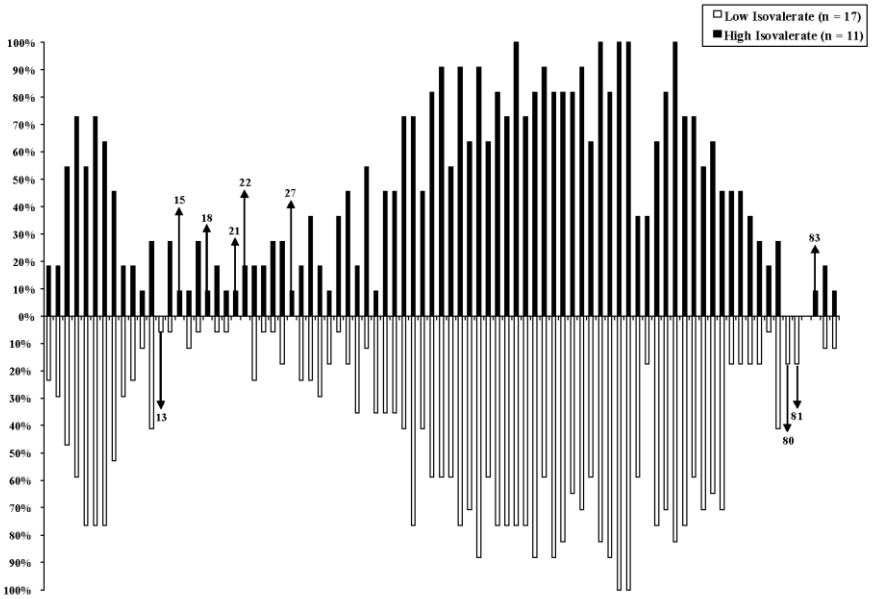


Figure 2.4. Frequency of PCR-DGGE bands in animals categorized on the basis of (A) Acetate, and (B) Propionate using PROC CATMOD analysis. The x axis represents 85 identified bands and the order of the bands reflects the migration locations on the PCR-DGGE gel from top to the bottom. The arrows indicate the frequency of bands detected in the tested the population of each trait.

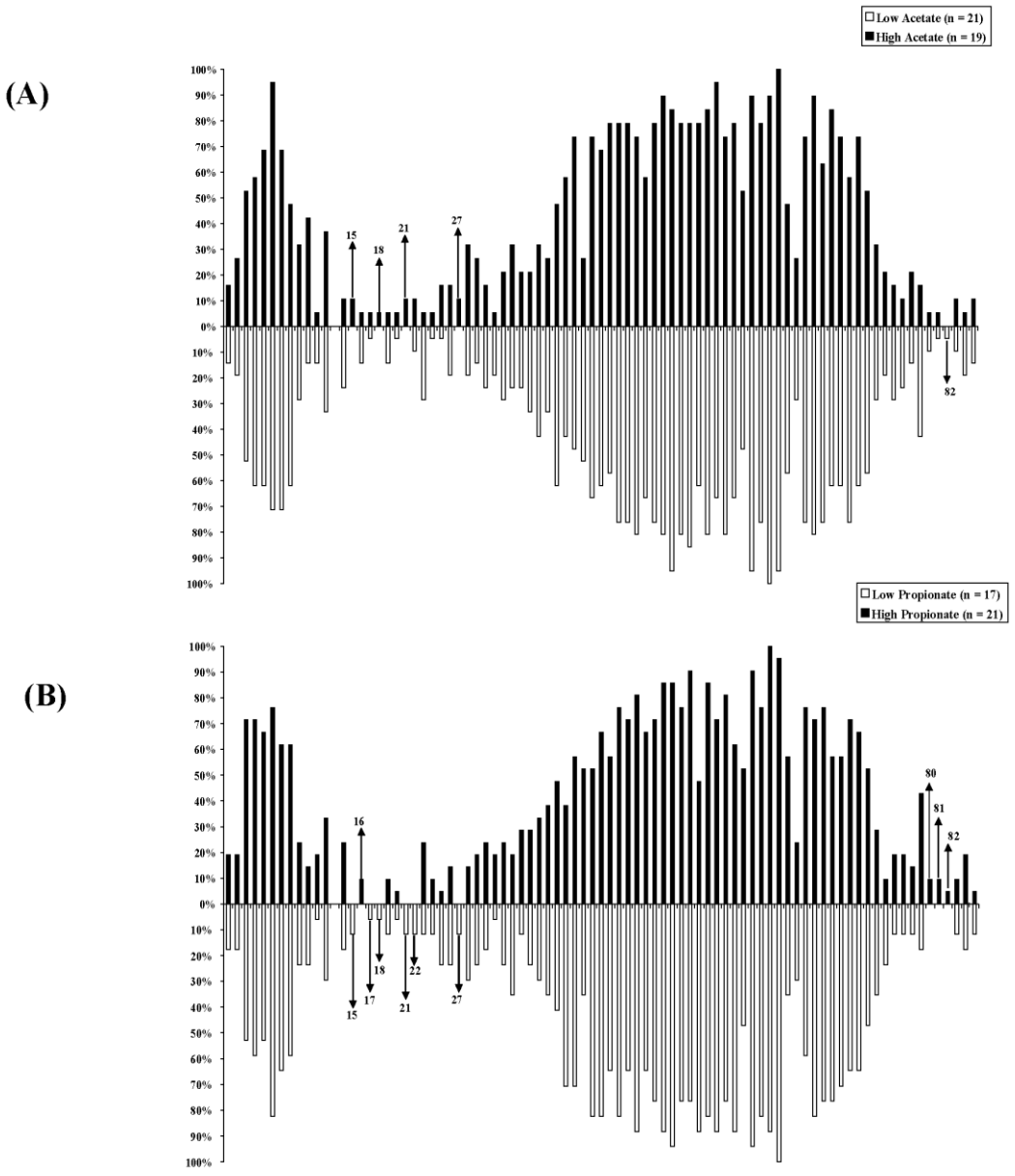


Figure 2.4. Frequency of PCR-DGGE bands in animals categorized on the basis of (C) Valerate, and (D) Total VFA concentration using PROC CATMOD analysis. The x axis represents 85 identified bands and the order of the bands reflects the migration locations on the PCR-DGGE gel from top to the bottom. The arrows indicate the frequency of bands detected in the tested the population of each trait.

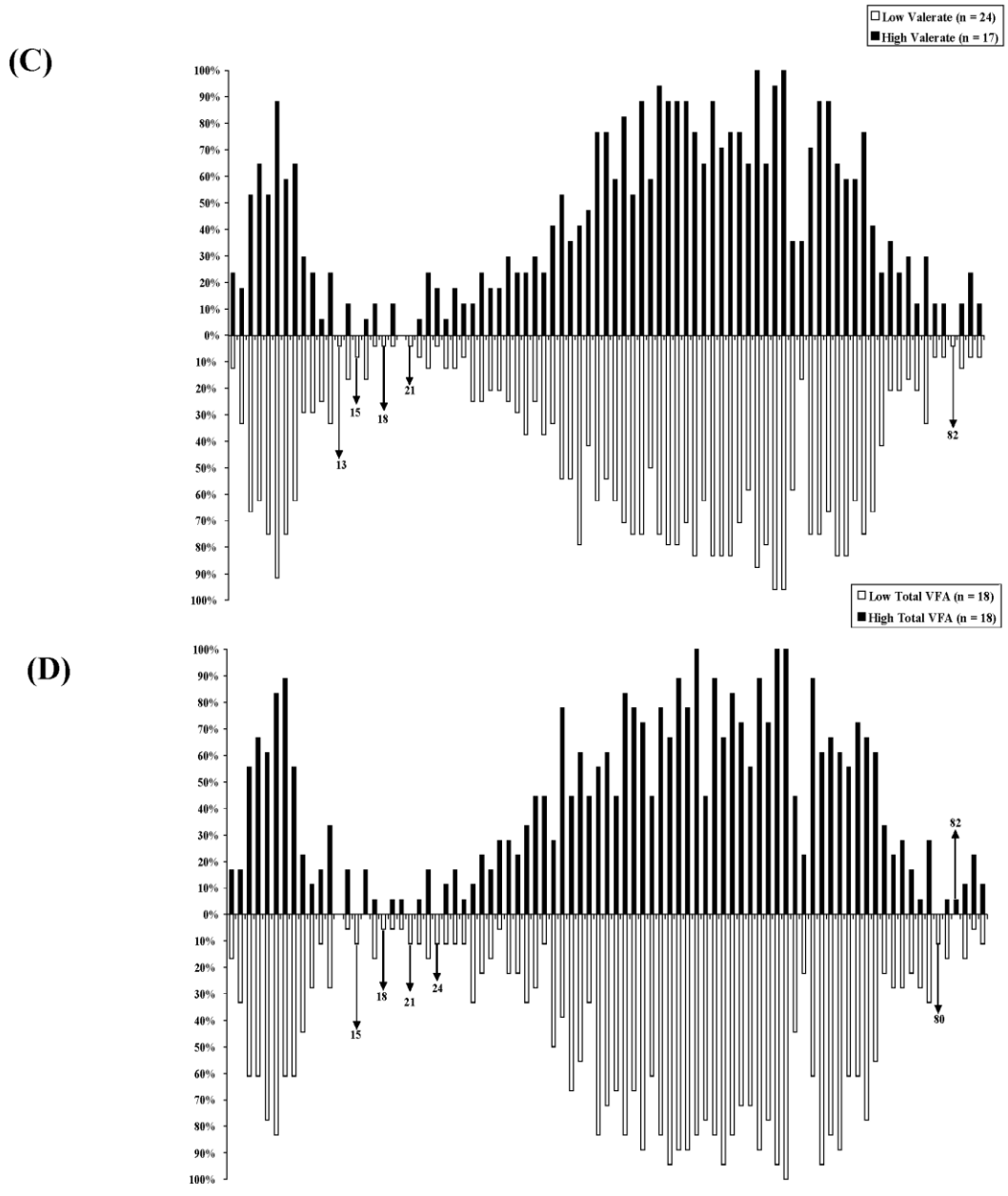
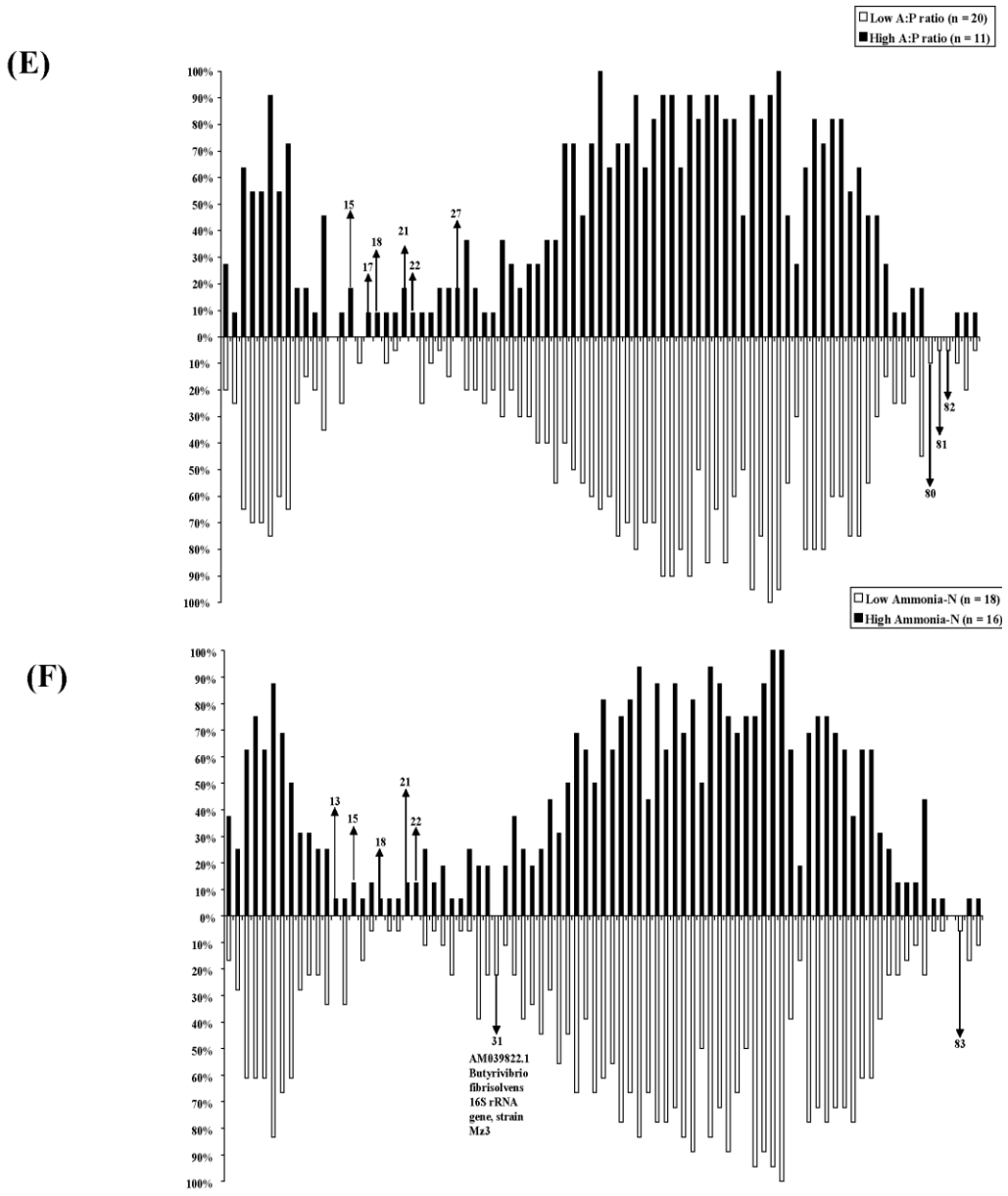


Figure 2.4. Frequency of PCR-DGGE bands in animals categorized on the basis of (E) Acetate to Propionate ratio, and (F) NH_3-N using PROC CATMOD analysis. The x axis represents 85 identified bands and the order of the bands reflects the migration locations on the PCR-DGGE gel from top to the bottom. The arrows indicate the frequency of bands detected in the tested the population of each trait.



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Chapter 3. Impact of Feed Efficiency and Diet on the Adaptive Variations in the Bacterial Community in the Rumen Fluid of Cattle Identified by PCR-DGGE and Quantitative Real Time PCR Analysis. [2]

3.1. INTRODUCTION.

The complex symbiotic microbiota in the rumen are responsible for the breakdown of feed components, enabling ruminants to derive approximately 70% of their metabolic energy from the microbial fermentation of feedstuffs (Bergman, 1990). Previous research has demonstrated that many factors influence the composition of rumen microbiota, affecting the population of certain bacterial groups (Fonty et al., 1987; Crater et al., 2007; Fonty et al., 2007; Romero-Pérez et al., 2011). Microbial diversity and activities have also been influenced by modifications in the diet (Bevans et al., 2005; Sun et al., 2009). Molecular techniques such as denaturing gradient gel electrophoresis (DGGE) have demonstrated that changes in diet can impact microbial composition in the rumen (Kocherginskaya et al., 2001; Regensbogenova et al., 2004; Duval et al., 2007). However, some studies have reported significant bacterial diversity among individuals (Brulc et al., 2009; Hernandez-Sanabria et al., 2010). Because individuals may respond differently to diet changes, identifying relationships between the differences in bacterial diversity in the rumen and host's phenotypic variations is particularly challenging. Moreover, the effect of bacterial population

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dynamics on host productive characteristics, such as feed efficiency, has not been well established.

The adaptability and structural complexity of the microbial community within the ruminal ecosystem allow ruminant animals to consume a wide variety of feedstuffs (Leedle et al., 1982). The type and amount of feedstuffs consumed by the host affect the nutritional supply to ruminal microbes and the end–products synthesized, thereby influencing the nutrients absorbed by the host. Animals with poor feed efficiency have an increased environmental impact and cost of production (Nkrumah et al., 2006; Hegarty et al., 2007). In beef feedlots, feed accounts for up to 80% of costs (Arthur et al., 2004), and up to 75% of total dietary energy consumed is used for non-productive purposes (Kelly et al., 2010). Improving feed efficiency without increasing maintenance energy expenditure may reduce the excretion of nutrients into the environment and has become economically relevant. Residual Feed Intake (RFI) has been used as a measurement of feed efficiency and has been defined as the difference between an animal's actual feed intake and its predicted intake, although the major biological mechanisms controlling RFI have yet to be fully elucidated (Moore et al., 2008; Herd et al., 2009). Recent studies have suggested that bacterial structure in the rumen may be associated with cattle feed efficiency (Guan et al., 2008), and PCR–DGGE band patterns have been linked to this phenotypic trait (Hernández-Sanabria et al., 2010; Zhou et al., 2010). Although probable associations among ruminal ecology or activities and cattle feed efficiency have been identified, there is little information on how specific bacterial groups impact whole microbial

profiles and functions and whether diet affects the relationship between microbial populations and host RFI.

In this study, I hypothesized that specific bacterial groups are associated with cattle feed efficiency and that these relationships can be impacted by host diet. Culture-independent methods (PCR-DGGE and quantitative Real Time-PCR) were used to characterize particular bacterial groups or phylotypes associated with host feed efficiency under divergent diet and RFI conditions. Multivariate statistical analysis was used to link the diversity of bacterial communities with the dynamics of bacterial groups, and host productivity under two different diets (low-energy density and high-energy density). Three phylotypes identified as RFI-associated bacteria were selected for validation in the steers that remained under the same RFI category on both diets. The evaluated productive measurements were dry matter intake (DMI), average daily gain (ADG), feed conversion ratio (FCR) (Feed:Gain), and residual feed intake (RFI).

3.2. MATERIALS AND METHODS.

3.2.1. Animals and sampling.

The animal protocol was approved by the Animal Care and Use Committee for Livestock at the University of Alberta. All animals were raised at Kinsella Research Station (University of Alberta) following the guidelines of the Canadian Council of Animal Care (CCAC, 1993).

One hundred and eighty Hereford × Aberdeen Angus steers (10 months old) were fed a total mixed ration, low-energy density (LE) feedlot diet composed

of 74% oats, 20% hay, and 6% feedlot supplement [32% CP beef supplement containing Rumensin (400 mg/kg) and 1.5% canola oil (ME 2.6 Mcal/kg)] for 90 days. After one week of adaptation, animals were switched to a high-energy (HE) density feedlot diet composed of 28.3% oats, 56.7% barley, 10% alfalfa pellets, and 5% feedlot supplement [32% CP beef supplement containing Rumensin (400 mg/kg), and 1.5% canola oil (ME 2.9 Mcal/kg)] for another 90 days. Feeding intake data were collected using the GrowSafe automated feeding system (GrowSafe Systems Ltd., Airdrie, AB, Canada). Feed efficiency traits (DMI, ADG, FCR and RFI) were obtained from all steers through both experimental periods following procedures outlined by Basarab et al. (2003). RFI was determined based on DMI and metabolic weight as per Nkrumah et al. (2006). Sixty steers were selected for their extreme RFI values (30 highest and 30 lowest) on LE diet (n=60). In both trials steers were ranked and assigned to the following groups: high RFI (H-RFI, mean plus 0.5 SD) or "inefficient", medium RFI (M-RFI, between the mean minus 0.5 SD and mean plus 0.5 SD), and low RFI (L-RFI, below mean minus 0.5 SD) or "efficient".

Rumen fluid samples (~150–200 ml) were collected via orogastric tubing from all steers in both LE and HE trials during the last week of the trial (day 83–90) before feeding using the method described by Hernandez-Sanabria et al. (2010).

3.2.2. DNA Extraction and PCR DGGE analysis.

Total DNA was extracted from rumen samples using physical disruption with the beads beating method (Guan et al., 2008). The concentration and quality of DNA were measured and 50 ng of total DNA was used as a template for PCR amplification of the V2-V3 region (~200 bp) of the 16S rRNA gene of bacteria using universal bacterial primers HDA1-GC/HDA-2, as outlined by Walter et al. (2000). Protocols for the purification of PCR products and the PCR-DGGE analysis followed previously reported studies (Hernández-Sanabria et al., 2010).

Similarities between the PCR-DGGE band patterns of the rumen fluid samples were analysed using the Dice similarity coefficient (D_{sc}). Similarity was determined by comparing and clustering the whole profiles using BioNumerics software v5.1 (Applied Maths, Austin, TX, USA). Herein hierarchical cluster comparisons were performed to group similar profiles and to generate a binary matrix of band classes. Dendrograms were generated using the unweighted pair–group method (UPGMA) at 1% position tolerance. The average of the D_{sc} (%) values was calculated to compare the profiles between the H–RFI and L–RFI group. Because RFI has been reported to be the most desirable measure of the feed efficiency (Arthur et al., 2004), we selected RFI to be correlated with the bacterial DGGE profiles.

3.2.3. Analysis of fermentation profiles: VFA and NH3-N.

Rumen fluid was subjected to VFA profiling using gas chromatography analysis following standard procedures. An enzymatic assay was performed to

measure NH_3–N (R-Biopharm Kit, Roche Inc., South Marshall, MI, USA), as in Hernandez-Sanabria et al. (2010).

To account for the possible dilution of rumen fluid by saliva and the time elapsed since the last meal (2010), the proportion of each VFA (acetate, propionate, butyrate, isobutyrate, valerate and isovalerate) relative to the total VFA concentration was obtained and used as the dependent variable for the microbial metabolite analysis. Differences in VFA composition and NH_3–N within the RFI group between the two diets and within each diet in the two RFI groups were compared using the simple covariance mixed model in SAS (SAS System, version 9.2; SAS Institute, Cary, NC). Statistical correlations were performed to identify interactions between the metabolites. Significance was assumed at $P < 0.05$.

3.2.4. Quantitative real-time PCR.

To verify the relationship between specific bacterial populations with RFI and diet, total rumen fluid DNA only from steers with the same RFI ranking under both diets (L–RFI, n = 13; H–RFI, n = 6) was subjected to qRT–PCR analysis to estimate the copy number of the 16S rRNA gene in each of the following bacterial phylotypes: *Robinsoniella peoriensis*, *Eubacterium rectale* and *Succinivibrio dextrinosolvens*.

For the quantification of total bacterial 16S rRNA gene copy number, a standard curve was constructed using serial dilutions of plasmid DNA from a clone identified as *Butyrivibrio hungateii* using a method previously described by

Li et al. (2009). Briefly, universal bacterial primers 27F and 1492R (48) were used to amplify the full length 16S rRNA gene from the plasmid DNA of a *Butyrivibrio hungateii* clone. The resultant PCR product was purified using the QIAquick PCR Purification Kit (Qiagen, Carlsbad, CA, USA). The mass concentration of the PCR product was measured using a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and converted to the molecule concentration using the following equation: DNA (number of molecules) = $(NL \times A \times 10^{-9})/ (660 \times n)$, where NL is the Avogadro constant (6.02×10^{23} molecules per mol), A is the molecular weight of the molecule in the standard, and n is the length of the amplicon (bp). The copy numbers of total bacteria in 50 ng of DNA were determined by relating the CT values to the standard curves based on the following equation: $Y = - 3.193 \times \log X + 35.003$ (Y, CT value; X, copy number of 16S rRNA gene) ($r^2 = 0.996$).

For the qRT-PCR analysis of the three bacterial phylotypes, primer Express v2.0 (Applied Biosystems, Foster City, CA, USA) was used to design primers (Table 3.1.) targeting the sequence of the DGGE bands corresponding to bacterial groups related to the genus *Robinsoniella*, potentially associated with RFI (Tables 3.5A, 3.55B and 3.10). To check for specificity, designed primers were compared to the available sequences in the BLAST database. PCR was performed to amplify the plasmid containing the PCR-DGGE sequence insert using the following conditions: initial denaturation for 10 min at 95 °C; 30 cycles at 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 60 s; and final elongation for 7 min at 72 °C. qPCR conditions included 95 °C for 10 min, 40 cycles of 95 °C for

30 s, 58 °C for 1 min and 95 °C for 15 s and a final step of 60 °C for 1 min. The *Eubacterium rectale* (Balamurugan et al., 2008) and *Succinivibrio dextrinosolvens* (Stevenson et al., 2007; Milinovich et al., 2008) PCR assays were performed as previously described. The specificity of all primers was further verified by the amplification of amplicons of the correct size from the target products in all rumen fluid samples. Three PCR products from different samples corresponding to each of the three targeted bacterial phylotypes were selected, purified and sequenced. When the primers amplified sequences with identities higher than 95% to the sequences of reported species in the GenBank database and to the PCR-DGGE bands, they were considered for further qRT-PCR analysis of the the bacterial groups above mentioned.

The standard curve for each bacterial phylotype was constructed using the plasmid DNA containing the inserts of the identified PCR-DGGE band from each of the three phylotypes. Because the sequences obtained from this study only had 95% identity with *Eubacterium rectale* and *Succinivibrio dextrinosolvens*, *Eubacterium* sp. and *Succinivibrio* sp. were used to represent the corresponding phylotypes. The copy numbers of each standard curve from the three targeted bacterial groups were calculated based on the equation used for the total bacterial population. The proportion of each phylotype was obtained after dividing the total copy number of 16S rRNA genes by the copy numbers of the 16S rRNA gene of each targeted phylotype. The efficiencies of RT-PCR were calculated from the given slopes in StepOneplus software, using the following equation: $E = [10^{(-1/slope)} - 1] \times 100\%$. qRT-PCR was performed using a StepOnePlus real-time PCR

system and SYBR GREEN (Applied Biosystems, Foster City, CA, USA). Data generated from reactions with efficiencies between 90 and 110% were used for further analysis (Zhou et al., 2009).

Analysis of variance using a mixed model in SAS was used to identify significant differences in total bacterial 16S rRNA gene copy number and in the proportion of each of the three specific bacterial phylotypes (*Succinivibrio* sp., *Eubacterium* sp., and *Robinsoniella* sp.) between RFI categories (High, n = 6, or Low, n = 13) within each diet (LE/HE, n = 19) as well as between diets within a particular RFI group. Correlations were determined among proportions of bacterial phylotype, total copy number, ruminal metabolites and feed efficiency traits (RFI, ADG, DMI and FCR), using the CORR procedure in SAS. Correlations were also performed separately within diet and within RFI groups. Significance was assumed when $P < 0.05$.

3.2.5. Statistical Analysis.

Data collected from only 55 steers were used for all analyses, because five animals were removed: two samples had saliva contamination, two had missing feed efficiency data for the HE diet and one lacked sharpness in the PCR-DGGE image.

To identify the association between the PCR-DGGE profiles obtained from both HE and LE trials and RFI, bands were identified based on the positions of each band in the PCR-DGGE gels using BioNumerics Software and fitted to previously reported categories (Hernandez-Sanabria et al., 2010). PROC

CATMOD (SAS System, version 9.2; SAS Institute, Cary, NC) was used to analyse the interaction between the recorded feed efficiency traits and the band frequency (presence or absence) on both HE and LE diets. The CATMOD procedure performs categorical data modelling that can be represented by contingency tables and calculates Chi–square values for linear models of response frequencies (presence/absence of a band). To assess the relationships between PCR-DGGE bands, feed efficiency and rumen metabolites, animals were classified under each feed efficiency/rumen fermentation variable using the criteria described above for High (H) and Low (L) groups. The presence of particular bands representing specific bacteria for each H or L category of the described variables was analysed only in steers that maintained H– or L–RFI rank in both diets and were classified within the High/Low group of each metabolite.

To detect the RFI–associated bands within diets, the frequency of all detected bands on each RFI group was compared for the HE and LE diets using Chi-square analysis. Similarly, the frequency of all bands between each diet was compared for the H– and L–RFI groups, using the same statistical model. Two-way contingency tables of cross classifications containing the frequencies of the bands per category (High/Low) and in both diets (LE/HE) were obtained using PROC FREQ in SAS. Diet–associated bands within the RFI categories and RFI–associated bands within the diets were revealed in two-way contingency tables created for each RFI group and for each diet, respectively. When the count of any of the cells was below five, the Fisher Exact test was used to calculate the

probability of the table. For each of the variables, the frequency of all bands was plotted for H and L groups within the diets.

3.3. RESULTS .

3.3.1. PCR-DGGE profiling of rumen bacterial dynamics between diets.

Different RFI rankings were observed for the 55 steers under LE and HE diets. In the LE diet trial, 20 steers were allocated to H-RFI, 14 to M-RFI, and 22 to L-RFI groups. In the HE diet trial, 14 steers were allocated to H-RFI, 24 to M-RFI, and 18 to L-RFI groups. Table 3.2. summarized the changes in RFI ranking in the HE diet for the steers that were ranked H- or L- RFI on the LE diet (n = 41). Within the latter group, 19 animals (L-RFI, n = 13; H-RFI, n = 6) retained the same RFI classification during both diets and were used for the study of the association between feed efficiency variables and bacterial populations.

Evaluation of the predominant bacterial diversity in the rumen of steers with different feed efficiency and diets was first compared with the UPGMA dendograms generated. The overall comparison of bacterial profiles showed a similarity of 74.5% among all individuals in both diets, and the steers tended to group according to RFI within the HE diet cluster (Figure 3.1.). A separate PCR-DGGE dendogram for the bacterial community on the HE diet alone confirmed the aforementioned grouping trend: average similarity among HE profiles was 69.8% (Figure 3.2.). Clustering tendency in LE was reported in the previous chapter (Figure 2.1.).

Steers ranked as H-RFI for both diets were compared, and the similarity among them was 66.3%, whereas similarities among the L-RFI and M-RFI animals were 70.5% and 59.1%, respectively (Figure 3.3.). Only two individuals switched from L- to H-RFI (D_{sc} = 46.6%) and one from H- to L-RFI (D_{sc} = 81.7%, Figure 3.4.). Due to the small sample size, further analyses were not performed. Because our objective was to identify the microbes associated with the H- or L-RFI animals, M-RFI steers were not selected for further analysis, as some inconsistencies in their clustering trends were observed (e.g., nine M-RFI steers grouped with L- or H-RFI steers) and the M-RFI category included steers that changed their RFI dramatically when fed HE diet (from L-RFI to M-RFI or from H-RFI to M-RFI).

3.3.2. Characterization of fermentation metabolites in response to diet and RFI classification.

Concentrations of VFAs and ammonia-nitrogen (NH_3-N) were assessed in rumen fluid samples to provide information on the associations between bacterial diversity and its functions in the rumen of animals fed LE and HE diets. Total VFA concentration was higher ($P < 0.0001$) in H-RFI steers on HE diet, while isobutyrate was lower ($P < 0.05$) in this group (Table 3.4.). Propionate, butyrate and isovalerate remained unchanged in the L-RFI animals for both diets. DMI and ADG were significantly different between the diets in both L- and H-RFI animals; in L-RFI individuals, RFI and FCR tended to improve when the HE diet was fed ($P < 0.0001$). In addition, significant differences were observed in NH_3-N among L-RFI animals ($P < 0.05$, Table 3.4). Although valerate and total VFA were

significantly different in both RFI groups, only acetate was found to differ in L-RFI steers ($P < 0.05$). This difference did not have an impact on the acetate to propionate (A: P) ratio or the straight to branched (St: Br) VFA ratio of L-RFI steers; in H-RFI animals, however, St: Br VFA ratio was significantly different. Furthermore, the correlations among fermentation metabolites and feed efficiency traits in the steers that remained either L-RFI or H-RFI in both diets were evaluated to provide information on the variations in the fermentation characteristics related to RFI. Results confirmed that for L-RFI steers, propionate, valerate and total VFA were negatively related to RFI ($P < 0.05$, Table 3.7A). In H-RFI individuals, RFI was positively correlated with NH_3-N ($P < 0.0001$), which in turn was linked with higher DMI ($P < 0.05$, Table 3.7B).

3.3.3. Interactions between PCR-DGGE profiles, fermentation characteristics and host feed efficiency under different diets.

Using a Chi-square based procedure (PROC CATMOD in SAS), the effect of fermentation and feed efficiency measurements on the presence/absence of all bands was determined in the steers that remained in the same RFI category. As revealed by the analysis of the frequencies within the steers that remained L-RFI in both diets, some bands tended to be more frequent in one diet than in the other. For instance, band 63 (*Eubacterium rectale*-like, 98% identity) was the most frequent band in LE diet, whereas band 83 (*Selenomonas ruminantium*) was not identified in any of the L-RFI steers (Table 3.3A). Band 1 (*Prevotella* sp.) was more frequently present ($P < 0.05$) in L-RFI steers than in H-RFI steers for both the LE and HE diets.

In total, 28 bands were related to RFI, but only band 1 (*Prevotella* sp.), band 5 (Uncultured *Succinivibrio* sp.), band 31 (*Butyrivibrio fibrisolvens*), band 32 (*Prevotella ruminicola*), band 54 (*Succinivibrio dextrinosolvens*), band 58 (*Moryella indoligenes*), band 68 (*Succinivibrio dextrinosolvens*), band 74 (Uncultured *Succinivibrio* sp.) and band 84 (*Bifidobacterium ruminantium*) were significantly different between RFI categories in LE diet ($P < 0.05$). For the HE diet, band 1 (*Prevotella* sp.), band 3 (*Prevotella maculosa*), band 6 (*Prevotella* sp.), band 35 (Uncultured *Prevotella* sp.), band 49 (Uncultured *Prevotella* sp.), band 50 (*Prevotella ruminicola*), band 67 (*Succinivibrio dextrinosolvens*), band 72 (*Clostridium indolis*), band 77 (*Succinivibrio dextrinosolvens*), band 79 (*Succinomonas amylolytica*) and band 83 (*Selenomonas ruminantium*) were significantly different between RFI groups ($P < 0.05$). Thus, for LE diet, bands 1, 5, 54 and 58 can be considered L-RFI-associated, and bands 31, 32, 68, 74 and 84 were H-RFI-associated. In animals fed HE diet, bands 1, 3, 50 and 77 were L-RFI associated, and bands 6, 35, 49, 67, 72, 79 and 83 were H-RFI associated (Table 3.3A, Figure 3.5).

When analyzing bands affected by the diet switch within L-RFI steers, bands 6 (*Prevotella* sp.), 35 (Uncultured *Prevotella* sp.), 49 (Uncultured *Prevotella* sp.), 65 (*Robinsoniella peoriensis*), 67 (*Succinivibrio dextrinosolvens*), and 77 (*Succinivibrio dextrinosolvens*) were significantly different between diets ($P < 0.05$). Therefore, in L-RFI steers, bands 6, 35, 49 and 67 were LE diet-associated, whereas bands 65 and 77 were HE diet-associated within. In H-RFI animals, bands 2 (*Prevotella* sp.), 54 (*Succinivibrio dextrinosolvens*), 58

(*Moryella indoligenes*), 79 (*Succinomonas amylolytica*) and 83 (*Selenomonas ruminantium*) were significantly different between diets ($P < 0.05$). All of these bands were HE diet-associated (Table 3.3B). There were no bands exclusively related to the HE diet or to the H-RFI steers on either diet (Figure 3.5).

For the animals that remained L-RFI on both diets, some trends in feed efficiency measurements were observed. For example, L-RFI steers were also allocated to the L-DMI, H-ADG and L-FCR groups (Table 3.6). In contrast, H-RFI individuals had H-DMI, L-ADG and L-FCR. Therefore, frequency plots of the bands present in such groups were created to determine the particular phylotypes related to positive feed efficiency traits. Twenty bands were associated with L-DMI in LE diet, whereas 13 were related in the HE diet. Only 5 bands were associated with H-DMI in the LE diet while 3 were associated with H-DMI in the HE diet (Figures 3.6A and 3.6B). Low RFI steers showed H-ADG, and 10 bands were associated with this trait in the LE diet group, while 6 bands were present in HE diet. For H-RFI individuals, three bands were associated with L-ADG in the LE diet, whereas 7 bands were present in HE diet. Sixteen bands were linked to L-FCR in LE diet, and 11 were correlated in HE diet. High FCR was observed on H-RFI steers: 3 bands were related in the LE diet but 6 in the HE diet (Figures 3.6A and 3.6B). In the acetate proportions, L-RFI individuals were classified under the High category in the LE diet, while H-RFI animals were ranked in the Low category. Four bands were associated with L-acetate category, and 10 were prevalent in H-acetate (Figure 3.6A). In the LE diet, H-isobutyrate was recorded in the L-RFI steers, and 9 bands were correlated to this

characteristic; however, no clear trend could be reported in H-RFI steers. As seen in Table 3.4, High NH_3-N was observed in L-RFI steers on the LE diet, and Low NH_3-N was detected in H-RFI individuals on the same diet; six bands were shown to be related to High NH_3-N and 11 to the Low NH_3-N (Figure 3.6A and 3.6B).

3.3.4. Comparison of specific and total bacterial populations.

To validate the above identified associations revealed by the multivariate statistical analysis of PCR-DGGE profiles, three bacterial groups were selected for qRT-PCR analysis (*Succinivibrio* sp., *Eubacterium* sp., and *Robinsoniella* sp.). The results of the quantification of the total bacteria and of the specific phylotypes selected were compared for both diets and for differing RFI categories by measuring the copy numbers of total and targeted 16S rRNA genes. Only the proportion of *Succinivibrio* sp. adapted to the differences in diet ($P < 0.05$, Table 3.5A). In the LE diet, total bacteria tended to be increased in H-RFI animals but the difference was not significant, while *Robinsoniella* sp. only showed a trend ($P < 0.1$) for increased proportion in L-RFI steers. For the HE diet, the proportion of *Eubacterium* sp. was significantly different between L- and H-RFI steers ($P < 0.05$), and the total bacterial population did not change between RFI groups (Table 3.5B). Correlation analysis showed a tendency for a negative association between RFI and *Robinsoniella* sp. ($P < 0.1$) in the LE diet (Table 3.8). *Eubacterium* sp. was positively associated with RFI ($P < 0.05$) and tended to be positively correlated with DMI under HE diet (Table 3.9).

3.4. DISCUSSION.

Feed consumed by animals is partitioned to meet host energetic requirements for production and maintenance (Archer et al., 1999). A wider understanding of the contributing factors will provide information on the physiological processes responsible for variations in the metabolic phenotype. During rumen fermentation, short chain VFA and microbial proteins are formed and utilized as energy and protein sources, respectively, by the host animal (Russell et al., 1981). The efficiency of nutrient utilization is determined by the balance of such fermentation products, which can ultimately be controlled by the ruminal microbiota.

The energetic metabolism in beef cattle with different RFI has been reported to be significantly different (Nkrumah et al., 2006). In fact, L-RFI (efficient) cattle have lower DMI and less methane emissions (Nkrumah et al., 2006; Hegarty et al., 2007). As differences in digestion contribute to approximately 10% of the differences in RFI (Herd et al., 2009), ruminal microbial fermentation must play a key role in this trait. Guan et al. (2008) reported a potential association between butyrate and RFI in cattle under high energy diet. Butyrate is mainly used as an energy source for the host and increases when animals are fed high energy diets (Russell et al., 1981; France et al., 1993). In our study, L-RFI steers had a lower proportion of butyrate in LE, which increased when the diet changed, suggesting that different butyrate metabolic pathways might contribute to RFI in different diets (Table 3.4). In our study, valerate was negatively related to RFI and FCR in L-RFI steers ($P < 0.05$, Table 3.7A). This observation may have been the result of decreased fermentation of

amino acids in the rumen, which has been associated with inhibited methane formation (Zhou et al., 2009, 2010). Lower production of branched-chain fatty acids can be a consequence of the inhibition of bacterial NADH-H hydrogenases (Russell et al., 1984), indicating high concentratons of H in the ruminal medium due to potential inhibition of methanogenic Archaea or H utilisation by these microorganisms (Russell, 2002). These findings may also relate to our observation of a lower proportion of isovalerate and a higher St: Br VFA ratio in L-RFI steers, suggesting that efficient animals might not divert much energy to methanogenesis (Hegarty et al., 2007; Zhou et al., 2009) and that more efficient N retention may potentially occur in L-RFI steers, improving feed efficiency (Chaucheyras-Durand et al., 2008). This trend was further confirmed by the correlation between NH_3–N and RFI in H–RFI steers ($P < 0.05$, Table 3.7B), suggesting inefficient incorporation of NH_3–N into microbial protein in the rumen. Nevertheless, additional studies on wasteful degradation of N by particular bacteria are needed.

As in our previous study linking PCR-DGGE band patterns to host RFI under LE diet, similar associations were observed on HE diet. Due to the diet impact on rumen microbial diversity, some of these associations were altered. For instance, we observed that, although some steers remained in the same RFI rank, the bacterial phylotypes associated with RFI and other phenotypic traits varied. This observation suggests that microbiota adaptation to the diet in some animals may not necessarily lead to changes in overall productive performance. In contrast,

in other individuals, either a positive or negative impact of the diet on feed efficiency was evident.

Three specific phylotypes identified as RFI-associated bacteria were selected for validation in the steers that remained under the same RFI category on both diets (n = 19): *Succinivibrio dextrinosolvens* (band 67, H-RFI associated), *Robinsoniella peoriensis* (band 65, H-RFI associated), and *Eubacterium rectale* (band 63, L-RFI associated). *Succinivibrio dextrinosolvens* is the predominant isolate when ruminants are fed high starch diets (O'Herrin et al., 1993). The proportion of this phylotype was higher when L-RFI steers were fed LE diet; however, their numbers decreased when animals were switched to the HE diet and its frequency of presence increased in H-RFI steers ($P < 0.05$). Previous reports mention acetate and succinate (a precursor of propionate) as the major products of *Succinivibrio dextrinosolvens* (Russell et al., 1981); acetate proportions were higher in L-RFI steers in both diets (Table 3.4). This rumen inhabitant might play a key role in the propionate synthesis in efficient steers, which could be readily absorbed from the rumen for hepatic gluconeogenesis, thereby improving feed efficiency when animals are fed a LE diet. Another net product from this bacterial group is formate, which is metabolized to CO_2 + H_2 and methane by rumen methanogens (Lovley et al., 1984). This unfavourable effect might lead to a loss of dietary energy and subsequent decreased feed efficiency in H-RFI steers as a result of increased *Succinivibrio* sp. population under HE diet.

Robinsoniella peoriensis is a bacterium isolated from anaerobic environments (Cotta et al., 2009); our previous research reported this species in

the rumen for the first time (Hernandez-Sanabria et al., 2010), although its function is not yet known. Cotta et al. (2009) revealed that acetate and succinate are its major metabolic end-products and, to a lesser extent, formate and lactate. In LE diet, the proportion of *Robinsoniella* sp. tended to be decreased in H-RFI steers ($P = 0.06$, Table 3.5B) and negatively correlated with RFI ($P < 0.05$, Table 3.8). The mechanisms underlying this relationship have yet to be explained, but they might be related to the production of formate and the consequent effects on methane production.

Eubacterium rectale has been isolated from human (Flint et al., 2007; Balamurugan et al., 2008) and pig (Leser et al., 2002) gut, and has been identified here for the first time in bovine rumen. Sequences from the *Eubacterium* group might comprise up to 8% of the total bacterial diversity in the pig gut (Leser et al., 2002). In our study, this phylotype was present in all steers fed LE diet, and it was the predominant bacterial phylotype in L-RFI steers after the diet was changed, probably be due to the bacterium's tolerance to low pH (Duncan et al., 2009). *E. rectale* is also abundant in human intestine (Aminov et al., 2006), where it produces butyrate and utilizes acetate (Flint et al., 2007). For ruminants, acetate is directly converted to ketone bodies and constitutes the major energy source for oxidation (France et al., 1993). In L-RFI steers, the acetate proportion was positively correlated to RFI ($P < 0.05$, Table 3.7A). Increases in acetate (as found in this study, Table 3.4) might stimulate butyrate production, and this fermentative pathway might be favoured because of its role as a hydrogen sink (Pryde et al., 2002).The function of acetate in L-RFI steers might also be related

to potential hydrogen transfer mechanisms to reductive acetogenesis by homoacetogenic bacteria (Breznar et al., 1986; Bernalier et al., 1996). Therefore, in L-RFI individuals, the acetate utilization characteristic of *Eubacterium* sp. may interact with the acetate-producing capacity of *Succinivibrio* sp., probably towards the consumption of excessive hydrogen, which would be otherwise directed to methanogenesis (Wolin et al., 1997; Chassard et al., 2006). Janssen (2010) proposed that microorganism that are more efficient to produce biomass will cause shifts away from H_2-producing fermentation pathways, which result in lower CH_4 formation or even lower methogen activity. Turnbaugh et al. (2006) also found that increased concentrations of acetate could be associated with improved dietary energy extraction by the gut microbiota. Since high acetate proportions were found in the L-RFI animals on both diets of our study (Table 3.4), future studies regarding the role of homoacetogenic bacteria on feed efficiency as well as in the shifts of pathways that result in a smaller energy change and larger production of biomass will provide more evidence of these speculations.

Based on the above observations, it is possible that cross-feeding interactions among different bacterial groups with the functions represented by the above three phylotypes (Figure 3.7) may be different on LE or HE diet. As a result, their association with RFI varied when diet changed. It has also been proposed that *Succinivibrio dextrinosolvens* reduces CO_2 to acetate (O'Herrin et al., 1993). Because many butyrate producers are hydrogen-producers, these cross-feeding interactions may influence the energetic metabolism of the host and, as in

this case, improve feed efficiency (Figure 3.7). It must be noticed that the three groups of bacteria studied herein do not alone account for the total variation in RFI; rather, they are representative of the phylotypes that potentially contribute to such differences. Methanogen diversity from the same group of animals has been associated with RFI (Zhou et al., 2009), and the above interactions may interfere with the CO_2-H_2 methanogenic pathway, explaining the altered methanogenic diversity observed (Zhou et al., 2010). However, only a small number of the 85 phylotypes was validated and thus, future studies on the interactions among bacteria and other ruminal inhabitants are necessary to clarify the above speculations.

Due to limited sampling access to the herd used in our study, rumen fluid samples used for bacterial diversity analysis were collected at a single time point before feeding, whereas feed efficiency traits were recorded for a longer period. However, previous studies suggest that pre-feeding VFA concentrations are similar between diets (Peters et al., 1990; Soto-Navarro et al., 2000) and individuals (Welkie et al., 2010) and are the lowest before feeding (Bevans et al., 2005), representing a suitable baseline indicator (Hernandez-Sanabria et al., 2010). Moreover, longer periods of adaptation lead to more stable rumen environments and fermentation characteristics (Hristov et al., 2001; Sun et al., 2009; Welkie et al., 2010). Thus, our VFA data are valid to illustrate potential associations between specific bacterial phylotypes and fermentation profiles of individual steers and their corresponding feed efficiency traits. However, for a broader explanation of the relationships between fermentation characteristics and feed

efficiency, it is essential to record intake variables and collect samples at multiple time points during the period as well as to include a defined control group.

3.5. CONCLUSIONS.

We identified probable associations between specific bacterial phylotypes and feed efficiency traits under HE and LE diets in beef cattle. Three bacterial phylotypes (*Succinivibrio* sp., *Eubacterium* sp., and *Robinsoniella* sp.) have been identified to be potentially associated with RFI based on their sequences and their predicted metabolic mechanisms in feed efficiency (propionate synthesis, formate production and cross-feeding interaction with methanogens) (Figure 3.7) and may be indicative of wider changes in the microbial population. Future efforts to isolate these bacteria from rumen and to study their metabolic pathways by whole genome sequencing will be necessary for a full understanding of their roles in feed efficiency. Our study provides a framework to identify variations in the population of rumen bacterial groups that are influenced by diet and play a major role in the energetic metabolism in the host, thereby influencing feed efficiency. The demonstrated relationships between diet, feed efficiency and rumen microbes also contribute to the knowledge of functional microflora and their potential for manipulation and further improvement of animal performance.

Table 3.1. Primers used for detection of particular functional bacterial in Study 2.

Target bacterium	Primer sequences (5'→ 3')	Annealing temperature (°C)	Amplicon size (bp)	Reference
Universal bacteria (U2)	**F:** ACTCCTACGGGAGGCAG **R:** GACTACCAGGGTATCTAATCC	50	468	64
Succinivibrio dextrinosolvens 0554	**F:** CGTCAGCTCGTGTCGTGAGA **R:** CCCGCTGGCAACAAAGG	60	80	44, 64
Eubacterium rectale ATCC 33656	**F:** AAGGGAAGCAACGCTGTGAA **R:** CGGTTAGGTCACTGGCTTC	60	200	8, 9
Robinsoniella peoriensis HGUE-09/9434	**F:** AAACGGATTTCTTCGGAATGAA **R:** TCTGTCTGTTATCCCCCTGTATGA	58	98	This paper

*F and R indicate forward and reverse primers, respectively.

Table 3.2. Variations in Residual Feed Intake classification of steers under two different diets. For illustration purposes, only the steers classified as L- or H-RFI in LE diet were included.

Animal_ID	Diet	RFI_class	Diet	RFI_class
1	LE	H	HE	M
11	LE	H	HE	H
13	LE	L	HE	M
23	LE	L	HE	L
31	LE	L	HE	H
33	LE	L	HE	L
39	LE	H	HE	M
65	LE	L	HE	M
69	LE	L	HE	H
75	LE	L	HE	L
91	LE	L	HE	L
95	LE	H	HE	H
97	LE	H	HE	M
101	LE	L	HE	L
111	LE	L	HE	L
119	LE	L	HE	L
121	LE	L	HE	M
129	LE	L	HE	L
151	LE	H	HE	M
159	LE	H	HE	M
161	LE	H	HE	M
163	LE	H	HE	M
167	LE	H	HE	H
173	LE	H	HE	H
197	LE	L	HE	M
205	LE	L	HE	L
225	LE	H	HE	H
249	LE	L	HE	M
259	LE	L	HE	L
271	LE	L	HE	L
279	LE	H	HE	L
287	LE	H	HE	H
293	LE	L	HE	M
311	LE	H	HE	M
403	LE	H	HE	M
407	LE	H	HE	M
411	LE	H	HE	M
423	LE	L	HE	L
427	LE	H	HE	M
39	LE	H	HE	M
451	LE	L	HE	L

Table 3.3A. Taxonomical identification of RFI-associated PCR – DGGE bands within particular diets (n = 19).

PCR-DGGE Band Category	Low Energy Diet		*P* value	High Energy Diet		*P* value	Taxonomy (GenBank Accesion No.)
	L-RFI (n = 13)	H-RFI (n = 6)		L-RFI (n = 13)	H-RFI (n = 6)		
1	46.15%	0.00%	0.0517	76.92%	0.00%	0.0021	*Prevotella* sp. (AF218619)
3	61.54%	83.33%	0.2846	84.62%	33.33%	0.0431	*Prevotella maculosa* strain W1609 (EF534315)
5	76.92%	16.67%	0.0270	76.92%	66.67%	0.3689	Uncultured *Succinivibrio* sp. clone EMP_B23 (EU794184)
6	84.62%	83.33%	0.4830	46.15%	100.00%	0.0277	*Prevotella* sp. BP1-56 (AB501155)
10	30.77%	0.00%	0.1483	7.69%	50.00%	0.0671	*Clostridium symbiosum* strain 69 (EF025909)
12	7.69%	50.00%	0.0671	23.08%	16.67%	0.4427	*Prevotella denticola clone WWP_SS6_P23 (GU409439)*
31	0.00%	33.33%	0.0347	0.00%	0.00%	0.8603	*Butyrivibrio fibrisolvens*, strain Mz3 (AM039822)
32	0.00%	33.33%	0.0347	7.69%	0.00%	0.5965	*Prevotella ruminicola (AB219152)*
35	38.46%	33.33%	0.3831	0.00%	33.33%	0.0347	Uncultured *Prevotella* sp. clone JD9 (FJ268952)
41	53.85%	66.67%	0.3406	61.54%	16.67%	0.0836	*Ruminococcus* sp. ZS2-15 (FJ889653)
42	61.54%	50.00%	0.3406	61.54%	98.36%	0.0899	*Prevotella* sp. 152R-1a (DQ278861)
46	84.62%	50.00%	0.1342	61.54%	100.00%	0.0899	Uncultured *Prevotella* sp. clone JD9 (FJ268952)
48	92.31%	50.00%	0.0671	84.62%	83.33%	0.4830	*Lactobacillus* sp. DI71 (AB290831)
49	84.62%	83.33%	0.4830	46.15%	100.00%	0.0277	Uncultured *Prevotella* sp. clone Gull85-50 (FJ220908)

50	76.92%	83.33%	0.4427	100.00%	50.00%	0.0068	*Prevotella ruminicola* strain TC2-3 (AF218617)
52	92.31%	50.00%	0.0671	69.23%	100.00%	0.1483	*Succiniclasticum ruminis* strain DSM 9236 (NR_026205)
54	69.23%	16.67%	0.0464	92.31%	100.00%	0.5965	*Succinivibrio dextrinosolvens* strain 0554 (NR_026476)
58	69.23%	16.67%	0.0464	84.62%	100.00%	0.3706	*Moryella indoligenes* strain AIP 220.04 (DQ377947)
65	7.69%	50.00%	0.0671	46.15%	83.33%	0.1362	*Robinsoniella peoriensis* strain HGUE-09/9434 (GU322806)
67	84.62%	50.00%	0.1342	46.15%	100.00%	0.0277	*Succinivibrio dextrinosolvens* strain 0554 (NR_026476)
68	46.15%	100.00%	0.0277	69.23%	50.00%	0.2838	*Succinivibrio dextrinosolvens* strain 0554 (NR_026476)
70	46.15%	83.33%	0.1362	76.92%	33.33%	0.0851	*Succinivibrio dextrinosolvens* strain 0554 (NR_026476)
72	61.54%	66.67%	0.3831	30.77%	83.33%	0.0464	*Clostridium indolis* (AF028351)
74	30.77%	83.33%	0.0464	30.77%	33.33%	0.3953	Uncultured *Succinivibrio* sp. clone EMP_V30 (EU794288)
77	7.69%	0.00%	0.5965	76.92%	16.67%	0.0134	*Succinivibrio dextrinosolvens strain 0554 (NR_026476)*
79	23.08%	0.00%	0.2362	0.00%	83.33%	0.0002	*Succinimonas amylolytica* strain DSM 2873 (NR_026475)
83	0.00%	0.00%	0.8603	23.08%	83.33%	0.0227	*Selenomonas ruminantium*, strain: S211 (AB198441.1)
84	0.00%	50.00%	0.0068	15.39%	0.00%	0.3706	*Bifidobacterium ruminantium* strain KCTC 3425 (GU361831)

Table 3.3B. Taxonomical identification of diet-associated PCR – DGGE bands within particular RFI groups (n = 19).

PCR-DGGE Band Category	L-RFI (n = 13)		*P* value	H-RFI (n = 6)		*P* value	Taxonomy (GenBank Accesion No.)
	LE diet	HE diet		LE diet	HE diet		
1	46.15%	76.92%	0.0924	0.00%	0.00%	0.5455	*Prevotella* sp. (AF218619)
2	46.15%	46.15%	1.0000	16.67%	83.33%	0.0390	*Prevotella* sp. (AF218619)
6	84.62%	46.15%	0.0428	83.33%	100.00%	0.3633	*Prevotella* sp. BP1-56 (AB501155)
9	23.08%	15.39%	0.3391	50.00%	0.00%	0.0909	*Blautia* sp. BM-C2-0 (GQ456220)
10	30.77%	7.69%	0.1413	0.00%	50.00%	0.0909	*Clostridium symbiosum* strain 69 (EF025909)
14	15.39%	15.39%	0.4070	50.00%	0.00%	0.0909	*Lachnospiraceae genomosp*. C1 (AY278618)
34	23.08%	30.77%	0.3109	50.00%	0.00%	0.0909	*Ruminococcus gauvreaui* strain CCRI 16110
35	38.46%	0.00%	0.0196	33.33%	33.33%	0.4545	Uncultured *Prevotella* sp. clone JD9 (FJ268952)
42	61.54%	61.54%	1.0000	50.00%	100.00%	0.0909	*Prevotella* sp. 152R-1a (DQ278861)
43	69.23%	38.46%	0.0953	66.67%	66.67%	0.4545	Uncultured *Roseburia* sp. clone M2-35 (EU530245)
46	84.62%	61.54%	0.1526	50.00%	100.00%	0.0909	Uncultured *Prevotella* sp. clone JD9 (FJ268952)
49	84.62%	46.15%	0.0428	83.33%	100.00%	0.5000	Uncultured *Prevotella* sp. clone Gull85-50 (FJ220908)
52	92.31%	69.23%	0.1413	50.00%	100.00%	0.0909	*Succiniclasticum ruminis* strain DSM 9236 (NR_026205)

54	69.23%	92.31%	0.1413	16.67%	100.00%	0.0076	*Succinivibrio dextrinosolvens* strain 0554 (NR_026476)
58	69.23%	84.62%	0.2422	16.67%	100.00%	0.0076	*Moryella indoligenes* strain AIP 220.04 (DQ377947)
65	7.69%	46.15%	0.0339	50.00%	83.33%	0.2424	*Robinsoniella peoriensis* strain HGUE-09/9434 (GU322806)
66	61.54%	92.31%	0.0727	66.67%	83.33%	0.4091	Uncultured *Succinivibrio* sp. clone EMP_B23 (EU794184)
67	84.62%	46.15%	0.0428	50.00%	100.00%	0.0909	*Succinivibrio dextrinosolvens* strain 0554 (NR_026476)
68	46.15%	69.23%	0.1588	100.00%	50.00%	0.0909	*Succinivibrio dextrinosolvens* strain 0554 (NR_026476)
70	46.15%	76.92%	0.0924	83.33%	33.33%	0.1136	*Succinivibrio dextrinosolvens* strain 0554 (NR_026476)
72	61.54%	30.77%	0.0953	66.67%	83.33%	0.4091	*Clostridium indolis* (AF028351)
73	61.54%	23.08%	0.0476	66.67%	50.00%	0.3788	Uncultured *Succinivibrio* sp. clone EMP_V30 (EU794288)
76	15.39%	23.08%	0.3391	0.00%	50.00%	0.0909	*Moryella indoligenes* strain AIP 220.04 (DQ377947)
77	7.69%	76.92%	0.0004	0.00%	16.67%	0.5000	*Succinivibrio dextrinosolvens* strain 0554 (NR_026476)
79	23.08%	0.00%	0.1100	0.00%	83.33%	0.0076	*Succinimonas amylolytica* strain DSM 2873 (NR_026475)
83	0.00%	23.08%	0.1100	0.00%	83.33%	0.0076	*Selenomonas ruminantium*, strain: S211 (AB198441.1)
84	0.00%	15.39%	0.2400	50.00%	0.00%	0.0909	*Bifidobacterium ruminantium* strain KCTC 3425 (GU361831)

Table 3.4. Rumen metabolites and feed efficiency traits in steers differing RFI and diet (n = 19).

Variable	H-RFI (n = 6)			L-RFI (n = 13)		
	Low Energy diet Mean ± SEM	**High Energy diet** Mean ± SEM	*P* value	**Low Energy diet** Mean ± SEM	**High Energy diet** Mean ± SEM	*P* value
Acetate (%) [a]	54.41 ± 1.34	50.16 ± 1.70	**0.06**	55.21 ± 1.02	52.12 ± 1.10	**0.04**
Propionate (%)[a]	31.57 ±1.75	36.39 ± 2.20	**0.10**	33.17 ± 1.62	34.06 ± 1.72	**0.71**
Butyrate (%)[a]	9.56 ± 0.96	7.83 ± 1.20	**0.27**	7.64 ± 0.73	8.87 ± 0.77	**0.25**
Isobutyrate (%)[a]	0.94± 0.06	0.67 ± 0.08	**0.01**	0.85 ± 0.06	0.72 ± 0.07	**0.19**
Valerate (%)[a]	1.01 ± 0.10	2.46 ± 0.12	**<0.0001**	1.05 ± 0.11	2.27 ± 0.11	**<0.0001**
Isovalerate (%)[a]	2.38 ± 0.40	2.29 ± 0.51	**0.89**	1.96 ± 0.21	1.81 ± 0.23	**0.62**
Total VFA (mM)	58.86 ± 5.53	113.19 ± 6.96	**<0.0001**	62.12 ±5.59	100.29 ± 6.52	**<0.0001**
A : P Ratio	1.86 ± 0.17	1.53 ± 0.21	**0.24**	1.70 ± 0.18	1.79 ± 0.19	**0.74**
St VFA: Br VFA ratio	31.04 ± 4.78	53.49 ± 6.01	**0.007**	37.12 ± 6.73	54.84 ± 7.11	**0.08**
Ammonia (mM)	0.10 ± 0.16	0.20 ± 0.02	**0.0002**	0.11 ± 0.01	0.17 ± 0.02	**0.007**
DMI (kg DM)	8.65 ± 0.19	11.52 ± 0.23	**<0.0001**	6.96 ± 0.15	9.55 ± 0.17	**<0.0001**
ADG (kg)	1.26 ± 0.05	1.73 ± 0.06	**<0.0001**	1.22 ± 0.04	1.81 ± 0.04	**<0.0001**
FCR (feed: gain)	6.98 ± 0.20	6.81 ± 0.25	**0.60**	5.75 ± 0.14	5.33 ± 0.15	**0.05**
RFI	0.78 ± 0.07	0.98 ± 0.09	**0.09**	-0.74 ± 0.08	-1.29 ± 0.09	**<0.0001**

[*] Values are given as a proportion of the total concentration of Volatile Fatty Acids.

Table 3.5A. Differences in particular bacterial species between Low and High Energy density diets within RFI group (n = 19).

Variable	H – RFI (n = 6)			L – RFI (n = 13)		
	Low Energy diet Mean ± SEM	**High Energy diet** Mean ± SEM	***P* value**	**Low Energy diet** Mean ± SEM	**High Energy diet** Mean ± SEM	***P* value**
Total bacteria (copies/ml)	7.56E10 ± 2.2E10	4.44E10 ± 2.2E10	**0.34**	4.07E10 ± 1.52E10	6.34E10 ± 1.52E10	**0.30**
***Succinivibrio* sp. (%)**	8.45 ± 1.88	0.21 ± 1.72	**0.01**	12.49 ± 2.34	0.09 ± 2.34	**0.001**
***Eubacterium* sp. (%)**	0.13 ± 0.19	0.55 ± 0.19	**0.15**	0.22 ± 0.09	0.09 ± 0.09	**0.28**
***Robinsoniella* sp. (%)**	0.001 ± 0.001	0.002 ± 0.001	**0.62**	0.005 ± 0.000	0.002 ± 0.000	**0.13**

Table 3.5B. Differences in particular bacterial species population between L – or H – RFI group within Low and High Energy density diets (n = 19).

Variable	Low Energy diet (n = 19)			High Energy diet (n = 19)		
	L-RFI (n = 13) Mean ± SEM	**H-RFI (n = 6)** Mean ± SEM	***P* value**	**L-RFI (n = 13)** Mean ± SEM	**H-RFI (n = 6)** Mean ± SEM	***P* value**
Total bacteria (copies/ml)	4.07E10 ± 1.28E10	7.56E10 ± 1.89E10	**0.15**	6.34E10 ± 1.71E10	4.44E10 ± 2.52E10	**0.54**
***Succinivibrio* sp. (%)**	12.49 ± 3.00	8.45 ± 4.83	**0.49**	0.09 ± 0.05	0.21 ± 0.07	**0.20**
***Eubacterium* sp. (%)**	0.22 ± 0.10	0.13 ± 0.15	**0.64**	0.09 ± 0.09	0.55 ± 0.14	**0.02**
***Robinsoniella* sp. (%)**	0.005 ± 8.33E-6	0.001 ± 0.001	**0.06**	0.002 ± 0.001	0.002 ± 0.001	**0.98**

Table 3.6. Changes by diet in classification assigned to ruminal metabolites (n = 19). * Diet 1: LE diet, Diet 2: HE diet, RFI: Residual Feed Intake, DMI: Dry Matter Intake, ADG: Average Daily Gain, FCR: Feed Conversion Ratio, Acet: acetate, Prop: propionate, But: butyrate, Isobut: isobutyrate, Val: valerate, Isoval: isovalerate, Total: total VFA, A: P ratio: acetate to propionate ratio, St: Br ratio: straight to branched – chain VFA, NH_3 – N: Ammonia – N.

ID	Diet	RFI	DMI	ADG	FCR	Acet	Prop	But	Isobut	Val	Isoval	Total	A:P ratio	St:Br ratio	NH_3-N
11	1	H	H	M	H	M	M	M	M	L	M	H	M	M	L
11	2	H	H	L	H	M	L	H	M	L	M	H	M	M	H
23	1	L	L	M	L	H	L	L	H	L	L	L	H	M	H
23	2	L	L	M	L	H	M	M	L	H	L	M	M	H	L
33	1	L	M	H	L	M	H	L	L	M	L	H	M	H	H
33	2	L	L	L	M	M	H	L	M	M	M	H	M	M	M
75	1	L	M	H	L	H	M	M	H	L	M	L	M	L	M
75	2	L	L	L	M	H	L	H	M	L	H	M	H	L	M
91	1	L	L	L	H	H	L	M	H	M	M	L	M	L	M
91	2	L	H	H	M	M	H	L	M	L	M	L	M	M	L
95	1	H	M	L	H	L	H	L	L	H	L	M	L	H	L
95	2	H	H	L	H	M	H	L	L	L	L	L	M	H	L
101	1	L	M	H	L	L	H	M	L	H	M	M	L	H	L
101	2	L	M	M	M	M	H	L	M	L	L	M	L	M	M
111	1	L	L	M	M	H	M	L	L	L	L	H	M	H	L
111	2	L	M	H	L	M	H	L	L	L	L	H	M	H	L
119	1	L	L	L	L	M	M	L	H	M	M	L	M	L	M
119	2	L	L	H	L	L	H	L	L	M	L	H	L	H	H
129	1	L	L	L	M	L	H	M	L	H	M	M	L	M	M
129	2	L	M	M	M	H	L	H	H	L	H	M	H	L	M
167	1	H	H	H	M	L	H	M	L	H	M	H	L	M	M
167	2	H	H	L	H	M	H	M	L	M	L	H	M	H	H
173	1	H	M	L	H	L	H	M	H	M	M	M	L	L	L
173	2	H	L	L	H	L	H	L	L	L	L	M	L	M	H
205	1	L	L	M	L	H	M	L	M	M	M	M	M	L	H
205	2	L	L	H	L	L	H	M	M	M	L	M	L	M	M
225	1	H	M	H	L	M	H	L	H	L	M	M	L	M	L
225	2	H	H	H	M	L	H	L	M	M	M	L	L	M	L
259	1	L	M	H	L	M	M	L	H	L	L	L	M	M	H
259	2	L	L	H	L	L	H	M	L	L	L	M	L	H	H
271	1	L	M	M	M	M	M	M	M	M	M	M	M	M	H
271	2	L	L	L	L	M	M	H	H	M	M	L	M	L	L
287	1	H	H	M	M	H	L	M	M	L	M	L	H	M	H
287	2	H	H	M	H	L	H	L	M	H	M	M	L	M	M
423	1	L	L	H	L	M	M	M	L	M	H	H	M	L	L
423	2	L	L	M	L	L	H	M	L	H	M	M	L	M	L
451	1	L	L	L	M	H	L	M	H	L	M	M	H	M	L
451	2	L	L	H	L	M	M	H	M	L	M	H	M	M	M

Table 3.7A. Correlation (r) of fermentation metabolites in the rumen of Low RFI steers with indicators of feed efficiency (n = 13) * *P* < 0.0001, **P* < 0.05.**

Variable	RFI	DMI	ADG	FCR
Acetate	0.586**	-0.491**	-0.684**	0.528**
Propionate	-0.388**	NS	0.536**	-0.430**
Butyrate	NS	NS	NS	NS
Isobutyrate	NS	-0.505**	-0.644**	0.462**
Valerate	-0.660**	0.652***	0.758***	-0.427**
Isovalerate	NS	NS	NS	NS
Total VFA	-0.465**	0.618***	0.759***	-0.525**
Branched VFA : Straight VFA ratio	NS	0.389**	0.458**	NS
Acetate : Propionate ratio	0.396**	NS	-0.467**	0.441**
NH_3-N	NS	NS	NS	NS
RFI		-0.397**	-0.605***	-0.497**
DMI			0.855***	NS
ADG				-0.637***

Table 3.7B. Correlation (r) of fermentation metabolites in the rumen of High RFI steers with indicators of feed efficiency (n = 6) * *P* < 0.0001, ** *P* < 0.05).**

Variable	RFI	DMI	ADG	FCR
Acetate	NS	NS	NS	NS
Propionate	NS	NS	NS	NS
Butyrate	0.504*	NS	NS	NS
Isobutyrate	NS	-0.814**	-0.557**	NS
Valerate	NS	0.724***	NS*	NS
Isovalerate	NS	NS	NS	NS
Total VFA	NS	0.815**	NS	NS
Branched VFA : Straight VFA ratio	NS	NS	NS	NS
Acetate : Propionate ratio	NS	NS	NS	NS
NH3-N	0.728**	0.643**	NS	0.765**
RFI		0.672**	NS	NS
DMI			0.780**	NS
ADG				NS

Table 3.8. Correlation (r) of fermentation metabolites in the rumen of steers differing RFI under LE with indicators of feed efficiency (RFI, DMI and FCR) and bacterial population (n = 19), * *P* < 0.0001, ** *P* < 0.05, *trend.**

[a] VFA are given as proportions of the total Volatile Fatty Acids concentration.

Variable	Total bacteria	*Succinivibrio* sp.	*Eubacterium* sp.	*Robinsoniella* sp.
Acetate [a]	NS	NS	NS	NS
Propionate [a]	NS	NS	NS	NS
Butyrate [a]	NS	NS	NS	NS
Isobutyrate [a]	NS	NS	NS	NS
Valerate [a]	NS	NS	NS	NS
Isovalerate [a]	NS	NS	NS	NS
Total VFA	NS	NS	NS	NS
Straight VFA: Branched VFA ratio	NS	NS	NS	NS
Acetate : Propionate ratio	NS	NS	NS	NS
NH_3-N	NS	NS	NS	NS
RFI	NS	NS	NS	-0.437*
DMI	NS	NS	NS	NS
ADG	NS	NS	NS	NS
FCR	NS	NS	NS	NS
Total bacteria		NS	NS*	-0.474**
***Succinivibrio* sp.**			NS	0.542**
***Eubacterium* sp.**				NS

Table 3.9. Correlation (r) of fermentation metabolites in the rumen of steers differing RFI under high energy diet with indicators of feed efficiency (RFI, DMI and FCR) and bacterial population (n = 19), * *P* < 0.0001, ** *P* < 0.05, *trend.**

[a] VFA are given as proportions of the total Volatile Fatty Acids concentration.

Variable	Total bacteria	*Succinivibrio* sp.	*Eubacterium* sp.	*Robinsoniella* sp.
Acetate [a]	NS	NS	NS	NS
Propionate [a]	NS	NS	NS	NS
Butyrate [a]	NS	NS	NS	NS
Isobutyrate [a]	NS	NS	NS	NS
Valerate [a]	NS	NS	NS	NS
Isovalerate [a]	NS	NS	NS	NS
Total VFA	NS	NS	NS	NS
Straight VFA: Branched VFA ratio	NS	NS	NS	NS
Acetate : Propionate ratio	NS	NS	NS	NS
NH_3-N	NS	NS	NS	NS
RFI	NS	NS	0.491**	NS
DMI	NS	NS	0.417*	NS
ADG	NS	NS	NS	NS
FCR	NS	NS	NS	NS
Total bacteria		NS	NS	NS
***Succinivibrio* sp.**			NS	NS
***Eubacterium* sp.**				NS

Table 3.10A. Correlation (r) of fermentation metabolites and feed efficiency indicators in the rumen of Low RFI steers with functional bacterial populations (n = 13), ***$P < 0.0001$, **$P < 0.05$, * $P < 0.1$.

Variable	Total bacteria	*Succinivibrio* sp.	*Eubacterium* sp.	*Robinsoniella* sp.
Acetate [a]	NS	NS	NS	NS
Propionate [a]	NS	NS	NS	NS
Butyrate [a]	NS	NS	NS	NS
Isobutyrate [a]	NS	NS	NS	NS
Valerate [a]	NS	-0.435**	NS	NS
Isovalerate [a]	NS	NS	NS	NS
Total VFA	NS	-0.385**	NS	-0.340*
Branched VFA : Straight VFA ratio	NS	NS	NS	NS
Acetate : Propionate ratio	NS	NS	NS	NS
NH_3-N	NS	-0.409**	NS	NS
RFI	NS	0.379*	NS	NS
DMI	NS	-0.533**	NS	NS
ADG	NS	-0.564**	NS	NS
FCR	NS	0.337*	NS	NS
Total bacteria		NS	NS	NS
***Succinivibrio* sp.**			NS	0.493**
***Eubacterium* sp.**				NS

[a] VFA are given as proportions of the total Volatile Fatty Acids concentration.

Table 3.10B. Correlation (r) of fermentation metabolites and feed efficiency indicators in the rumen of High RFI steers with functional bacterial populations (n = 6), ***$P < 0.0001$, **$P < 0.05$, * $P < 0.1$.

Variable	Total bacteria	*Succinivibrio* sp.	*Eubacterium* sp.	*Robinsoniella* sp.
Acetate [a]	NS	NS	NS	NS
Propionate [a]	NS	NS	NS	NS
Butyrate [a]	NS	NS	NS	0.743**
Isobutyrate [a]	NS	NS	NS	NS
Valerate [a]	NS	-0.629**	NS	NS
Isovalerate [a]	NS	NS	NS	NS
Total VFA	-0.605**	NS	NS	0.534*
Branched VFA : Straight VFA ratio	NS	NS	NS	NS
Acetate : Propionate ratio	NS	NS	NS	NS
NH_3-N	-0.769**	-0.765**	NS	NS
RFI	NS	NS	NS	0.736**
DMI	NS	-0.546*	NS	NS
ADG	NS	-0.573*	NS	NS
FCR	NS	NS	NS	NS
Total bacteria		NS	NS	NS
***Succinivibrio* sp.**			NS	NS
***Eubacterium* sp.**				NS

.[a]VFA are given as a proportions of the total Volatile Fatty Acids concentration

Figure 3.1. PCR-DGGE profiles generated from ruminal fluid DNA from fifty-five steers fed with low and high energy density diet, using primers HDA1-GC and HDA2 (22 to 55% DGGE). H, M and L represent the steers with high RFI (H-RFI, mean plus 0.5 SD, inefficient), medium RFI (M-RFI, -0.5 SD < mean RFI < 0.5 SD) and low-RFI (L-RFI, < mean minus 0.5 SD, efficient), respectively. RFI, residual feed intake, is a parameter to measure feed efficiency in cattle (Basarab et al., 2003). The comparison of the PCR-DGGE profiles was generated with the BioNumerics software package using UPGMA (unweighted pair-group) method as described in the text; the first column indicates the ID of the steer, the second column corresponds to the diet and the third column represents the RFI classification when steer was either under LE or HE.

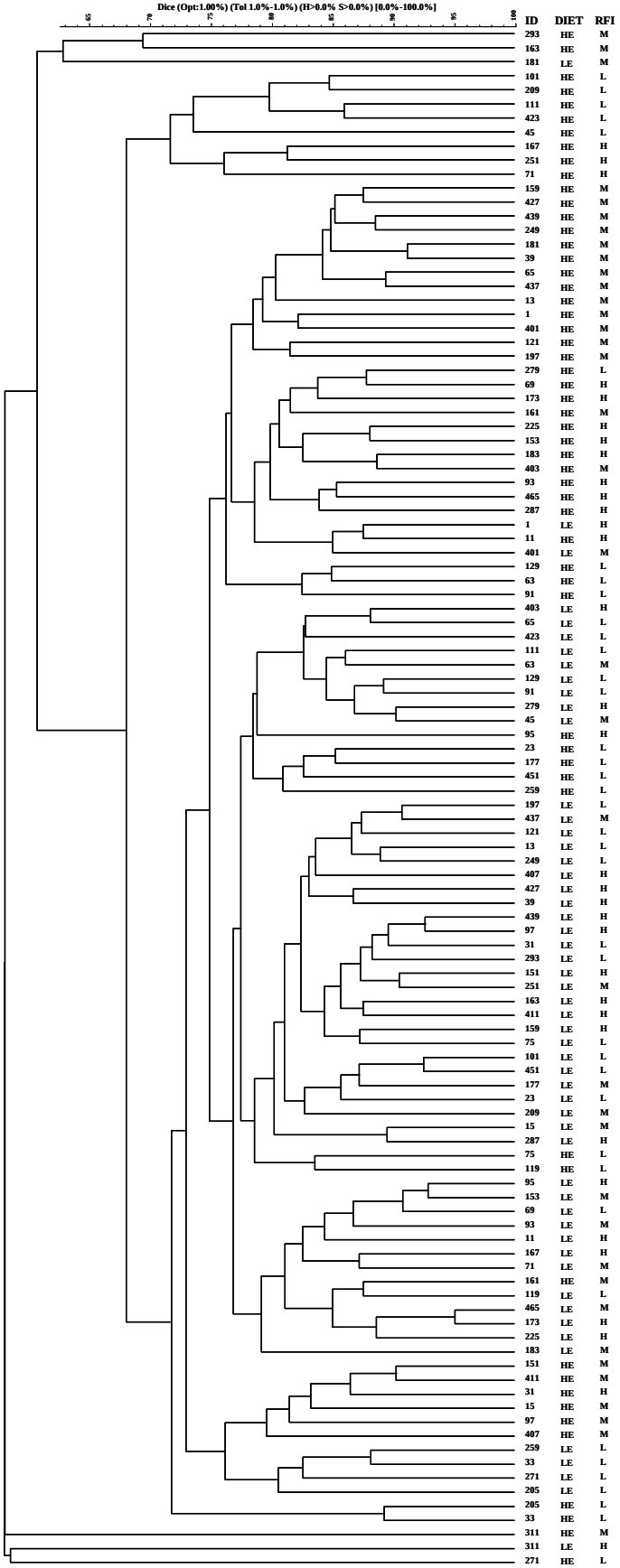

Dice (Opt:1.00%) (Tol 1.0%-1.0%) (H>0.0% S>0.0%) [0.0%-100.0%]
ID DIET RFI
293 HE M
163 HE M
181 LE M
101 HE L
209 HE L
111 HE L
423 HE L
45 HE L
167 HE H
251 HE H
71 HE H
159 HE M
427 HE M
439 HE M
249 HE M
181 HE M
39 HE M
65 HE M
437 HE M
13 HE M
1 HE M
401 HE M
121 HE M
197 HE M
279 HE L
69 HE H
173 HE H
161 HE M
225 HE H
153 HE H
183 HE H
403 HE M
93 HE H
465 HE H
287 HE H
1 LE H
11 HE H
401 LE M
129 HE L
63 HE L
91 HE L
403 LE H
65 LE L
423 LE L
111 LE L
63 LE M
129 LE L
91 LE L
279 LE H
45 LE M
95 HE H
23 HE L
177 HE L
451 HE L
259 HE L
197 LE L
437 LE M
121 LE L
13 LE L
249 LE L
407 LE H
427 LE H
39 LE H
439 LE H
97 LE H
31 LE L
293 LE L
151 LE H
251 LE M
163 LE H
411 LE H
159 LE H
75 LE L
101 LE L
451 LE L
177 LE M
23 LE L
209 LE M
15 LE M
287 LE H
75 HE L
119 HE L
95 LE H
153 LE M
69 LE L
93 LE M
11 LE H
167 LE H
71 LE M
161 HE M
119 LE L
465 LE M
173 LE H
225 LE H
183 LE M
151 HE M
411 HE M
31 HE H
15 HE M
97 HE M
407 HE M
259 LE L
33 LE L
271 LE L
205 LE L
205 HE L
33 HE L
311 HE M
311 LE H
271 HE L

Figure 3.2. PCR-DGGE profiles generated from ruminal fluid DNA from fifty-eight steers fed with high energy density diet using primers HDA1-GC and HDA2 (22 to 55% DGGE). H and L represent the steers with high RFI (H-RFI > 0.5, inefficient), M-RFI (-0.5 < RFI < 0.5) and low-RFI (L-RFI < -0.5, efficient), respectively. RFI, residual feed intake, a parameter to measure feed efficiency in cattle (Basarab et al., 2003). The comparison of the PCR-DGGE profiles was generated with the BioNumerics software package using UPGMA (unweighted pair-group) method as described in the text.

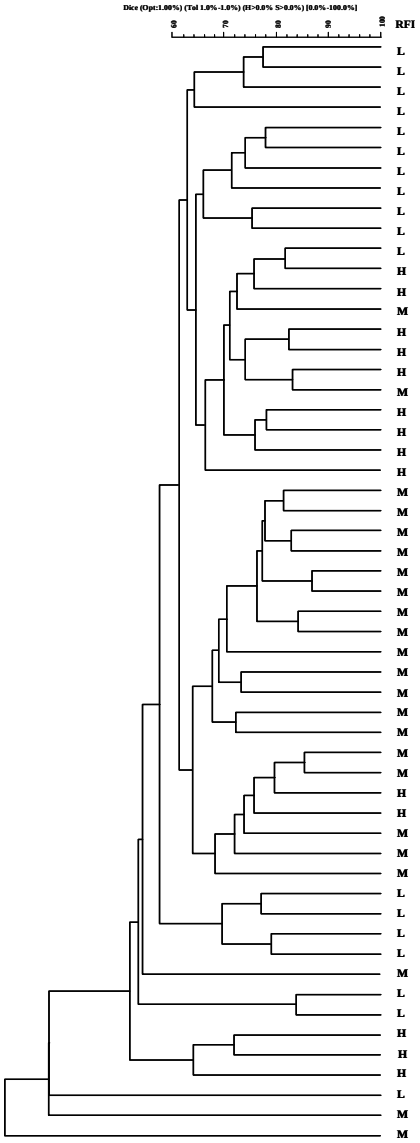


Figure 3.3. Comparison of PCR – DGGE bacterial profiles for different RFI groups, from nineteen steers (H–RFI, n = 6; M–RFI, n = 4 and L– FI, n = 13) fed low and high energy density feedlot diets. (A) Individuals grouped in the H–RFI group throughout diets, (B) steers belonging to the M–RFI category and (C) animals in L–RFI classification under both feed trials. Dice similarity coefficient is indicated for each clustering analysis.

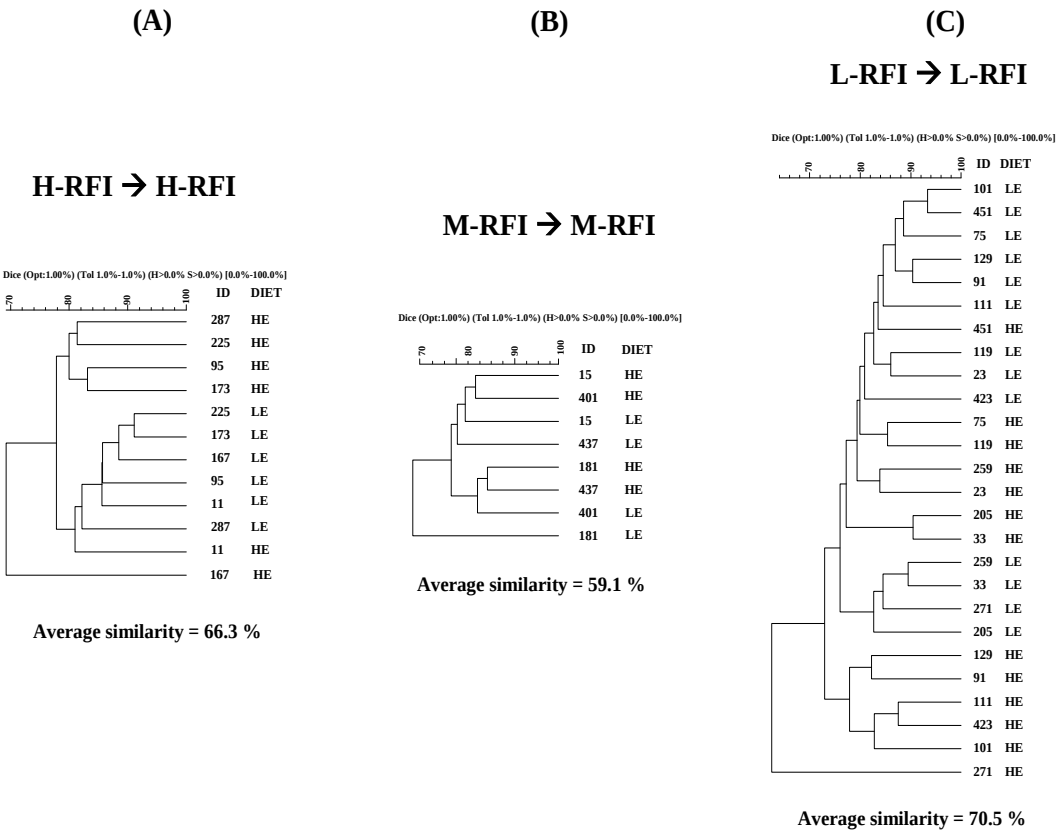
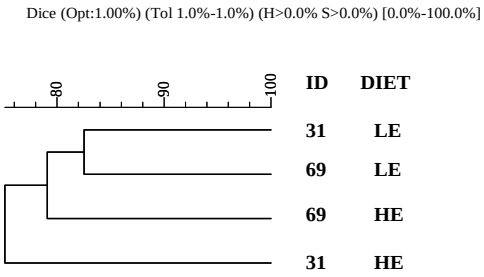


Figure 3.4. Comparison of PCR – DGGE bacterial profiles for different RFI groups from nineteen steers (H–RFI, n = 6; M–RFI, n = 4 and L–RFI, n = 13) fed low and high energy density feedlot diets. (A) Individuals with L-RFI in LE diet that switched to H-RFI in HE, and (B) steer that switched from H-RFI in LE to L-RFI in HE. Dice similarity coefficient is indicated for each clustering analysis.

(A)

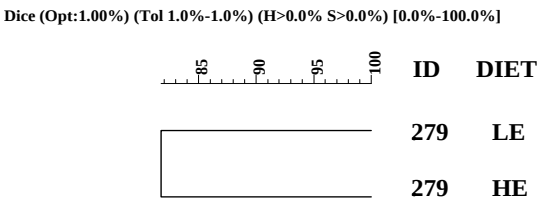
L-RFI → H-RFI



Average similarity = 46.6 %

(B)

H-RFI → L-RFI



Average similarity = 81.7 %

Figure 3.5. Specific bacterial species correlated to LE and HE diet and to L– and H–RFI groups. Asterisks indicate the number of species significantly correlated to each group; taxonomy is indicated in the squares corresponding to each quadrant.

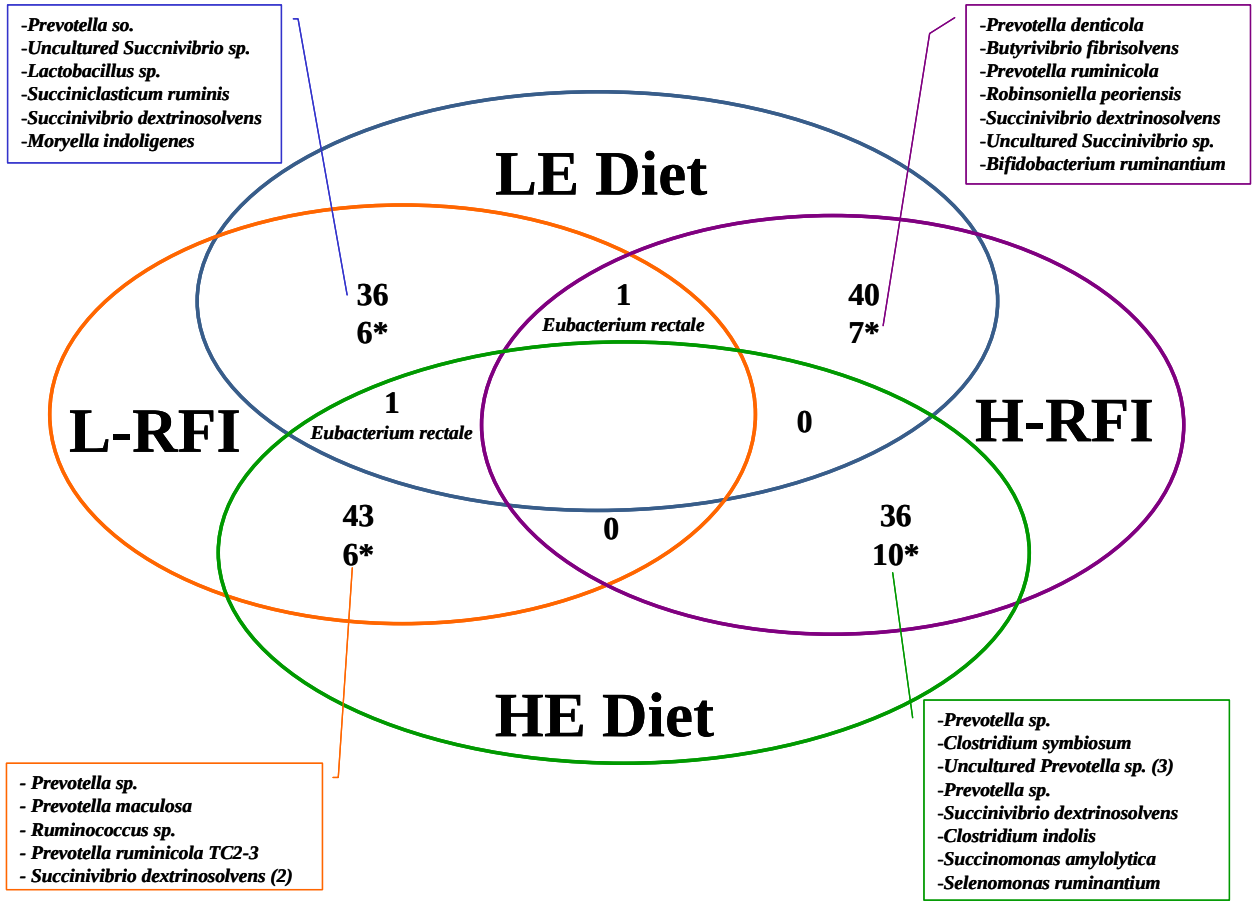


Figure 3.6A. Frequency of PCR-DGGE bands in animals categorized on the basis of Dry Matter Intake (DMI), Average Daily Gain (ADG), Feed Conversion Ratio (FCR, F:G), Acetate, Isobutyrate, and NH_3 – N, under Low Energy diet (A) using PROC CATMOD analysis. The x-axis represents 85 identified bands and the symbols plotted reflect the frequency of the bands detected in the tested the population of each trait.

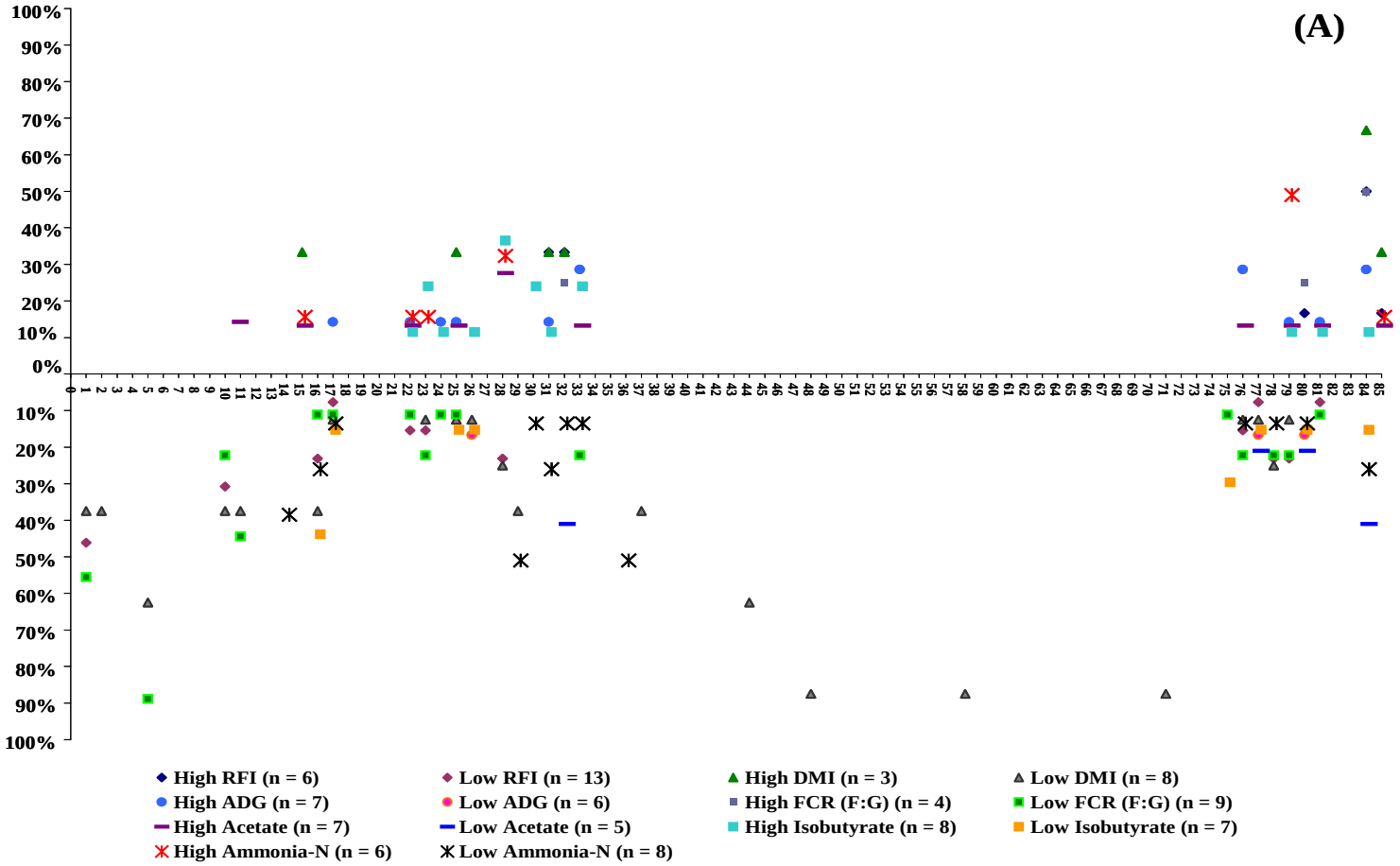


Figure 3.6B. Frequency of PCR-DGGE bands in animals categorized on the basis of Dry Matter Intake (DMI), Average Daily Gain (ADG), Feed Conversion Ratio (FCR, F:G), Acetate, Isobutyrate, and NH_3–N, under High Energy diet (B) using PROC CATMOD analysis. The x-axis represents 85 identified bands and the symbols plotted reflect the frequency of the bands detected in the tested the population of each trait.

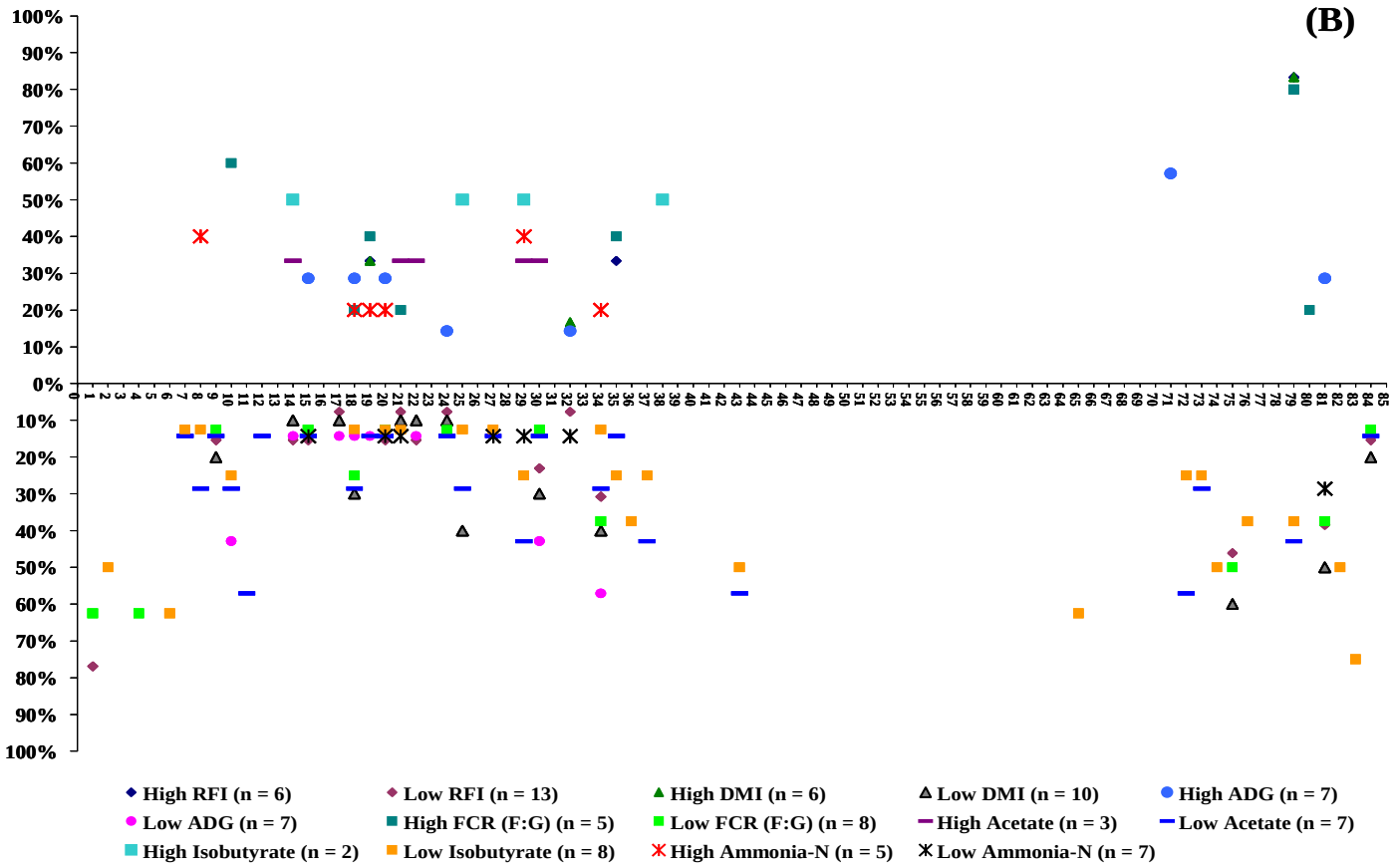
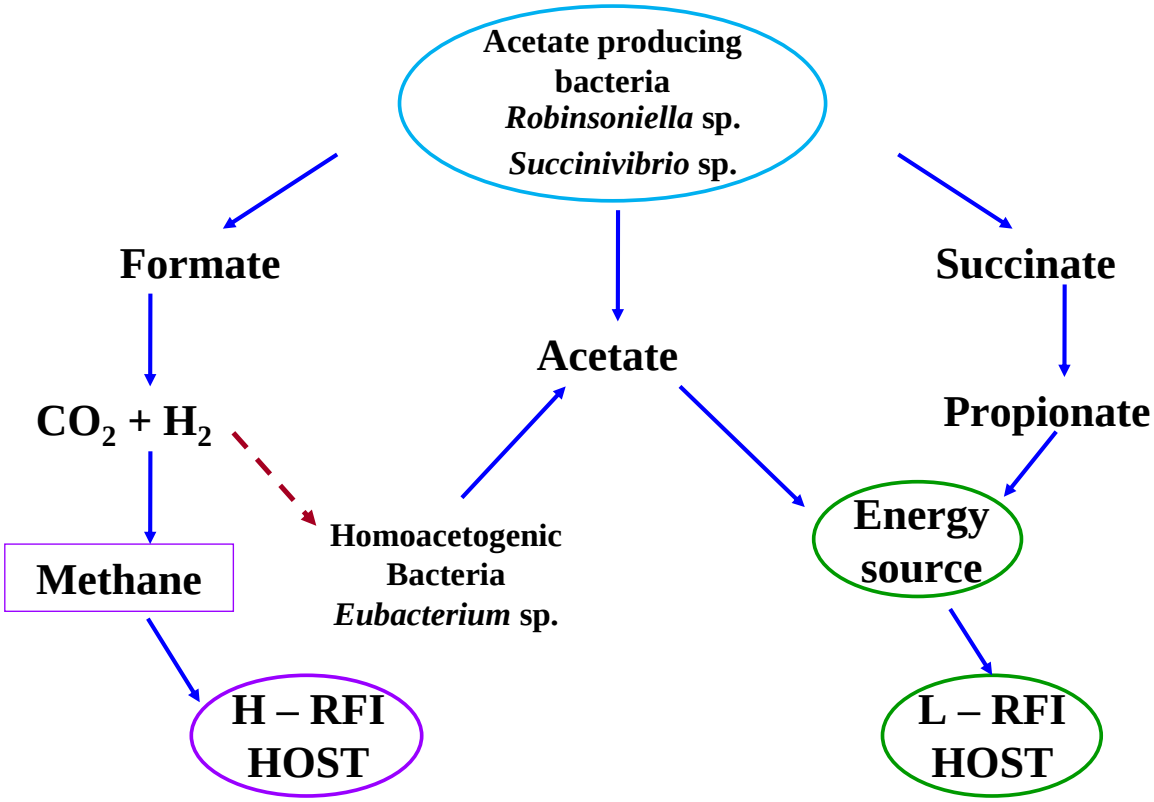


Figure 3.7. Proposed cross-feeding interactions among three bacterial groups with similar functions (*Succinivibrio* sp., *Eubacterium* sp., and *Robinsoniella* sp.) and their potential associations with RFI.



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Chapter 4. Potential Associations between Ruminal Bacterial PCR–DGGE Profiles and Host Breeds in Beef Cattle. [3]

4.1. INTRODUCTION.

Although it has been acknowledged that ruminal microbiota impacts cattle performance, the underlying features of the bacterial communities involved in these functions remain largely undefined. Traditionally, three different subpopulations can be distinguished in the rumen (Edwards et al., 2008): a planktonic population composed of "free" bacteria in the rumen fluid, a population attached to feed particles and a population attached to the rumen epithelium, named "epimural" (Cheng et al., 1981; Mead et al., 1981). Bacteria associated with feed particles are considered numerically predominant and essential for feed degradation (Koike et al., 2003), whereas epimural populations represent less than 1% (Czerkawski, 1986). Interactions between the more resilient particle–associated bacteria with the dynamic free–floating population have also been documented (Edwards et al., 2005; Shinkai et al., 2009). It has been reported that the bacterial community attached to the epithelium was not affected by diet, as opposed to the other populations (McCowan et al., 1980; Sadet et al., 2007). Due to their close contact with the ruminal tissue, the host might have a strong influence on their structure (Larue et al., 2005). Ecologically, a greater diversity has been considered a positive attribute for a more stable and resilient community (Zoetendal et al., 2004) and epimural bacteria shows a high

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inter-animal variation (Lukas et al., 2010). Because rumen epithelium is the location of the intermediate metabolism (Žitňan et al., 1993), it is likely that the epimural community is more closely related to the metabolic activity of the host than the bacteria from the rumen contents (Wallace et al., 1979). Although microbial activities in the rumen and interactions among bacterial communities appear to be previously examined (Edwards et al., 2008) as well as the potential relationships between host breed and ruminal bacterial structure in liquid and contents (Guan et al., 2008), little has been reviewed about the fluctuations in bacterial populations influenced by the host genotype.

Feed consumption drives profitability and sustainability in beef production systems. The efficiency of the nutrient utilisation in ruminants is mainly determined by a balanced rumen fermentation, which is ultimately controlled by the ruminal microbiota (Russell et al., 1981; McSweeney et al., 1994). Because ruminal microbes play essential roles in feed conversion, their structure and activities may be associated with host feed efficiency (Hernandez-Sanabria et al., 2010).

In the present study, it was hypothesised that sire breed can have impact on the specific bacterial groups associated with a particular rumen fraction of the offspring, even under equal diet conditions. Therefore, I focused on the investigation of the associations among rumen bacteria and host feed efficiency variables in steers diverging genetic background, aiming to identify a potential bacterial community linked to a particular breed in beef cattle. Culture independent methods were used to screen the diversity of the bacterial

populations in rumen liquid, contents and tissue and further multivariate statistical analysis permitted establishing correlations among productive measurements such as dry matter intake (DMI), average daily gain (ADG), feed conversion ratio (FCR) (Feed:Gain), and residual feed intake (RFI) with bacterial species. Analysis was carried out independently for each fraction since their bacterial structures are unique, as suggested by previous work (Larue et al., 2005; Firkins et al., 2010).

4.2. MATERIALS AND METHODS.

4.2.1. Animals and sampling.

Animals were selected from a herd of 180 steers raised under feedlot conditions at the Kinsella Research Station, following the guidelines of the Canadian Council on Animal Care (CCAC, 1993) and the protocol approved by the Animal Care and Use Committee for Livestock at the University of Alberta. Data consisted of forty eight beef steers (10 months old), offspring of a cross between a composite dam line and Angus (ANG), Charolais (CHA) or University of Alberta hybrid bulls (HYB). The dams used (Mujibi et al., 2010) were produced from crosses among Hereford (60%) and other breeds (40%). Animals were fed a totally mixed ration, high-energy density feedlot diet composed by 28.3% oats, 56.7% barley, 10% alfalfa pellets, and 5% feedlot supplement [32% CP beef supplement containing Rumensin (400 mg/kg), and 1.5% canola oil (ME 2.9 Mcal/kg)] for 90 days. Feed intake data were collected using the GrowSafe automated feeding system (GrowSafe Systems Ltd., Airdrie, AB, Canada).

Feed efficiency traits including DMI, ADG, FCR and RFI were obtained from each steer through the whole testing period, as outlined by Basarab et al. (2003) and Nkrumah et al. (2006). RFI was calculated based on DMI, ADG and metabolic weight data as per Nkrumah et al. (2006). Steers were classified into three groups based on their RFI: high (H–RFI, above mean plus 0.5 SD) or "inefficient", medium (M–RFI, between the mean minus 0.5 SD and mean plus 0.5 SD), and low (L–RFI, below mean minus 0.5 SD) or "efficient". Steers for this study were selected based on their extremely low or high RFI ranking. Initial number of samples included 30 L–RFI and 30 H–RFI individuals but due to unsuitable quality and exclusions in the sampling process, only 48 steers were taken for further analysis (H–RFI, n=13; M–RFI, n=21; L–RFI, n=14).

Rumen fluid samples were collected from all steers via oro-gastric tubing on the same day during the last week of the trial (day 83-90) before feeding using the method described by Hernandez-Sanabria et al. (2010). Rumen particulate contents and rumen tissue samples were obtained following slaughter and stored in RNAlater (Ambion, Carlsbad, CA, USA) at -80 °C for further analysis.

4.2.2. DNA Extraction and PCR DGGE analysis.

Total DNA was extracted from rumen samples using the beads beating method described by Guan et al. (2008). Liquid fraction was processed as in Hernandez-Sanabria et al. (2010). Solid digesta and rumen tissue were recovered after centrifugation and further removal of RNAlater; two grams from digesta and one gram of epithelium were used for the respective total DNA extraction

procedures. After measuring the concentration and quality of DNA, 50 ng of total DNA were used as template for PCR amplifications of the V2-V3 region of the bacterial 16S rRNA gene (~200 bp) using universal bacterial primers HDA1-GC/HDA-2 (Walter et al., 2000). Purification of PCR products and PCR-DGGE analyses followed previously described protocols (Hernandez-Sanabria et al., 2010). All PCR–DGGE bands were sequenced as previously reported (Hernandez-Sanabria et al., 2010).

Similarities among all PCR–DGGE band profiles from the three fractions (liquid, contents or tissue) and within each rumen fraction were calculated using the Dice similarity coefficient (D_{sc}) in BioNumerics software v5.1 (Applied Maths, Austin, TX, USA). Hierarchical cluster comparisons were generated using the unweighted pair–grouping method (UPGMA) at 1% position tolerance to group similar profiles and to generate a binary matrix of band classes. Multi–dimensional Scaling (MDS) module in BioNumerics was used to spatially display PCR–DGGE profiles and to observe clustering trends among rumen fractions. Average D_{sc} (%) was calculated and used to compare the clustering trends between RFI groups and between breeds (ANG, CHA or HYB) within each rumen fraction. Since RFI has become the most desirable measure for characterising feed efficiency, it was selected to be correlated with the PCR-DGGE profiles (Moore et al., 2008).

4.2.3. Analysis of ruminal fermentation products.

Rumen fluid was subjected to VFA analysis using standard procedures for gas chromatography. An enzymatic assay was carried out to measure NH_3–N (R-Biopharm Roche Inc., South Marshall, MI, USA) as in Hernandez–Sanabria et al. (2010).

Proportions of each short-chain VFA relative to the total VFA concentration were obtained and used for the microbial metabolites statistical analysis (Hernandez-Sanabria et al., 2010). Rather than raw data, proportions were preferred to account for the sampling method influence, the dilution rate by the saliva and the differences in fermentation stage due to the time elapsed since last meal. Differences in VFA composition and NH_3–N between breeds (ANG, CHA or HYB) were compared using the simple covariance mixed model in SAS (SAS System, version 9.2; SAS Institute, Cary, NC). Significance was assumed at the $P < 0.05$.

4.2.4. Statistical Analysis.

Bands from all PCR–DGGE band profiles on each rumen fraction (liquid, contents or tissue) were obtained with BioNumerics Software, based on the positions assigned in the PCR–DGGE gel. Liquid fraction showed 85 band categories whereas the solid fraction presented 82 and tissue fraction had 83. These band categories were fitted to the previously reported categories (Hernandez-Sanabria et al., 2010). Associations between PCR–DGGE bands and breed were identified using a Chi-square analysis (PROC CATMOD in SAS), after which band frequencies (presence or absence) among breeds in a particular

rumen fraction were analysed. Relationships between PCR–DGGE bands and feed efficiency traits were determined after classifying individuals into High (H) and Low (L) groups under each variable using the CATMOD procedure. The dependant variable was the count of particular bands falling into H or L groups. The Medium (M) group was not further analysed because of the variability in their RFI values.

Frequency of breed–associated bands within rumen fraction was compared using 3×2 contingency tables of cross classifications containing the frequencies of the bands per breed, obtained with the FREQ procedure in SAS. Feed efficiency–associated bands within rumen fraction were obtained by 3×2 contingency tables created for each H or L group of the corresponding feed efficiency variable (DMI, ADG, FCR and RFI). Table probabilities were calculated using Fisher test when the count of any of the cells was below 5, otherwise Chi-square was preferred. For each band, to detect significant differences between any pair of frequencies, pair wise comparisons between breeds (ANG vs. CHAR, ANG vs. HYB and CHAR vs. HYB) were performed using the CATMOD procedure in SAS. Significant differences were declared at $P < 0.05$. Frequency of all bands was plotted for L–DMI, H– ADG, L–FCR, and L–RFI groups within rumen fraction.

4.3. RESULTS.

4.3.1. Evaluation of PCR – DGGE profiles among rumen fractions.

Structure of the bacterial community attached to feed particles and rumen epithelium was initially determined by PCR–DGGE analysis. Comparison of

bacterial profiles in rumen contents showed a similarity of 75.5% based on Dice similarity coefficient (D_{sc}); a trend for breed–related clusters was observed. Average similarity in rumen tissue profiles was 77.7% and no clear clustering tendency associated with breed was recorded in the dendogram, although some individuals tended to group according to RFI classification (Figure 4.1). Because dendograms failed to identify clear clustering tendencies, multidimensional scaling analysis (MDS) was performed using BioNumerics Software, showing a clustering trend according to rumen fraction (Figure 4.2).

4.3.2. Assessment of fermentation profiles and feed efficiency variables among breeds.

Volatile Fatty Acids (VFA) and Ammonia–N (NH_3–N) concentrations were measured to find associations between bacterial fermentation products and feed efficiency variables among different breeds. Total VFA and isovalerate proportion tended to be higher in Angus steers than in the other breeds, although they were not significantly different (Table 4.1). Angus steers tended to have lower DMI but higher ADG; however, high SD within the small number of samples did not allow detection of significant differences. Similarly, FCR tended to be the highest in hybrid steers ($P < 0.1$) and the lowest in Angus individuals (Table 4.2). Even though significant difference was not measurable, RFI in Angus steers could be classified as Low (RFI < 0.5) whereas Charolais and Hybrid steers could be considered within the Medium RFI group (see materials and methods).

Correlations among fermentation metabolites and feed efficiency measurements in steers from the same breed were obtained to provide insight on the variations in fermentation pathways potentially impacted by the breed (Table 4.3). In Hybrid steers, high ammonia was associated with high RFI ($P < 0.05$) whereas low ammonia was linked to high DMI in Angus animals ($P < 0.05$). No significant correlations among feed efficiency and ruminal fermentation variables in Charolais individuals were recorded.

4.3.3. Linkage of PCR–DGGE profiles, ruminal fermentation and feed efficiency measures with diverging breeds.

As described in Hernandez–Sanabria et al. (2010), the relationship between specific bands and fermentation/feed efficiency variables in different beef breeds was determined for each rumen fraction. Frequency analysis of the bands present in liquid showed that 35 bands in total were significantly different among breeds ($P < 0.05$), but a number of them were not associated with either breed (Figure 4.3.). Band 35 (Uncultured *Prevotella* sp.), band 42 (*Prevotella* sp.), band 64 (*Butyrivibrio fibrisolvens*) and band 76 (*Moryella indoligenes*) were absent in Angus steers. In contrast, band 2 (*Prevotella* sp.) and band 57 (*Eubacterium xylanophilum*) were the most frequent for that breed. Band 9 (*Blautia* sp.) and band 12 (*Prevotella denticola*) were not recorded in rumen liquid of Charolais steers while band 50 (*Prevotella ruminicola*) was the most frequent. No bands were exclusive of Hybrid steers in the liquid fraction.

In the solid fraction of the rumen, 17 bands were different among breeds ($P < 0.05$). Band 3 (*Prevotella maculosa*) was not present in Angus and Charolais steers, whereas band 51 (*Robinsoniella peoriensis*) was the most frequent in these breeds. Band 42 (*Prevotella* sp.) and band 67 (*Succinivibrio dextrinosolvens*) were also present in all Angus steers. Even if no band was particularly related to Hybrid steers, band 53 (*Ruminobacter amylophilus*) was the most frequent in this group, but it was not significantly different from other breeds. In rumen tissue, band 42 (*Prevotella* sp.), band 44 (Uncultured *Prevotella* sp.) and band 47 (*Hespellia porcina*) were common among all Angus and Hybrid steers. but only band 72 (*Clostridium indolis*) could be directly linked to Charolais individuals. In addition, band 63 (*Eubacterium rectale*) was exclusive of Angus steers and band 25 (*Prevotella oulora*) was absent in this breed (Figure 4.3.). Comparisons among rumen fractions within a particular breed were not performed because rumen liquid samples were not collected at the same time.

Within animals with high feed efficiency (Low RFI), trends in metabolic and feed efficiency indicators (DMI, ADG and FCR) have been reported (Tedeschi et al., 2006; Sadet et al., 2007). Therefore, frequency tables of the bands associated with all the positive feed efficiency traits (L–FCR, H–ADG, L–DMI and L–RFI) were created. In rumen liquid, seven bands were linked with L–DMI; from those, band 1 (*Prevotella* sp.) was associated with Angus steers and non-existent in Hybrid animals. Conversely, band 75 (Uncultured *Succinivibrio* sp.) was the most frequent in the Hybrid and missing in Angus breed (Figure 4.4A). Twenty bands were related to H–ADG in rumen liquid: 3 were Angus–

associated (Figure 4.4A), one Charolais–associated (Uncultured *Succinivibrio* sp.) and none was particularly related to Hybrid steers. Only 5 bands were related to L–FCR in liquid: band 2 (*Prevotella* sp.) was Angus–associated and missing in Hybrid individuals. In contrast, band 76 (*Moryella indoligenes*) was the most frequent in Hybrid but absent in Angus. Twenty–two bands were associated with L–RFI in liquid, 4 were Angus–associated and one was shared among all Hybrid and Charolais steers (Band 51, *Robinsoniella peoriensis*).

Four bands were related to L–DMI in the solid fraction: band 5 (Uncultured *Succinivibrio* sp.) was the most frequent in Hybrid steers while band 45 (Unknown) was related to Angus; band 72 (*Clostridium indolis*) was associated, but not exclusively, with Charolais individuals (Figure 4.4B). Twelve bands were linked to H–ADG in rumen contents; from them, 2 bands were related but not restricted to Angus steers and 3 bands had increased frequencies in Hybrid animals (Figure 4.4B). Eight bands were associated with L–FCR in contents: out of them, band 42 (*Prevotella* sp.) was shared among all Angus and Hybrid steers and one band (Band 51, *Robinsoniella peoriensis*) was common for Charolais and Angus animals. Seven bands were associated with L–RFI in contents; bands 46 (Uncultured *Prevotella* sp.) and band 50 (*Prevotella ruminicola*) were the most frequent in Hybrid whilst band 72 (*Clostridium indolis*) was predominant in Charolais.

Rumen tissue showed the highest number of bands related to L–DMI, with a total of 8 (Figure 4.4C). Four bands were linked to Angus but not to this breed alone; two bands were Charolais–associated and one was Hybrid–associated

(Band 10, *Clostridium symbiosum*). From the 15 H–ADG associated bands in tissue, four were shared among all Angus and Hybrid individuals (Figure 4.4C). Four bands were associated with L–FCR in tissue and only one (band 74, Uncultured *Succinivibrio* sp.) was the most frequent in Angus and had low or null association with the two other breeds. Thirteen bands were associated with L–RFI in rumen tissue; from them, band 30 (*Prevotella maculosa*) and band 46 (Uncultured *Prevotella* sp.) were shared among all Hybrid and Angus individuals whereas band 38 (*Clostridium indolis*) and band 43 (Uncultured *Roseburia* sp.) were Charolais–associated (Figure 4.4C).

4.4. DISCUSSION.

It was speculated that PCR–DGGE bacterial profiles may cluster based on the genetic background of the host as previously reported (Guan et al., 2008). Instead, it was observed that profiles tended to form rumen fraction-related clusters when all profiles were plotted together, suggesting that the majority of the dominant bacteria were not common to more than one spatial location (Figure 4.2.). This observation is in agreement with recent findings on the segregation of bacterial communities from ruminal solid digesta and epithelial tissue in beef cattle (Li et al., unpublished). Host factors are fundamental to determine the presence of particular microbial communities in the gastrointestinal tract (Wallace, 2008); however, the interactions of these communities with the host mechanisms responsible for the variations on the metabolic phenotype are completely unknown.

The observation of insignificant differences in VFAs among breeds might indicate that increased diversity of microbial communities in the rumen may enhance the resistance of the network of metabolic pathways, due to the use of alternative pathways (Firkins et al., 2007) to compensate differences in microbial fermentation products. Yet, this apparent diversification might negatively impact the energetic economy of the host, because FCR tended to be increased in Hybrid steers while Angus individuals could be considered more efficient according to their RFI value (RFI = -0.7, Table 4.2).

As in the previous study on the association between PCR–DGGE bands and host RFI (Hernandez-Sanabria et al., unpublished), similar linkages were observed with breed. PCR–DGGE bands specific from each rumen fraction varied when breed differed (Figure 4.3). Rumen liquid of Angus steers presented a high number of species belonging to the *Bacteroidales* family whereas *Clostridiales* were more abundant in Charolais. None of the bands was highly frequent (> 90%) in Hybrid individuals. The higher number of bands in Angus might be indicator of a more diverse community and, therefore, of a potentially more resistant microbial ecosystem (Firkins et al., 2007).

Bands from Charolais steers associated with rumen contents were restricted to bacterial species belonging to the *Clostridiales,* but an equal number of bands from *Clostridiales*, *Aeromonadales,* and *Bacteroidales* was noticed in Angus animals. Hybrid individuals showed a high frequency of *Prevotella* sp. (*Bacteroidales*). Solid-associated microbial community plays the most important role in feed digestion (McAllister et al., 1994), and recent evidence showed that

this community is organised as biofilms (Mayorga et al., 2007), an advantageous strategy that provides structural stability and a wider variety of substrates for their members. Stevenson et al. (2007) have reported that up to 60% of the total particle–attached bacterial population belongs to the *Prevotella* genus. While *Prevotella* sp. might predominate in biofilms of Hybrid steers, Charolais animals showed association of various species from the same bacterial family. Thus, it is likely that biofilm organization follows different strategies in different breeds. In Angus individuals, the diversity of bacterial families in the biofilm might be a mutualistic strategy of the community that had a favourable impact on feed efficiency of the host.

PCR–DGGE bands associated with rumen tissue among Hybrid and Angus were mostly from the *Clostridiales* family. Charolais animals showed a low number of bands representing this family and only one (*Clostridium indolis*) was present in all the steers from this breed. Like in the other two fractions, Angus steers had a greater number of bands than the other breeds and from different bacterial families. Higher bacterial diversity did not have any significant influence on the rumen fermentation variables among breeds. Hence, further studies using a larger number of animals and sequences would aid to clarify whether there is redundancy in the functions of particular bacterial populations, and thus, feed efficiency is not affected negatively.

Previous research has suggested that highly efficient steers had similar growth rates and final live weights but ate less per day and had lower RFI than low efficiency steers (or H–RFI) (Richardson et al., 2004). Cultured and

uncultured *Prevotella* spp. were highly frequent in the rumen liquid of Angus steers with L–DMI, H–ADG and L–RFI (Figure 4A). Conversely, the number of *Prevotella* sp. present in H–ADG, L–FCR and L–RFI was increased in the solid fraction of Hybrid individuals. Because high diversity in the *Prevotella* sp. has been suggested in previous reports (Purushe et al., 2010), it would be expected that their functions might be also diversified.

In rumen contents of Hybrid steers there seems to be a core population of particle–associated bacteria formed by *Prevotella* spp. that appears to be associated with positive feed efficiency traits, as mentioned above. *Prevotella* sp. is a proteolytic species that degrades dietary protein to peptides and ammonia–N to be used as a source of N (Baldwin et al., 1983; Purushe et al., 2010). In fact, the main peptidase activity in ruminal contents comes from an ammonia–degrading peptidase associated with *Prevotella* sp. (Purushe et al., 2010). It has been suggested that *Prevotella* sp. can contribute to hemicellulose degradation (Firkins, 2010). However, the disadvantage of that beneficial contribution is that the proteolytic capacity of *Prevotella* sp. might influence the NH_3–N metabolism. Because Hybrid steers tended to be more inefficient than their Angus peers and showed a negative correlation between NH_3–N and RFI, *Prevotella* sp. might negatively influences the assimilation of microbial protein in the rumen of Hybrid steers.

Bands representing *Clostridium indolis* were the most prevalent in rumen tissue of steers with L–RFI regardless breed (Band 38 in Charolais steers and Band 72 in Angus and Hybrid animals, Figure 4.4A–4.4C). This bacterial species

has been reported to possess nuclear dehydrogenases and mucinolytic activity (Karjalainen et al., 1997). As rumen epithelium represents the surface available for interactions between host and microbiota, the capacity of *C. indolis* to hydrolyse host mucin might help to build a mutually beneficial host–bacteria relationship. The advantages of such association are evident: mucin is constantly replenished due to cell turnover, its fermentation yield carbon and acetate for the host (Hooper et al., 2002) and competition is limited due to niche specificity. Some researchers (Larue et al., 2005) showed novel particle-attached bacterial species related to *Clostridia* (which use amino acids and peptides) and such species might have potential decreased deaminase activity. These mechanisms might be common among steers considered efficient and influence the metabolic phenotype. Uncultured *Roseburia* sp. (band 43) was highly frequent in Charolais animals with positive feed efficiency characteristics (excepting for H-ADG). The positive impact of this phylotype might be indirectly related to the potential regulation of gene expression and growth of rumen epithelial cells (Firkins, 2010).

Correlation analysis provided additional information on possible pathways that could be influenced by the microbial ecosystem interactions in a particular breed. Ruminal NH_3–N predicts the efficiency of dietary N conversion into microbial N (Firkins et al., 2007); when this concentration decreases, blood urea–N is transferred into the rumen (Russell et al., 1981). Low blood urea concentration has been associated with lean growth (Herd et al., 2009), L–RFI (Richardson et al., 2004) and decreased feed intake (Richardson et al., 2004);

hence H–RFI steers have higher rate of protein degradation than L–RFI individuals (Richardson et al., 2004). In our study, low DMI correlated with increased ruminal NH_3–N in Angus steers, contrasting with the positive correlation between NH_3–N and RFI in Hybrid individuals (Table 4.3). These contradictory scenarios might reflect differences in the efficiency of N incorporation into the microbial protein synthesis among individuals with diverging genetic background but similar RFI (Table 4.3). However, to achieve a more complete understanding of the associations between fermentation and feed efficiency measurements, it is necessary to include multiple sampling points during the collection period of feed intake data, as well as a defined control group. In addition, RFI is an index that could be influenced by a number of factors (Moore et al., 2008; Herd et al., 2009) and the above mentioned interactions among bacterial populations might represent only a component of the total variation in feed efficiency.

4.5. CONCLUSIONS.

In summary, probable relationships between bacterial species and feed efficiency measurements in beef cattle with different sire breed were identified. To my knowledge, this is the first study attempting to describe potential interactions between bacterial PCR-DGGE band patterns in the rumen, host metabolic phenotype and genetic composition. Bacterial species such as *Prevotella* sp. might play a decisive role on the NH_3–N metabolism in the rumen. The development of molecular tools have revealed the extraordinary richness of bacterial species in the rumen (Michelland et al., 2008) and metagenomic

techniques have provided additional knowledge of the bacterial community and their potential functions impacting host performance (Hegarty et al., 2007; Zhou et al., 2010). Future studies to characterise their specific ecological functions within a community and interactions with host factors are necessary to reveal effective strategies to manipulate and improve animal performance.

Table 4.1. Feed Efficiency and Ruminal Metabolic Measurements in steers differing breed and Residual Feed Intake (n = 48).

Variable	Breed			*P* value
	Angus Mean ± SEM (n = 5)	Charolais Mean ± SEM (n = 19)	Hybrid Mean ± SEM (n = 24)	
Acetate (%) *	49.19 ± 1.90	50.10 ± 0.97	49.66 ± 0.86	0.89
Propionate (%) *	36.89 ± 3.38	36.42 ± 1.73	37.09 ± 1.54	0.96
Butyrate (%) *	7.73 ± 1.53	8.45 ± 0.79	8.39 ± 0.70	0.91
Isobutyrate (%) *	0.66 ± 0.13	0.67 ± 0. 07	0.63 ± 0.06	0.90
Valerate (%) *	2.45 ± 0.28	2.44 ± 0.15	2.50 ± 0.13	0.99
Isovalerate (%) *	2.90 ± 0.74	1.78 ± 0.38	1.59 ± 0.34	0.28
Total VFA (mM)	121.41 ± 11.70	109.97 ± 6.00	106.92 ± 5.34	0.53
Acetate : Propionate ratio	1.48 ± 0.26	1.46 ± 0.13	1.44 ± 0.11	0.99
Straight VFA: Branched VFA ratio	49.35 ± 14.43	49.32 ± 7.40	62.43 ± 6.59	0.38
Ammonia (mM)	0.19 ± 0.03	0.17 ± 0.02	0.20 ± 0.01	0.40
Dry Matter Intake (kg DM)	10.11 ± 0.48	10.72 ± 0.25	11.02 ± 0.22	0.21
Average Daily Gain (kg)	1.87 ± 0.10	1.81 ± 0.05	1.80 ± 0.05	0.72
Feed Conversion ratio (F:G)	5.46 ± 0.37	5.82 ± 0.20	6.21 ± 0.17	0.12
Residual Feed Intake	-0.76 ± 0.40b	-0.18 ± 0.20a	0.11 ± 0.18a	0.13

* Values are given as a proportion of the total VFA concentration. Different superscripts indicate significant differences among groups.

Table 4.2. Feed Efficiency Measurements in steers from diverging sire breed and differing Residual Feed Intake (only H-RFI and L-RFI individuals, n = 27).

Variable	Breed			*P* value
	Angus Mean ± SEM (n = 5)	Charolais Mean ± SEM (n = 10)	Hybrid Mean ± SEM (n = 12)	
Dry Matter Intake (kg DM)	10.11 ± 0.56	10.32 ± 0.40	10.92 ± 0.36	0.37
Average Daily Gain (kg)	1.87 ± 0.10	1.81 ± 0.07	1.71 ± 0.07	0.39
Feed Conversion ratio	5.46 ± 0.45	5.73 ± 0.32	6.53 ± 0.29	0.09
Residual Feed Intake	-0.76 ± 0.53	-0.31 ± 0.37	0.21 ± 0.34	0.28

* Values are given as a proportion of the total VFA concentration.

Table 4.3A. Correlation (r) of fermentation parameters in the rumen of Angus steers differing RFI with indicators of feed efficiency (RFI, DMI, ADG and FCR). n = 5, ******P* **< 0.0001, *****P* **< 0.05, *trend.

Variable	RFI	DMI	ADG	FCR
Acetate	NS	NS	NS	NS
Propionate	NS	NS	NS	NS
Butyrate	NS	NS	NS	NS
Isobutyrate	NS	NS	NS	NS
Valerate	0.834*	NS	NS	NS
Isovalerate	NS	NS	NS	NS
Total VFA	NS	NS	NS	NS
A:P ratio	NS	NS	NS	NS
Straight VFA : Branched VFA ratio	NS	-0.814*	NS	NS
NH_3-N	-0.816*	-0.961**	NS	-0.830*
RFI		0.884**	NS	NS
DMI			NS	NS
ADG				NS

Table 4.3B. Correlation (r) of fermentation parameters in the rumen of Charolais steers differing RFI with indicators of feed efficiency (RFI, DMI, ADG and FCR), n = 19. * *P* < 0.0001, ** *P* < 0.05, *trend.**

Variable	RFI	DMI	ADG	FCR
Acetate	NS	NS	NS	NS
Propionate	NS	NS	NS	NS
Butyrate	NS	NS	NS	NS
Isobutyrate	NS	NS	NS	NS
Valerate	NS	NS	0.445*	NS
Isovalerate	NS	NS	-0.528**	NS
Total VFA	NS	NS	NS	NS
A:P ratio	NS	NS	NS	NS
Straight VFA : Branched VFA ratio	NS	NS	0.477**	NS
NH_3-N	NS	NS	NS	NS
RFI		0.761**	NS	0.548**
DMI			0.522**	NS
ADG				-0.615**

Table 4.3C. Correlation (r) of fermentation parameters in the rumen of Hybrid steers differing RFI with indicators of feed efficiency (RFI, DMI, ADG and FCR), n =24. * *P* < 0.0001, ** *P* < 0.05, *trend.**

Variable	RFI	DMI	ADG	FCR
Acetate	NS	NS	-0.390*	NS
Propionate	NS	NS	NS	NS
Butyrate	NS	0.361*	NS	NS
Isobutyrate	NS	NS	NS	NS
Valerate	NS	NS	NS	NS
Isovalerate	NS	NS	NS	NS
Total VFA	NS	NS	NS	NS
A:P ratio	NS	NS	NS	NS
Straight VFA : Branched VFA ratio	NS	NS	NS	NS
NH_3-N	0.477**	NS	NS	0.345*
RFI		0.491**	-0.445**	0.745***
DMI			0.344*	NS
ADG				-0.788***

Figure 4.1A. PCR-DGGE profiles generated from ruminal contents DNA from forty-eight steers fed high energy density diet using primers HDA1-GC and HDA2 (22 to 55% DGGE). H and L represent the steers with high RFI (H-RFI > 0.5, inefficient), M-RFI (-0.5 < RFI < 0.5) and low-RFI (L-RFI < -0.5, efficient), respectively. RFI, residual feed intake, a parameter to measure feed efficiency in cattle (Basarab et al., 2003). The comparison of the PCR-DGGE profiles was generated with the BioNumerics software package using UPGMA (unweighted pair-group) method as described in the text.

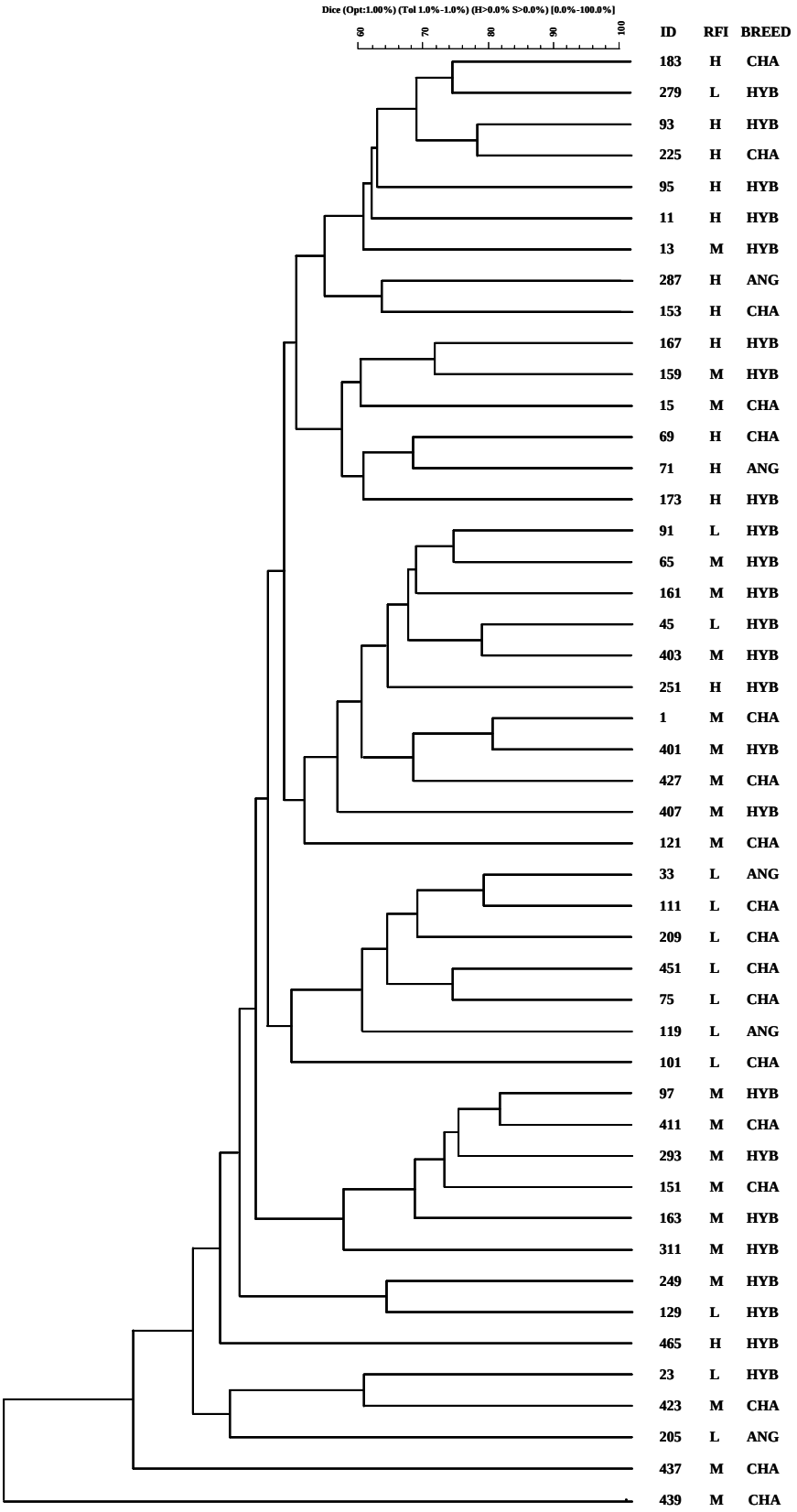

Dice (Opt:1.00%) (Tol 1.0%-1.0%) (H>0.0% S>0.0%) [0.0%-100.0%]
60
70
80
90
100
ID RFI BREED
183 H CHA
279 L HYB
93 H HYB
225 H CHA
95 H HYB
11 H HYB
13 M HYB
287 H ANG
153 H CHA
167 H HYB
159 M HYB
15 M CHA
69 H CHA
71 H ANG
173 H HYB
91 L HYB
65 M HYB
161 M HYB
45 L HYB
403 M HYB
251 H HYB
1 M CHA
401 M HYB
427 M CHA
407 M HYB
121 M CHA
33 L ANG
111 L CHA
209 L CHA
451 L CHA
75 L CHA
119 L ANG
101 L CHA
97 M HYB
411 M CHA
293 M HYB
151 M CHA
163 M HYB
311 M HYB
249 M HYB
129 L HYB
465 H HYB
23 L HYB
423 M CHA
205 L ANG
437 M CHA
439 M CHA

Figure 4.1B. PCR-DGGE profiles generated from ruminal tissue DNA from forty-eight steers fed high energy density diet using primers HDA1-GC and HDA2 (22 to 55% DGGE). H and L represent the steers with high RFI (H-RFI > 0.5, inefficient), M-RFI (-0.5 < RFI < 0.5) and low-RFI (L-RFI < -0.5, efficient), respectively. RFI, residual feed intake, a parameter to measure feed efficiency in cattle (Basarab et al., 2003). The comparison of the PCR-DGGE profiles was generated with the BioNumerics software package using UPGMA (unweighted pair-group) method as described in the text.

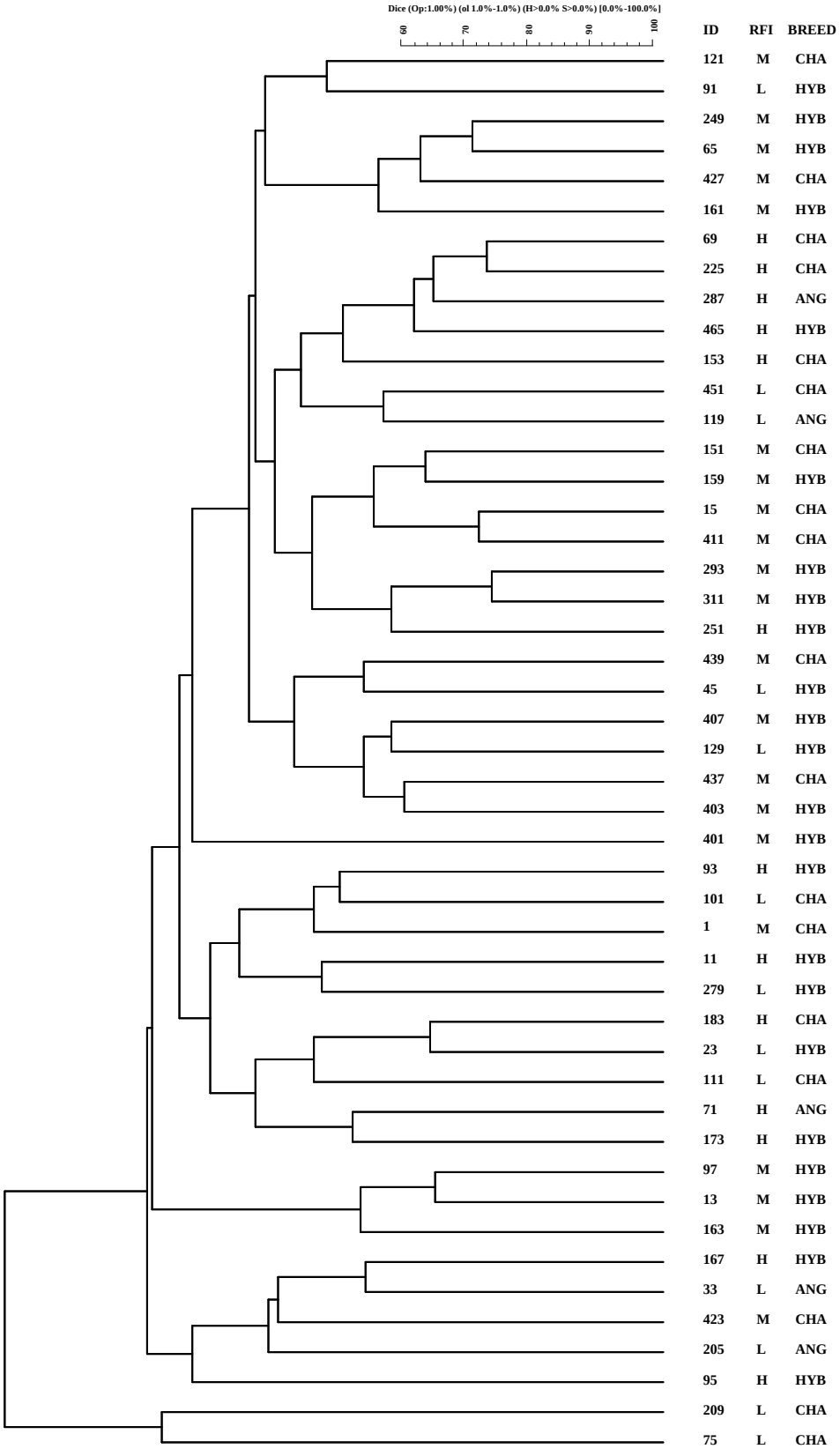

Dice (Op:1.00%) (ol 1.0%-1.0%) (H>0.0% S>0.0%) [0.0%-100.0%]
60
70
80
90
100
ID RFI BREED
121 M CHA
91 L HYB
249 M HYB
65 M HYB
427 M CHA
161 M HYB
69 H CHA
225 H CHA
287 H ANG
465 H HYB
153 H CHA
451 L CHA
119 L ANG
151 M CHA
159 M HYB
15 M CHA
411 M CHA
293 M HYB
311 M HYB
251 H HYB
439 M CHA
45 L HYB
407 M HYB
129 L HYB
437 M CHA
403 M HYB
401 M HYB
93 H HYB
101 L CHA
1 M CHA
11 H HYB
279 L HYB
183 H CHA
23 L HYB
111 L CHA
71 H ANG
173 H HYB
97 M HYB
13 M HYB
163 M HYB
167 H HYB
33 L ANG
423 M CHA
205 L ANG
95 H HYB
209 L CHA
75 L CHA

Figure 4.2. Multidimensional Scaling Analysis of the PCR-DGGE profiles generated from ruminal fluid, digesta and tissue DNA from forty-eight steers fed with low and high energy density diet, using primers HDA1-GC and HDA2 (22 to 55% DGGE). Colours represent a particular rumen fraction: blue, rumen liquid; green, rumen contents and pink, rumen tissue. The comparison of the PCR-DGGE profiles was generated with the BioNumerics software package using UPGMA (unweighted pair-group) method as described in the text; comparison was optimised using the default settings of the software.

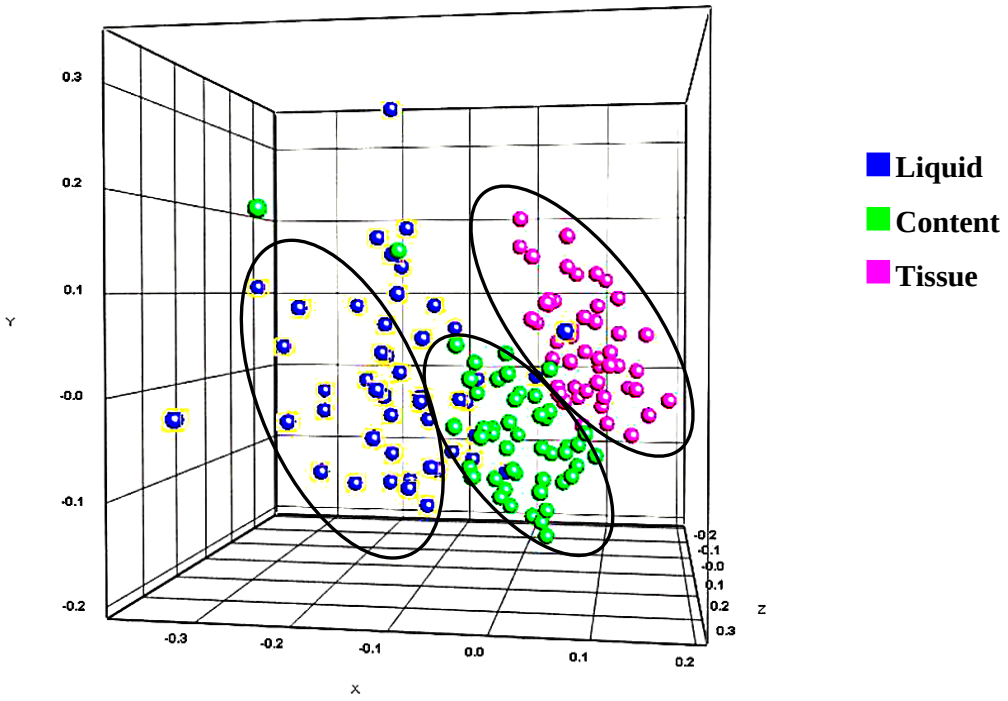


Figure 4.3. Specific bacterial species correlated to particular rumen fractions. Asterisks indicate the number of species significantly different within each group; taxonomy of the species with high frequency among breeds (>90%) is indicated in the squares corresponding to each quadrant.

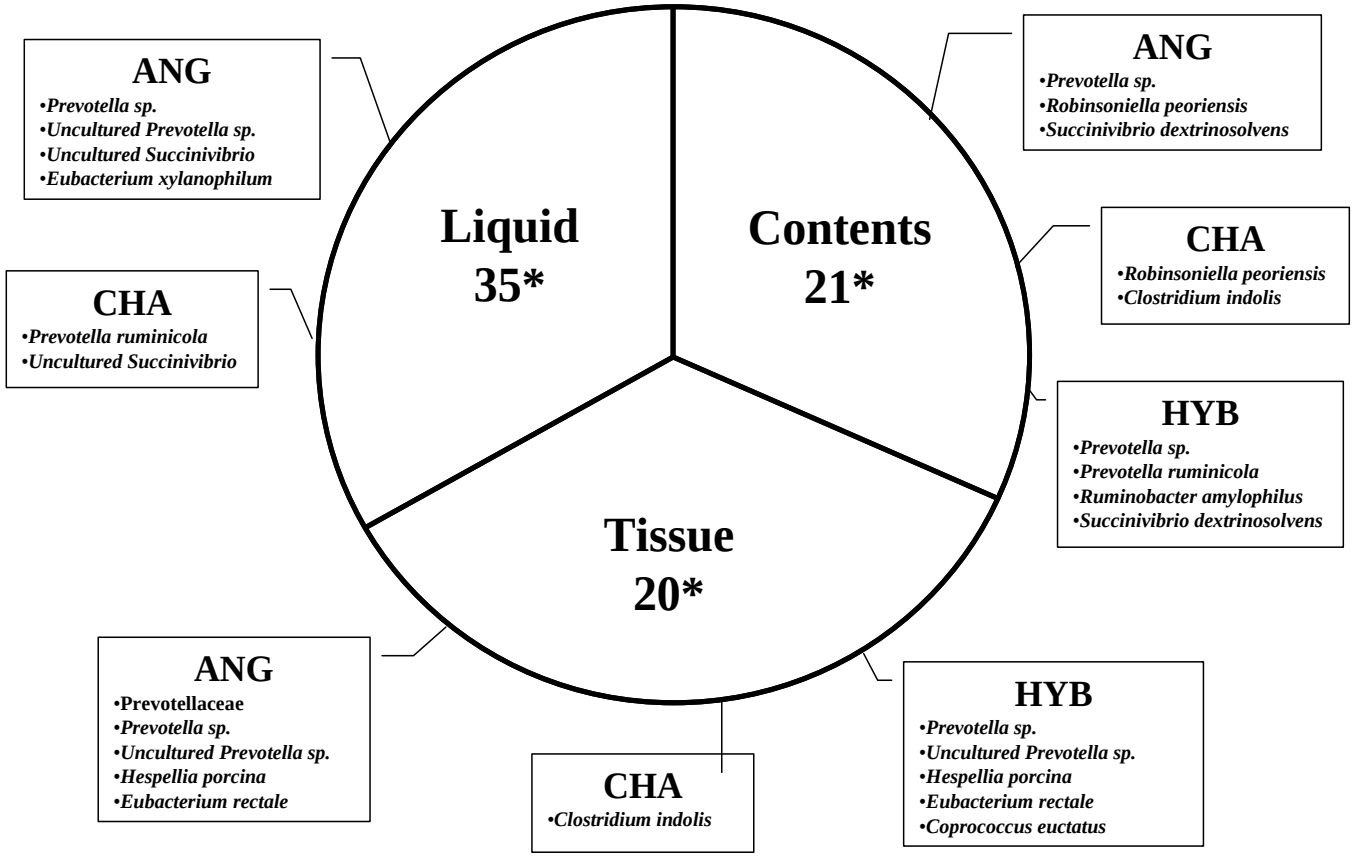


Figure 4.4A. Frequency of PCR-DGGE bands in animals categorized on the basis of Low Dry Matter Intake (L–DMI), High Average Daily Gain (H–ADG), and Low Feed Conversion Ratio (L–FCR, F:G), in rumen liquid using PROC CATMOD analysis. The x-axis represents 85 identified bands and the symbols plotted reflect the frequency of the bands detected in the tested the population of each trait. * $P < 0.05$.

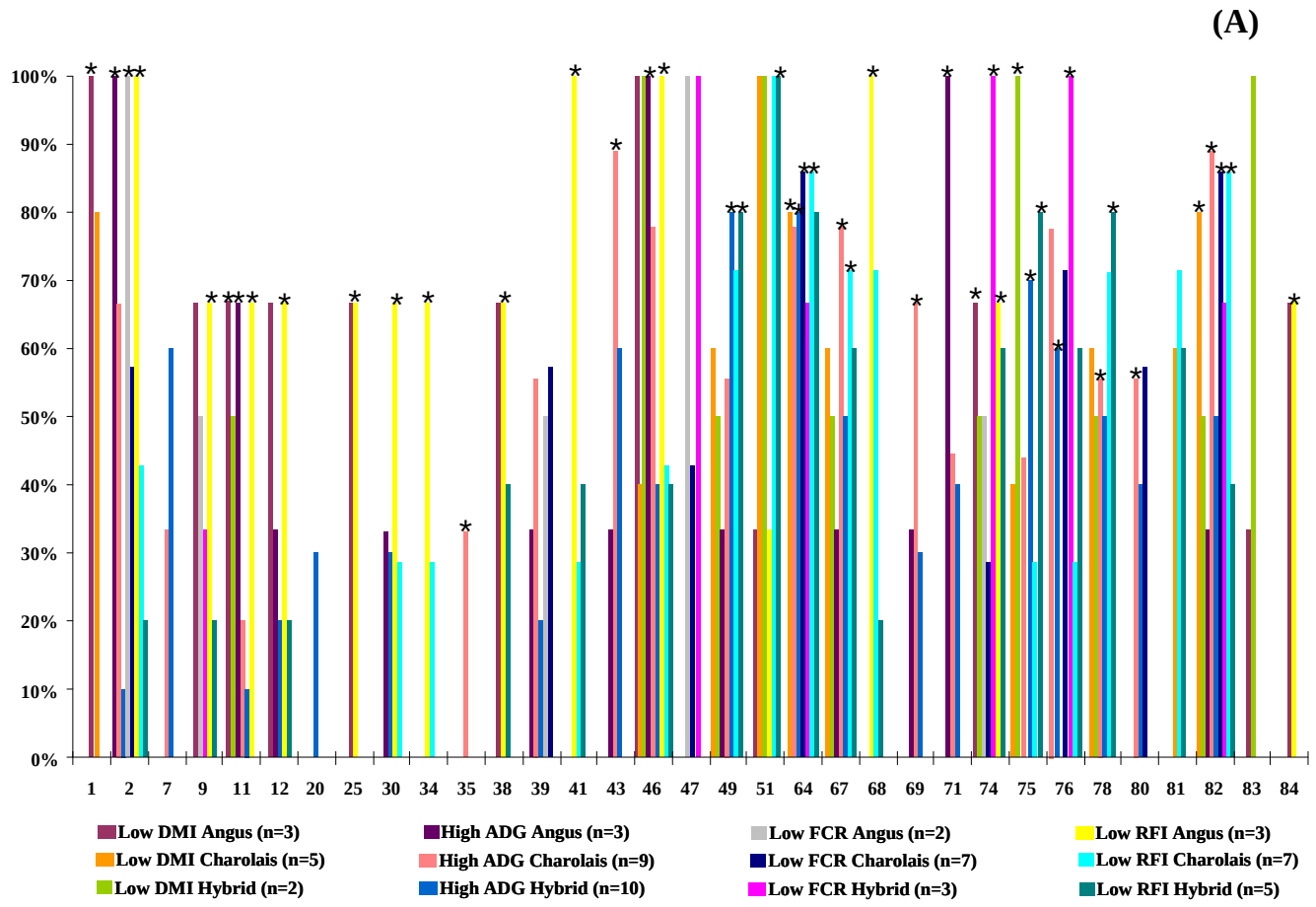


Figure 4.4B. Frequency of PCR-DGGE bands in animals categorized on the basis of Low Dry Matter Intake (L–DMI), High Average Daily Gain (H–ADG), and Low Feed Conversion Ratio (L–FCR, F:G), in rumen digesta using PROC CATMOD analysis. The x-axis represents 85 identified bands and the symbols plotted reflect the frequency of the bands detected in the tested the population of each trait. * *P* < 0.05.

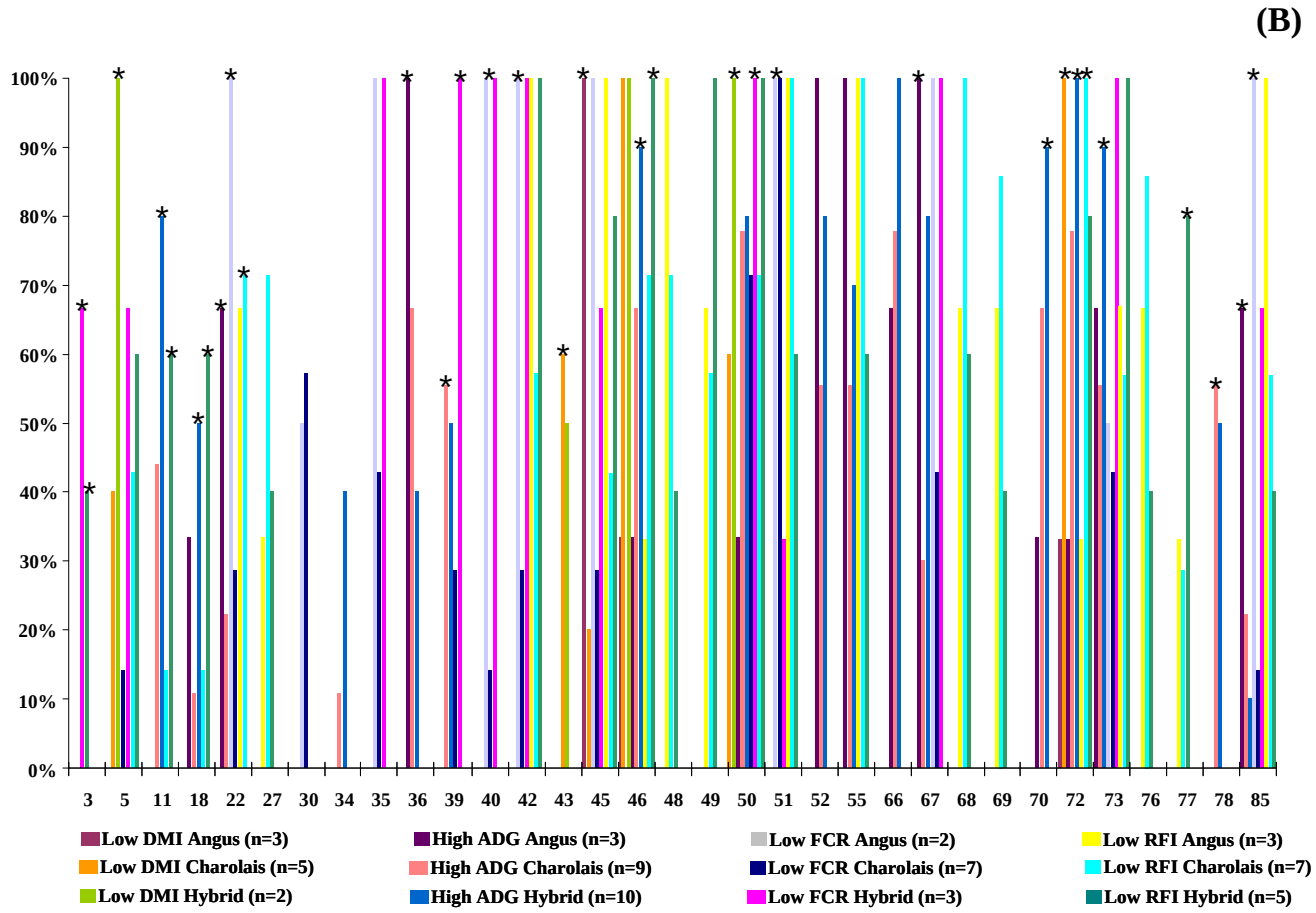
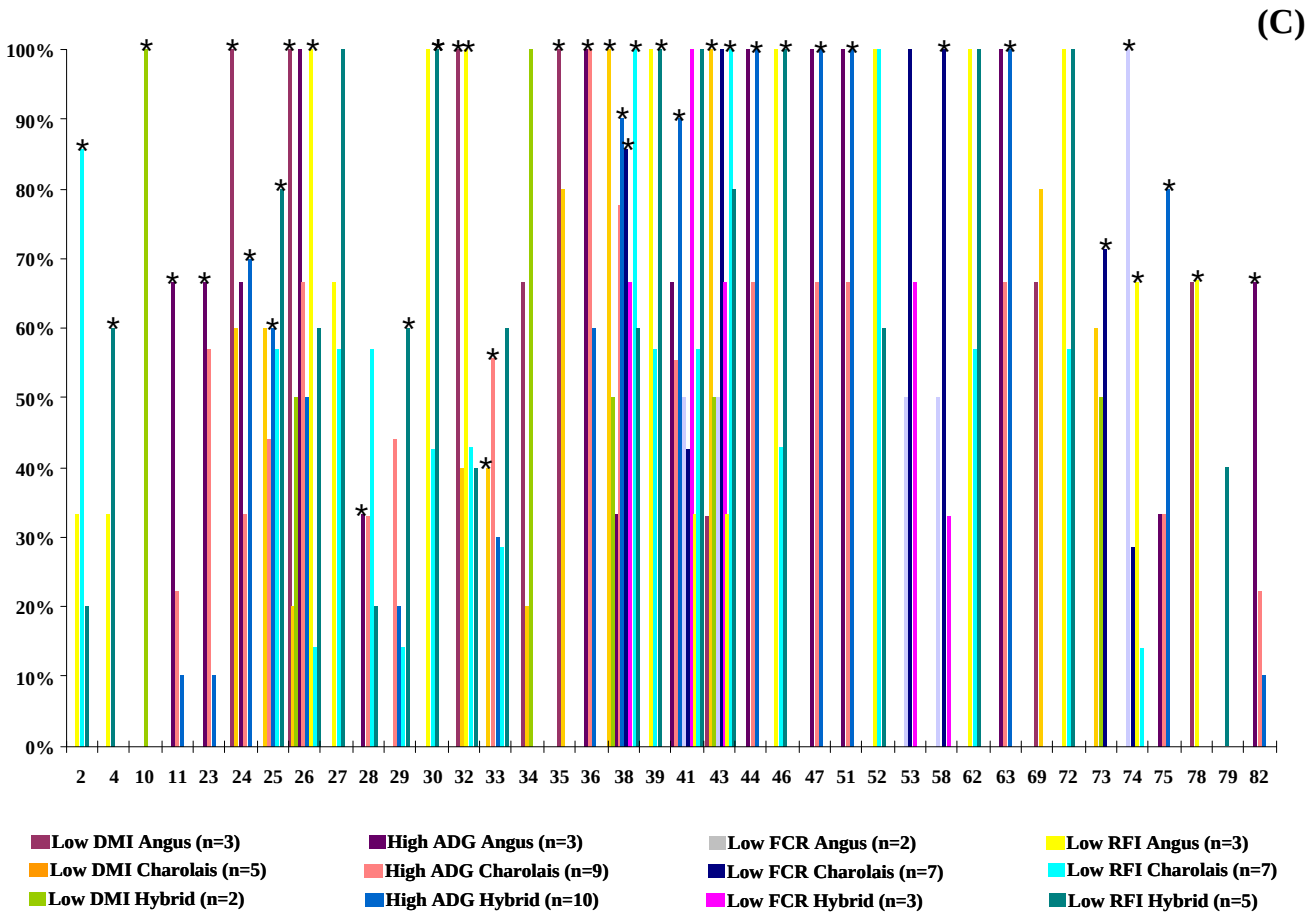


Figure 4.4C. Frequency of PCR-DGGE bands in animals categorized on the basis of Low Dry Matter Intake (L–DMI), High Average Daily Gain (H–ADG), and Low Feed Conversion Ratio (L–FCR, F:G), in rumen tissue using PROC CATMOD analysis. The x-axis represents 85 identified bands and the symbols plotted reflect the frequency of the bands detected in the tested the population of each trait. * $P < 0.05$.



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Chapter 5. General Discussion.

5.1. Development of a statistical analysis to link the PCR-DGGE bacterial profiles to phenotypic parameters.

Fingerprinting methods such as PCR-DGGE have been extensively applied to screen environmental microbial communities. Until recently however, the interpretation of these band patterns was constrained to a visual assessment, which impedes to accurately evaluate the statistical significance and to interpret the outcome from the analysis. These limitations were overcome by developing a statistical approach to link the PCR-DGGE profiles to host phenotypic measurements such as feed efficiency and rumen fermentation parameters. Therefore, the relationship between rumen bacteria under both feed trials and RFI was evaluated using a multivariate statistical analysis in this project. In DGGE analysis, band patterns are considered to reflect the diversity of a bacterial community, each band theoretically representing a unique sequence or phylotype (Muyzer et al., 1998). Co-migration of bands corresponds to identical sequences (Kowalchuck et al., 1997). Nevertheless, as each bacterial species or strain may generate multiple band patterns due to the PCR bias (Muyzer, 1999) and because most bacterial species may have multiple copy number of 16S rRNA gene, assigning a single band to a single bacterial population should be interpreted cautiously. In fact, PCR-DGGE analysis must be accompanied by sequencing techniques to attain a precise identification of the members of the bacterial community, given that DGGE patterns outlines the community structure rather than the actual richness of the bacterial population.

The initial approach to evaluate differences among DGGE patterns involved observing changes in the presence/absence of single bands; thus, banding patterns were converted to a binary matrix. Similarities between banding patterns can be performed through pairwise comparisons and expressed as a percentage of a similarity coefficient (van der Gucht et al., 2001). The Dice coefficient (D_{sc}) assigns a double weight to the bands that are present on both entries; it gives the overlap between both samples ignoring the bands that are absent on these both entries but taking into account the bands that are present on other samples from the comparison. This method has been used to compare the similarity of PCR-DGGE profiles so to assess the differences in the detectable microbial diversity. The D_{sc} is the same as the Nei-Li coefficient, known to be the most suitable coefficient to determine genetic relatedness based upon DNA restriction fragment patterns (Nei et al., 1979) and which can be applied to other amplified fragments, such as DGGE bands. This coefficient has a straightforward biological interpretation because it measures the proportion of fragments that two samples share, as they have been inherited from a common ancestor. Similar to D_{sc}, it is computed as the proportion of positive bands shared by both samples, divided by the average of the proportion of bands present in sample *i* and sample *j*. In this way, D_{sc} indicates similarity among species present and it is identical to the Sørensen similarity index. The initial analysis of the bacterial diversity was performed using D_{sc} to compare the band patterns to the total profile. Nevertheless, this analysis only provided grouping trends and disregarded the potential importance of individual bands.

Similarity matrices can be graphically displayed as hierarchical cluster comparisons, namely dendograms. To join similar band patterns, techniques such as the unweighted pair group method using arithmetic averages (UPGMA) have been reported. UPGMA uses an algorithm to calculate the arithmetic average from all the individual similarities between samples of a given cluster and samples of a new cluster (Gronau et al., 2007). Hence, band patterns representing the numerically dominant species of the bacterial population are clustered in groups according to the relatedness of the patterns. This methodology overlooked which operational taxonomic units or particular bands were important and only stated that differences existed. Therefore, alternative strategies were evaluated in this study to find the associations between rumen bacteria and feed efficiency/metabolic variables.

Although variance analysis can determine the cause and effect of a dependable variable at a time, for instance, whether the presence/absence of a band had impact on feed efficiency measurements, joint effects can better be determined by a multivariate approach, such as the principal component analysis (PCA). Furthermore, PCA transforms the original variables into new variables or axes called principal components, which are linear combinations of the original variables with coefficients consolidating the correlations (or loadings) within PCs and the original variables (Goonewardene et al., 2004). Each PC is independent from each other and explains the dispersion of the samples, but only for linear relationships. The first PC accounts for the majority of the variation and the following PCs account for decreasing percentage of the total variation. The size of

the variation for each component (vector) is indicated by the latent root or eigen value. This method was useful to detect potential sources of variation among fermentation and feed efficiency measurements. Although components were independent from each other, the variables within the components were not. This is because the underlying model of PCA assumes that there are linear relationships among components and thus, that biological populations have a linear response, which might not be necessarily accurate. Further, PCA was unable to handle large numbers of categorical variables (presence/absence), as the 85 band categories detected in the present studies. Therefore, PCA was only considered a method to sort sources of variation and potential relationships among data.

As opposed to the regression analysis, correlations were preferred to find associations among rumen fermentation and feed efficiency variables. The variability of each measurement within either H- or L-RFI groups was already determined using the analysis of variance. Other techniques such as multiple regression analysis have been utilised to elucidate the relative importance of each variable, within a number of independent variables, in determining the magnitude of a single dependent variable to be estimated. Because we were not assigning dependence or indepedence to all the variables, which is one of the main assumptions of any regression analysis, multiple linear regression was not employed. Moreover, one should be aware that there is always internal correlation between variables. The variable with the greater range in values will account for a larger portion of the variation and may mask the values with more important

biological significance. Further, the number of measurements obtained in this study that could be used to the predict RFI might not be adequate, because there was only one unreplicated RFI value and the significance of the regression could not be quantified; indeed, this fitted equation could be only valid for the current group of animals and would not be useful to predict trends in similar cohorts, if only using multiple regression analysis. Finally, the relationship with the bacterial phylotypes could not be assessed using only this model.

Therefore, a maximum likelihood approach was used to reveal the direct linkage between a specific group of bands and particular fermentation/feed efficiency estimates because the above PCA and regression analyses failed to include all the bands as variables as well as to show the direct linkage among different variables. Using a chi-square model, the effect of all variables on the prevalence of every band was determined based on the transformation of the cell probabilities (response function). This model analysed a data matrix containing either the averaged Gaussian position of the band or zero, indicating class. The CATMOD Procedure in SAS performed categorical data modelling that was represented by a contingency table and calculated chi-square values for Linear models of response frequencies (presence/absence of a band in our study). Chi-square was an appropriate analysis because we had count data: within each band category we had a number of animals either showing the band or not; the response variable was then categorical and dichotomous (presence/absence). Indeed Chi-square analysis reveals interactions rather than differences between categories, which was one of the main objectives of the project. Because the data did not fit

the assumptions of a non-parametric ANOVA model, such approach was not suitable. The estimation of the linear model type in CATMOD was using the default maximum-likelihood method, which is an iterative procedure and is considered to be the best method when the counts are small.

Categorical data with more than two factors at a time can be also analysed using the FREQUENCY procedure in SAS. Therefore, we obtained two-way contingency tables of cross classifications containing the frequencies of the bands per category (for instance, High/Low RFI). By having cross classifications, we attempted to address two objectives: first, whether feed efficiency/metabolic variables were independent or associated with the band presence/absence and, second, whether the distribution of the bands was consistent for each variable. Analysis of band frequencies did not determine the species present, but provided information about the interactions between presence/ absence of a particular band in a given RFI category, for example. The same analysis was applied to observe interactions with the rest of the feed efficiency and rumen fermentation variables. The band frequency analysis also pinpointed how often a particular band appeared in the tested population.

However, DGGE gels can display divergent banding patterns because of small but significant differences among conditions that could not be overcome with the normalizing procedure from BioNumerics. The only way to correctly identify co-migrating bands from different gels was by excising and sequencing DNA from all the detected bands. In addition, because some bands had low frequency, validation techniques were applied to verify the presence of bacterial

populations. PCR-DGGE analysis without any confirming sequence information as well as an appropriate statistical approach provides little evidence to support ecological hypotheses attempting to describe the relationships between microbial diversity in the rumen and their ecological niches.

5.2. IDENTIFICATION OF BACTERIAL SPECIES ASSOCIATED TO RESIDUAL FEED INTAKE (RFI) IN THE RUMINAL LIQUID.

The rumen ecosystem is found to be an increasingly complex system of vital importance for the productivity of ruminant livestock. Previous research performed by Guan et al. (2008) revealed potential correlations between the rumen microbial ecology with feed efficiency in beef cattle. Therefore, one of the objectives of this study was to determine whether there was any association between bacterial diversity and RFI in beef cattle as well as to observe the diet-related changes in the bacterial population. Because the majority of the rumen bacteria have not been identified or cultured, PCR-DGGE analysis and further sequencing could enable the detection of microorganisms at deeper level. As cited by Zhou et al. (2009), there is potential to detect variations even at species or strain level using the techniques applied in this study. Based on these observations and in the non-conclusive trends in the hierarchical clustering, it was hypothesised that variations in the fermentation and feed efficiency variables may be associated with some particular bacterial species (bands) rather than with the whole bacterial structure. Ultimately a number of specific bacterial bands on the DGGE gels were shown to be correlated to different feed efficiency and fermentation characteristics. The results of Study 1 showed that a fraction of the bacterial

phylotypes was continuously present throughout individuals, which suggested that rumen microbiota in cattle, like in the gut of humans, consists of a stable core of colonising microbes. Despite the differences in band frequency among individuals with diverging RFI, there were indicators that microbial phylotypes are shared, indicating that the ruminal microbiota is diverse but dominated by a number of conserved bacterial species.

Despite the differences observed in band frequencies in terms of the distribution in the RFI categories, the proportions of the fermentation variables were not significantly different between groups of animals. One of the fermentation measurements, VFA in the rumen, are the net result of balancing microbial production and host epithelial absorption. Therefore, the concentrations of VFA were considered as one of the indicators of microbial-microbial as well as host-microbial interactions. Although possible associations between some VFA and feed efficiency traits were demonstrated, data were limited and could be biased when looking at the results only under the low energy diet. In fact, from Study 1 it could not be concluded whether the whole microbial profile was more important than particular bacterial phylotypes. Moreover, the diet effect needed also to be taken into account to observe how it affected the relationship between microbial populations and host RFI. Thus, in Study 2, variations in the population of rumen bacterial species that were influenced by diet and had a potential role in the feed efficiency of the host were identified. In the LE diet, the predominant bands in the majority of the steers were identified as *Eubacterium* sp. (band 63, in 100% of animals) and as *Robinsoniella peoriensis* (band 62, in 96.4% of steers).

Least frequent bands included *Prevotella ruminicola* (Band 18), and *Pelotomaculum thermopropionucum* (band 21). In contrast, *Succinivibrio dextrinosolvens* (band 54) was present in more than 90% of the animals when they were switched to HE diet. These results are in line with previous reports; this species has been detected when diets containing large amounts of rapidly fermented carbohydrates are fed (Gomez-Alarcon et al., 1982). From these observations, it was evident that when animals were fed a LE diet, they showed higher bacterial diversity and high frequency of *Firmicutes* species (54.5% vs. 27.3% of *Bacteroidetes* and 18.2% of both *Proteobacteria* and *Actinobacteria*); and under HE diet, the abundance of *Firmicutes* was even higher (62.5%). Because band patterns from efficient steers showed a higher similarity between diets than those from inefficient steers, we can infer that several bacterial phylotypes fill specific ecological niches, and that each niche is fulfilled differently from animal to animal (Durso et al., 2010). Hence, regardless the changes in the diet, the composition of the microbiota in the ruminal ecosystem of the efficient steers function in the same stable manner, for example. The plasticity of the rumen microbiota in efficient steers offers a competitive advantage over inefficient individuals, because they are able to adapt to variations in the environment and their functional redundancy permits continuous nutrient supply for the ecosystem. Plain detection of bacterial species in a rumen fluid sample reveals little of their ecological role in the rumen, or even if they are metabolically active; it is fundamental to take into account that DGGE patterns only summarise the community structure rather than the actual richness of the

bacterial population. However, the phenotypic data recorded and their statistical correlations with specific bacterial species demonstrated in Study 2 provided evidence of the impact that bacterial interactions have on host functions. Such relationships may be resulting of interactions among the different bacterial groups in the rumen; nevertheless, DGGE analysis coupled with generation of confirming sequences increases the robustness of the technique.

5.3. Assessment of the relationships among the three bacterial populations inhabiting the rumen and host's Residual Feed Intake (RFI).

Although Study 1 provided an overview of the consortia present in the liquid phase of the rumen, it did not provide sufficient information about the total ruminal RFI-associated microbiota. Therefore, a section of Study 3 aimed to provide a more complete insight on the interactions among rumen microflora that had an impact on host's performance. The interactions among planktonic, particle feed-attached, and epimural bacteria in the rumen of steers under HE diet were assessed by hierarchical cluster comparisons and multivariate statistical analysis. This enabled me to observe the structure of the bacterial community and to determine the influence that both host and RFI played in the clustering of PCR-DGGE profiles. Differences in the band patterns from the three bacterial populations were detected within each individual. As a result, when profiles were plotted together, they tended to cluster by similar fraction rather than by individual, suggesting that the majority of the dominant bacteria were not common to more than one spatial location. In fact, recent studies demonstrated

that bacterial populations between rumen fractions within the same diet are significantly different (Kong et al., 2010b). This indicates that, although the differences in bacterial species in liquid fraction among animals are important (Studies 1 and 2), the differences in the whole bacterial profile between fractions are greater but not always have a direct impact on host phenotype. Study 2 was fundamental to establish that variations in the population of particular bacterial phylotypes are influenced by diet and may impact feed efficiency.

Because the comparison of the three fractions altogether could not be performed since the rumen liquid samples were collected at different time points and results could be biased, frequency tables of the bands associated with all the positive feed efficiency traits (L–FCR, H–ADG, L–DMI and L–RFI) were created for each rumen fraction. In this way, the whole feed efficiency-associated microbiota could be assessed separately to obtain a general overview. *Butyrivibrio* sp., *Prevotella* sp. and *Clostridium* sp. were associated to all feed efficiency traits in rumen liquid, particulate fraction and in tissue, respectively. Future studies to characterise their specific ecological functions within the rumen community and interactions with host factors are necessary to reveal effective strategies to understand their impact on animal performance. Once diet effect was explored, the need to consider the host influence on these variables occurred. As previously mentioned, host factors are fundamental to determine the presence of particular microbial communities in the gastrointestinal tract (Wallace, 2008); however, the interactions with the host mechanisms responsible for the variations on the metabolic phenotype were completely unknown.

5.4. Evaluation of the impact of host genetic background and Residual Feed Intake (RFI) on microbial populations in the rumen.

Previous molecular studies had demonstrated that the composition of the bacterial community is host-specific and stable over time (Seksik et al. 2003; Vanhoute et al. 2004), indicating that the host effects on the microbial community cannot be neglected (Hooper et al. 2002). Furthermore, bacterial community is species-specific under the same diet and environmental conditions (Shi et al., 2008). From the two previous studies, it was not possible to find whether the identified RFI-associated phylotypes in the rumen fluid were also present within the other two ruminal subpopulations under the same diet; the bacterial diversity under the same diet needed to be determined as well. And further, the potential impact of sire breed on the bacterial diversity of the offspring was still unrevealed. Thus, Study 3 attempted to assess the role that host genetic background plays on the differences in rumen microbiota. One common trend observed in Studies 1 and 2 was the insignificant differences in VFA among steers with same RFI, but fed different diets. The fact that there were not differences in VFA among breeds in Study 3 also indicated that microbial communities could diversify their ecological roles to enhance their resilience, and maintain a stable rumen environment. In addition, the number of bands that were significantly different in rumen liquid among breeds was greater than in contents or tissue. Moreover, even the composition of the breed-specific microbiota differed within fractions, reinforcing the hypothesis that the host has a fundamental role in the composition of the

rumen ecosystem. Angus steers tended to have the highest FCR of the individuals we sampled to represent the three breeds and showed a larger number of shared breed-specific species in rumen liquid and tissue. The subpopulation associated with the ruminal fluid subsists on soluble feed components within ruminal fluid (McAllister et al., 1994) and is fundamental for initiating feed digestion; epimural community has been proposed to be intimately related to the metabolic activity of the host (Wallace et al., 1979). Bacterial community in Angus steers had redundancy in their functions so to maintain a favourable, resilient and balanced ecosystem (Firkins et al., 2007). In consequence, in Angus steers, these groups of bacteria were more efficient at extracting energy from the feedstuff, as reported in studies in mice gut (Turnbaugh et al., 2006). Although Angus and Hybrid individuals had in common bacterial species in the rumen tissue, there were differences in the species associated with rumen contents. Solid-attached bacterial community is the most abundant and organised as biofilms; again, beyond the differences in the whole microbial profiles, particular families or taxa are determinant and can have diverging impact on host feed efficiency. In this way, Study 3 allowed to explore potential relationships between particular bacterial phylotypes and feed efficiency measurements in cattle differing sire breed. To my knowledge, this is the first study describing the interactions among PCR-DGGE profiles, host metabolic phenotype and sire breed.

The analysis described in the current dissertation highlighted potential metabolic pathways that could be impacted due to the interactions of the three bacterial subpopulations in the rumen. Nevertheless, these interrelationships may

represent only a component of the total variation in feed efficiency. In fact, the identified phylotypes may represent the same species, or the same strains of different species; moreover, the identified phylotypes only represent small portion of the whole rumen microbiome. Future studies using pyrosequencing are necessary Current function-directed metagenomic techniques, such as pyrosequencing, as well as additional future studies including multiple sampling points and larger number of samples and sequences may prove to be useful in shedding additional information on the impact of specific phylotypes/taxa on host feed efficiency and will aid to identify the whole rumen microbiome and their relationships with residual feed intake.

5.5. LITERATURE CITED.

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Chapter 6. General Conclusions.

This is the first study linking rumen microbial diversity and microbial metabolites to host feed efficiency traits and their implications on individual variations in RFI of beef cattle. Several studies have been published on the use of culture-independent techniques to investigate the profiles of ruminal bacteria but these microbial profiles have not been linked to the host functions yet. Thus, this study will contribute to the development and application of molecular techniques to assess rumen microbial ecology and their effects on host phenotype. Because the impact of host variation on ruminal microflora and, more specifically, on the microflora associated to feed efficiency has not yet been fully established, the developed statistical method provides a novel approach to pin down particular species prevailing, and influencing the metabolic phenotype of the host. While this study limits itself to a small number of detected phylotypes, the underlying DGGE data provided a comprehensive framework appropriate for accomplishing the obejectives proposed. Molecular based techniques are likely to provide a better insight into the interactions among rumen microorganisms, which will enable us to overcome current limitations in rumen biotechnology. Gene-based technologies have the potential to improve the knowledge of the composition and metabolic activity of ruminal bacteria, therefore quantitative Real-Time PCR (qPCR) and PCR-DGGE are robust approaches to quantify the importance of functional microbial groups.

The relevance of this project for cattle production and feed efficiency research is enlarged because it provides an improved understanding of the contribution of the rumen microflora to the RFI and it also allows reaching insight into the mechanisms that cause feed efficiency variations between animals. Nevertheless, it is fundamental to elucidate the functions of the identified phylotypes associated with each particular rumen fraction and to explore the additional phylotypes reported using high-throughput techiques. Further validation of the hypotheses proposed in this thesis using a larger population of samples will aid to consolidate the role of ruminal microbiota in cattle RFI. In this way, the information provided should assist in the development of strategies to influence feed efficiency, either through a directed manipulation of the rumen microbiota or by improving production by means of identifying and selecting efficient steers. The utilization of this information can contribute to decrease feed costs in beef production.

The future of rumen microbiology research depends upon the adoption of molecular technologies, and the challenge is how we utilize this knowledge to improve ruminant production through a better understanding of microbial functions and interactions. To address such questions properly, the development of a novel research area involving our current knowledge of microbiology, animal genomics and animal nutrition towards understanding the importance of how bacterial species affect RFI, is fundamental.