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In vivo and in vitro nutrient balance and assessment of PCR and biophotonics as techniques for evaluating ruminal bacteria

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IN VIVO AND IN VITRO NUTRIENT BALANCE AND ASSESSMENT OF PCR AND BIOPHOTONICS AS TECHNIQUES FOR EVALUATING RUMINAL BACTERIA

By

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To better understand the facets of nutrient utilization, a series of *in vivo* and *in vitro* studies were undertaken to elucidate the effect of supplementation on utilization of moderate-quality bermudagrass hay and to identify mechanisms to evaluate the role of rumen bacterial populations on feedstuff utilization.

A digestion trial was conducted using 6 ruminally cannulated steers receiving bermudagrass hay supplemented with soybean hulls (HULLS), cracked corn (CORN), or soybean hulls and cracked corn (MIX; 75% and 25%, respectively) in a 3x3 Latin Rectangle arrangement. Additionally, ruminal fluid was continuously cultured using the BioFlo® 110 fermentation system to evaluate the *in vitro* fermentive parameters of ground moderate-quality bermudagrass hay either alone (HAY; 20 g DM L^{-1} d^{-1}) or supplemented (7 g DM L^{-1} d^{-1}) with corn (CORN), soybean hulls (SBH), or both (25:75; MIX) in a randomized complete block. Genomic DNA from continuous culture as well as from pure bacterial culture samples were sought to differentially enumerate select

bacterial strains via real-time PCR using specie-specific DNA primers. The information is to be used for elucidating responses in ruminal digestibility of varying feed-types. Finally, as an alternative to PCR, bioluminescence of transformed *Escherichia coli* was evaluated by measuring extent of photonic emission with and without antibiotic selection over time. Evaluations were also made of photonic emission by *E. coli* grown in ruminal fluid with and without additional feed particles.

Data seem to indicate that replacing a portion of corn with soybean hulls may successfully improved fiber digestion and improved ruminal N-utilization. Real-time PCR shows potential for evaluating ruminal bacteria where as biophotonics may need further modification before meaningful *in situ* evaluations of live ruminants can be employed.

Key Words: Ruminal bacteria, Soybean hulls, Corn, Digestibility, Bermudagrass, PCR, Bioluminescence, Biophotonics

DEDICATION

In memory of the late Dr. R. Allen Scott (1946 – 2007) retired faculty member and former head of the department of Animal and Horticultural Sciences, Berry College, Mount Berry Georgia. Dr. Scott initially sparked my interest in rumen bacteriology by stating: "To be a ruminant nutritionist, you have to first become a microbiologist."

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TABLE OF CONTENTS

DEADICATION ..... *ii*

ACKNOWLEDGEMENTS ..... *iii*

CHAPTER

I. INTRODUCTION 1

II. LITERATURE REVIEW 5

Ruminant Diets 5
Ruminal Bacteria 6
Cellulolytic Bacteria 7
Ruminococcus albus 7
Fibrobacter succinogenes 14
Butyrivibrio fibrisolvens 16
Ruminococcus flavefaciens 23
Amylolytic Bacteria 26
Streptococcus bovis 26
Selenomonas ruminantium 28
Ruminobacter amylophilus 32
Microbial Yield 35
Microbial Markers 37
Amino Acids 37
2,6-Diaminopimelic acid 37
2-Aminoethylphosphonic acid 39
D-Alanine 41
Leucine 41
Lysine 42
Amino Acid Combinations 42
Adenosine Triphosphate 42
Nucleic Acids 45
DNA & RNA 45
Total Purines 46

Radioisotopes 49
^{15}N Enrichment 49
^{35}S Enrichment 50
^{32}P Enrichment 51
Bioluminescence 52
Fluorescence 58
DNA Isolation 60
Polymerase Chain Reaction 61
Real-time PCR 64
DNA-binding Dyes 67
SYBR Green 67
Ethidium Bromide 68
Amplicons, Primers, and Probes 68
PCR of Ruminal Microflora 69
Continuous Culture 71

III. IMPACT OF SUBSTITUTING CORN WITH SOYBEAN HULLS UPON DIGESTIBILITY OF A FORAGE-BASED DIET OFFERED TO BEEF CATTLE 75

Objectives 75
Materials and Methods 76
Digestibility Trial 76
Laboratory Analysis 78
Calculations and Statistical Analysis 78
Results and Discussion 78
Implications 86

IV. SEMI-CONTINUOUS CULTURE 87

Objective 87
Materials and Methods 88
Experimental Conditions 88
Diets 91
Apparent Digestibility 91
VFA, NH3-N, & pH 92
Statistical Analysis 92
Results and Discussion 93
Conclusion 99

V. REAL-TIME PCR 100

Objective 100
Bacterial Cultivation 100
DNA Isolation 101

Real-time PCR 101
Results and Discussion 102
Bacterial Cultivation 102
DNA Isolation 105
Real-time PCR 106

VI. EVALUATING BIOLUMINESCENT APPLICATIONS IN NUTRITION 107

Objective 107
Materials and Methods 107
Experiment 1 107
Experiment 2 110
Statistics 112
Experiment 1 112
Experiment 2 112
Results and Discussion 112
Experiment 1 112
Trial 1 113
Trial 2 121
Experiment 2 129
Conclusions 133

LITERATURE CITED 135

APPENDIX

ANAEROBIC MICROBIOLOGICAL GROWTH MEDIA 166

LIST OF TABLES

TABLE Page

1. Nutrient composition of bermudagrass hay, corn, and soybean hulls consumed by beef steers during a confined digestibility trial 79

2. Least square means for body weight, intake, and apparent digestibility of bermudagrass hay supplemented with soybean hulls, cracked corn, or both soybean hulls and cracked corn consumed by beef steers during a confined digestion trial 80

3. Least square means for ruminal volatile fatty acids, pH, and ammonia-N concentration among beef steers consuming bermudagrass hay supplemented with soybean hulls, cracked corn, or both soybean hulls and cracked corn during a confined digestion trial 82

4. Composition of mineral buffer 89

5. Actual dry matter and anticipated quantity of feedstuffs supplied to semi-continuously cultured ruminal fluid. 91

6. Nutrient content, supply, and balance of continuously cultured ruminal fluid maintained on one of four diet combinations 94

7. Specie-specific, rDNA-targeting primers for evaluation of ruminal bacteria by PCR and Real-time PCR 103

8. Plasmids and corresponding antibiotic concentrations used to assess emission loss by transformed *Escherichia coli* MM294 109

9. (Trial # 1) Biophotonic emission, viable cell densities, and photonic stability over time of *Escherichia coli* MM294 transformed with one of three plasmids, with and without antibiotic selection.[1] 117

10. (Trial # 2) Biophotonic emission, viable cell densities, and photonic stability over time of *Escherichia coli* MM294 transformed with one of three plasmids, with and without antibiotic selection.[1,2,3] 123

11. Evaluation of redced photonic detection and growth of *E. coli* MM294 in ruminal fluid or LB. 131

LIST OF FIGURES

FIGURE **Page**

1. Combined species production of propionate from cellulose via succinate. 30
2. Combined species production of propionate from starch or soluble sugars via succinate. 30
3. A possible mechanism of the stimulatory effect of starch on cellulose digestion in the rumen ecosystem. 35
4. Illustration depicting the glycan subunits of peptidoglycan and the tetrapeptide crossbridge that serves to link two conjoining chains 38
5. Relationship between fluorescence and number of cycles. 65
6. Detection of FLT3 ITD by high-resolution melting analysis. 66
7. SYBR Green a dsDNA-binding dye.....67
8. Relationship between period, adaptation, and data collection 90
9. Ruminal NH_3-N as influenced by feedstuff during in vitro ruminal culture. 96
10. Polynomial regression analysis of Ruminal NH_3-N as influenced by feedstuff during in vitro ruminal culture. 97
11. Ruminal pH as influenced by feedstuff during in vitro ruminal culture. 98

12. Restriction and gene maps for pAK1lux, pXen1 and pXen5. 108

13. (Trial #1) Biophotonic emission of *Escherichia coli* MM294 transformed with one of three plasmids, with and without antibiotic selection. 114

14. (Trial #1) Biophotonic stability of *Escherichia coli* MM294 transformed with one of three plasmids, with and without antibiotic selection. 115

15. (Trial 1) Polynomial modeling of biophotonic emission when *Escherichia coli* MM294 is transformed with one of three plasmids (pAk1*lux*; pXen1; pXen5) with antibiotic pressure (Ampicillin or Erythromycin). 118

16. (Trial 1) Polynomial modeling of biophotonic emission when *Escherichia coli* MM294 is transformed with one of three plasmids (pAk1*lux*; pXen1; pXen5) without antibiotic pressure. 119

17. (Trial #1) Fit of polynomial models by biophotonic stability when *Escherichia coli* MM294 is transformed with one of 3 plasmids (pAk1*lux*; pXen1; pXen5) and cultured without antibiotic pressure (Ampicillin or Erythromycin). 120

18. (Trial #2) Biophotonic stability of *Escherichia coli* MM294 transformed with one of three plasmids (pAk1*lux*; pXen1; pXen5), with and without antibiotic selection (Ampicillin or Erythromycin). 124

19. (Trial # 2) Biophotonic emission of *Escherichia coli* MM294 transformed with one of three plasmids (pAk1*lux*; pXen1; pXen5), with and without antibiotic selection (Ampicillin or Erythromycin). 125

20. (Trial 2) Polynomial modeling of biophotonic emission when *Escherichia coli* MM294 is transformed with one of three plasmids (pAk1*lux*; pXen1; pXen5) with antibiotic pressure (Ampicillin or Erythromycin). 126

21. (Trial 2) Polynomial modeling of biophotonic emission when *Escherichia coli* MM294 is transformed with one of three plasmids (pAk1*lux*; pXen1; pXen5) without antibiotic pressure. 127

22. (Trial #2) Fit of polynomial models by biophotonic stability when *Escherichia coli* MM294 is transformed with pXen1 or pXen5 and cultured with antibiotic pressure (Ampicillin or Erythromycin). 128

23. Viable cell density of *lux*-transformed *Escherichia coli* MM294 grown in LB or ruminal fluid. 130

24. Effect of ruminal fluid and feed particles on photonic emission of *lux*-transformed *Escherichia coli* MM294.....132

CHAPTER I

INTRODUCTION

Ruminants possess a direct symbiotic relationship with microflora and microfauna residing in the rumen, a relationship essential for sustaining life of the foregut-fermenters. Because of this relationship, ruminants are able to harvest nutrients from fibrous feedstuffs that are predominantly unavailable to other simple-stomached species. Pregastric microbial fermentation provides ruminants with monomers and polymers of structural carbohydrates (e.g., cellobiose, xylans, pectins, dextrins, arabinose), high quality protein (e.g., microbial protein), a steady supply of microbial-derived B-vitamins, and nutritive organic acids (i.e., volatile fatty acids) that serve as precursors for gluconeogenic and lipogenic pathways for the animals (Yokoyama and Johnson, 1988; 0.37% to 2.01%; Krause and Russell, 1996).

Functional advantages of pre-gastric fermentive digestion are a slower rate of passage resulting in greater feed retention time, nutrient digestion, and nutrient absorption (Udén et al., 1980). For these reasons, rumen function and ruminal microbiology have been investigated since at least the 1940's (Hungate, 1944; Niven et al., 1948), and continue to be an area of extensive investigation with potential for tremendous impact on animal agriculture for years to come.

When starch-based supplements are provided in conjunction with forage-based diets, negative associative effects often result, reducing dry matter intake (DMI) and diminishing fiber digestion (Chase and Hibberd, 1987; Pordomingo et al., 1991; Fieser and Vanzant, 2004). Soybean hulls have been estimated to have a similar feeding value as corn (Hibberd et al., 1987; Anderson et al., 1988a), and, in some cases, have alleviated adverse impacts on forage utilization often exhibited by cereal grains (Klopfenstein and Owen, 1988; Martin and Hibberd, 1990; Galloway et al., 1993a). Garcés-Yépez (1997) concluded that corn or soybean hull supplementation, less than 0.5% BW, had no adverse effect on intake or digestibility of chopped bermudagrass. However, when supplementation was increased from 0.5% BW to 0.8 % BW, intake was decreased with no effect upon digestion. When contrasting corn-soybean meal mixture with soybean hulls, Garcés-Yépez (1997) reported that supplementing with soybean hulls increased neutral detergent fiber (NDF) digestibility.

Beneficial effects conferred by supplementation and supplementation type (e.g., starch-based vs. fiber-basesed systems) can be altered by the quality of forage in the diet. Fieser and Vanzant (2004) reported a trend for greater NDF digestibility when soybean hulls rather than corn was fed in conjunction with mature tall fescue (*Festuca arundinacea* Schreb.) hay. Similarly, supplementing low-quality fescue hay with soybean hulls increased apparent NDF and ADF (acid detergent fiber) digestibility (Highfill et al., 1987). Similar results where reported when soybean hulls supplemented moderate quality bermudagrass hay (Orr et al., 2007). Apparent NDF digestibility was reportedly greater for orchardgrass (*Dactylis glomerata* L.) hay than bermudagrass, both with and without soybean hull supplementation (Galloway et al., 1993a).

In an effort to provide the benefit of additional energy, yet optimize fiber digestion, some studies have combined both corn and soybean hulls (Galloway et al., 1993b; Ludden et al., 1995; Hejazi et al., 1999). Grigsby et al. (1993) fed bromegrass (*Bromus* L.) hay supplemented with soybean hulls and(or) corn, resulting in a tendency ($P = 0.06$) toward a linear decrease in apparent NDF digestibility when feeding corn. Additionally, corn concentration quadratically decreased ruminal NDF digestibility. True ruminal-N digestibility was reported greatest ($P = 0.01$) when soybean hulls were fed at 0.42% BW in conjunction with ground corn at 0.21% BW.

Knowledge of the nitrogen, mineral, and carbon requirements for growth of ruminal bacteria is essential for better understanding the protein, carbohydrate (structural and non-structural), and mineral requirements of ruminants and is ultimately key for optimization of animal agriculture. With growing concerns for nutrient management (i.e., excretion of excess minerals, nitrogen, methane, carbon dioxide, etc.), rising grain cost due to ethanol research, and overall environmental impact of commercial agricultural operations, nutritionists are needed to investigate efficiency of nutrient utilization. It is currently known that altering feedstuff combinations and ratios can have a direct impact on digestibility, efficiency, and performance of livestock species (Scheifinger and Wolin, 1973; Wells et al., 1995; Matulova et al., 2001). However, information regarding these interactions is still limited.

It is known that a plethora of bacterial species reside within the rumen conferring a large diversity of degradative capabilities (Yokoyama and Johnson, 1988). However, the equilibrium among bacterial species is easily disrupted due to variation in substrate

preferences and restrictions or limitations. Existing mechanisms of bacterial fermentation are not completely efficient, allowing monomeric to polymeric molecules to be released from degrading feedstuffs, allowing secondary bacteria to thrive that would otherwise die-off during periods of environmental and/or dietary transition. However, dramatic shifts among bacterial populations still occur with noticeable effects in livestock performance (Highfill et al., 1987; Martin and Hibberd, 1990; Grigsby et al., 1992, 1993).

It is my hypothesis that better understanding rumen bacterial populations and their interdependence combined with a growing knowledge of feedstuff composition, will allow for wise and informed ration formulation. The potential long-range impact from this line of research is: 1) improved efficiency and profitability of agricultural enterprises, 2) greater output to help meet the growing global demand for quality foodstuffs on a receding farmscape, 3) help offset nitrogen, phosphorus, methane, carbon dioxide, etc. excretion into the environment 4) incorporation of nutritive, digestible feedstuffs with greater quantities of fibrous by-products, traditionally considered to have little to know value, as a means of decreasing cereal grain usage and to provide disposal of products that would otherwise be burned or disposed in landfills.

CHAPTER II

LITERATURE REVIEW

Ruminant Diets

Grains fed to cattle consuming forage-based diets have been shown to cause substitutive (i.e., replacement of forage by concentrates in diet) and negative associative effects (i.e., phenomenon whereby starch-based supplements decrease fiber digestibility), limiting the efficient utilization of lower quality forages (Campling and Murcoch, 1966); (Klopfenstein and Owen, 1988; Galloway et al., 1993a). Performance of cattle consuming soybean hulls have been shown to be comparable to cattle consuming corn, implying similar availability of energy from both feedstuffs (Anderson et al., 1988b). Despite similarities in performance, unlike corn, soybean hulls are considered a roughage (i.e., > 18 % crude fiber) although readily digested within the rumen (Streeter and Horn, 1983; Hsu et al., 1987). Negative associative effects that impede forage utilization, have been minimized when feeding soybean hulls, as opposed to corn (Highfill et al., 1987; Martin and Hibberd, 1990; Grigsby et al., 1992, 1993). Previously published data indicates that substitutive effects of soybean hulls are minimal (Martin and Hibberd, 1990; St. Louis et al., 2002), whereas feeding corn often exhibits greater degrees of forage replacement (Mertens and Loften, 1980; Chase and Hibberd, 1987). For these

reasons, feeding soybean hulls, a by-product of soybean processing, may benefit both row crop farmers and cattle producers alike. However, the mechanisms by which soybean hulls are more efficiently utilized than other fibrous feeds and the means by which they produce results similar to corn-fed cattle, has yet to be elucidated.

Ruminal Bacteria

Ruminal bacteria exist in concentrations of 10^{10} per g of ruminal content. The majority of these bacteria are obligate anaerobes, but a portion of the bacterial populations are facultative amounting to around 10^{7} cells per g ruminal content (Yokoyama and Johnson, 1988). Ruminal bacteria are classified based upon size, shape, presence of flagella, structure, and most often by preferred substrate(s) fermented and subsequent by-products produced (Yokoyama and Johnson, 1988; Krause and Russell, 1996). Substrate classifications include, but are not limited to cellulose, hemicellulose, starch, sugars, pectin, intermediate acids, protein, amino acids, lipids, methane, and ammonia (Yokoyama and Johnson, 1988). These designations are important in understanding each bacterium's role in ruminant digestion and to what extent they contribute to, or detract from, digestion, sometimes referred to as positive and negative associative effects. Through fermentation of ingested feed, microorganisms breakdown feeds into VFA and other non-volatile acids (e.g., propionate, acetate, butyrate, valerate, isobutyrate, isovalerate, 2-methylbutyric, or lactate) and depending upon which VFA is produced, NH_3 and/or CH_4 may also be released. The final product will either benefit or impede the growth and production of other bacteria and ultimately the ruminant host. Bryant and Robinson (1961), noting this interaction, stated that the key to greater

understanding of feed utilization and metabolic disorders of ruminants lies in the study and of rumen microbiology. Rumen bacteriology can be devided into two main categories, cellulolytic andc amylolytic.

Cellulolytic Bacteria

Ruminococcus albus. Cellulolytic bacteria have been considered the most important classification of bacteria to the ruminant, and among those, the genus *Ruminococcus* is likely the most important from the cellulolytic groups (Bryant, 1959; Bryant and Robinson, 1961). *Ruminococcus albus*, a gram-variable bacterium, is considered one of the most active cellulolytic species within the rumen, and its fermentive substrates are comprised of glucose, cellulose, and cellobiose while yielding H_2, CO_2, ethanol, acetic acid, formic acid, and/or lactic acid and succinic acid as end products of digestion (Iannotti et al., 1973; Hungate, 1975; Dehority, 2003). Growth was shown to occur more rapidly on cellobiose than glucose, and when both substrates are present, preferential selection for cellobiose was observed (Thurston et al., 1993). As a requirement for growth, branched-chain VFA such as isobutyrate, 2-methylbutyrate, or 2-ketoisovalerate must be incorporated into growth media (Allison et al., 1962b; Dehority et al., 1967a). Isobutyric acid has also been reported as a nutritional requirement, but can be substituted with 2-methylbutyric acid (Miura et al., 1980). Further, research also indicated that a positive growth response was observed upon the inclusion of acetic acid (Dehority et al., 1967a) in combination with brach-chain acids.

Leatherwood (1965) reported 20% of all ruminal cellulolytic activity was attributed to the activities of *R. albus.* However, discrepancy may exist in their report because Hungate (1963) as well as Fusee and Leatherwood (1972) reported that when cellobiose was present, cellulose was not degraded until all cellobiose was completely utilized. Further, Smith et al. (1973) reported a numerical increase of *R. albus* cell growth in the presence of cellobiose as opposed to cellulose, indicating a preference for cellobiose by *R. albus.* Statistical comparisons were not provided to discern the relevance of the reported values. However, later work reported a constitutive synthesis of *cellobiose phosphorylase* by *R. albus* (Thurston et al., 1993), substantiating the initial conclusion by Smith et al. (1973).

Pentoses such as xylose and arabinose are used inefficiently and non-preferentially by *R. albus.* In fact, *R. albus*, is not well adapted for glucose, xylose, or arabinose utilization, but of these there seems to be a preference for hexose sugars. Which, as Thurston et al. (1994) pointed out, is very logical, knowing that cellobiose and glucose are products of cellulose hydrolysis where pentoses are traditionally products of hemicellulose hydrolysis. Despite this fact, there is a lag period in bacterial growth when switching cultures from pentose to cellobiose-based media, presumably to shift metabolic and enzymatic gene expression, as implied by the reduction in intracellular ATP (Thurston et al., 1994). The change in intracellular ATP also provide strong evidence that pentose uptake is not driven by proton motive force, rather, by ATP hydrolysis. Thurston et al., (1993) reported a high-affinity ATP-driven system for glucose transport. Interestingly, a shift from pentose to glucose does not involve a lag phase as occurs with a xylose-cellobiose shift. It appears that xylose and glucose share a common transport

system due to their similar pyranose ring structure. Other ruminal bacteria (e.g., *Selenomonas ruminantium* and *Prevotella ruminicola*) have demonstrated pentose transport via proton and sodium-dependent mechanisms (Strobel, 1993a; Strobel, 1993b). The preference of hexoses over arabinose has been previously documented (Greve et al., 1984a; Greve et al., 1984b), and the non-competitive inhibition of arabinose transport was speculated to result from a glucose-associated inactivation of *arabinose permease* (Thurston et al., 1994), as has been documented in *Butyrivibrio fibrisolvens* (Strobel and Dawson, 1993).

Shi and Weimer (1997) grew *R. albus* 7 in co-culture along with *Fibrobacter succinogenes* S85 or *Ruminococcus flavefaciens* FD-1 to test competition for cellobiose utilization. *Ruminococcus albus* 7 was able to out-compete both *F. succinogenes* S85 and *R. flavefaciens* FD-1, for this carbon sources, resulting in the demise of the competing bacterium. No known bacterosins or metabolic products have been isolated from *R. albus* 7 that would interfere with the growth or metabolism of *F. succinogenes*; other strains, however, are known to produce bacterosins (e.g., albusin A and B) specific for *R. flavefaciens* (Chen et al., 2004).

Judging from phylogenetic comparisons of 16S rDNA, all bacteria within the genus *Ruminococcus* sp. reside in the subphylum *Bacillus*/*Clostridium* in one of two clusters. The first grouping contains *R. flavefaciens*, *R. albus,* and colonic isolates of *R. bromii* and *R. callidus*, which are all characterized by a low G+C content. A second group, unrelated to the first, contains *R. flavus*, *R. lactaris*, *R. obeum*, *R. productus*, and *R. torques* (Rainey and Janssen, 1995). Morrison and Miron (2000) sought to elucidate molecular components of *R. albus'* cellulose hydrolysis due to its uniqueness compared

to other cellulolytic bacteria. Hydrolyzing enzymes of *R. albus* are provisionally organized and produced based upon the inclusion of clarified ruminal fluid within the culture media. In fact, interdependence has been documented during co-culture of *R. albus* and *Prevotella ruminicola* or *Megasphera elsdenii* in which *P. ruminicola* and *M. elsdenii* supplied essential branched-chain fatty acids from deamination of brached-chain amino acids (Bryant and Wolin, 1975; Miura et al., 1980).

Since at least the early 1960's, it has been known that clarified ruminal fluid is an essential component of growth media when culturing *Ruminococcus* species, as reported by Bryant and Robinson (1961) and reiterated by Leatherwood (1973). The reason, deamination of amino acids by proteolytic bacteria yield branch-chain fatty acids (i.e., valine, leucine, and isoleucine, etc) that are required for amino acid synthesis by celluloytic bacteria (Bryant and Doetsch, 1955; Dehority et al., 1967a; Miura et al., 1980). Not only is this fact related to a need for amino acid precursors, such as branch-chain fatty acids, the resulting bacterial growth and cellulose fermentation has been directly correlated to phenylpropanoic acid and phenylacetic acid concentrations (Hungate and Stack, 1982; Stack et al., 1983; Stack and Cotta, 1986). The reason being, 3-phenylpropanoic acid and phenylacetic acid have a direct provisional effect upon cellulosome formation and production of extracellular cellulase enzymes (Stack et al., 1983; Stack and Hungate, 1984). Therefore, as long as these two compounds (i.e., phenylpropanoic acid and phenylacetic acid) are contained within the culture media, inclusion of ruminal fluid is not necessary.

Hungate and Stack (1982) isolated phenylpropanoic acid, phenylacetic acid, and benzoic acid from strained rumen fluid and tested them for their ability to promote the

growth of cultured *R. albus.* Of these, included at a concentration of 1.5 x 10^{-4} *M*, only phenylpropanoic acid exhibited any stimulatory capacity. Phenylpropanoic acid is a non-volatile fatty acid contained within ruminal fluid (Tappeiner, 1886) known to originate form during normal plant metabolism (Koukol and Conn, 1961) but the concentration of which is influenced by ruminal metabolism. Hungate and Stack (1982), citing unpublished work, stated the absence of L-Tyrosine reduced phenylpropanoic acid concentrations required within ruminal fluid, a fact substantiated by work from Scott et al. (1964). Despite a common biochemical pathway between L-Tyrosine and L-Phenylalanine, only metabolism of L-Tyrosine yielded phenylpropanoic acid (Hungate and Stack, 1982). Scott et al. (1964) noted that the ruminal concentrations of phenylpropanoic acid where such that L-Tyrosine metabolism could not account for its formation. Phenylpropanoic acid, however, is an intermediate compound formed from the degradation of ferulic acid, a precursor in the lignin biosynthetic pathway (Hungate and Stack, 1982; Jung and Fahey, 1983), indicating a potential mechanism for lignin degradation. This information accounts for the genesis of phenylpropanoic acid within the rumen, but not its degradation, so, the exact mechanism is still unknown. However, Scott et al. (1964) reported that data suggest a phenylpropanoic role in phenylalanine biosynthesis, on the microbial level.

Traditionally, cellulose attachment and hydrolyses is attributed to the celluosome, a large cell-associated, multi-enzyme complex (Bayer et al., 1998a; Bayer et al., 1998b). Examination of mutant representatives from *R. albus* SY3 and *R. albus* 8 that both lack adhesive and cellulolytic properties has led to genetic evidence confirming the presence of cellulosome-like structures, previously only characterized in *Clostridium* spp.

Specifically, loss in functionality was equated with the disruption of cellulose binding-protein D and E (i.e., CbpD and CbpE) as well as xylanases and endoglucanases (Morrison and Miron, 2000). However, only partial loss in adhesion was noted indicating a possible derivation from *Clostridial* sp. adhesion mechanisms.

Previously, fimbrae-like structures have been found to extend as far as 600 nm from the cellular surface of *R. albus* (Patterson et al., 1975; Stack and Hungate, 1984; Kim et al., 2001). Specifically, cellulose binding protein type C (CbpC) has been isolated from the *R. albus* cell wall and does not exhibit sequence similarities to cellulose binding domains of traditional cellulosomes. However, the CbpC amino-terminus contains leader peptide, cleavage site, and motif characteristics of Pili-family proteins, specifically type-4 fimbrial subuit proteins from at least *Dichelobacter nodosus*, *Morazella bovis*, *Neisseria gonorrhoeae*, and *Pseudomonas aeruginosa* (Morrison and Miron, 2000). At the carboxy termini a short hydrophobic domain binds to the cell (Dalrymple and Mattick, 1987). Comparison of CbpC from *R. albus* and fimbrial proteins from aforementioned pathogens have similar hydrophilic sequences at the carboxy-terminus, an occurrence not duplicated in other Pili-family proteins (Pegden et al., 1998). It is speculated that this carboxy-terminal region aids *R. albus* in adhesion to particulate matter within the rumen, in addition to cellulosomal attachment. This is supported by the fact that mutant *R. albus* with minimal adhesive and cellulolytic characteristics reacted positively when subjected to Western immunblot assays utilizing polyclonal antibodies targeting CbpC proteins (Pegden et al., 1998). These findings adequately explain why only partial mitigation of fibrolytic activity occurred among mutant bacteria lacking cellulosomal structures.

Morrison and Miron (2000) reported that in addition to the classic cellulosome complex consisting of basal glycosylated protein extending from bacterial cell wall (with multiple functional domains facilitating anchoring to bacterial cell wall, enzyme attachment, and cellulose binding), *R. albus* also uniquely utilizes vesicular protuberances as well as cellulose adhesion proteins that resemble Pil-proteins such as the type-4 fimbrial proteins of pathogenic bacteria. Interestingly, cellulolysis and production of vesicular and fimbrial-like structures were directly correlated with micromolar concentrations of two ruminal organic acid isomers (i.e., phenylacetic acid and phenylpropionic acid; Stack et al., 1983; Stack and Hungate, 1984; Morrison and Miron, 2000).

As previously discussed, fimbrae-like structures of *R. albus* have shown improved adherence to fiber particles, a process regulated by acetate and propionate isomer concentrations. Though not yet understood, these unique characteristics of *R. albus* may explain the competitive advantage *R. albus* has toward adhesion when grown in co-culture with *Fibrobacter succinogenes* S85 and *Ruminococcus flavefaciens* FD1. Mosoni et al. (1997) using radiolabels (i.e., ^{14}C and ^{3}H) compared adhesion efficiency in both mono-cultured and co-cultured *R. albus*, *F. succinogenes*, or *R. flavefaciens*. However, their findings may be biased in that cell cultures were based upon equal volumes of culture media exhibiting equal optical density for each bacterium. No indication was given to correlate optical density with colony forming units (i.e., CFU). As such, a simple increase in CFU concentration could yield results artificially indicating greater adhesion properties.

With cellulose concentrations between 0.2 to 1%, fiber surface area should have been limited enough to cause competition among selected bacteria. Upon adding co-cultured bacteria simultaneously, adhesion of *R. albus* was not inhibited by either *F. succinogenes* or *R. flavefaciens* yet *R. albus* successfully inhibited adhesion of both *F. succinogenes* and *R. flavefaciens.* During the sequential study, a monoculture was allowed access to cellulose for 45 min. After culture was centrifuged and the second culture added, fresh media was introduced. The co-culture was incubated for 45 additional minutes, and under this scenario, *R. albus* successfully inhibited *R. flavefaciens* but not *F. succinogenes* from adhering to cellulose when compared to controls.

Fibrobacter succinogenes. Formerly Bacteroides succinogenes, *F. succinogenes* is a predominant cellulolytic bacterium that plays an important role in ruminant digestion. It is a pleomorphic (i.e., multi-shaped) gram-negative rod that produces considerable quantities of succinate and lesser amounts of acetate upon fermentation of glucose, cellulose, and cellobiose with NH_3 as its preferred source of N (Stewart and Flint, 1989; Russell, 2002; Dehority, 2003). As with many ruminal bacteria, *F. succinogenes* readily hydrolyzes hemicellulose, but the resulting pentose by-products are not well utilized (Dehority, 1973). *In vitro* cultures of *F. succinogenes* require small molecular weight fatty acids, both straight (i.e., 5 to 8 C including valeric or caproic, etc.) and branch-chain (i.e., isobutyric, isovaleric, or 2-methylbutyric acid; Bryant and Doetsch, 1955).

Mass and Glass (1991) reported that cellular transport of cellobiose and glucose by *F. succinogenes* occurred simultaneously, with glucose taken up more efficiently. The

population of *F. succinogenes* is not as extensive as *Ruminococcus albus* (Russell, 2002); nevertheless, this bacterium is vital for the persistence and survival of other bacteria across variable feeding regimens, resulting from cellodextrin and maltodextrin release into the media (e.g., cellobiose and maltose polymers, respectively). Both *F. succinogenes* and *Ruminococcus flavefaciens* were reported to grow on cellopentose and cellotetraose by first hydrolyzing the polymers into glucose, cellobiose, and celotriose that were later fermented (Russell, 1985; Shi and Weimer, 1996). It has been suggested that this initial hydrolysis is mediated by a periplasmic *cellodextrinases* by *F. succinogenes* or extracellular *cellodextrinases* of *F. succinogenes* and *R. flavefaciens* (Huang and Forsberg, 1988; Brown et al., 1993). Interestingly, the accumulation of cellopentose was noted when *F. succinogenes* cultures were grown on cellotetraose. Similarly, cellotriose accumulation was observed when *R. flavefaciens* was cultured with cellobiose (Wells et al., 1995; Shi and Weimer, 1996). These authors speculated that the biosynthesis of cellodextrins may be typical for cellulolytic bacteria containing *cellobiose phosphorylase* or *cellodextrin phosphorylase*.

Fibrobacter succinogenes has contributed to the growth *in vitro*, of at least *Streptococcus bovis* and *Selenomonas ruminantium* (Scheifinger and Wolin, 1973; Wells et al., 1995; Matulova et al., 2001). It has been speculated that this leakage of carbon units from fiber-adherent bacteria is to support their own species existent in the planktonic phase (non-adherent and potentially "immature" bacteria; Shi and Weimer, 1996) and is only by consequence that other species receive benefit. It is also thought that the ability by *F. succinogenes* to store polysaccharides intracellularly aids survival during the planktonic phase (Wells and Russell, 1994).

In a trial investigating the competition between *F. succinogenes* S85 and *Ruminococcus flavefaciens* FD-1 for cellobiose utilization, *R. flavefaciens* FD-1 was found dominant (Shi and Weimer, 1997). When grown in co-culture with *Ruminococcus albus* 7, *F. succinogenes* S85 was not able to persist under cellobiose-limiting conditions (Shi and Weimer, 1997). However, it must be remembered that while *R. albus* has a greater affinity toward cellobiose, *F. succinogenes* and *R. flavefaciens* are better able to metabolize cellulose (Pavlostathis et al., 1988a, b; Shi and Weimer, 1992; Weimer, 1993). Comparing *F. succinogenes* and *R. albus*, it is generally accepted that *F. succinogenes* has a greater cell density, a lesser maintenance requirements, and a greater affinity for both glucose and cellobiose than *R. albus*. However, *R. albus* is known to more completely utilize glucose than *F. succinogenes* (Pavlostathis et al., 1988a, b; Weimer, 1993).

Energetically, *F. succinogenes* along with *Ruminococcus flavefaciens*, are valuable sources of succinate that ruminants may use to feed the TCA Cycle and ultimately gluconeogenesis. Further, succinate can be decarboxylated to form propionate by bacteria or mammalian metabolism. Therefore, in the ruminant economy, *F. succinogenes*, supporting gluconeogenesis via succinate and/or succinyl-CoA (Doetsch and Robinson, 1953; Scheifinger and Wolin, 1973).

Butyrivibrio fibrisolvens. A curved rod-shaped bacterium, *B. fibrisolvens* is most often referred to as a gram-negative organism even though Cheng and Costerton (1977) examined the cell wall via electron microscopy and concluded that *B. fibrisolvens* contained a gram-positive cell wall. Trilamellar outer membrane structures consistent

with gram-negative bacteria could not be identified, which is in agreement with publications citing the presence of glycerol teichoic acids, and lipoteichoic acids in the cell wall (Sharpe et al., 1975; Hewett et al., 1976), characteristics traditionally associated with gram-positive bacteria. At the time of publication, Sharpe et al. (1975) and Hewett et al. (1976) did not question the gram-negative nature of this bacteria, but considered the aberrations, exceptions to the rule. Cheng and Costerton (1977) concluded that even though *B. fibrisolvens* are structurally gram-positive bacteria, a weak gram-positive reaction is returned because of its exceptionally thin cell wall, resulting in ambiguous or variable gram stains.

Further, *B. fibrisolvens* is a motile bacterium that can be described as a "generalist," fermenting a wide variety of substrates (Russell, 2002). This versatile bacterium effectively ferments pentoses, hexoses, pectin, starch, xylans, hemicellulose, and cellulose, with butyrate as the primary end product of digestion, as implied by the genus (Hungate, 1963; Margherita and Hungate, 1963; Russell, 2002).

Butyrivibrio fibrisolvens is unique among ruminal cellulolytic bacteria in that it is free-floating, where as *R. flavefaciens, R. albus*, *F. succinogenes* are adherent to fiber particles (Weimer, 1996). This versatility directly aids survivability, as shown by *B. fibrisolvens* presiding as a large percentage of bacterial counts when cattle consumed low-quality diets (Margherita et al., 1964). These factors may contribute to the minimal maintenance requirement reported previously (Russell and Baldwin, 1979). It is, however, considered the most prevalent xylan degrading bacteria within the rumen (Dehority, 1966; Morris and van Gylswyk, 1980), but unlike other ruminal bacteria, *B. fibrisolvens* is capable of growing on all products of hemicellulose hydrolysis (Dehority,

1973; Hespell, 1988). Hemicellulose comprises a relatively large portion of the plant cell wall fractions, which is composed of a conglomeration of polysaccharides such as xylan, glucan, mannans, glactans, and arabinans.

The backbone of hemicellulose is composed largely of a xylanose polymer connected by β-1-4 linkages from which side chains comprised of 3-O-linked L-arabinose and 2-O-linked 4-O-methylglucuronate, glucuronate, or acetate. To the arabinoses, there may be ester-linked phenolic acids and/or other lignin-like polymers (Hespell and O'Bryan, 1992). Specifically, xylans, a heteropolymer composed of β 1-4 linked xylose with variable arabinose, glucose, galatose, uronic acid, and other sugar side chains (Cotta and Zeltwanger, 1995). As a result, xylan fermentation requires multiple enzymes (*xylanase*, *xylosidase*, *glucuronidase*, *arabinofuranosidase, acetylxylan esterase*, and *phenolic acid esterase*), in some cases, just to gain access to the xylans (Hespell, 1988; Wong et al., 1988). This is primarily the reason xylans are the least efficient fiber competent to be broken down with in the rumen (Cotta and Zeltwanger, 1995). *Xylanase* functions extracellularly, *xylosidase* is cell-associated, and *arabinofuranosidase* functions both extracellularly and as a cell-associated enzyme (Hespell et al., 1987; Hespell and Whitehead, 1990), and the regulation among these enzymes seem to vary among strains of *B. fibrisolvens*.

It should be noted that not all bacterium classified as *B. fibrisolvens* are physiologically similar. There is considerable variation among the mechanisms of butyrate production leading to the alternative production of lactate, by certain strains, which are related to the expression of the enzyme, *butyrate kinase* (Russell, 2002). It has been proposed that *Butyrivibrio* spp. be divided into two genera, *Butyrivibrio*, which use

a *kinase* in butyrate formation and *Pseudobutyrivibrio* that utilize a *transferase* in butyrate formation (Russell, 2002).

Aside from fermenting various carbohydrates, *B. fibrisolvens* produces extracellular proteases, regardless of growth phase, however, growth conditions have been shown to modulate proteolytic activity (Cotta and Hespell, 1986). Conversely, proteolysis of *Bacteroides amylophilus* and *Prevotella ruminicola* is almost exclusively cellular-based, and proteolytic activity of *Bacteroides amylophilus*, by contrast, is not altered by media conditions but is effected by bacterial growth phase (Blackburn, 1968b, a; Cotta and Hespell, 1986). This characteristic of *B. fibrisolvens* allows for both protein and peptide utilization by most strains of this bacterium, making it an important proteolytic bacterium, more numerous than any other proteolytic organism in the rumen (Blackburn and Hobson, 1962; Fulghum and Moore, 1963; Hazlewood et al., 1983b; Cotta and Hespell, 1986). Its flexibility and stability over a wide range of dietary conditions has contributed to its role as one of the most abundant bacteria within the rumen (Scott et al., 1997).

Dehority (1967) reported that several ruminal bacteria are capable of rapidly degrading xylan but the subsequent fermentation did not proceed with vigor. Because of this, intermediate products (e.g, xylooligosaccharides) of the initial enzymatic hydrolysis become concentrated within culture, providing a means for cross-feeding among bacteria. *Selenomonas ruminantium* is an example of an amylolytic bacterium incapable of using xylan but capable of utilizing xylooligosaccharides.

Isolates of *B. fibrisolvens* have demonstrated esterase activities in hydrolyzing tributyrin, galactolipids, and short-chain fatty acids (Lanz and Williams, 1973;

Hazlewood and Dawson, 1979; Hazlewood et al., 1983a). In addition, strain S2 exhibited *phospholipase* and *galactolipase* activities (Hazlewood et al., 1983a). Hespell and O'Bryan-Shah (1988) evaluated 22 strains of *B. fibrisolvens* for the presence of esterase enzymes targeting ester, naphthyl ester, and/or *p*-nitrophenyl ester bonds. All strains evaluated demonstrated some degree of esterase capability. The authors noted that enzymatic activity was proportional to bacterial growth and decreased proportionally to increase in carbon-chain length.

Conjugated linoleic acid (CLA) is known to be synthesized by biohydrogenation reactions (Polan et al., 1964) of bacteria such as *B. fibrisolvens* (Kepler et al., 1966), *Eubacterium lentum* (Verhulst et al., 1986), *Propinibacterium freudenreichi* (Jiang et al., 1998), *Lactobacillus acidophilus* (Ogawa et al., 2001), *Lactobacillus reuteri* (Lee et al., 2003), *Megasphaera elsdenii* (Kim et al., 2002), and *Bifidobacterium breve* (Coakley et al., 2003). Specifically, the ruminal bacterium *B. fibrisolvens*, has been shown to produce greater concentrations of CLA than other bacteria studied to date (Polan et al., 1964; Kepler and Tove, 1967; Hunter et al., 1976). During the summer months, there is a greater milk dienoic fatty acid content (Riel, 1963), which includes CLA. This is important because CLA may function as a nutrapseutical. In some studies, CLA has inhibited chemically induced tumors (Kavanaugh et al., 1999), helped reverse and prevent arthrosclerosis (Nicolosi et al., 1997), improve hyperinsulinemia (Houseknecht et al., 1998), modulate immune function, improve ratio of LDA and HDL cholesterol (Lee et al., 1994), and improved protein-to-fat ratios in lab animals (Chin et al., 1992; Dougan et al., 1997), indicating a potential health benefit for dairy consumers. By better

understanding the rumen microbial ecology, it is thought that CLA content might be selectively increased by feeding cattle to support *B. fribrisolvens* proliferation.

As a result, ruminant nutritionists have sought to alter feeding regimens and ruminal fermentation to increase CLA content within meat and milk products (Franklin et al., 1999; Chouinard et al., 2001; Duckett et al., 2002). However, their work may be premature and based upon a false premise. While *B. fibrisolvens* has demonstrated ability toward converting linoletic acid to *cis*-9, *trans*-11 CLA (Kepler et al., 1966; Hughes et al., 1982), Griinari and Bauman (1999) question its contribution and significance because mammalian tissues are also capable of synthesizing *cis*-9, *trans*-11 CLA form *trans*-octadecenoic acid (*trans*-$C_{18:1}$). It was originally thought that biohydrogenation is a detoxification processes imposed by ruminal bacteria on polyunsaturated fats (Dawson and Kemp, 1969). Kim et al. (2000) reported that linoleic acid and conjugated linoleic acid both inhibited the growth of *B. fibrisolvens* A38, unless the cultures were actively growing and slowly adapted to greater concentrations of linoleic acids. This data corresponds with reported sensitivity by *B. fibrisolvens* to other fatty acids (Henderson, 1973). Later, Kim et al. (2000) was able to show that production of CLA by pure cultures of *B. fibrisolvens* was not a product of biohydrogenation, as previously thought. On the contrary, CLA production only occurred when biohydrogenation of polyunsaturated fatty acids was inhibited and the viability of cultures were lost. In fact, *B. fibrisolvens* readily produces hydrogenated end products other than CLA. The production of CLA then, is not an end product of cellular lipid metabolism but the release of membrane-bond CLA into culture media upon bacterial lysis. It has been reported that unlike many bacteria, upon reaching stationary phase, *B.*

fibrisolvens readily lyse. This data is substantiated by Kim et al. (2000) who when isolating a cell membrane fraction, found 10 times more CLA present than with the cytosolic fraction. Further, *linoleic acid isomerase* has been isolated from the membrane fraction of prepared *B. fibrisolvens* cells (Kepler and Tove, 1967). Despite this seeming negative data, it still implicates *B. fibrisolvens* with fluctuations in CLA milk and meat content, at least in part.

A hemicellulose degrading bacterium, *B. fibrisolvens* will thrive when cattle consume a digestible forage-based diet (Bryant and Small, 1956), and with linoleic acid present, actively dividing cells will convert it into CLA (Kim et al., 2000), presumably for incorporation into the cell's cytosolic and/or outer membrane. When cattle consume fiber-rich diets, milk CLA content will often increase to a concentration less than 20 mg/g (Riel, 1963). However, when cattle are consuming predominantly grain-based diets fortified with linoleic acid, total milk CLA content often exceeds 20 mg/g (Kelly et al., 1998). Therefore, the exact role of *B. fibrisolvens* in this process is not yet clear. It is known that other ruminal and colonic bacteria are capable of producing CLA. It is also know that mammalian cells can also synthesize CLA. Any *trans*-$C_{18:1}$ (i.e., oleate) that escapes the rumen can be readily converted by mammary and adipose tissues into CLA. To better understand and elucidate these processes, additional work will need to be done, but what is clear is that *B. fibrisolvens* is an important participant in the ruminal ecosystem.

Ruminants are known to be less severely affected by mycotoxins than are monogastrics due to ruminal detoxification. Pure cultures of ruminal bacteria have been able to detoxify T-2 toxin (i.e., trichothecene mycotoxin), and some of its derivatives, by

deacetylation to form HT-2 toxin, which is further deacetylated to form T-2 triol and neosolaniol (Westlake et al., 1987a, b). Proteinaceous cell preparations from *B. fibrisolvens* CE51 cell membrane successfully degraded T-2 toxin to HT-2 and T-2 triol (Westlake et al., 1987b).

Ruminococcus flavefaciens. A gram-variable, cellulolytic cocci, *Ruminococcus flavefaciens* is an obligate anaerobe capable of fermenting cellulose and cellobiose, while utilizing less readily fructose and mannose, to yield acetic acid, lactic acid, ethanol, succinic acid, formic acid, and H_2. *Ruminococcus* sp. in general are incapable of utilizing glucose, and this holds true for *R. flavefaciens*, which on hydrolyzing cellulose, releases cellobiose rather than glucose, contrary to previous thought (Ayers, 1958b). Under certain conditions, growth was reported to be greater in the presence of the cellodextrin, cellopentaose, or cellotriose than on cellobiose; it was further observed that pure cellodextrinis were hydrolyzed to cellobiose or cellotriose (Rasmussen et al., 1988).

To satisfy the nitrogen requirement, amino acids or peptone must be provided (Sijpesteijn, 1951; Ayers, 1958a). Essential growth factors were evaluated and determined to include CO_2, folic acid, adenine, guanine, and a yet to be identified water-soluble factor present in yeast extract (Ayers, 1958a). This bacterium requires branched-chain VFAs such as isovalerate or isobutyrate, for synthesis of branched-chain amino acids, specifically leucine (Allison et al., 1962a; Allison et al., 1962b). However, there is great disparity among *R. flavefaciens* strains with regard to VFA requirements. Strain C94 has an absolute requirement for valeric, isobutyric, isovaleric, and 2-methylbutyric acids, whereas strain C1a had an absolute requirement for only isobutyric (Dehority et al.,

1967a). *Ruminococus flavefaciens* B34b is unique in that it does not require any VFA so long as casein hydrolysate is present (Dehority, 1963), yet even under these conditions although growth will occur, it is characterized by a prolonged lag phase (e.g., 48 to 72 h).

Ruminococcus flavefaciens has been shown to have a strong affinity for, and rate of adherence to, fiber particles, more so than either *Fibrobacter succinogenes* or *Ruminococcus albus* (Morris, 1988; Bhat et al., 1990; Shi et al., 1997). As a consequence, *R. flavefaciens* was able to out compete *R. albus* and *F. succinogenes* for cellulose utilization when grown in co-culture (Shi et al., 1997). However, this is a forage-dependent response (Latham et al., 1978) as determined by the ratio of structural to non-structural carbohydrates. Greater binding affinity by *R. flavefaciens*, while a competitive advantage, should be considered at least partially beneficial to *Ruminococcus albus* and *Fibrobacter succinogenes* in that they are better apt to utilize glucose monomers produced by *R. flavefaciens* during cellulose hydrolysis (Shi et al., 1997). In fact, Helaszek and White (1991) reported that *R. flavefaciens* is unable to grow on media with glucose as the sole carbon source. Ayers (1958b) working with dried cell preparations identified *cellobiose phosphorylase*, which concurs with reports on *Clostridium thermocellum*, a cellobiose degrading bacterium that does not utilize glucose (Sih et al., 1957).

Xylanase from *R. flavefaciens* 17 was found to be a complex compilation of enzymes containing more than one catalytic domain and two conserved non-catalytic domains (Zhang and Flint, 1992; Flint et al., 1993; Zhang et al., 1994). These dockerin sequences strongly resemble that of *Clostridium thermocellum*, giving molecular support for cellulosome-like enzymatic organization, which is understandable given the

previously identified relationship between *Ruminococcus* sp. and *Bacillus* as well as *Clostridium* (Rainey and Janssen, 1995).

Though an important ruminal bacterium, *R. flavefaciens* reside at concentrations consistently less than that of *Rumnococcus albus*. When grown in co-culture with, or on the supernatant from, *Ruminococcus albus*, the growth of *R. flavefaciens* was impeded (Chen et al., 2004) by a protein isolate identified as albusin B. Sensitivity of albusin B by other ruminal bacteria and *Escherichia coli* was investigated resulting in no apparent effect upon growth (Chen et al., 2004). Due to its proteinaceous and selective inhibitory nature, it was concluded that albusin B is a bacteriosin (Klaenhammer, 1993; Chen et al., 2004), rather than a random anti-growth factor. Similar support was obtained by Odenyo et al. (1994a) who investigated growth inhibitors produced by *R. albus* 8 that affected *R. flavefaciens* FD-1 during co-culture; no adverse affects where noted upon co-culture with *Fibrobacter succinogenes*.

When grown in co-culture with *F. succinogenes* S85, it has been documented that *R. flavefaciens* FD-1 was able to outcompete in cellobiose utilization, as well (Shi and Weimer, 1996, 1997). Judging from calculated Monod saturation constants for cellobiose affinity, *R. flavefaciens*, in at least the planktonic phase, will likely be able to outcompete other planktonic cell of other species (Shi and Weimer, 1997). It is thought that one reason for cellodextran leakage by fiber-adherent bacteria is to provide a simplified free-floating carbon source to planktonic or non-adherent and potentially "immature" cells of it own specie (Shi and Weimer, 1996).

According to the finding of Odenyo et al. (1994a) testing inhibitor stability against *pronase E* and *α-chymotrypsin* resulted in the elimination of antagonism while

boiling at 100°C for 10 min, did not. To assess the prevalence of bacteriosin production among ruminal bacteria, Chan and Dehority (1999) evaluated *R. albus* strains 7, MO2a, and MO3g, *R. flavefaciens* B1a, B34b, and C1a, *Fibrobacter succinogenes* S85, and *Butyrivibrio fibrisolvens* H17c. Strains 7, MO2a, and MO3g of *R. albus* effectively inhibited *R. flavefaciens* strains B1a and C1a where as stain B34b was inhibited by *R. albus* MO2a, and MO3g. *R. flavefaciens* R13e2 was unaffected by any exposure (i.e., co-culture or filtrate) to the three *R. albus* strains (Chan and Dehority, 1999). While Chan and Dehority (1999) acknowledge the limited effect bacteriosins likely have on the whole of ruminal fermentation, they do point out their potential function among interacting bacteria in the locality of fiber attachment, indicating a need for further investigation.

Amylolytic Bacteria

Streptococcus bovis. A predominant facultative bacteria found in the rumen is *S. bovis*, which has a highly dynamic growth curve with the ability for rapid growth (Russell and Robinson, 1984; McAllister et al., 1990). This gram-positive bacterium primarily digests starch, but is additionally capable of fermenting maltose, cellobiose, sucrose, glucose, fructose, galactose, mannose, and lactose (Stewart et al., 1997). Though amylolytic, the ability to persist within the rumen of forage-fed cattle is related to the ability of *S. bovis* to utilize water-soluble cellodextrins derived from crystalline cellulose (Russell, 1985). Other contributing factors would include the ability by some strains to ferment phloroglucinol and similar compounds from bioflavonoids.

Additionally, Wojciechowicz and Ziolecki (1984) identified a strain capable of degrading pectin via *endopolygalacturonate lyase*, enabling the utilization of arabinose, xylose, galactose, and rhamnose (i.e., products of plant cell wall hydrolysis) by these strains.

An ability to alter its metabolic efficiency has been observed via manipulation of biochemical pathways to yield varied end products of fermentation. When energy is scarce, acetate, formate, and ethanol are released providing 3 and 4 moles of ATP/glucose. However, when energy sources are abundant, less efficient lactate is produced, which only provides 2 ATP/glucose to the bacteria (Marounek and Bartos, 1987; Russell, 1998, 2002). Carbon dioxide is the gaseous byproduct of fermentation for this bacterium.

Analysis of the cell wall has revealed variation among *S. bovis* strains. Latham et al. (1979) noted that the peptidoglycan of *S. bovis* to have lysine, alanine, threonine, and serine in addition to glutamic acid, but not any diaminopimelic acid. Six strains, however, were later identified to, in fact, containe diaminopimelic acid (Russell and Robinson, 1984). It is uncertain whether CO_2 is required or is merely stimulatory to growth, but biotin was shown to be essential while thiamine and arginine are growth stimulants (Latham et al., 1979). Further, according to experiments conducted by Cotta and Hespell (1986) *S. bovis* does not possess proteolytic activity, but N-sources need not consist solely of amino acids, ammonia-N has successfully sustained *S. bovis* as the only source of N (Wolin et al., 1959).

Exteracellular α-*amylase* is used for starch hydrolysis (Freer, 1993) whereby *S. bovis* is then capable of passively transporting glucose into the cell. However, during periods of glucose surplus, facilitated diffusion becomes the preferred mechanism

(Russell, 1990). Energy-spilling is supported when glucose is taken up in excess, limiting substrates for other amylolytic bacteria (Cook and Russell, 1994).

Upon excessive starch consumption by cattle, *S. bovis* rapidly becomes a dominant bacterial species within the rumen, and buildup of lactate drastically reduces ruminal pH. Such reductions in ruminal pH may lead to the decline of other ruminal microorganisms as well as potential death of the ruminant (Russell, 2002). Acid tolerance is achieved by *S. bovis* by an adaptive mechanism through which intracellular pH is caused to parallel that of the environment (Russell, 1991a, b). Therefore, through pH reduction, *S. bovis* has the potential to drastically influence the microbial ecology of the rumen.

Selenomonas ruminantium. This bacterium is a gram-negative anaerobic bacterium native to the rumen. Most strains of *S. ruminantium* are curved flagellated-rods 0.8 to 1.0 µm wide by 2 to 7 µm long, with flagella attached laterally (Dehority, 2003). Primary fermentive substrates are starch along with most other soluble carbohydrates, and cellulodextrins with lactic acid as the primary by-product of digestion. A graded transition toward acetic and propionic acid is also observed with traces of butyric, formic, and succinic acids (Russell and Baldwin, 1979; Dehority, 2003). Nitrate is not reduced by this bacterium and neither does it produce catalase or indole. Further, Scheifinger et al. (1975b) identified traces of H_2 productivity by *S. ruminantium* strains HD4, HD1, PC18, GA31, but not GA192.

Cellulose and xylan are typically not fermented, contributing to a lack of persistence by *S. ruminantium* when soluble carbohydrates are scarce (Bryant, 1956).

Dehority (1967) reported that several ruminal bacteria are capable of rapidly degrading xylan, but at a relatively slow rate. Because of this, intermediate products (e.g, xylooligosaccharides) of the initial enzymatic hydrolysis become concentrated within culture, providing a means for cross-feeding among bacteria. *Selenomonas ruminantium* is one amylolytic bacterium incapable of using xylan but capable of utilizing xylooligosaccharides (Figure 1), providing a means of survivability within a changing ruminal environment.

Proteolytic activity was measured by Cotta and Hespell (1986), by spectrophotometric assessments of enzyme activity with no indication that *S. ruminantium* can affectively utilize proteins. However, the ability of *S. ruminantium* to ferment RNA to a greater extent than other ruminal bacteria has been noted previously and is a result of its ability to ferment ribose (Cotta, 1990). Ureolytic strains have been identified by John et al. (1974), and are potentially a major source of urease in the rumen. Though equipped with ureases, surprisingly, *S. ruminantium* prefers NH_4 over NH_3 (Dehority, 2003). Further, *S. ruminantium* plays an important role in decarboxylating succinate to propionate within the rumen (Figure 1; Figure 2), both of which may contribute to gluconeogenesis by the ruminant (Scheifinger and Wolin, 1973; Strobel and Russell, 1991).

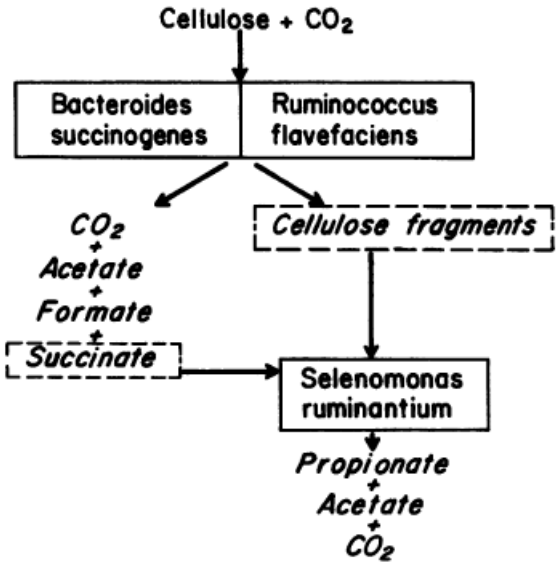

Figure 1. Combined species production of propionate from cellulose via succinate.

(Reprinted from Scheifinger and Wolin, 1973)

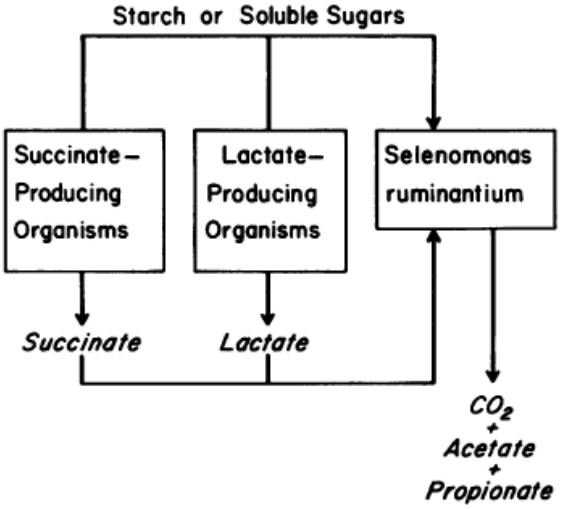

Figure 2. Combined species production of propionate from starch or soluble sugars via succinate.

(Reprinted from Scheifinger and Wolin, 1973)

An ability to synthesize glutamate from ammonia has been reported (Smith et al., 1980) and may occur in one of two known pathways. First, NH_4 is coupled with an NADPH-linked *glutamate dehydrogenase* or secondly, NH_4 is incorporated into the amide of glutamate via ATP-dependent *glutamine synthase.* The latter pathway is most active during N-limiting conditions. *Glutamine synthase* activity is correlated with *urease* activity among *S. ruminantium* strains studied (Smith et al., 1981).

To aid survival within constantly changing rumen, *S. ruminantium* possesses the functional advantage of being able to store intracellular polysaccharides consisting of 98% glucose in a 12 subunit branched-glycogen form. Other sugars include 0.5% rhammose, 0.6% arabinose, and 0.2% xylose (Wallace, 1980). This ability may contribute to the relatively low maintenance requirement (Russell and Baldwin, 1979).

In addition, some strains can affectively ferment lactate (i.e., *S. ruminantium* var. *lactilyticas*), often only after soluble carbohydrates are depleted, yielding acetate, propionate, and butyrate (Scheifinger et al., 1975a; Russell, 1985; Dehority, 2003). An increased ability to ferment lactate correlated with an increase in countable lactilytic bacteria (e.g., *S. ruminantium* and *Megasphera elsdenii*) was observed by incorporating distillers byproducts into an energy-dense ration, indicating a potential preconditioning strategy for deterring acidosis among feedlot cattle (Fron et al., 1996). Others showed a response from malate, fumarate, and yeast culture infusions toward ruminal lactate utilization (Nisbet and Martin, 1990; Nisbet and Martin, 1991).

Additionally, *S. ruminantium*, is noted for its ability to persist at a minimum pH of 4.3 (Dehority, 2003). Many ruminal bacteria that produce lactate are able to persist at relatively low pH. A potential reason for this was proposed by Van Golde et al. (1975) in their identifying cellular morphology unique to lactate-producing bacteria (i.e., *Megasphaera elsdenii*, *Veillonella parvula*, *Anaerovibrio lipolytica*, and *S. ruminantium*). In these strains, the phospholipids diachlphosphatidylethanolamine and/or ethanolamine plasmalogens are found in large concentrations, whereas a survey of non-lactate utilizing bacteria revealed only trace amounts (< 1%) of these phospholipids, regardless of whether or not they were anaerobic, aerobic, ruminal, or non-ruminal bacteria (Van Golde et al., 1975).

Methanogens, gaining a significant quantity of carbon from CO_2, cannot persist in pure culture without added H_2 or formate, byproducts released from other ruminal bacteria (i.e., *S. ruminantium*) to facilitate CO_2 reduction. Scheifinger et al. (1975b) confirmed this among the methanogenic bacteria *Methanobacillus omelianskii* and *Methobacterium ruminantium* PS. This is of interest because as methane production increases within the rumen, carbon is largely lost and not sequestered by the ruminant, greatly reducing rumen energy efficiency.

Ruminobacter amylophilus. Formerly *Bacteroides amylophilus* (Hamlin and Hungate, 1956), *R. amylophilus* was reclassified into a new genera by Stackerbrandt and Hippe (1986) based upon 16S rRNA sequencing. It is a pleomorphic rod 0.9 to 1.6 µm wide by 1 to 4 µm long.

This gram-negative anaerobic bacterium is identified as being a predominant amylolytic and proteolytic bacterium in the rumen. Substrates include starch, maltose, dextrin, and glycogen to produce formate, acetate, and succinate with a limited amount of lactate and ethanol yielded (Stewart et al., 1997; Dehority, 2003). Carbon dioxide and ammonia are essential for growth (Hungate, 1966). There is no requirement for supplemental fatty acids (Bryant and Robinson, 1962; Blackburn, 1968b). For optimal growth, relatively large concentrations of sodium are required (Caldwell et al., 1973). Wetzstein et al. (1987) reported an intracellular Na concentration of 0.2 m*M* maintained by Na/2H antiport system.

It is thought that the mode of starch degradation involves binding and translocation of subunits across cell wall prior to hydrolysis (i.e., oligomers; Anderson, 1995). Receptors for such a process have initially been identified in *Bacteroides thetaiotaomicron* (Anderson and Salyers, 1989) and in this case, hydrolytic enzymes are found periplasmiclly, allowing glucose to then cross the cytoplasmic membrane. Anderson (1995) has identified the first evidence for starch-binding proteins on a ruminal bacterium or any non-*Bacteroides* specimen. These receptors from *R. albus* have an affinity for α 1-4 glucose polymers with the vast majority of enzymatic activity noted among the membrane and periplasmic regions (Anderson, 1995).

Unlike *Butyrivibrio fibrisolvens*, the proteolytic activities of *R. amylophilus* are not influenced by the presences or absence of extra cellular amino acids or peptides (Cotta and Hespell, 1986). In fact, *R. amylophilus*, can synthesize protein and amino acids *de novo* from starch and NH_4 and are not dependent upon external nitrogen sources (Bryant and Robinson, 1962; Blackburn, 1968b). In addition to NH_4, pure-cultures

enriched with CO_2 revealed that gas was depleted and incorporated into succinate by *R. amylophilus* grown on maltose. However, the extent of radio-labeled succinate was less than would be expected if all succinate was formed by direct fixation with CO_2, such as by carboxlylation of phosphoenol pyruvate or pyruvate (Caldwell et al., 1969) indicating another pathway may be involved.

Ruminobacter amylophilus also holds a pivotal role in symbiotically sustaining other ruminal bacteria. The ability of *R. amylophilus* to produce branch-chained amino acids ensures a readily available source of the needed substrate for deaminase-capable bacteria, such as *Megasphaera elsdenii* (Allison, 1978). At least *in vitro*, *M. elsdenii* and *P. ruminicola* require supplemental amino acids, and *R. albus* requires branch-chain fatty acids, setting the sage for a harmonious interrelationship requiring collaborative contribution of all three species. In co-culture, *R. amylophilus* and *M. elsdenii* were capable of growing on simple media containing starch. Production of branch-chain fatty acids increased upon starch supplementation. Specifically, the increase in isobutyric acid was proportional to the increase in supplemental starch (Miura et al., 1980). When *M. elsdenii* and *Ruminococcus albus* were grown in co-culture, in the presence of amino acids, growth of *M. elsdenii* followed that of *R. albus*. However, as amino acids concentrations decreased, growth of *M. elsdenii* decreased and conversely growth of *R. albus* increased (Miura et al., 1980). The co-culture of *R. amylophilus* and *R. albus* with starch and cellobiose resulted in growth of only *R. amylophilus* (Miura et al., 1980). Finally, when culturing *R. amylophilus*, *R. albus*, and *M. elsdenii* in tandem on media containing starch, glucose, and cellobiose, all three strains thrived. Growth patterns were similar when either *Ruminococcus* sp. were cultured individually with *M. elsdenii* (Miura

et al., 1980). Figure 3, a schematic reproduced from Miura et al. (1980), depicts the possible roles of interrelationship among the three ruminal bacteria.

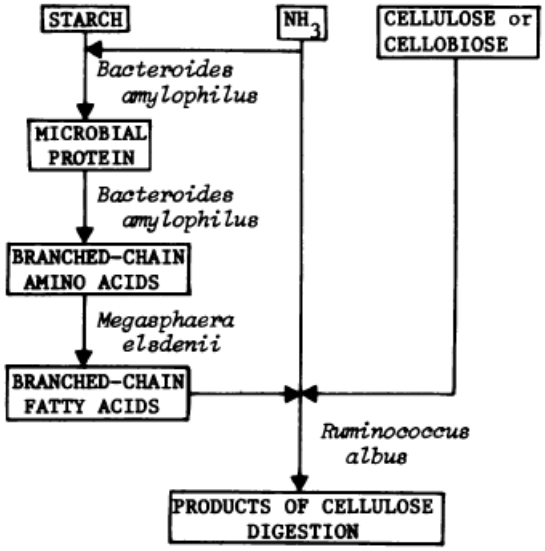

Figure 3. A possible mechanism of the stimulatory effect of starch on cellulose digestion in the rumen ecosystem.

(Figure reprinted from Miura et al., 1980)

Microbial Yield

Although influenced by ruminal environment (i.e., pH, bacterial fluctuations, passage rate, etc), the majority of protein consumed by ruminants is metabolized by ruminal microorganisms (Bach et al., 2005). Under normal rates of passage, bacterial cells are the primary source of protein for ruminants (from 50 to 80%), and therefore the extent to which a diet supports microbial populations has a direct affect upon the ruminant (Storm and Ørskov, 1983; Cotta and Hespell, 1986; Bach et al., 2005).

When evaluating a diet, adequate means of measuring microbial growth are needed. Production of bacterial cells per unit of ATP has been used to estimate microbial efficiency (Bauchop and Elsden, 1960). Bauchop and Elsden's report of 10.5 g cell yield per mole ATP, though considered a constant for many years, has been shown to be unreliable (Marr et al., 1963; Hobson, 1965; Pirt, 1965; Hobson and Summers, 1967). These measurements may still be valuable estimates, provided they are interpreted within the context of the individual trial. Hoover, et al. (1984) proposed correlating ATP yield with VFA production citing 2 mol ATP/mol acetate and butyrate, 3 mol ATP/mol propionate and valerate, and 1 mol ATP/mol lactate.

Bacterial yield is also measured by the efficiency of microbial protein synthesis, which includes g microbial-N per unit of available energy (Bach et al., 2005). Bacterial-N has been measured through crude protein, specific amino acids, DNA, RNA, total purines, and purine derivatives (Zinn and Owens, 1986; Calsamiglia et al., 1996; Obispo and Dehority, 1999; González-Ronquillo et al., 2004). However, such methods for isolating and measuring microbial-N apart from somatic and plant (i.e., feed) nitrogen have proven difficult due to the lack of internal markers unique to bacteria or the lack of assay specificity (Dehority, 2003). Several reports indicate that total purines can be successfully used when used in conjunction with ^{15}N salts (Broderick and Merchen, 1992; Calsamiglia et al., 1996). Most often, total purines are used, and it is generally understood that it is not foolproof and care should be taken when comparing results from different trials (Calsamiglia et al., 1996; Obispo and Dehority, 1999).

Microbial Markers

Amino Acids

In an attempt to track and quantify the flow of bacterial protein from the rumen (i.e., and essential component of a ruminant's protein balance), a number of makers have been evaluated. Peptidoglycan within the bacterial cell wall is composed of repeating glycan polymers consisting of alternating *N*-acetylmuramic acid and *N*-acytylglucosamine. Each *N*-acetylmuramic acid has a tetrapeptide attached to the number 3 carbon (Figure 4). The peptide contains: L-alanine, D-glutamate, L-R_3, and D-alanine. The third amino acid (i.e., L-R_3) varies with specie and may include: L-alanine, L-homoserine, L-diaminobutyric acid, L-glutamic acid, L-ornithine, L-lysine, LL-diaminopimelic acid, or *meso*-diaminopimelic acid.

2,6-Diaminopimelic acid. Peptidoglycan often contains diaminopimelic acid (DAPA), but contrary to popular belief, this non-protein amino acid is not found exclusively within bacteria, as originally thought. All samples analyzed by Rahnema and Theurer (1986) contained DAPA, which included bacteria, protozoa, and select feedstuffs. However, they also reported that bacterial samples contained 2 to 5 times more DAPA on a N-basis and 3 to 27 times more per gram of sample than do protozoa or select feedstuffs, and may, therefore, have value as an internal microbial marker.

Rahnema and Theurer (1986) reported limitations in accurate DAPA measurements. Similar findings were found by Spackman et al. (1958) who noted that, during hydrolysis, traces of isoleucine formed allo-isoleucine via racemization that may

"contaminate" assay standards. Allo-isoleucine will elute at essentially the same time as DAPA resulting in an overlay of two peaks. Further, the accuracy of DAPA requires a constant ratio of bacterial species present and/or DAPA to N ratios that remain constant among microbes (Stern and Hoover, 1979). The reason being, DAPA to N ratios are known to be variable among species (Work and Dewey, 1953).

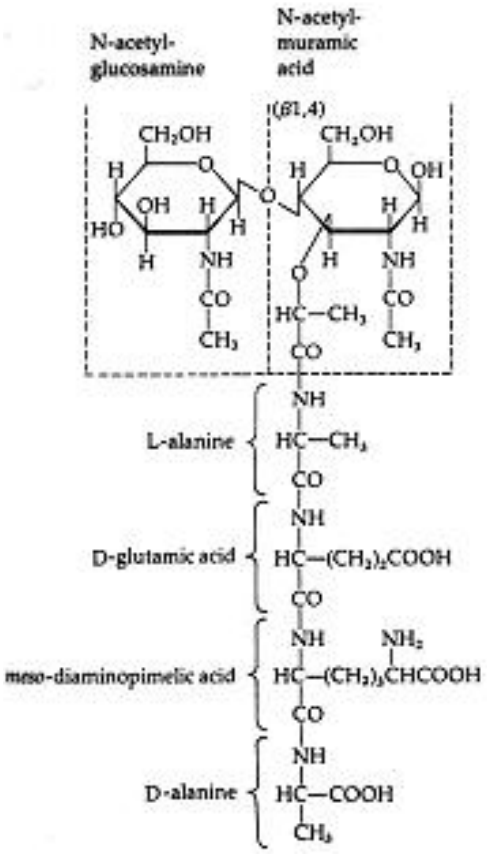

Figure 4. Illustration depicting the glycan subunits of peptidoglycan and the tetrapeptide crossbridge that serves to link two conjoining chains

File reprinted from:
http://www.textbookofbacteriology.net/Bacillus.html

Masson et al. (1991) published data from an *in vivo* experiment showing DAPA metabolism by protozoa, bacteria, and ruminant animals. Upon radiolabeling DAPA with [^{3}H] the authors could demonstrate the decarboxylation of DAPA to yield Lysine, which in turn was incorporated into bacterial and protozoal cells. These results are in agreement with preliminary *in vitro* experiments suggesting lysine formation as likely avenue of DAPA disappearance (Masson and Ling, 1986; Denholm and Ling, 1989). After correcting for cell density, Masson et al. (1991) compared their data with the *in vitro* data of Masson and Ling (1986) to show great continuity in [^{3}H]-lysine incorporation into protozoa and bacteria. Since only [^{3}H]-DAPA was infused in either case, the theory of DAPA decarboxylation into lysine was verified. Within 24 h, 42% of [^{3}H]-DAPA passed into the duodenum as [^{3}H]-DAPA while 23% passed in the form of [^{3}H]-lysine. Across the trial, 41% of DAPA was degraded in the rumen while 81% was degraded between the duodenum and the rectum (Masson et al., 1991). This data indicate that DAPA is readily metabolized within the alimentary tract. It is a component of cellular debris and is not a reliable marker for tracing true microbial protein along the gastrointestinal tract.

2-Aminoethylphosphonic acid. Aminoethylphosphonic acid (AEP), is an organophosphoric compound with characteristic C–P bonds that are very resistant to acid hydrolysis (Czerkawski and Faulds, 1974; Ankrah et al., 1989). First identified in ruminal ciliate protozoa by Horiguchi and Kandatsu (1959), the compound is sometimes referred to as ciliatine. While AEP is the most abundant, other aminophosphonic acids have been found in rumen protozoa (Broderick and Merchen, 1992).

In protozoa, AEP is found in both lipids and proteins, while in animals it has only been isolated from lipids. Because AEP was believed to occur exclusively in protozoa (e.g., lipids and protein) and minimally in animal tissues (e.g., lipids), it has been used as an indication of rumen protozoal dynamics apart from bacterial populations (Horiguchi and Kandatstu, 1959; Abou Akkada et al., 1968; Czerkawski and Faulds, 1974). However, it was later shown that AEP is found within bacteria, protozoa, animals, as well as dietary components (Ling and Buttery, 1978) calling into question its value as an protozoal internal marker. Further, considerable variability in AEP concentrations among ruminal ciliate protozoa has been previously noted (Whitelaw et al.), limiting is reliability.

Ankrah et al. (1989) developed a quantitative technique for analyzing AEP that was employed to evaluate rumen metabolism of AEP as well as the content of AEP in ruminal bacteria and feed samples. Ruminal fermentation of AEP ranged from 95 to 100% in 24 h, which is in agreement with reports by others (Cockburn and Williams, 1982) indicating greater disappearance of ruminally infused AEP than was accounted for by that flowing from the rumen. For AEP to be an adequate marker of protozoal protein flowing into the lower tract, it would be important for any free-floating AEP to be readily fermented within the rumen. However, Ankrah et al. (1989) working with defaunated sheep detected AEP in the ruminal fluid, feedstuffs, among mixed and pure-cultured bacteria, and duodenal digesta.

Every bacterium evaluated contained sizable concentrations of AEP, suggesting that AEP is synthesized by bacteria. Among those where, *Fibrobacter succinogenes* B21a and *Ruminococcus flavefaciens* B1a containing 60 to 80% less AEP than *Prevotella*

ruminicola H2b; *Butyrivibrio fibrisolvens* H10b, H4a, H17c; *Lachnospira multiparus* D15d; or *Streptococcus bovis* ARD-5d (Ankrah et al., 1989). Their findings concurred with earlier reports (Ling and Buttery, 1978; Cockburn and Williams, 1984). Numerous citations were provided by Ankrah et al. (1989) indicating the universal presence of AEP in nature (i.e., mollusks, echinoderms, arthropods, coelenterates, liver, brain, and milk) would prohibit the use of AEP as a microbial marker.

D-Alanine. The tertrapeptides of bacterial peptidoglycan contain D-alanine. It has been proposed as an effective alternative to diaminopimelic acid for use as an internal marker to measure flow of bacterial cells (i.e., bacterial protein). D-Alanine it is found in greater concentrations within bacterial cells and it would provide greater consistency across species than diaminopimelic acid. Garrett et al. (1987) reported an absence of D-alanine in feed samples tested, possibly indicating potential use as an internal marker. However, observed discrepancies may nullify its perceived promise (Quigley and Schwab, 1988).

Leucine. Bacterial leucine content is similar to most feedstuffs, and would likely limit its use as a bacterial marker under most feeding systems. Sorghum grain and casein, however, contain twice as much leucine as bacteria. In general, leucine could be used to monitor bacterial flow under concentrate diets, but not roughage diets (Rahnema and Theurer, 1986). However, Sinclair et al. (1995) compared [^{3}H]Leucine and Cytosine as markers to determine microbial-N in duodenal samples of sheep receiving diets

consisting of approximately 50:50 concentration to roughage. In this case, there were no differences between the two markers.

Lysine. Within bacteria, lysine represents the greatest proportion of amino-N. When comparing lysine and diaminopimelic acid, similar measurements of bacterial yield were noted by Rahnema and Theurer (1986).

Amino Acid Combinations. Rahnema and Theurer (1986) conducted a trial to evaluate amino acids independently and in combination. Diaminopimelic acid returned a greater (67.1%) estimation of percent abomasal bacterial protein than lysine, leucine, or combinations of DAPA, lysine, or leucine. Similar results were obtained when DAPA, lysine, or lysine x leucine markers were used. Diaminopimelic acid x lysine and leucine markers returned unrealistic values of less than zero or greater then 100, and should be used with caution. The most continuity was observed when DAPA and lysine were used individually.

Adenosine Triphosphate

It has been accepted that ATP can serve as an estimator of microbial biomass associated with aquatic and terrestrial ecosystems (Ausmus, 1971; Christian et al., 1975), and unlike 2-aminoethylphosphonic acid and 2,6-diaminopimelic acid that differentiate between bacteria and protozoa biomasses, use of ATP will give a holistic assessment of both protozoan and bacterial biomass (Forsberg and Lam, 1977). A further benefit of using ATP to evaluate microbial biomass is that all other methods (i.e., radiometric,

amino acids, etc.) will also measure dead cells, where as ATP will only evaluate viable cells because of ATP's relation to cellular metabolism (Wallace and West, 1982). However, it has also been noted that when *Escherichia coli* is cultured on a glucose-limiting substrate, a shift toward a smaller ATP pool occurs (Cole et al., 1967). The efficacy of using ATP as a biomass marker as opposed to an indicator of metabolic activity needs to be considered when using this method.

Forsberg and Lam (1977) conducted a comprehensive trial to 1) identify the best method for extracting ATP from whole or fractionated ruminal fluid, 2) evaluate the difference in total ruminal ATP-pools among cattle receiving hay, corn, or rolled oat diets, and 3) to determine the ATP-pool from 9 dominant ruminal bacteria grown in pure-culture. The method resulting in the greatest ATP-yield was determined to be a modification of protocols previous proposed by Christian et al. (1975) and Lee et al. (1971). When evaluating the effect of diet upon ruminal ATP-pools, Forsberg and Lam (1977) reported greater ATP yields when cattle received grain in their diet. Specifically, when cattle received grain, ATP was predominantly isolated from the strained ruminal fluid fraction and when solely hay was fed, ATP was predominantly isolated from the particulate fraction, indicating a shift from free-floating amylolytic bacteria to particulate-attached cellulolytic bacteria.

Forsberg and Lam (1977) wanted to evaluate the distribution of ATP among bacteria and protozoa as well as from pure bacterial-cultures. They determined that removing protozoa by slow-speed centrifugation (175 x *g*) resulted in a removal of 74% of extracted ATP. Finally, nine dominant ruminal bacteria were selected and evaluated for individual ATP-pool by growing pure-cultures on artificial media. Selected species

included: *Streptococcus bovis* Sb3, *Prevotella* (*Bacteroides*) *ruminicola* subsp. *ruminicola* 23, *Selenomonas ruminantium* D, *Butyrivibrio fibrisolvens* D1, *Megasphera elsdenii* B159, *Ruminococcus albus* B199, *Fibrobacter* (*Bacteroides*) *succinogenes* S85, *Bacteroides amylophilus* 70, and *Eubacterium ruminantium* GA195. Most cells investigated exhibited a consistent ATP yield during early exponential phase (ranging from 1.1 to 17.6 µg ATP/mg dry cell, across bacterial species investigated). The greatest ATP yield was associated with *S. bovis* and *P. ruminicola* subsp. *ruminicola* (17.6 and 6.5 µg ATP/mg dry cell, respectively), with the remaining bacterial ATP ranging from 1.1 to 4.5 µg ATP/mg dry cell. By late exponential phase, ATP production reduced by half followed by a precipitous decline throughout stationary phase. This was true for all bacteria investigated except *R. albus* and *B. amylophilus* cultures which did not show any signs of ATP decline during late exponential growth.

To definitively assess the contribution of ruminal ATP pools by bacteria and protozoa separately, Nuzback et al. (1983) conducted an *in vitro* trial to culture and compare faunated and defaunated ruminal fluid (i.e., with our without protozoan populations). As a control, results were compared to pure-cultured *S. bovis.* Within 3.5 h the ATP pool for *S. bovis* increased from 0.1 to 1.2 µg ATP/mL, but throughout the stationary phase concentrations decreased to 0.4 µg ATP/mL. Conversely, ruminal fluid was taken from cattle consuming one of two diets: 1) 70% alfalfa hay and 30% grain, 2) 50% alfalfa hay and 50% grain. During the fist 4 h of culture, ATP concentrations increased 0.1 to 0.5 µg ATP/mL and 0.5 to 2.5 µg ATP/mL, respectively. When cultured ruminal fluid contained protozoa, the ATP yields were 40 times greater than cultures of similar bacterial counts devoid of protozoa. Specifically, when cattle consumed 70%

alfalfa hay and 30% grain, 50% alfalfa hay and 50% grain, or 100% alfalfa hay, protozoa contributed 95, 85, and 64% of ruminal ATP, respectively (Nuzback et al., 1983).

Concentrations of ATP were correlated with bacterial counts during the exponential growth phase, but during stationary phase variable results were recorded with no strong correlations observed. This may be due to the fact that there is a specie-dependent variability among ruminal bacteria that is only partially related to diet (Forsberg and Lam, 1977; Erfle et al., 1979; Nuzback et al., 1983). Ruminal ATP pools are consistently correlated with protozoal concentrations, rather than bacterial densities (Nuzback et al., 1983). As a result, ATP would likely not be the best tool for assessing rumen bacterial densities, much less the total rumen microbial biomass.

Nucleic Acids

DNA & RNA. Both DNA and RNA and their products of metabolism have been used to evaluate the flow of microbial protein from the rumen. Often urine excretion is evaluated for purine derivatives, which will be discussed in the section entitled "Total Purines," below.

Potential problems related to nucleic acid markers were noted by McAllan and Smith (1973) and included that nucleic acids were readily degraded in whole and cell-fee ruminal fluid. The DNA and RNA degradation was likely a combination of extracellular nucleases (from lysed cells) and oxidative damage, for which RNA is especially susceptible. From McAllan and Smith's 1973 study, essentially all RNA had disappeared within 1 h of infusion into ruminal fluid, conversely after 4 h, as much as 36% of the

DNA he was still detectible. This was confirmed by Razzaque and Topps (1972) who indicated that little to no DNA or RNA passed beyond the abomasum of lambs or the rumen of cattle, respectfully. Other work seems to indicate RNA detection methods tend to overestimate microbial concentrations reaching the duodenum (Smith et al., 1978). Often, ruminal RNA is evaluated in relation to total N, however, the RNA to total-N of mixed bacteria will fluctuate due to dietary and environmental factors.

Other researchers calculated nucleic acids entering the duodenum of steers to be 15 to 35 g/kg DM, of which 60 to 70% was from RNA (Smith and McAllan, 1971). The source of nucleic acid was believed to be largely from microbial origin. The necessity of protection, and giving credence to a microbial-origin theory (as opposed to ruminal lysate), was demonstrated previously when free-form nucleic acids infused into the small intestine were degraded at a rate of 97% (McAllan, 1980). The majority of DNA and RNA added to the proximal duodenum of sheep and cattle disappeared within the first 25% of the small intestine (McAllan, 1980). Nucleic acid disappearance is likely a combination of of enzymatic degradation and oxidative stress (McAllan, 1982). If a large quantity of nucleic acids, and specifically microbial-RNA, are being degraded in the intestine and subsequently in the enterocytes and liver, metabolites may be detectible in the urine and/or feces. This has lead to a field of study investigating the concentration of purines and purine derivatives as a means of assessing flow of microbial protein from the rumen.

Total Purines. Microbial protein makes a significant contribution to the protein balance of cattle, and measuring total purine has been suggested as a microbial marker to

track the production and flow of non-feed protein to the intestine where it can be assimilated by the animal (Zinn and Owens, 1986). Obispo and Dehority (1999) noted a lack of uniformity in published literature and proposed a set of standard conversion factors, which are: RNA 22% purine; DNA 20.5% purine; Purines 49% N; RNA 10.8% N; Bacterial protein 15% N (i.e., N x 6.667). These proposed conversion factors were based upon previously published literature (Marshak and Vogel, 1951; Van Soest, 1994; Dehority, 1995).

Obispo and Dehority (1999) modified the procedures of Zinn and Owens (1986) and Ushida et al. (1985) for use in low-density rumen bacterial cultures. Both pure cultures and mixed-cultures were evaluated for purine, protein, and cell density content. Obispo and Dehority (1999) reported, purine, protein, and purine:protein ratio content of pure cultures that varied considerably (0.69 to 5.57% DM basis) yet were strongly correlated and could be used to predict cell density within the respective pure culture. Slightly less, were the results reported by Arambel et al. (1982; 0.37 to 2.01%) when evaluating 17 pure-cultured rumen bacteria. By recording cell density and purine concentrations of ruminal fluid, Obispo and Dehority (1999) were able to take purine values and back-calculate cell density, obtaining a value within range of their reported viable cell counts. The authors urged caution when applying these data to predict duodenal microbial protein. Purine:protein ratios from pure cultures was 0.0883, while that of the mixed cultures was 0.0306. Noting the discrepancy, a potential for overestimating microbial protein is potential when may when using the mixed-culture purine:protein ratio. It was sepeculated that the resulting discrepancy was due to contamination by trace plant particulate matter.

Purines are known to be efficiently absorbed and are only partially metabolized, yielding purine derivates. So, as an alternative to measuring purines from digesta, it has been proposed to evaluate purine-derivatives (e.g., allantoin, and uric acid) as metabolic markers secreted in urine and milk to indicate microbial protein utilization (González-Ronquillo et al., 2004). Reportedly, mammary excretion of purine derivatives was minor and not correlated with changes in feed intake, while total purine derivatives secreted in urine increased as feed intake increased. Previous work has validated purine derivative excretion in urine upon abomasal or duodenal purine infusion (Balcells et al., 1991; Vagnoni et al., 1997). However, extent that feedstuff and host cells contribute to purine metabolism will vary based upon physiologic and dietary status, and should be used with caution.

Reynal et al. (2005) sought to compare flow of non-amino N to the omasum by using total purines, complete amino acid profiles, purine derivatives, and ^{15}N as microbial markers when comparatively feeding 4 concentrations of ruminally degradable protein (RDP). As RDP decreased, total omasal non-amino N decreased linearly. Amino acid profiles and ^{15}N showed a decrease in non-amino N for fluid-associated bacteria, fiber-associated bacteria, and total bacteria. Total purine derivative and uric acid were not impacted by RDP concentrations, but allantoin decreased linearly as RDP decreased. When total purines where used to monitor bacterial flow, no measurable difference was observed RDP decreased.

Previous investigators suggested a combination of both ^{15}N and total purine techniques as the best method for quantifying microbial yield (Broderick and Merchen, 1992; Stern et al., 1994; Calsamiglia et al., 1996). However, Cecava et al., (1990) noted

a shift in purine:N ratio between fluid-associated and particulate-associated bacteria over time after feeding.

Radioisotopes

^{15}N Enrichment. Inorganic ^{15}N has been used previously as a marker to monitor the production and flow of microbial protein in ruminants *in vivo* and *in vitro* (Broderick and Merchen, 1992) to differentiate from endogenous and dietary-N sources. Dosing the rumen with ^{15}N-containing ammonium salts has been shown to solely label microbial protein in short-term studies. In long-term evaluation, host tissues will be label through the assimilation of microbial protein and will skew estimates of microbial nitrogen. Similarly, additional variation can be introduced into this method by bacteria receiving non-labeled feed nitrogen rather than then enriched infusate. Further, protozoal-protein will also become labeled via predation of labeled bacteria. A report by Firkins et al. (1987) indicated N of free-floating and adherent bacteria comprised 73 and 70% NH_3-derived N, whereas protozoa obtained only 51%, indicating enrichment may be a reliable means of assessing flow of microbial protein into the duodenum. It should be remembered that data will be variable due to relative energy, protein, and ammonia availability within the rumen. Protozoal-N leaves the rumen to a far less degree than bacterial-N and comprises approximately only 27% of duodenum non-ammonia N, so even if protozoa are labeled, they should contribute very little, if any, toward duodenal studies.

Calsamiglia (1996) evaluated and compared the use of purines and ^{15}N as microbial markers with *in vitro* rumen-cultures receiving diets formulated with variable protein degradability and purine concentrations. It was noted that almost all dietary purines were degraded by ruminal bacteria, and the remnant purines evading digestion did not complicate calculation of microbial-protein flow. When ^{15}N was used, less variation among samples occurred, indicating a more precise estimation of microbial protein.

Firkins et al. (1987) infused $(^{15}NH_4)_2SO_4$ into the rumen of cannulated steers and compared flow of microbial-N and N turnover among protozoa, particulate-associated bacteria (PAB), and fluid-associated bacteria (FAB). Nitrogen and ratios of N to purines and N to DAPA were not influenced by DMI or urea supplementation among protozoa, PAB or FAB. Further, enrichment of ruminal microbes with ^{15}N was similar among bacteria regardless of intake, but was greater ($P< 0.05$) when steers received the urea-supplemented high-intake diet. Ratios of purines to N were similar among free and adherent bacterial pools, however, DAPA to N ratios varied among bacterial populations. Similarly, ^{15}N was compared to ^{35}S and was determined to result in an estimation of microbial protein 10% less than enriched sulfur (Kennedy and Milligan, 1978).

^{35}S Enrichment. Initial studies involved $Na_2{}^{35}S$ to label the sulfide pool of the rumen with the assumption that S used in microbial protein would first transition as H_2S (Walker and Nader, 1968), which was later shown not to be true (Stern and Hoover, 1979). To evaluate flow of microbial protein, ^{35}S-labeled inorganic sulfate (i.e., $Na_2{}^{35}SO_4$) has been continuously infused into the rumen. This method is preferred

because it does not rely on relatively unstable sulfide and does not require that all microbial protein use H_2S as the sulfur source, as there are many in the rumen (e.g., dietary amino acids). In this form, $^{35}SO_4$ is reduced to ^{35}S and incorporated into bacterial protein via *de novo* synthesis of cystine and methionine (Beever et al.; Broderick and Merchen, 1992). Protozoa will be largely dependent on external amino acids sources and would be indirectly labeled through predation of labeled-bacteria and will bias results slightly. Walker and Nader (1975) found a correlation (r = 0.68) between ^{35}S and DAPA when used as a marker for monitoring microbial protein. The results were consistently 30% less for DAPA, presumably because of protozoal protein flow.

In another study, RNA, ^{35}S, DAPA, and AEP were compared (Ling and Buttery, 1978). Their findings lead them to conclude that AEP is insufficient as a protozoal maker due to the fact that significant concentrations of AEP can be found in bacteria, feedstuffs, and protozoa. As a general marker of rumen microbiota as a whole, DAPA can be used, but should not be considered reliable as strictly a bacterial marker due to the fact that protozoa also contribute DAPA to the duodenal samples. Progressively, RNA was seen as the next most reliable maker of those evaluated, allowing general comparisons among treatments. However, the most accurate marker was seen as ^{35}S.

Any impact on heath or environmental when using ^{35}S are relatively minimal due to weak β-emissions and short half-life (87 d); however, accumulation within milk and tissues would preclude their use as a food source (Broderick and Merchen, 1992).

^{32}P Enrichment. In an attempt to measure microbial protein synthesis and flow, Bucholtz and Bergen (1960) monitored microbial phospholipid synthesis by infusing

$H_3{}^{32}PO_4$ into the rumen of sheep and found that phospholipid incorporation of ^{32}P was correlated (r = 0.98) to synthesis of microbial protein. Free inorganic-^{32}P was later incorporated by growing flora. A strong correlation occurred between cell growth and phospholipid synthesis with a diet-protein response. When comparing the ratio of ^{32}P incorporated into nucleic acids against non-amino-N to estimate microbial protein derived from RNA in comparison to total RNA, ^{32}P-labeled RNA values were 15% less (Smith et al., 1978), possibly due to dietary RNA contamination.

A study to compare the efficacy of using ^{15}N, ^{35}S, and ^{32}P as microbial markers was conducted and indicated extensive degradation and exchange of cellular material among microbes within the rumen. None of the three isotopes were deemed useful in measuring periods of an absence of growth, negative growth, or normal growth (Stern and Hoover, 1979), where negative net growth was defined by Naga and Harmeyer (1975) as the condition where degradation of microbial matter exceeds that of synthesis.

Although use of ^{32}P shows strong promise, environmental implications associated with its hazardous nature limit its widespread usage.

Bioluminescence

Bioluminescence is the phenomenon of natural low-light emission from select dinoflagellates, fungi, bacteria, bacterial nematodes, insects, fish, shrimp, and squid (Meighen, 1991; Węgrzyn and Czyż, 2002). Bioluminescence is an exergonic, chemiluminescent reaction that involves at least two physiologically stable molecules. The substrate known as luciferins (i.e., light bearers), are oxidized by an oxygenase enzyme called *luciferase* (Hastings, 1996). This oxidation yields non-reactive

oxyluciferin and electronically excited intermediates which interact with long-chain aldehydes to release energy in the form of blue-green light (Meighen, 1991; Hastings, 1996; Greer and Szalay, 2002). Luciferins and *luciferase* enzymes are diverse and varying with respect to cofactors and/or coenzymes that are required for the reaction to proceed, and in some cases, no cofactors or enzymes are required (Greer and Szalay, 2002). As a result, the excited intermediate and color spectra emitted, will vary as the molecular conformation changes thus altering spectral properties (Hastings, 1996). The wavelength emission is often associated with the native habitat of the organism. Deep sea organisms will often have peak emission in blue (450 to 490 nm), costal marine life typically emit within the green spectrum (490 to 520 nm), land and fresh water life often emit within orange (550 to 580 nm), deep violate (400 nm), or far red (620 nm) spectra (Hastings, 1996).

Luciferins and *luciferases* are represented in numerous organisms, taxa, and phyla, yet they can all be summed up in five basic luciferin-*luciferase* systems, all of which require oxygen (Meighen, 1991; Hastings, 1996). (1) Bacterial luciferin involves the oxidation of reduced riboflavin phosphate ($FMNH_2$) by *luciferase* in the presence of oxygen and long-chain aldehydes. In addition to bacteria, this category of luciferins can also be found in fish, pyrosomes, and squid. The cDNA coding for luciferins from the "bacterial luciferin" category are often obtained from the bacteria *Vibrio harveyi* and *Photorhabdus luminescens* and are referred to as the *lux* gene. (2) Dinoflagellate luciferin bears a resemblance to porphyrin of chlorophyll. This luciferin is unique in that it is shielded from *luciferase* at pH 8 and above due to conformational changes in the tertiary structure of the molecule. However, under moderately acidic conditions (pH 6),

this luciferin is readily oxidized by *luciferase.* (3) Vargulins are a type of luciferin found in *Vargula,* a marine ostracod. This luciferin, also found in some types of fish, is thought not to occur naturally but occur by ingestion from some other source. (4) Coelenterazine is among the most studied luciferin. Coelenterazine can be found in Cnidarians, Copepods, Chaetognath, Ctenophores, Decapod shrimp, Mysid shrimp, Radiolarians, and some fish. The activity of the associated *luciferase* is tightly controlled by Ca^{+2} concentration, and actually resembles Calmodulin. (5) Firefly luciferin is exclusively found within fireflies (*Photinus* or *Luciola*). This luciferin is unique in that it requires ATP for its activation, and as a result has been used in ATP assays. The cDNA encoding for firefly *luciferase* is designated as the *luc* gene, not to be confused with the bacterial luciferin gene, *lux.*

Among luminescent organisms, bacteria are the most abundant and are represented by free-living, symbiotic, and parasitic bacteria (Meighen, 1991, 1994) with *Vibrio harveyi, V. fischeri*, *Photobacerium phosphoreum, P. leiognathi,* and *Xenorhabdus luminescens* being studied the most commonly (Meighen, 1991). As much as 20% of the bacteria's energy is expended in promoting luminescence and, until recently, was of no known value to the bacterium independent of a symbiotic relationship that attracts prey to a host organism (Meighen, 1991; Węgrzyn and Czyż, 2002). However, Czyż et al. (2000) made a presumption that luminescence provided UV protection, a hypothesis based upon random mutagenesis in *V. harveyi* (a common bioluminescent bacterium). Others have indicated that luminescence might be a function of oxygen detoxification (Rees et al., 1998), an idea supported by later work subjecting wild-type and variant *lux* bacteria to H_2O_2 (Węgrzyn and Czyż, 2002).

Bacterial *luciferase* is a herterodimer composed of an α and β subunit, and is designated as *lux*AB in the *lux* operon. One common bacterial *luciferase* reaction (2-1) includes luciferin, riboflavin phosphate ($FMNH_2$), which is oxidized to FMN, and the oxidation of long chain aldehydes to fatty acids (Meighen, 1991; Węgrzyn and Czyż, 2002). The resulting fatty acids are then reduced back to aldehydes forming $NADP^+$ and ADP to continue the cycle (Ziegler and Baldwin, 1981).

$$RCHO + FMNH_2 + O_2 \longrightarrow RCOOH + FMN + H_2O + h\upsilon \quad (490\ nm) \qquad (2\text{-}1)$$

Similarly, the *lux*CDE gene of the *lux* operon code for aldehyde synthesis is essential for the bioluminescence reaction (Reaction 2-2). The *lux*CDE codes for a trimeric fatty acid reductase complex containing a reductase, transferase, and synthetase, respectively. The transferase initiates the transfer of acyl groups to water or other oxygen or thiol acceptors during a process of acylation (Meighen, 1991).

$$RCOX + HOH\ (HSR') \longrightarrow RCOOH\ (RCOSR') + XH \qquad (2\text{-}2)$$

The reductase enzyme causes the reduction of fatty acids to aldehydes while *synthetase* activates fatty acids by forming acyl-AMP. When *reductase* present, acyl groups are transferred to *synthetase* then to *reductase* before being reduced by NADPH to aldehyde (Reaction 2-2; Wall et al., 1986; Meighen, 1991).

$$RCOOH + ATP + NADPH \longrightarrow NADP + AMP + PP_i + RCHO \qquad (2\text{-}3)$$

In 1985, Engebrecht et al. (1985) demonstrated that the full bacterial *luciferase* operon, *lux*CDABE, from *Vibrio fischeri* could be expressed in *E. coli*, thereby eliminating the necessity to add luciferin substrates (Greer and Szalay, 2002). Since that time numerous researchers have successfully used the complete *lux*CDABE operon in gram-negative bacteria, but not gram-positive bacteria, which are not compatible with the *lux*CDABE operon. Winson et al. (1998) reported that gram-positive ribosomes are unable to translate mRNA with gram-negative ribosomal binding sites. All naturally occurring luminescent bacteria are gram-negative and have served as the source or pattern for operon use. Therefore, the operons being used were only suitable for gram-negative bacteria.

The *lux*CDABE operon contains two main genes. The *lux*AB gene encodes for *luciferase* while the *lux*CDE gene encodes for a fatty acid *reductase* complex that replenishes the aliphatic aldehydes (Meighen, 1991; Francis et al., 2000). The *lux*AB gene has been successfully incorporated into gram-positive bacteria, but luciferin substrates must be manually added to the media, limiting effective use in natural environmental conditions. That is, until, Francis et al. (2000) successfully transform gram-positive bacteria with a complete functioning operon. Their success was attributed to slight modifications made to the gram-negative *lux* operon obtained from *Photorhabdus luminescens.* Modifications included the addition of gram-positive ribosome binding sites to each gene (*lux*A, -B, -C, -D, and -E); then, Francis et al. (2000)

rearranged the genes from *lux*CDABE to *lux*ABCDE, and finally a promoter was added upstream of the gene cassette.

Lux bioreporters are genetically modified organisms containing the *lux* reporter gene. Dorn et al. (2003) cited numerous authors who indicated a correlation between luminescence of the bioreporters and either physiological or environmental conditions. In fact, Dorn et al. (2003) reported from personal research that luminescence of *lux* bioreporters was susceptible to temperature, pH, cell growth stage, substrate concentration, and dissolved oxygen concentrations. As temperature decreased, luminescence declined. Further, luminescence was relatively stable from pH 5 to 6.6, but was less stable as pH increased from 6.6 to 8.

Some *luciferases* are reported to be heat labile at temperatures in excess of 30ºC (Manukhov et al., 1999). To investigate intrinsic characteristics of bacteria on stability of *luciferase,* Mackey et al. (1994) inserted the *lux*AB gene from either *Vibrio fischeri* or *Xenorhabdus luminescens* into *Escherichia coli*, *Listeria monocytogenes*, *Salmonella typhimurium, and Brochotrix thermosphacta.* Thermal stability and activity of *luciferase* was determined. When *V. fischeri luciferase* was inserted into *E. coli*, enzymatic stability and viability was detectible at temperatures up to 44ºC. However, *luciferase* activity diminished at temperatures in excess of 30ºC, with 36.3ºC as the 50% inactivation temperature. Thermal stability studies involving *S. typhimurium* showed a 50% inactivation temperature of 35.2ºC and no significant differences compared to *E. coli*. However, the 50% inactivation temperatures for *L. monocytogenes* and *B. thermosphacta* were 38.8 and 40.5 ºC, respectively.

This work by Mackey et al. (1994) reveals that there is both a bacterial and gene affect on thermal stability. *Luciferase* from *X. luminescenes* was less heat labile than *luciferase* from *V. fischeri* when expressed in *E. coli* or *L. monocytogenes*, but regardless of *luciferase* source, *L. monocytogenes* showed added heat resistance compared to *E. coli*. Often, the cytoplasmic environment can affect heat stability of expressed *luciferase* and may be a contributing factor in the stability of *L. monocytogenes*. However, despite this fact, even though *S. typhimurium* is more heat-resistant than *E. coli*, there was no difference in *luciferase* thermal stability. Further, the least heat-resistant bacterium studied was *B. thermosphacta* for which *luciferase* was least affected by temperature (Mackey et al., 1994). Numerous internal and external environmental factors could affect the stability of expressed *luciferase*, but it is interesting to note that *luciferase* was most stable in gram-positive bacteria studied by Mackey et al. (1994).

Among the many bacterial markers that can be used, luminescence can prove advantageous by allowing quantitative measurements of bacterial growth and proliferation (Cook et al., 1993). Furthermore, response time with *lux* or *luc* is much faster than fluorescence incurred by using green fluorescent protein (Dorn et al., 2003).

Fluorescence

Green fluorescent protein (GFP) was first isolated from the marine hydroid *Obelia*, and shortly after in the jellyfish *Aequorea aequorea* in 1962 (Shimomura and Johnson, 1975; Hastings, 1996). It was found that the protein aequorin would emit light in the presence of Ca^{+2}; oxygen is not necessary for this reaction as in other bioluminescent reactions. Aequorin was therefore dubbed a photoprotein (Hastings,

1996); however, this designation was later showed to be flawed. In fact, aequorin is an intermediate compound formed from the binding of apo-aequorin (*luciferase*) with coelenterazine (luciferin). This luciferin-*luciferase* complex then reacts with oxygen to form aequorin that is stored. Upon binding to calcium, a conformational change alter spectral properties resulting in fluorescent emission (Hastings and Gibson, 1963; Hastings and Morin, 1969; Shimomura and Johnson).

Some organisms produce both fluorescence and luminescence such as dinoflagellates. This single-celled algae produces much of the visible luminescence of the ocean, which results from small luciferin-containing organelles called scintillons (Fogel and Hastings, 1972; Nicolas et al., 1987; Hastings, 1996) that emitt blue light. Co-localized with luminescence, fluorescence is caused by red pigment in the chloroplast (Johnson et al., 1985; Hastings, 1989; Desjardins and Morse, 1993). Upon excessive growth, the red fluorescence from the chlorophyll is clearly seen, producing "read tides" (Hastings, 1996). The fluorescent compound of dinoflagellates is actually the luciferin compound responsible for luminescence.

Since its discovery by Chalfie et al. (1994), GFP has been used extensively as a visual indicator, or reporter, of gene transcription. However, it has also been employed as a marker of bacterial growth in the presence of a desired substrate or toxin as well as to map fluctuations in bacterial populations (March et al., 2003). However, this marker is not without its shortcomings. The stability of GFP is unusually high, with relatively limited proteolysis, and up to 2 h or more is needed before the first detection of GFP can be achieved (Tsien, 1998; March et al., 2003). Further, Tsien (1998) reported that oxygen is required for GFP fluorescence limiting its efficacy in the study of obligate

anaerobes, a finding that was confirmed by previous work (Hastings and Gibson, 1963; Shimomura and Johnson, 1975; Hastings, 1996). However, though protein fluorescence may be more difficult in obligate anaerobes, it may not impossible and has been demonstrated by growing transformed *E. coli* in an anaerobic environment (Banning et al., 2002). Another difficulty that must be overcome with GFP use is the "background noise" or autofluorescence, which occurs often at the same wavelength as GFP (March et al., 2003). Tsien (1998) suggested at least 1 μmol of GFP was needed to produce twice as much fluorescence as background emission. However, variants of GFP now exist that function at a wide range of wavelengths (colors) and may offer advantageous alternatives to traditional GFP (March et al., 2003).

DNA Isolation

Previously, real-time PCR has been preformed on ruminal samples (Schofield et al., 1997; Tajima et al., 2001; Klieve et al., 2003; Ozutsumi et al., 2006; Stevenson and Weimer, 2007). Wang et al. (2005) amplified 16S rRNA and used PCR end-point analysis to evaluate bacterial diversity of the human intestinal tract. However, with any such sample analysis involving diverse bacterial populations and/or opportunities for DNA contamination, specificity of PCR may be compromised. This is of greater concern when isolation of bacterial DNA is complicated by the presence of numerous DNA sources as within the rumen (i.e., bacteria, feedstuff, protozoa, fungi, and host animal). To circumvent these adorations, the DNA Stool Mini Kit (QIAGEN®) was recommended because of its ability to isolate DNA while removing PCR-inhibitive impurities from fecal material (Yu and Morrison, 2004).

Others have used general chemical procedures (Denman and McSweeney, 2006; Mosoni et al., 2007) for cell lysis while still others have used bead-beating methods (Tajima et al., 2000; Tajima et al., 2001).

Polymerase Chain Reaction

Polymerase chain reaction (PCR), is the classic and revolutionary innovation of Dr. Kary Mullis, enabling the near exponential amplification of RNA and DNA. For this discovery, Dr. Mullis was awarded the Nobel Prize for chemistry in 1993, and rightly so, for since its inception, PCR has facilitated timely research and lead to untold discoveries both in molecular biology, microbiology, and medical sciences to name a few.

PCR requires sample DNA, buffer, $MgCl_2$, DNA primers, and deoxynucleotidephospates (dNTPs; e.g., GTP, ATP, CTP, and TTP) for DNA extension. Finally, to carry out the reaction, *DNA polymerase* is added. Most often, *Taq polymerase* from *Thermophilus aquaticus* is used because this bacterium can naturally survive extreme temperatures. As a result, *Taq polymerase* remains functional at temperatures many proteinaceous enzymes are heat labile. *Taq polymerase* is a very active enzyme polymerizing in the 5'-3' direction at a rate of 30 to 70 bp per second (Jeffreys et al., 1988) but lacks a 3'-5' exonuclease proofreading ability, making it prone to error (Bustin, 2000). To help minimize this prolificacy at room temperature, "hot-start *Taq*" was developed to enhance the specificity, sensitivity and yield of the DNA sequence of interest. Hot-start *Taq* has been modified with heat labile amino groups that at room temperature inhibit the functionality of the enzyme until denaturing temperatures for dsDNA have been reached. A second *polymerase* used, *Tth polymerase*, is derived from

the bacterium *Thermus thermophilus*, and is less thermostable and efficient than *Taq polymerase* (Grabko et al., 1996). Unique to *Tth polymerase* is a reverse transcriptase capability in the presence of Mn^{2+}, making it possible to amplify RNA by first converting it to cDNA (Myers and Gelfand, 1991; Chiocchia and Smith, 1997), a process known as reverse transcriptase PCR (RT-PCR).

In vitro polymerization of DNA depends upon an established thermal cycle consisting of three phases: 1) "melting" or denaturation of double stranded DNA (dsDNA) into single stranded DNA (ssDNA; 95°C), 2) primer annealing (55°C), and 3) primer elongation (75°C). Efficiency of the reaction will vary based upon melting temperature (T_m) which is directly affected by the G/C content of the DNA, salt concentrations (Mg^{2+} or Mn^{2+}), and length of oligonucleotide probes (if any). Further, PCR efficiency is influenced by dNTP concentration and primer concentration (Bustin, 2000). Due to the disparity in number of hydrogen bonds made between G/C and A/T, as a general rule, T_m can be calculated by the following formula: $T_m = 4 \cdot (C + G) + 2 \cdot (A + T)$.

Even though PCR efficiency should be considered, it must be realized that shortcomings exist regardless of efficiency of DNA amplification. These biases include: 1) contaminant inhibition of PCR, 2) differential amplification, 3) amplification of undesired DNA artifacts, 4) DNA contamination, and 5) variations in 16S rRNA due to *rrn* operon heterogeneity (von Wintzingerode et al., 1997). In addition, loss of specificity and accuracy of PCR assay has been reported when an excessive number of PCR cycles are used (Wilson and Blitchington, 1996; Whitford et al., 1998).

Inhibition of PCR has resulted from humic acids extracted along with nucleic acid isolation and strongly decrease efficacy of DNA modifying enzymes (von Wintzingerode

et al., 1997). As little as 0.08 to 0.64 ug/mL humic acid has been shown to inhibit *Taq polymerase* (Tebbe and Vahjen, 1993; Jacobsen, 1995). Humic acid is derived from the soil organic matter, humus, and would primarily be problematic for environmental microbiologist investigating soil bacteria. Other sources of inhibition exist including increased concentrations of polysaccharides, proteins, and tannins, all of which will be of concern to the rumen microbiologist (Sharma et al., 2003). In addition to inhibition of amplification, phenolic compounds have degraded DNA samples, thereby reducing PCR efficiency (Sakagami et al., 1995). To circumvent inhibition and degradation of DNA samples, Moreira (2001) proposed, as an alternative, to use electrophoresis as a means of isolating pure DNA samples.

Differential amplification is of concern when multiplex reactions are of unequal efficiency. Amplification of artifacts can divert primers away from target sequences and potentially out compete target for dNTPs and *DNA polymerase* resulting in biased real-time PCR analysis. Contamination of DNA can nullify controls, and lead to co-amplification of target and secondary DNA samples. Non-specific DNA may cause ambiguity, or create diversity that is artificial. Elimination of DNA contamination was investigated by Niederhauser (1994) and reported the most effective methods to be UV treatment and pre-PCR uracil *DNA glycosylase* digestion. Lastly, *rrn* operon (i.e., rRNA gene regions) heterogeneity has been identified as a source of extraneous error in PCR reactions. This is a reference to the degree of similarity or difference in 16S rRNA among bacterial species. Should sequences be fairly similar, precise differentiation will become problematic during PCR, necessitating both accuracy and precision when designing and constructing primers and probes.

Real-time PCR

Real-Time PCR allows for relative or absolute quantification of PCR products and/or gene expression. Relative quantification, for example, measures the change in gene expression among tissues or treatments in reference to a constitutively expressed housekeeping gene (e,g., glyceraldehyde-3-phosphate dehydrogenase, albumin, actins, tubulins, cyclophilin, hypoxantine phosphoribosyltransferase, 28S, and 18S rRNAs; Thellin et al.; Pfaffl, 2001). Absolute quantification depends upon an internal or external calibration curve that must be highly validated to ensure accuracy (Pfaffl, 2001).

Contrary to end-point PCR assays, the use of sequence-specific fluorescent probes complementary to the sequence of interest, allows enumeration of amplicon accumulation. To be successful, fluorescent dyes used must be bright and spectrally well-resolved (Lee et al., 1999). Fluorescence can be detected using either 1) emission scanning with fixed excitation wavelength or 2) scanning where multiple excitation and emission wavelengths are scanned simultaneously (Lee et al., 1999). Emission scanning with laser excitation, allows detection of target DNA even at low concentrations. Synchronous scanning by using multiple excitation wavelengths makes it possible to conduct multiplex PCR when several PCR reactions take place in a single sample. However, such multiplex systems are not as sensitive with small concentrations of amplicons and therefore not reliable for accurate quantification (Lee et al., 1999).

Fluorescent probes also have the added benefit of testing optimal PCR operating temperatures. By mapping fluorescence against temperature, the efficacy of current temperature gradient can be assessed by revealing completeness of DNA denaturation (e.g., melting; Figure 6). Melting curves can test the accuracy of PCR amplification

(Ririe et al., 1997); by comparing T_m against known nucleotide composition and/or sequence of desired amplicons, presence or absence of numerous artifacts can be elucidated. In real-time PCR, fluoresence is recorded after each amplification cycle to equate amount of PCR product produced (Figure 5). The greater amount of starting template present will result in a fewer number of cycles needed to reach threshold and concomitantly, a brighter signal.

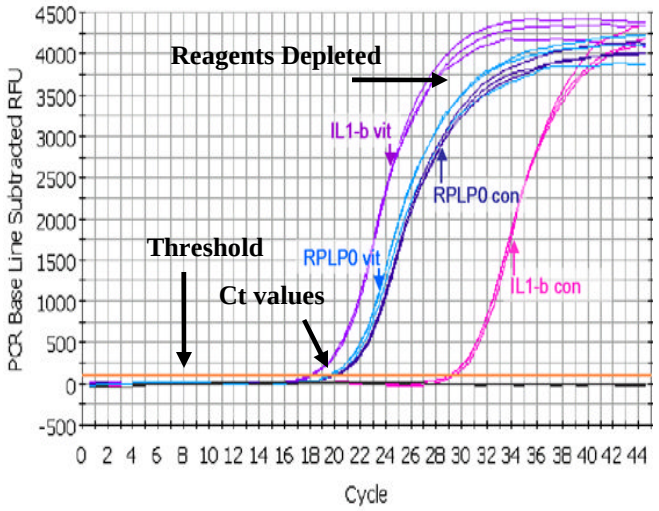

Figure 5. Relationship between fluorescence and number of cycles.

Adapted from:
http://pathmicro.med.sc.edu/pcr/realtime- home.htm.

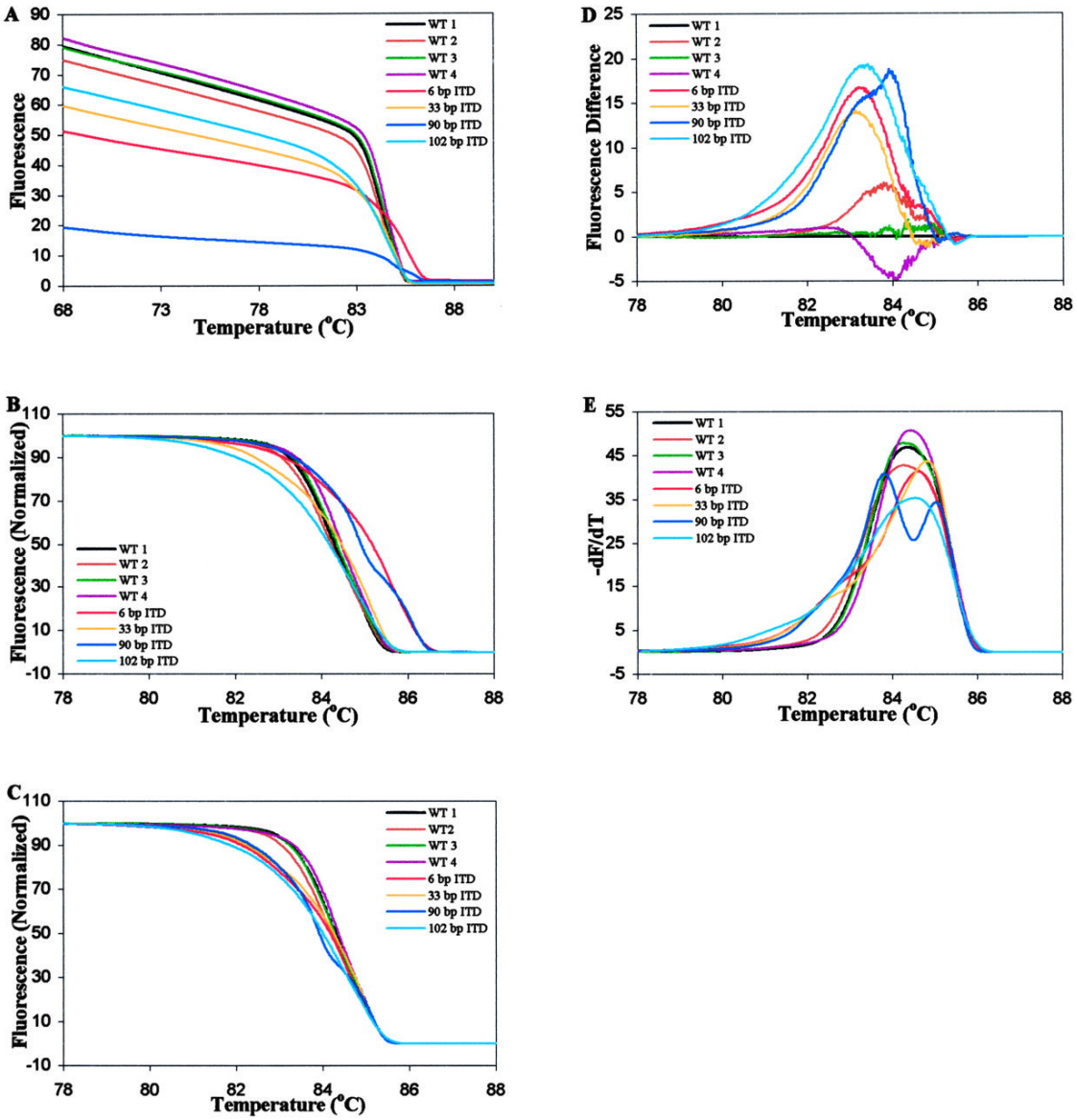

Figure 6. Detection of FLT3 ITD by high-resolution melting analysis.
Panel A: Fluorescence versus temperature melting curves using raw fluorescence data. Melting curves (panel B to E) after fluorescence-normalization, for the samples depicted in panel A.

Figure reprinted from: (Vaughn and Elenitoba-Johnson, 2004).

DNA-binding Dyes

SYBR Green. The simplest of fluorescence reporters for use in PCR is SYBR Green. This Fluorophore non-specifically binds to the minor groove (Figure 7) of any dsDNA (Morrison et al., 1998), and when unbound, SYBR Green exhibits very little fluorescence. Non-specificity of SYBR Green has the added benefit of allowing monitoring both annealing and amplification whereas probe only monitor amplicon formation. As amplification of desired sequence occurs, fluorescent signal will also increase, but artifacts, primer-dimers, and dsDNA bound to multiple SYBER Green dye molecules are also recorded. Since SYBR Green does not have any intrinsic specificity when binding, precision of quantitative results may be limiting. This is of greatest concern when comparing amplification of targets of varying lengths. If amplification efficiencies are constant, longer PCR products will generate a greater fluorescent signal than shorter amplicons, and if reaction efficiencies are not equal, data will be further confounded and unreliable. This problem may be over come by utilizing hybridization probes.

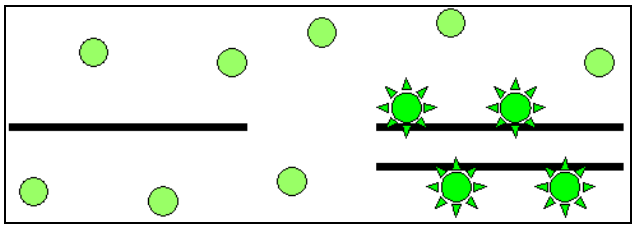

Figure 7. SYBR Green a dsDNA-binding dye.

Figure reprinted from:
http://pathmicro.med.sc.edu/pcr/realtime-home.htm

Ethidium Bromide. Ethidium bromide (EtBr) is a common dye used to detect dsDNA in agarose gel electrophoresis assays. Researchers have also used EtBr to monitor PCR reactions (1992; Higuchi et al., 1993). However, the efficacy of EtBr in PCR analysis is likely limited due to the greater fluorescence and dsDNA detection provided by SYBR Green (Bourzac et al., 2003), while EtBr has limited efficacy as a marker for small concentrations of DNA. Experimentation by Higuchi et al. (1993) involved serial dilutions of viral DNA, without which information early PCR reactions could not have been quantified much less starting with sample of unknown concentration. Finally, EtBr has been implicated as a carcinogen leading many investigators to seek alternative DNA binding dyes (Bourzac et al., 2003).

Amplicons, Primers, and Probes

To minimize error related to DNA contamination, primers should be constructed to span at least one intron of the genomic sequence (Bustin, 2000). Optimal primer length is approximately 15 to 20 bases while G/C content residing between 20 and 70% (Bustin, 2000). A PCR reaction should contain primer concentrations between 50 and 200 n*M* because as primer concentration increases, probability of mispairing and production of undesirable PCR product also increases (Bustin, 2000). Prolonged PCR reactions may result in insufficient primer availability, but this should not be of concern in real-time PCR reactions given that C_t values gained relatively early in the reaction are of primary importance for quantification. Further, efficacy can be contributed to amplicon length and should be between 80 and 100 base pairs (bp). Long amplicons may

necessitate increased Mn^{2+} or Mg^{2+} concentrations to facilitate stability of probe binding (Bustin, 2000).

As stated previously, probes are fluorigenic oligonucleotides designed to hybridize with a specific DNA sequence of interest in order to monitoring amplification as well as quantifying initial DNA sample. Optimal probe concentration is dependent upon background fluorescence and primer concentration, but should be approximately 100 n*M*. Fluorescence of un-annealed hybridization probes is more effectively quenched than uncleaved hydrolysis probes making detection of hybridized probes easier, a fact that should be taken into consideration when designing probes. Primers are extended immediately after annealing to form dsDNA preventing probe binding. As a result, probes must approximately 30 bp long, have a greater T_m (at least 10°C) than primers, and consist of a G/C content near 50% (Bustin, 2000). Further, target sequences for probe binding should be located toward the 3'-end of the amplicons to allow fluorescence detection prior to probe displacement by *Taq polymerase*.

PCR of Ruminal Microflora

While PCR has been used extensively in nearly all facets of modern science, applications to ruminal microbiology is less diverse. Among the first known instances where PCR was used to evaluate ruminal bacteria was published by Odenyo et al. (1994b) and others (Stahl et al., 1988; Lin and Stahl, 1995; Muetzel et al., 2003) who used 16S rRNA-specific probes labeled with [γ-^{32}P] ATP and attached to a nylon membrane to identify PCR amplicons. Later, Reilly and Attwood (1998) successfully

preformed competitive PCR to enumerate *Clostridium proteoclasticum*, a proteolytic bacterium isolated from the rumen of grazing cattle in New Zealand.

Similarly, competitive PCR (cPCR) has been employed to enumerate *Butyrivibrio fibrisolvens*, *Fibrobacter succinogenes*, *Ruminococcus albus*, and *Ruminococcus flavefaciens* (Kobayashi et al., 2000; Koike and Kobayashi, 2001; Koike et al., 2004). Others have investigated ruminal diversity across proteolytic bacteria (Reilly et al., 2002) and across both amylolytic and cellulolytic bacteria using cPCR (Edwards et al., 2004). Competitive PCR works by adding known amounts of standard DNA to the samples, which also contain identical primer binding sites as the target DNA. In this way, target DNA competes with controls for *Taq Polymerase*, dNTPs, and primers.

Real-time PCR uing SYBR Green has previously been used to investigate ruminal protozoa, *Entodinium* (Sylvester et al., 2004; Skillman et al., 2006), and numerous ruminal bacteria (Tajima et al., 2001; Peixoto et al., 2005; Ozutsumi et al., 2006; Stevenson and Weimer, 2007), Molecular beacon (Schofield et al., 1997). TaqMan probes have also been employed (Ouwerkerk et al., 2002; Klieve et al., 2003).

Similarly, numerous authors have sought to evaluate fecal bacteria from cattle. Inglis and Kalischuk (2004) quantified *Campylobacter jejuni* and *Campylobacter lanienae* using SYBR Green. Malinen et al. (2003) compared SYBR Green, and TaqMan applications of real-time PCR with γ-^{32}P tagged oligonucleotide probes, citing real-time PCR was easier and faster with greater sensitivity. Further, when using thermally activated *polymerase*, SYBR Green yielded similar results as TaqMan probes.

Continuous Culture

Various *in vitro* methods for evaluating extent of ruminal digestion have been employed. Among them are batch and continuous culture (Owens and Goetsch, 1988). Used most often are batch culture techniques initially described by Tilley and Terry (1963). Over 40 years later, this method is still in use because of its strong correlation with *in vivo* digestion and has become the basis for the *In Vitro* Dry Matter Digestibility (IVDMD) measurements. However, due to the complexity of ruminant digestion (e.g., dilution rate, rate of passage, nutrient absorption, ammonia recycling, microbial ecology, etc.), a method more closely related to *in vivo* conditions was needed (Teather and Sauer, 1988). For this reason, continuous culture was proposed to provide a steady state environment allowing for prolonged microbial growth and diet evaluation.

Both of these *in vitro* methodologies provide an anaerobic environment and substrates sufficient for bacterial growth, but the main difference and benefit gained from continuous culture is the attention to dilution and pH of culture media. Altering either dilution or pH can have significant implications upon microbial proliferation and digestibility estimates (Ørskov and Fraser, 1975; Crawford et al., 1980a; Crawford et al., 1980b; Hoover et al., 1982; Hoover et al., 1984). In addition, with continuous culture, substrates are replenished regularly to support bacterial growth, unlike batch culture systems. Further, byproducts of microbial fermentation, such as VFA, often reduce media pH below the physiological viable range of the bacteria, leading to inhibitory affects that reduce microbial growth and feedstuff digestion (Ørskov and Fraser, 1975; Crawford et al., 1980a; b; Hoover et al., 1982; 1984).

Ruminal pH is important for proper rumen function and microbial growth. Ruminal pH is often decreased when cattle are fed concentrate-based diets compared to forage-based diets because of a greater rate and extent of digestion, resulting in increased organic acid production (Wolin, 1969; Owens and Goetsch, 1988). Ruminal pH may also impact efficiency of fermentation because optimal pH for proteolytic enzymes range from 5.5 to 7.0 (Kopency and Wallace, 1982). Researchers using dual flow continuous culture with either increased concentrations of starch or fiber maintained pH at set values between 4.9 and 7.0. As pH decreased, proteolytic activity also decreased (Cordozo et al., 2000, 2002). Fiber digestion and ammonia production are directly related to bacterial responses to ruminal pH fluctuation (Calsamiglia et al., 2002). Most bacterial species thrive around pH 6.5 with optimal fiber digestion occurring above 6.0 (Owens and Goetsch, 1988). Russell (1992) differentiated cellulolytic and amylolytic bacteria by reasoning that structural carbohydrate-utilizing bacteria have low maintenance requirements, grow slowly, and use ammonia as the main source of N. On the other hand, non-structural carbohydrate-utilizing bacteria have greater maintenance requirements, grow rapidly, and use ammonia, peptides, and amino acids as N sources. Regardless of nutrient availability, when pH is less than 5.5, many ruminal microorganisms halt growth processes. Intracellular pH of bacteria is not affected by environmental pH because bacterial cells are not permeable to H^+ or OH^- (Owens and Goetsch, 1988). However, numerous reports indicate that lipophilic acids (i.e., lactic acid) present in the rumen can freely diffuse across the bacterial cell wall. Normal proton motive mechanisms responsible for a portion of ATP production are disrupted, a process known as uncoupling (Owens and Goetsch, 1988; Russell and Wilson, 1996). However,

most ruminal organic acids are hydrophilic and would not contribute to the uncoupling processes (Russell and Wilson, 1996), with the exception of butyric acid, which, when undissociated, is a lipolytic acid and may contribute to uncoupling of ruminal bacteria (White, 2000). If VFA had uncoupling properties, then all bacteria utilizing protonmotive forces as a means of energy transduction would find VFA cytotoxic. Instead, variable sensitivity to VFA accumulation has been noted (Russell and Wilson, 1996).

To compensate for these deleterious affects often observed *in vitro*, constant rates of dilution have been used to prevent organic acid buildup by creating turnover of culture media and prolonging viability of ruminal cultures. Various dilution rates have been employed in continuous culture resulting in both favorable and unfavorable fluctuations of pH, VFA production, feedstuff digestion, and formation of microbial DM (Crawford et al., 1980a; Crawford et al., 1980b; Hoover et al., 1984; Meng et al., 1999). Because there is no absorption of nutrients *in vitro*, dilution further serves to increase passage of media, feed particles, and bacteria from culture. If dilution rate is too low, then organic acids continue to accumulate, thereby limiting functionality of the culture, but if the dilution rate is too great, substrate (i.e., feedstuffs) and bacterial passage becomes prohibitive of optimal fermentation. A moderate dilution rate between 10 to 12% vessel volume per h helps optimize media pH and passage rate, promoting feed utilization and microbial growth (Isaacson et al., 1975; Hoover et al., 1984; Meng et al., 1999).

The rumen is a reduced, anaerobic environment, and previously *in vitro* rumen simulations, have utilized CO_2 gas to expel O_2 (Hungate, 1950; Bryant, 1959). Almost universally, CO_2 has been replaced with N_2 due to conversion of CO_2 to carbonic acid

and its deleterious effects upon culture pH (Hoover et al., 1976; Calsamiglia et al., 2002; Vallimont et al., 2004). In effort to mirror ruminal conditions and promote rapid establishment of ruminal obligate anaerobes, a reduced mineral buffer has been used in both batch and continuous culture (Tilley and Terry, 1963; Goering and Van Soest, 1970a; Isaacson et al., 1975), but according to Mould et al. (2005) this is unnecessary provided anaerobic conditions are established rapidly. Culture should be evaluated to insure adequate N availability with at least 25 mg N/g substrate (Mould et al., 2005). Most mineral buffering media used in batch or continuous culture contain urea, no more than 1/3 of total N should derive from non-protein nitrogen sources.

CHAPTER III

IMPACT OF SUBSTITUTING CORN WITH SOYBEAN HULLS UPON DIGESTIBILITY OF A FORAGE-BASED DIET OFFERED TO BEEF CATTLE

Objectives

Previously, growth and digestiblility among cattle consuming a forage-based diet supplemented with soybean hulls has shown to be considerable to cattle supplemnted with corn. Similarities include production of propionic acid, growth, and weight gain. Further, while corn-based supplemtns often decrease fiber utilization, supplementation of soybean hulls has been shown to improve fiber digestibility. To further investigate this effect and the potential befefits of replacing corn with soybean hulls, a trail was conducted to evaluate the digestibility of steers consuming bermudagrass hay and supplemented with corn, soybean hulls, or corn and soybean hulls.

Materials and Methods

Digestibility Trial

At the Leveck Animal Research Center of Mississippi State University, 6 ruminally-cannulated steers (initial BW 266 ± 36.9 kg; Angus, Hereford, and Hereford-Charolais cross) individually received *ad libitum* access to bermudagrass hay, a 70% bermudagrass and 30% dallisgrass (*Paspalum dilatatum* Poir) mixture. The basal hay diet was supplemented at 1.5% BW/d with either cracked corn (CORN), pelleted soybean hulls (HULLS), or both corn and soybean hulls (MIX; 75% pelleted soybean hulls and 25% cracked corn, as fed basis), during three 19-d periods running from August until November. Each period began with a 14-d adaptation phase followed by a 5-d collection phase.

Steers were transferred to individual stalls three d prior to data collection. Daily, supplements were fed at 0800 followed by *ad libitum* access to bermudagrass hay. Hay offered to each steer was first weighed for calculation of individual hay intake. Supplement remaining after 30 min was added to the rumen via the fistula. Hay and supplements offered and hay orts were sampled and composited by period. Each morning total fecal collections were mixed and weighed, retaining 5% aliquots. Fecal samples were dried in a forced-air oven at 70°C for 48 h and composited by steer, treatment, and period.

On d 3 of collection periods, ruminal fluid was sampled at 0800 (just prior to supplementation) and every 2 h thereafter for the next 8 h. To achieve a homogenate

sample, ruminal fluid was collected by sampling ruminal contents from 4 to 5 locations within the rumen, followed by filtering contents through 4 layers of cheesecloth. Acquired samples were immediately placed on ice. After chilling, pH was determined using a Thermo-Orion combination electrode meter (model 290A meter, Beverly, MA, USA). Ruminal fluid was sub-sampled and centrifuged (900 x g, 20 min). Supernatant was retained and acidified 5:1 (sample to acid) with m-phosphoric acid (25% wt/vol) containing 2-ethyl butyric acid (2 g/L), intended as an internal standard for VFA analysis. Samples remained on ice until frozen (-20°C).

For VFA analysis, frozen ruminal fluid was thawed and hand-shaken; 5-mL sub-samples were then centrifuged (30,000 x g, 4°C, 20 min). Supernatant was analyzed for VFA using combination gas chromatography and mass spectrophotomety based on procedures described by Grigsby et al. (1992) and temperature gradient program described by Bateman et al. (2002). Helium was the carrier gas with injection flow rate of 60 mL/min. Ruminal NH_3-N was analyzed using ruminal fluid samples containing 2-ethyl butyric acid. Samples were thawed, vortexed, and centrifuged (3,500 x g, 4°C, 15 min) followed by analysis using a direct colorimetric method described by McCullough (McCullough, 1967). Samples were incubated at 91% humidity, 5% CO_2, and 37°C for at least 35 minutes prior to colorimetric analysis. Preliminary data indicated a need to revise the procedure of McCullough (1967) such that samples were not deproteinated prior to NH_3-N determination.

Laboratory Analysis

All hay, supplements, and dried fecal samples were ground in a Thomas Wiley Mill (model 4, Thomas Scientific, Swedesboro, NJ) to pass a 2-mm screen and analyzed for DM, ash, CP, NDF, and ADF (Goering and Van Soest, 1970b; AOAC, 2003). Fiber fractions were isolated using the ANKOM fiber analyzer (ANKOM Technology, Macddon, NY); NDF analysis included sodium sulfite and heat stabile α-amylase to aid in the removal of complex proteins and starch, respectively.

Calculations and Statistical Analysis

Using total feedstuffs offered, total orts, and total fecal excretion, apparent digestibilities of DM, OM, CP, NDF, and ADF were determined. Using the General Linear Model procedure in SAS (SAS Inst. Inc., Cary, NC), data were analyzed as 3 x 3 Latin Rectangle to account for any dietary carryover affects that may have resulted from the crossover design and the order in which treatments were applied to individual steers. Ruminal pH, NH_3-N, and VFA were analyzed as a repeated measure using the Mixed procedures in SAS (SAS Inst. Inc., Cary, NC). When significant ($P < 0.05$), least square means from digestion and ruminal parameters were separated using Tukey's HSD. No interactions were found ($P < 0.05$).

Results and Discussion

Nutrient composition is reported in Table 1 for feedstuffs offered. Total DM intake and DM intake for hay alone (Table 2) did not differ among treatments. However, total DM intake tended to decrease when steers consumed HULLS as expressed as kg/d

(P = 0.06). Additionally, hay DM intake tended to decrease among steers receiving HULLS expressed as both kg/d (P = 0.07) and %BW/d (P = 0.06).

Table 1. Nutrient composition of bermudagrass hay, corn, and soybean hulls consumed by beef steers during a confined digestibility trial

	Feedstuff [1]			
	Hay, %	Corn, %	Soybean Hulls, %	Corn + Soybean Hulls, %
DM	91.69	87.54	88.88	87.72
OM	92.63	89.48	95.14	95.85
CP	7.41	12.42	11.24	11.48
NDF	71.85	13.7	64.26	52.92
ADF	41.54	2.57	46.08	34.74
Hemicellulose	30.32	11.13	18.18	18.18

[1] Hay = bermudagrass hay (70% bermudagrass and 30% dallisgrass); Corn = cracked corn; Soybean hulls = pelleted soybean hulls; Corn + Soybean hulls = 75% pelleted soybean hulls and 25% cracked corn, as fed.

Table 2. Least square means for body weight, intake, and apparent digestibility of bermudagrass hay supplemented with soybean hulls, cracked corn, or both soybean hulls and cracked corn consumed by beef steers during a confined digestion trial

	Treatment [1]				
Item	CORN	HULLS	MIX	S.E.	*P*-value
BW, kg	270.3	273.3	270.0	2.229	0.56
Total DM Intake					
kg/d	7.487	6.527	6.748	0.212	0.068
%BW/d	2.793	2.415	2.509	0.085	0.67
Hay DM Intake					
kg/d	3.938	2.883	3.196	0.222	0.079
%BW/d	1.480	1.082	1.193	0.085	0.065
Apparent Digestion, %					
DM	59.17	64.90	64.07	1.464	0.15
OM	59.22	66.96	65.71	1.518	0.09
CP	50.46	51.95	50.90	2.025	0.45
NDF	51.88 [a]	64.92 [b]	62.66 [b]	1.35	0.002
ADF	48.68 [a]	65.39 [b]	63.21 [b]	2.165	0.0006
Hemicellulose	55.05 [a]	64.01 [b]	61.78 [b]	1.299	0.001

[1] Treatment : Bermudagrass hay = 70% bermudagrass and 30% dallisgrass; CORN = bermudagrass hay supplemented with cracked corn offered at 1.5% initial BW; HULLS = bermudagrass hay supplemented with soybean hulls offered at 1.5% initial BW; MIX = bermudagrass hay supplemented with 75% soybean hulls and 25% corn, as fed, offered at 1.5% initial BW.

[a,b] Within row, means without common superscript differ ($P < 0.05$).

Apparent DM ($P = 0.15$), OM ($P = 0.09$), and CP ($P = 0.45$) digestibilities were not different among treatments (Table 2). Apparent NDF ($P < .01$), ADF ($P < 0.001$), and hemicellulose ($P = 0.001$) digestibilities were greater for HULLS or MIX than CORN. These findings concur with results published by Orr et al., (2007) reporting

greater apparent DM, CP, NDF ADF digestibility when bermudagrass hay was supplemented with soybean hulls. However, Orr et al. (2007) also reported no differences in NDF and ADF digestibilities among corn-supplemented and non-supplemented steers. In the present study, corn supplementation resulted in minimal depression of nutrient digestion, likely because the concentration of corn offered (1.5% BW MIX at 25% cracked corn = 0.375% BW cracked corn) was less than that fed by Orr et al. (2007; 0.455% BW). Forster et al. (1993), feeding graded amounts of corn and(or) rice bran, reported greater apparent OM digestibility and greater apparent NDF digestibility when cows consumed corn (0.3 or 0.6% BW) verses rice bran (0.38 or 0.76% BW). When cows received corn and rice bran (0.6 and 0.76 %BW, respectively), response mimicked that of cows consuming corn. Rice bran, like soybean hulls, is a fibrous by-product yet has shown to decrease apparent fiber digestion, possibly due to greater fat content. Unlike Forster et al. (1993), the current trial did not show a trend (P = 0.09) toward improving total tract OM apparent digestibility among steers consuming HULLS or MIX, demonstrating a benefit of feeding fibrous feedstuffs to cattle consuming forage-based diets. This is especially true when noting the changes in fiber digestibility between CORN and HULLS or MIX. While incorporating digestible fibrous products into the diet can itself increase the fiber digestibility, it should be also noted that addition of supplements did not affect hay intake at P = 0.05, but at the P = 0.06 and P = 0.07 level, intake was lessened. Intuitively, digestible, fibrous-products present in the diet would promote fibrolytic bacteria that should in turn aid fermentation of low-quality forages.

Simply incorporating digestible fiber into a diet may increase total tract apparent digestibility of fiber fractions. To better discern this potential, ruminal characteristics were evaluated (Table 3) with no difference in total ruminal VFA production ($P = 0.97$) among treatments. Similar results were present for individual VFA and ruminal pH. Nguyen et al. (2007), feeding corn or soybean hulls, reported an increase in total VFA and molar proportions of propionate that corresponded to reductions in ruminal pH. Nguyen et al. (2007) also reported a divergence in response among steers consuming soybean hulls and corn, a stark contrast to the findings of the current study, which may be related to greater supplement intake by steers of the present trial.

Table 3. Least square means for ruminal volatile fatty acids, pH, and ammonia-N concentration among beef steers consuming bermudagrass hay supplemented with soybean hulls, cracked corn, or both soybean hulls and cracked corn during a confined digestion trial

	Treatment [1]				
Item	CORN	HULLS	MIX	S.E.	*P*-value
Ruminal pH					
h 0	6.929	6.848	6.802	0.134	0.31
h 2	6.568	6.604	6.410	0.134	0.31
h 4	6.359	6.114	6.055	0.134	0.31
h 6	6.295	6.133	6.163	0.134	0.31
h 8	6.283	6.167	6.232	0.134	0.31
Ruminal NH_3-N, mg/dL					
h 0	0.570	0.663	0.662	0.150	0.56
h 2	0.878	0.983	1.043	0.160	0.30
h 4	0.593 [a]	0.910 [b]	0.614 [ab]	0.160	0.05
h 6	0.260 [a]	0.613 [b]	0.271 [a]	0.160	0.03
h 8	0.235 [ab]	0.479 [b]	0.173 [a]	0.160	0.06
Total Ruminal VFA, m*M*					
h 0	245.78	248.09	232.61	42.576	0.97
h 2	288.01	273.67	285.74	42.576	0.97
h 4	298.31	316.78	296.39	42.576	0.97
h 6	280.83	282.02	272.58	42.576	0.97
h 8	289.79	249.80	276.76	43.411	0.97

Table 3 Continued

Ruminal VFA, mol/100 mol					
Acetate					
h 0	71.937	72.205	73.599	1.326	0.71
h 2	71.335	71.161	72.360	1.326	0.71
h 4	70.770	71.270	71.823	1.326	0.71
h 6	70.975	71.827	71.544	1.326	0.71
h 8	70.717	71.795	71.679	1.338	0.71
Propionate					
h 0	17.252	17.571	15.743	1.349	0.47
h 2	18.402	18.753	17.157	1.349	0.47
h 4	19.034	19.199	17.738	1.349	0.47
h 6	19.213	18.819	17.698	1.349	0.47
h 8	19.211	18.690	17.734	1.355	0.47
Acetate:Propionate					
h 0	4.258	4.195	4.708	0.349	0.46
h 2	3.942	3.836	4.293	0.349	0.46
h 4	3.794	3.749	4.119	0.349	0.46
h 6	3.766	3.857	4.114	0.349	0.46
h 8	3.762	3.897	4.120	0.351	0.46
Butyrate					
h 0	7.954	7.628	7.620	0.549	0.70
h 2	7.577	7.514	7.600	0.5491	0.70
h 4	7.687	7.352	7.763	0.549	0.70
h 6	7.550	7.260	8.127	0.549	0.70
h 8	7.723	7.424	8.076	0.559	0.70
Isobutyrate					
h 0	1.125	1.085	1.338	0.202	0.50
h 2	1.004	0.969	1.169	0.202	0.50
h 4	0.914	0.742	0.993	0.202	0.50
h 6	0.795	0.741	0.997	0.202	0.50
h 8	0.834	0.754	0.964	0.204	0.50
Valerate					
h 0	0.717	0.694	0.741	0.050	0.62
h 2	0.857	0.841	0.866	0.050	0.75
h 4	0.848	0.813	0.873	0.050	0.47
h 6	0.767 [ab]	0.734 [a]	0.839 [b]	0.050	0.04
h 8	0.721 [a]	0.737 [a]	0.842 [b]	0.052	0.02

Table 3 Continued

Isovalerate					
h 0	1.125	1.085	1.338	0.202	0.50
h 2	1.005	0.969	1.169	0.202	0.50
h 4	0.914	0.742	0.993	0.202	0.50
h 6	0.795	0.742	0.997	0.202	0.50
h 8	0.834	0.753	0.964	0.204	0.50

[1] Treatment : Bermudagrass hay = 70% bermudagrass and 30% dallisgrass; CORN = bermudagrass hay supplemented with cracked corn offered at 1.5% initial BW; HULLS = bermudagrass hay supplemented with soybean hulls offered at 1.5% initial BW; MIX = bermudagrass hay supplemented with 75% soybean hulls and 25% corn, as fed, offered at 1.5% initial BW.

[a,b] Within row, means without common superscript differ ($P < 0.05$).

Ruminal concentrations of butyrate, isobutyrate, and isovalerate did not differ ($P = 0.22$) among diets. However, ruminal valerate concentrations were greater ($P = 0.04$) for CORN or MIX at 7 h post-supplementation. At 8 h post-supplementation, only steers consuming MIX had greater ($P = 0.02$) valerate concentrations, however, the physiological significance of this finding remains suspect. It is interesting to note that the bulk of VFA were not different in the present study when differences were readily reported by Nguyen et al. (2007), among others. The reason being, valerate, isovalerate, isobutyrate are typically required nutrients for fibrolytic bacteria native to the rumen (Bryant and Doetsch, 1955; Allison et al., 1958; Bryant and Robinson, 1961). Often within the literature, molar proportions of valerate decrease upon corn supplementation while others report an increase (Seal et al., 1992). Shart et al. (1982) showed that cattle receiving grown corn had greater molar propotions of valerate than those receiving whole corn. Processing is likely impacting degree and rate of fermentation thereby influencing

valerate molar proportions. In the present study, valerate concentrations tended to be elevated among steers receiving MIX, possibly indicating a vacillation among dominant bacterial populations as shown by reduced molar proportions of valerate 6 to 8 h post feeding, when steers received a fibrous supplement. Also of note, at least some bacterial strains require valerate in conjunction with isovalerate or isobutyrate (Dehority et al., 1967b), and even though fluctuations were observed in valerate proportions, molar proportions of isovalerate was not impacted in the present study. Nguyen et al. (2007) reported greater proportions of valerate when either soybean hull or corn were fed. However, isovalerate concentrations decreased from 0 to 8 h and may have limited utilization of valerate reported by Nguyen et al. (2007).

Fixed effects for ammonia-N were accepted at the $P = 0.06$ level, and comparisons were then made within time at the $P < 0.06$ level, as shown in Table 3. Ruminal ammonia-N decreased at least from 4 and 8 h post-feeding of supplements with steers receiving HULLS exhibiting greater ammonia-N concentrations ($P = 0.05$) than CORN or MIX. As a general assessment of the data, typical responses were observed even though differences were not widely indicated. After supplemental feeding each morning, molar proportions of propionate increased numerically while molar proportions of acetate tended to decrease numerically. Further, ruminal ammonia-N concentrations peaked approximately 2 h post-feeding and total VFA concentrations peaked approximately 2 h later implying synchronous use of energy and N within the rumen, as previously reported (Martin and Hibberd, 1990; Grigsby et al., 1992; Von et al., 2007).

Implications

As shown in the current trial, feeding fibrous by-products may be able to replace greater amounts of corn within rations offered to energy deficient cattle in the southeastern US. Feeding marginal amounts of cereal grains may provide sufficient energy to aid utilization of protein without impeding fiber digestion or feed intake. This is important in light of current trends regarding changing demands, availability, and affordability of feed-grade corn. Further investigation will be needed to elucidate the interaction between corn and fibrous feed sources and to understand the potential for effectively substituting or replacing corn with fibrous by-products.

CHAPTER IV

SEMI-CONTINUOUS CULTURE

Objective

Previously, unanticipated outcomes have been reported upon supplementing forage-based diets with soybean hulls in the diets of cattle. Specicfically, performance by beef cattle supplemtned with soybean hulls was similar to corn-based supplementation demonstrating improved digestibility, greater molar proportions of propionate, growth, and weight gain. However, soybean hulls often demonstrate great total-tract fiber digestibility than corn-supplemented cattle, indicating a potential greater feeding value of soybean hulls than corn.

The ruminal environment consists of a complex and diverse microbial ecosystem. Often, the degree of efficiency, and nuances observed in ruminant digestion can be directly attributed to microbial fermentation. Examples include, positive and negative associcative effects of forage digestibility, and transitionary diets decreasing the severity of lactic acidosis. In the later case, prevalence of *Megasphera elsdenii* and *Selenomonas ruminantium* (i.e., lactate utilizing bacteria) are increased.

To investigate the corn and soybean hulls further, an *in vitro* trial was conducted to form a baseline of *in vitro* digestibility and a sourse of diet-adapted ruminal cultures for evaluating prominence and shifts among the rumen microbial species.

Materials and Methods

Experimental Conditions

The BioFlo 110 fermentation system (New Brunswick Scientific, New Brunswick, NJ) was used for simulated *in vitro* ruminal digestion. Ruminal fluid was collected from two fistulated steers maintained on grass pasture and/or hay. An electric medical-pump (model 68MOD; C. M. Sorensen Co., Inc.) was used to collect 8 L of ruminal fluid (i.e., 4 L per steer). Ruminal fluid was harvested into a vacuum-safe glass jar, which was transferred to a pre-warmed thermos under flow of CO_2 gas to minimize O_2 exposure. A small portion of ruminal particulate matter was added to each thermos to ensure inclusion of particle-adherent bacteria.

Prior to inoculation, each culture vessel contained 400 mL of mineral buffer (Table 4) and 8 mL reszurin, pre-warmed to 39°C under constant flow of N_2-gas. Thermoses were shaken and decanted in 800 mL increments under flow of CO_2 gas (mixing 800 mL from each steer to yield a 1600 mL inoculum) and added to each of 4 vessels. Final working volume of each culture was 2 L, including the mineral buffer.

Upon inoculation, cultures were given hay and respective supplements, but no fluid was removed until d 2. Diet adaptation and stabilization of bacterial populations

was allowed from d 2 through d 6 (Figure 8), where cultures were maintained, fed, and diluted, as per protocol. Data collection occurred from d 7 through 9.

Table 4. Composition of mineral buffer

Buffer adaptation from McDougall (1948) and deVeth and Kolver (2001a).

Item	Concentration, g/L
CaCl2	0.0133
MgSO4	0.12
Na2HPO4 anhydrous	1.955
NaHCO3	5
KHCO3	1.6
KCl	0.6
NaCl	0.47
MnCl2 · 4 H20	0.010
CoCl2 · 6 $H_2$0	0.001
Urea	0.4

Each fermentation vessel, controlled by a central computer, was maintained at 39ºC under constant agitation (300 rpm) and N_2-gas sparging. Every three h (from 0700 to 1900) 500 mL of media was removed (i.e., effluent) in a single-flow manner (i.e., removal of both fluid and particulate matter simultaneously). Effluent was collected in plastic containers containing 210 mL 2 *N* H_2SO_4 (3 part 2 *N* H_2SO_4 to 50 parts effluent) to increase acidity and retention of ammonia-N and VFA as well as to inhibit further

microbial fermentation (Crawford et al., 1980b). At the end of d 7, 8, and 9, samples were retained and composited by treatment, day, and week for nutrient analysis.

		Day								
Fermenter	Block (week)	1	2	3	4	5	6	7	8	9
1 2 3 4	1 2 3 4 5	**CULTURE ADAPTATION**						**SAMPLE COLLECTION**		

Figure 8. Relationship between period, adaptation, and data collection

Culture pH was modulated by automated addition of $2N$ H_2SO_4 to prevent pH from rising above 6.5. However, culture pH was allowed to decrease at the dictates of fermentation patterns, without interference. Every 2 h for 12 h, pH was recorded and a 50-mL sample was taken from each fermentation vessel, acidified with $2N$ H_2SO_4 (containing 2-ethylbutyrate) at 6% of sample volume (3 part 2 N H_2SO_4 to 50 parts culture), pooling by week, treatment, and day. At each sampling, peristaltic pumps were used to add mineral buffer to restore cultures to 2 L.

Diets

Feedstuffs included bermudagrass hay, soybean hulls, and/or corn, ground to pass a 1-mm screen using a Thomas Wiley Mill (model 4, Thomas Scientific, Swedesboro, NJ). Each culture was intended to received (Table 5) bermudagrass hay (20g DM L^{-1} d^{-1}) either alone (HAY) or in conjunction with additional (7 g DM L^{-1} d^{-1}) corn (CORN), soybean hulls (SBH), or both (MIX; 25:75). Daily, hay was added to cultures 5 times (every three h; from 0700 to 1900 h), while supplements were added to once daily (0700 h).

Table 5. Actual dry matter and anticipated quantity of feedstuffs supplied to semi-continuously cultured ruminal fluid.

Feedstuff [a]	DM	$g \cdot L^{-1} \cdot d^{-1}$ DM	g/d DM	g/d As Fed	g/feeding As Fed
Corn	86.95	7	14	16.1	16.1
Soybean hulls	89.39	7	14	15.66	15.66
Hay	91.29	20	40	43.82	8.76
Mix – Corn	86.95	1.75	3.5	4.03	4.03
Mix – SBH	89.39	5.25	10.5	11.75	11.75

[a] All feedstuffs were ground through 1-mm screen. SBH = soybean hulls; Hay = bermudagrass hay.

Data Collection and Analysis

Apparent Digestibility. Apparent digestibility of the cultures was determined by subtracting nutrient content of the vessel effluent from feed offered. Feedstuffs and effluent samples were analyzed for DM, OM, CP, NDF, ADF, and ash (Goering and Van

Soest, 1970a; AOAC, 2003). Microbial carryover (weight and/or protein) was not accounted in the calculations.

VFA, NH3-N, & pH. Culture samples, not effluent, were used to evaluate ruminal paramaters. Ruminal pH was monitored in real time, but only recorded every 2 h for 12 h; at which time, 50-mL samples were obtained, acidified with 2*N* H_2SO_4 + 2-ethylbutyrate and frozen. At a later date, samples were thawed, centrifuged (30,000 x *g*, 4°C, 15 min), and anylized for ammonia-N determination following a direct colorimetric method described by McCullough (1967).

Organic acids and VFA (acetic, propionic, succinic, butyric, lactic, valeric, isovaleric, isobutyric, 2-methylbutyric, and formic acids) will be analyzed using combination gas chromatography and mass spectrophotometry. The protocol is currently under revision and validation by the Mississippi State Chemistry Laboratory.

Statistical Analysis

With vessel as the experimental unit, data was analyzed as a randomized complete block, blocking by week for a total of 5 weeks. Repeated Measures of the Mixed procedures of SAS were used for statistical comparisons and when significant, means were separated using Tukey's HSD ($P < 0.05$). The fit of data to the 1st through 4th order polynomial kinetics models were also evaluated using the Regression procedures of SAS. Adjusted-R^2 was used in evaluation and are reported herein.

Results and Discussion

Nutrient composition of feedstuffs offered, DM provided to cultures, and nutrient balance is presented in Table 6. Judging from CP and ADF content, the bermudagrass hay offered was of moderate-quality, ideal for assessing interactions among selected diets. However, the CP of corn (8.41%) is much lower than would be expected (10 to 12%). Further, hay samples were close to 10% ash and hay ADF was much lower than anticipated. Fiber fractions for hay and soybean hulls should have been much closer in relationship. These factors lead me to conclude that upon grinding samples to pass 1-mm, sample loss may have occurred. In addition, grinding may have contributed to a greater degree of settling during storage and that samples were not adequately mixed prior to feeding and/or analysis.

Intended addition of feedstuffs on a DM basis can be found in Table 5 while the actual DM addition is listed in Table 6. Addition of DM was in line with intended specifications. Average total DM added to fermenters for the entire trial was slightly greater ($P < 0.0001$) for CORN than SBH or MIX. It is doubtful that this would constitute a physiological effect given that CORN only received between 0.17 and 0.20 g more than SBH or MIX, respectively.

Dry matter and OM disappearance each followed a similar trend with greatest apparent disappearance occurring when cultures were maintained on a supplement, in addition to bermudagrass hay, a finding that is commonly observed *in vivo* (Orr et al., 2007; Von et al., 2007). No differences were observed for apparent disappearance of CP, NDF, or ADF. This may be due to not accounting for bacterial inoculation of effluent samples, leading to additional N and DM that could distort differential weighing.

However, assessing flow of microbial DM and protein is of great value because microbial DM is an important source of protein. Therefore, data should be viewed in this light.

Table 6. Nutrient content, supply, and balance of continuously cultured ruminal fluid maintained on one of four diet combinations

	Treatments [1]					
Item [2]	CORN	HAY	SBH	MIX	*P*-Value	SE
Nutrient Composition, %						
DM	90.83	93.02	91.65	.	.	.
Ash	1.14	9.16	5.05	.	.	.
CP	8.41	11.94	11.97	.	.	.
OM	89.69	83.85	86.60	.	.	.
NDF	14.52	65.21	63.43	.	.	.
ADF	1.62	29.25	46.14	.	.	.
DM Added, g						
Hay, g	39.14	39.07	39.13	39.14	0.34	0.0447
Total, g	53.78 [c]	39.07 [a]	53.61 [b]	53.58 [b]	<0.0001	0.0867
Nutrient Disappearance, g						
DM	0.0029 [bc]	-0.0058 [a]	0.0012 [c]	0.0045 [b]	< 0.0001	0.00093
OM	0.0081 [bc]	0.00021 [a]	0.0063 [c]	0.00935 [b]	< 0.0001	0.00092
CP	-0.2371	-0.2205	-0.2335	-0.1778	0.10	0.02593
NDF	-0.01994	-0.0395	-0.0197	-0.0063	0.25	0.0162
ADF	-0.0071	-0.0107	-0.0013	0.0221	0.22	0.01674

[abc] Within row, leastsquare means without common superscript differ ($P < 0.05$)

[1] Values are least square means. Corn = ground (1-mm) bermudagrass hay and corn; Hay = ground (1-mm) bermudagrass hay; Mix = ground (1-mm) bermudagrass hay, corn, and soybean hulls; SBH = ground (1-mm) bermudagrass hay and soybean hulls.

[2] Total = DM added to cultures including both bermudagrass hay and supplement (e.g., corn and/or soybean hulls).

By increasing the dilution rate of ruminal cultures, nutrient disappearance has been negatively affected (Meng et al., 1999), revealing a quadratic response between DM

digestibility and rate of dilution. The resulting reductions in retention time as well as the washing out of feed particles and bacteria were most likely the root causes. As much as 80 to 90% of omasal nitrogen was determined to be of microbial origin, with 55 to 85% being bacterial nitrogen (Weller et al., 1958). This may be a reason why, in the current trial, no differences were observed for CP, NDF, or ADF balance, otherwise, bacterial biomass may have been washed out of the cultures prior to sampling, limiting fermentive capabilities of cultures.

Ammonia-N was least (P<0.001) for cultures maintained with CORN within the 12 h span evaluated (Figure 9), indicating a greater efficiency of N utilization by this treatment-group. To further evaluate nitrogen utilization, data was evaluated for degree of fit to polynomial models (Figure 10). There was a quartic (P = 0.003) response for CORN, cubic (P = 0.003) response for SBH, and a quadratic (P = 0.004) response for MIX. No polynomial response was detected for HAY. Though supplement groups demonstrated a polynomial response, fit to the model, in all three cases, was very weak (Adj-R^2 ranging from 0.08 to 0.11).

Meng et al. (1999) fed soybean hulls, soy protein, or a mixture of ground corn (78%) + soybean hulls (14%) + soy protein (8%) *in vitro*. Cultures receiving soybean hulls responded quadraticly for NH_3-N and pH with respect to their ascribed dilution scheme. The corn-fed culture also had a quadratic response for pH and NH_3-N under the ascribed dilution scheme. A tendency for greater efficiency was observed by the culture provided with corn. The current trial also showed greater N-efficiency by cultures receiving CORN. A weak quartic response was recorded, indicating a need for further

investigation of this effect once the BioFlo 110 system is better optimized for ruminal fermentation.

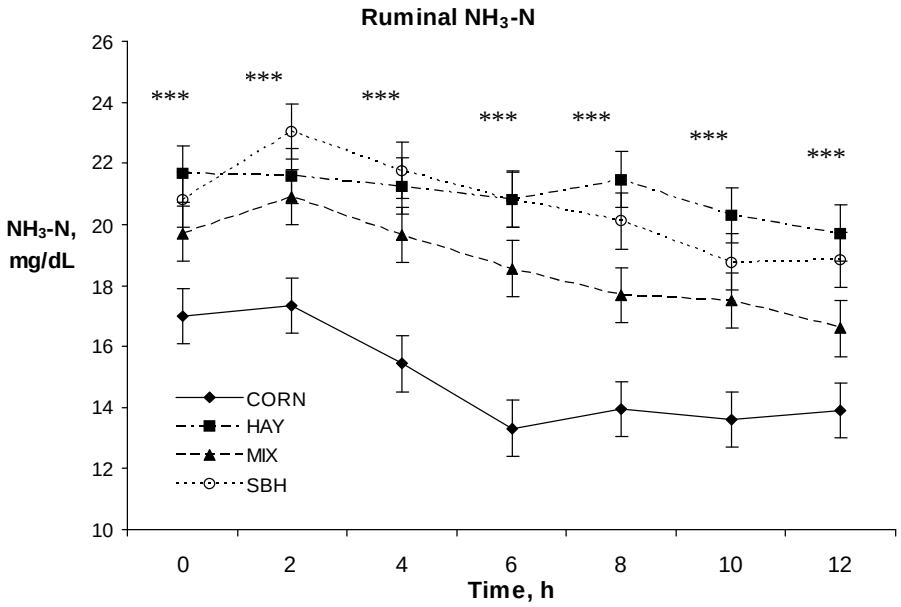

Figure 9. Ruminal NH_3-N as influenced by feedstuff during in vitro ruminal culture.

CORN = ground (1-mm) bermudagrass hay and corn; HAY = ground (1-mm) bermudagrass hay; MIX = ground (1-mm) bermudagrass hay, corn, and soybean hulls; SBH = ground (1-mm) bermudagrass hay and soybean hulls. Least square means within time differ: †P<0.10, *P< 0.05, **P< 0.01, ***P< 0.001.

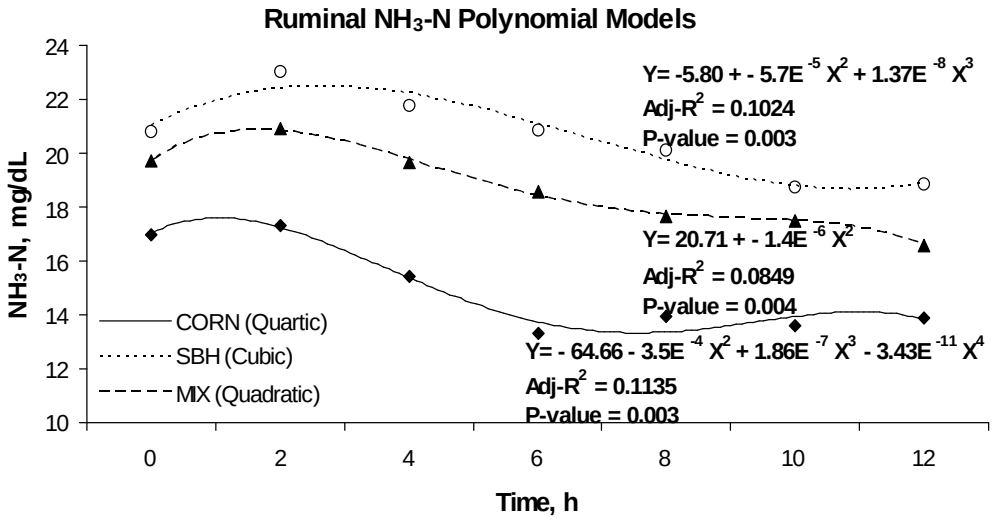

Figure 10. Polynomial regression analysis of Ruminal NH_3-N as influenced by feedstuff during in vitro ruminal culture.

CORN = ground (1-mm) bermudagrass hay and corn; MIX = ground (1-mm) bermudagrass hay, corn, and soybean hulls; SBH = ground (1-mm) bermudagrass hay and soybean hulls.

Similarly, ruminal pH (Figure 11) was least (P<0.001) for cultures receiving supplements prior to feeding (0700). Cultures provided MIX, SBH, quickly rebounded and maintained a pH of approximately 6.5, as was consistently maintained by HAY. From 2 to 6 h post-supplementation, CORN had the lowest (P<0.001) pH, which began increasing from 6 to 10 h before beginning to decline. No differences were observed for pH at 10 or 12 h maintenance.

The reduction of pH is commonly associated with the production and accumulation of organic acids, products of microbial fermentation and metabolism. To

date, this cannot be confirmed. Collaboration is underway with the Mississippi State Chemical Laboratory for VFA assay improvement and validation.

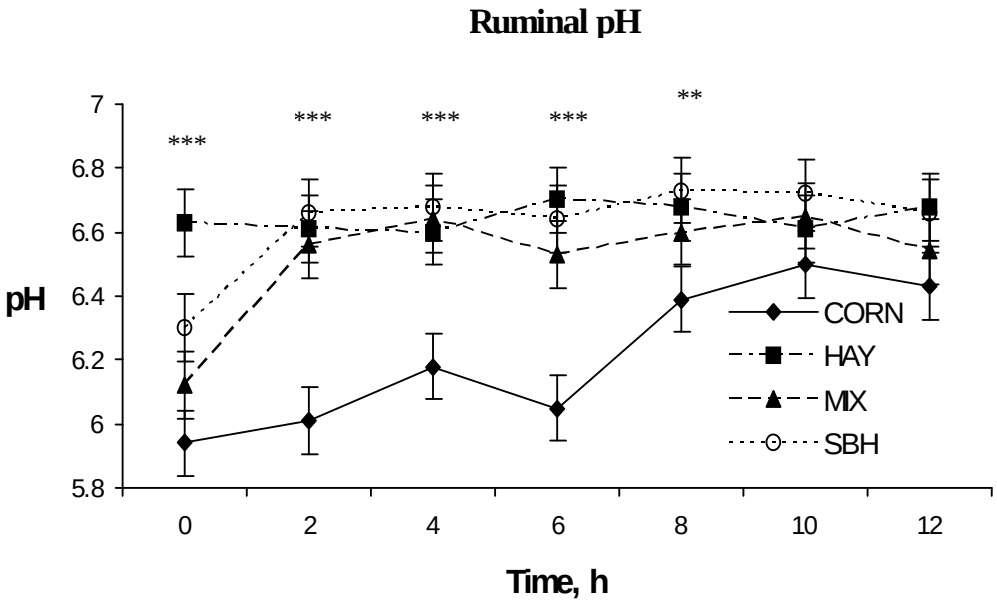

Figure 11. Ruminal pH as influenced by feedstuff during in vitro ruminal culture.

CORN = ground (1-mm) bermudagrass hay and corn; HAY = ground (1-mm) bermudagrass hay; MIX = ground (1-mm) bermudagrass hay, corn, and soybean hulls; SBH = ground (1-mm) bermudagrass hay and soybean hulls. Least square means within time differ: †P<0.10, *P< 0.05, **P< 0.01, ***P< 0.001.

It has been previously shown that pH in the range of 6.3 to 6.5 optimizes fibrous digestion (de Veth and Kolver, 2001b, a). In the current trial, due to a tendency of culture pH to exceed 6.5, an upper limit was set to prevent this increase; reductions in pH were not prevented.

Previously, the effect of pH upon bacterial growth and survivability was evaluated. Peak cell density was observed from pH 6.0 to 6.5 for *Selenomonas*

ruminantium, *Streptococcus bovis*, *Lactobacillius vitulinus*, *Butyrivibrio fibrisolvens*, *Ruminococcus albus*, *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, and *Prevotella ruminicola* B14, GA33. However, *Megasphaera elsdenii* reached peak cell density at pH 5.5 (Russell and Dombrowski, 1980). This information substantiates the report by de Veth and Kover (2001a, b) for optimal fermentation occurring from 6.0 to 6.5 and further adds credence to its adoption for the current trial.

Conclusion

Ruminal environment of living cattle cannot be directly compared to that of the chemostat because the rumen, by its very nature, is not a steady-state environment. We can, however gain insights to trends that may occur *in vivo*. It appears from data that the fermentation system has not been optimized. The dilution rates may have been too great and, therefore, did not allow sufficient time for bacterial populations to flourish and degrade feedstuffs to a reasonable extent that would parallel natural rumen function. The mineral buffer may need to be reformulated. Feeding rates could potentially be increased.

What can be gleaned from the data is the importance of N and energy synchrony. Cultures receving added supplements were capable of fermenting a greater extent of DM, and the cultures receiveing energy-dense supplementation demonstrated a greater utilization or efficiency of utilization of N among cultures. To completely evaluate the performance of the *in vitro* ruminal cucltures, organic acid and VFA data will be needed.

CHAPTER V

REAL-TIME PCR

Objective

Ruminants possess a symbiotic relationship with microorganisms, primarily in the forgut. It is known that relative ratios among bacterial species fluctuate with dietary alterations. These shifts, as well as the diverse and complex interactions ruminal bacteria, have a direct impact upon utilization of feedstuffs.

In order to determine to what extent this holds true when supplemnting cattle with corn, soybean hulls, or a mixture of corn and soybean hulls, samples were obtained from semi-continuously cultured ruminal fluid. To evaluate the microflora of these samples, real-time PCR was chosen of differentially monitoring and quantifying bacterial populations across 4 dietary conditions.

Bacterial Cultivation

Pure cultures of ruminal bacteria (Table 7) were obtained from American Type Culture Collection (ATCC®). *Streptoccus bovis* is a facultative anaerobe and was grown aerobically on commercically available media. All other pure cultures were cultivated in partially defined anaerobic media (Appendix A) with or without 23 g agar ganules.

Media was autoclaved and purged with CO_2 while cooling in an ice bath to expel O_2. Cultivation occurred within an anaerobic glove box. Meda was allowed to aquilibrate to gaseous concentrations of chamber prior to beginning cell cultivation.

DNA Isolation

Isolation and purificaction of genomic DNA from pure-culture bacteria as well as from mixed-bacterial culture samples were made using QIAamp® DNA Stool Mini Kit (Qiagen, Inc.) and the Gentra® Puregene® kit (Qiagen, Inc.). Manufacturer's specification were followed.

Real-time PCR

Pure-bacterial and mixed-bacterial ruminal culture samples were obtained for DNA isolation for use in verify primer and amplicon specificity as well as to define the optimal PCR assay conditions (i.e., salt concentrations, primer concentrations, annealing temperatures, denaturation temperatures, etc), prior to performing real-time PCR reactions.

Seven prominent ruminal bacteria were selected based upon characteristics published within the literature. Using GenBank (National Center for Biotechnology Information; NCBI) the rDNA sequence for each bacterium was obtained; using BeaconDesigner (PremierBiosoft) species-specific primers were designed for the rDNA sequences (Table 7). Primers were then subjected to computational evaluation of homology to ensure specificity of primers and to minimize, possibly prevent, cross reactivity. However, specificity was only evaluated among 7 species of interest.

Invitrogen was selected to manufacture primers and PCR premix as well as SYBER Green, a DNA binding dye, was obtained from BioRad.

Results and Discussion

Bacterial Cultivation

All cells were successfully cultured on liquid media, however, only *Streptoccus bovis* grew on solidified media. It is not completely clear why growth by other species was not observed on solidified media. It may be related to the fact that most ruminal bacteria have narrow growth constraints and specifications. In the future, it may prove helpful to use semi-solid media during cultivation. Simply reducting agar content by 10g may be beneficial. It must be remembered that results have shown molton agar to be detrimental to ruminal bacteria (Leedle and Hespell, 1980).

Table 7. Specie-specific, rDNA-targeting primers for evaluation of ruminal bacteria by PCR and Real-time PCR

ATCC No. [a]	NCBI Accession No. [b]	Bacteria	Forward Primer (5' to 3') [c]	Reverse Primer (5' to 3')
19171	U41172	*Butyrivibrio fibrisolvens*	GAA GAA CAT CAG TGG CGA AGG	AGG TGG AAT ACT TAT TGC GTT AGC
43856	M62696	*Fibrobacter succinogenes* S85	CGG AGG AAG GTG TGG ATG AC	AGG ACG GCT TTG AGG ATT GG
29744	Y15992	*Ruminobacter amylophilus*	GCT TGC TTC CTG GCT GAC	TCT GAA TGC GAC TGG TTG C
27210	AY445600	*Ruminococcus albus*	CAG TCT TAG TTC GGA TTG TAG	CTT CCT CCT TGC GGT TAG
19208	AY445593	*Ruminococcus flavefaciens*	GGC GAA GGC GGC TTA CTG	TTA ACT GCG GCA CGG A AG G
12561	M62702	*Selenomonas ruminantium* GA192	GCG TAG ATA TTA GGA GGA ACAC	CAG GCG GAA TGC TTA TTG C
33317	AB002482	*Streptococcus bovis*	GAA GCA ACC GCA AGA ACC	CTT ATG ATG GCA ACT AAC AAT AGG

[a] ATCC ® = American Type Culture Collection were bacteria were purchased. Numerical designations indicate the Trade Mark identifier of the ATCC product.

[b] NCBI = National Center for Biotechnology Information a subsidiary database of the National Library of Medicine (NLM) at the National Institutes of Health (NIH) as created by congress in 1988. This database is were whole rDNA sequences were derived to in turn, formulate appropriate primers.

[c] Primers were designed using BeaconDesigner™ (of Premier Biosoft International) and were synthesized by Invitrogen.

Table 7 Continued

ATCC No. [a]	NCBI Accession No. [b]	Bacteria	Target Sequence Length, bp	PCR Product Length, bp	G/C Content
19171	U41172	*Butyrivibrio fibrisolvens*	1,477	174	41.7
43856	M62696	*Fibrobacter succinogenes* S85	1,504	124	55
29744	Y15992	*Ruminobacter amylophilus*	1,539	140	52.6
27210	AY445600	*Ruminococcus albus*	1,503	170	55.6
19208	AY445593	*Ruminococcus flavefaciens*	1,505	142	57.9
12561	M62702	*Selenomonas ruminantium* GA192	1,486	189	52.6
33317	AB002482	*Streptococcus bovis*	1,457	175	37.5

[a] ATCC ® = American Type Culture Collection were bacteria were purchased. Numerical designations indicate the Trade Mark identifier of the ATCC product.

[b] NCBI = National Center for Biotechnology Information a subsidiary database of the National Library of Medicine (NLM) at the National Institutes of Health (NIH) as created by congress in 1988. This database is were whole rDNA sequences were derived to in turn, formulate appropriate primers.

[c] Primers were designed using BeaconDesigner™ (of Premier Biosoft International) and were synthesized by Invitrogen.

DNA Isolation

Many researchers have isolated rRNA for PCR analysis, but given the unstable nature of RNA samples, the current project sought to evaluate ruminal bacteria based upon rDNA evaluation, as previously reported (Wilson and Blitchington, 1996; Li et al., 2003; Rinttila et al., 2004). Attempts to isolated genomic DNA from bacteria grown in pure-culture or mixed-culture were unsuccessful with either of the nucleic acid extraction kits used. Stern and Hoover (1979) stated that separating ruminal contents into microbial fractions is one of the most difficult aspects of rumen microbial investigation. For this reason, they sought to isolate bacterial DNA from pelleted ruminal contents, which was found to be equally as difficult.

Many compounds within ruminal fluid may interfere with downstream amplification of DNA (Li et al., 2003). To help counteract this effect, the QIAamp® DNA Stool Mini Kit (Qiagen, Inc.) was selected for its proprietary additive designed to adsorb inhibitory compounds. When this kit failed to successfully produce genomic DNA, the Gentra® Puregene® kit was attempted, also failing to yield a favorable result.

Manufacturer's spcifications were followed initially, but were also modified to evaluate the efficiency of the DNA extraction. Modifications included incubation time and temperature, enzyme cocentrations, initial cell concentrations, and serial dilutions of samples. Neiter the recommended protocols nor modifications gave a faborable outcome.

The failure of DNA extraction kits may have been due to an inability to disrupt the bacterial cell wall, cellular concentrations that were too great or too little, or DNA concentrations that were resultingly too great or little (i.e., as when using spin colmns). Attempts to serially dilute samples had no effect upon the outcome. It is possible that the

need for further optimization of the continuous culture system limited cell density and thus compromised DNA extraction. However, this does not explain why extraction from pure-cucltures was unsuccessful.

Previously, scientists using bacterial DNA to monitor population dynamics have used a bead-beating method for DNA extraction. This method was not considered in the current tril due to concern for DNA shearing that might limit PCR sensitivity. Due to the failue of the two separate chemicacl techniques, in the future, this may need to be recocnsidered.

Real-time PCR

Because neither pure nor mixed-culture genomic DNA was obtained, PCR optimization was not prefored and real-time PCR was not attempted. However, judging from rDNA and primer design, the PCR efficiency can be roughtly anticipated. Bustin (2000) recommended that primers be between 15 and 20 bp with a G/C content between 20 and 70%. Current primer design falls either just with this specified range or exceeds it. The G/C content is well within the recommended range. Amplicon length has been recommended to not exceed 80 to 100 bp or 50 and 150 bp (Bustin, 2000). Under the current design, all PCR products exceed this recommendation. These factors indicate the efficiency of the reaction my be compromised and will require greater optimization and adustments temperature gradients as well as salt and primer concentrations.

CHAPTER VI

EVALUATING BIOLUMINESCENT APPLICATIONS IN NUTRITION

Objective

Bacterial fermentation is a primary component of ruminant digestion. Microbial populations are known to fluctuate due to dietary conditions. Bioluminescence is therefore proposed as a menas of evaluating ruminal bacteria.

The following two trials are intended to investigate the fesability of using bioluminescence in the live producing ruminant. Sepcifically, the need for antibiotics to sustain luminescence, the efficiency of luminescence and sustainability among 3 plasmids, and finally the constraints of ruminal fluid and its particulate matter on the detection of luminescence.

Materials and Methods

Experiment 1

The stability of photonic emissions were evaluated over time for 3 plasmids pAK1*lux* (Karsi et al., 2006), pXen1, and pXen5 (Xenogen Bioware™, Caliper Life

Sciences, Hopkinton, MA; Figure 12) with or without antibiotic selection (ampicillin or erythromycin; Table 8) during two trials. Use of bacteria transformed with plasmids that do not contain transposable elements, requires antibiotic pressure to maintain plasmids and photonic emission over time. Continuous infusion of antibiotics into the rumen is not practical. Therefore, the extent of emission loss will need to be accounted for prior to biophotonically evaluating ruminal microflora *in vivo.* The current two trials seek to address this issue.

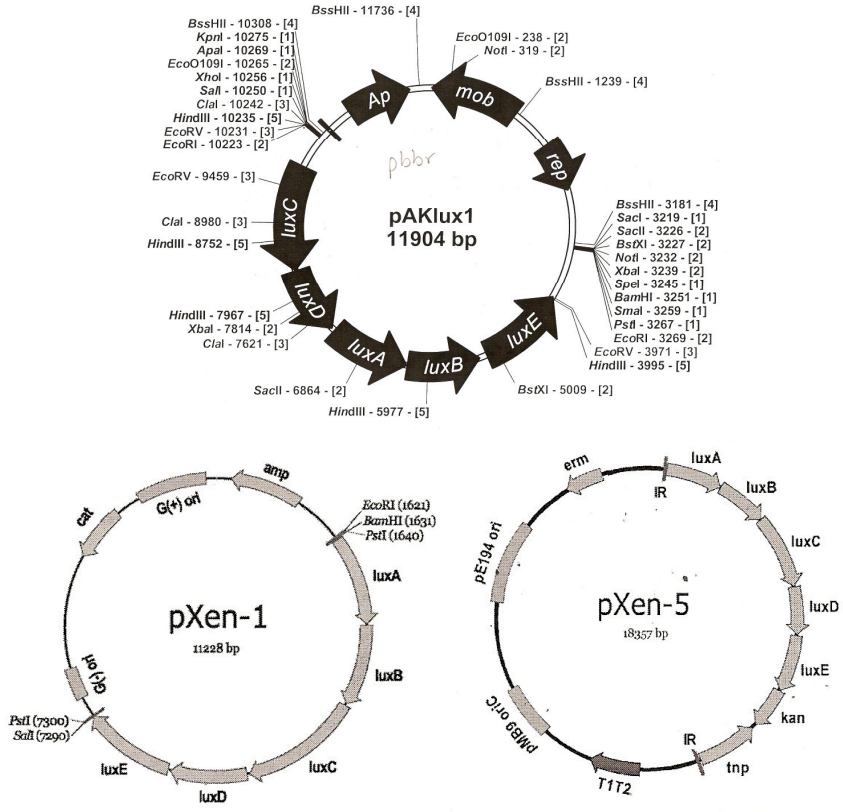

Figure 12. Restriction and gene maps for pAK1lux, pXen1 and pXen5.

Table 8. Plasmids and corresponding antibiotic concentrations used to assess emission loss by transformed *Escherichia coli* MM294

Plasmid [1]	Ampicillin	Erythromycin
pXen5 – A	-	150 µg/mL
pXen5 – B	-	-
pXen1 – A	100 µg/mL	-
pXen1 – B	-	-
pAk1*lux* – A	2 µg/mL	-
pAk1*lux* – B	-	-

[1]Plasmid type with (A) or without (B) respective antibiotic

Six cultures of *Escherichia coli* MM294 (ATCC 33625), one for each plasmid type, containing antibiotics were imaged. For the remaining 6 days, one of each plasmid type were cultured in absence of antibiotics in order to compare loss of luminescence over time. Images were taken once daily.

On day one of each trial, 6 overnight cultures (20 mL; 1 vial for each plasmid type) of transformed *Escherichia coli* MM294 containing a plasmid-specific antibiotic (Table 8) were pipetted into 8 wells of a 96-well plate (200 µL each). Images were obtained using the Stanford Photonics imaging system (XR/MEGA-10Z; Stanford Photonics, Inc., Palo Alto CA) at numerous acquisition times. The 96-well plates were kept on ice before and after imaging to prevent further growth prior to spread-plating. After imaging 96-well plates and spin-plating, a new overnight-culture (200 µL to 19.8 mL) was started using inoculum from previous day's culture. Beginning d 2 and

continuing through d 7, one culture for each plasmid type received antibiotic while one did not.

Wells 1 through 8, for each plasmid type (containing 200 µL), were used for duplicate spread-plates (100 µL per plate; 16 plates per treatment per d) to determine viable cell density and for determination of photonic stability over time (% colonies emitting) after removal of antibiotic pressure. For this, plates were incubated at 37°C for 24 h followed by imaging using a Berthold/Nightowl camera equipped with WinLight 32 version 2.51.111901 software (Berthold Technologies, Oak Ridge, TN). Total colony counts and number of emitting colonies were obtained.

Experiment 2

A preliminary experiment was conducted to assess the potential for using biophotonics to evaluate individual ruminal bacteria either *in vitro* or *in vivo.* Specifically, the loss of photonic emission due to the opaque nature of ruminal fluid with and without added feedstuffs was evaluated using the BioFlo 110 (New Brunswick Scientific, NJ) fermentation system.

To accomplish this, 3 fermentation vessels contained a basal medium of: 1) Luria-Bertaini (LB) broth, 2) Ruminal fluid + LB powder (R), or 3) Ruminal fluid + LB powder + Ground hay (RH). Prior to autoclaving, media pH was adjusted to 7.0 and after cooling, ampicillin (2 µg/mL) was added to all 3 vessels. Culture temperature was maintained at 39ºC to simulate ruminal temperature.

Specifically, 4 liters of ruminal fluid was collected from a single fistulated steer, using an electric medical-pump (model 68MOD; C. M. Sorensen Co., Inc.). Luria-Bertaini (LB) broth powder was added to either distilled water or ruminal fluid at 20g/L and transferred to respective fermentation vessels, at which time they were autoclaved at 121℃ for 20 min. *Escherichia coli* MM294 transformed with pAk1*lux* was cultured, and a 5-mL (containing 1.2 x 10^8 CFU/mL) inoculate was added to each fermentation vessel. Vessel 3 contained 20 g bermudagrass hay ground to pass a 1-mm screen that was previously autoclaved at 121°C for 20 min, and was aseptically added to fermentation vessel.

Images were taken over a 12-h period using the Stanford Photonics imaging system (XR/MEGA-10Z; Stanford Photonics, Inc., Palo Alto CA) using 'real-time' image acquisition. Camera was mounted extemally, and images were taken, peering through the water jacket of each vessel. Black photographer's drapes were used to exclude external light from affecting detection of sample emission. To evaluate growth and proliferation of cultures for determing when or how often to image, absorbace readings were obtained for LB (data not shown). Absorbence was attempted for R and RH but the media from each was too dark to facilitate transmittance.

For each image obtained, the time was recorded, and a sample was taken and spread-plated (100 µL per plate) on LB solidified-medium (containing 2 µg/mL ampicillin) for cell enumeration.

Statistics

Experiment 1. Experiment was designed as a Randomized Complete Block, blocking by trial. A treatment by day interaction occurred. Because of this and a lack of continuity in acquired images, treatments were compared within trial and day. Summary statistics are reported but *P*-values were not obtained. Data were analyzed for their fit to polynomial models ($P< 0.05$) using Adj-R^2 in assessing model fit.

Experiment 2. Data was analyzed as a Randomized Complete Block, blocking by time at imaging. Relative light units were standardized by dividing by time, in minutes, since culture inoculation. Using the Mixed Procedures of SAS, means were separated using Tukey's HSD ($P< 0.05$). No interactions were noted.

Results and Discussion

Experiment 1

Photonic stability data is inconclusive, due to a lack of repeatability (i.e., inconsistent acquisition time, inconsistent cell density, missing data). Each culture was plated and imaged in multiples each morning. However, these are subsamples and can only be averaged, not treated as replicates. Trial 1 and 2 were intended to be pooled to obtain adequate replication. However, due to unforeseen difficulties with camera and culture responses, images were not taken under identical acquisition times (within and across trials), precluding any meaningful comparisons.

Trial 1. It can be noted from trial 1 that, in general, LuxA tended to maintain photonic emission throughout with only slight loss of photonics in absence of ampicillin (Figure 13). Emission by P5-cultures tended to be stable throughout, but upon removal of erythromycin, rapid loss of emission was observed. Cultures transformed with P1 did not produce consistent emission with or without ampicillin on solidified media, but was consistent with other plasmids when bacteria were grown on liquid media (Figure 14). Very little emission was observed by P1 early in the week, but by week's end, a considerable numeric increase in emission was observed. A logical reason is that cell density increased as the week progressed (Table 8), thereby concentrating bacteria and thus intensifying photonic emission.

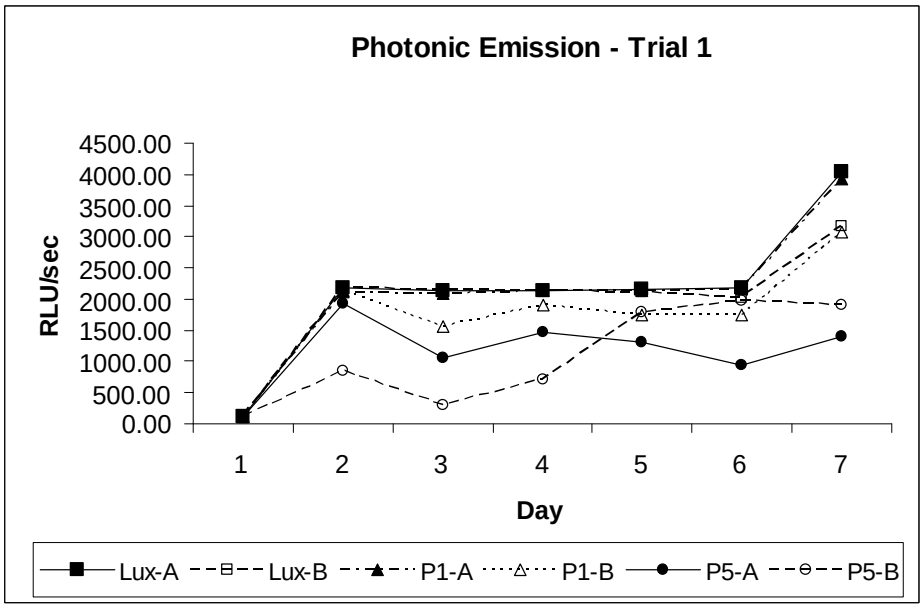

Figure 13. (Trial #1) Biophotonic emission of *Escherichia coli* MM294 transformed with one of three plasmids, with and without antibiotic selection.

Plasmids = pAk1*lux*, pXen1, and pXen5; A= with respective antibiotic; B= without respective antibiotic (Ampicillin or Erythromycin).

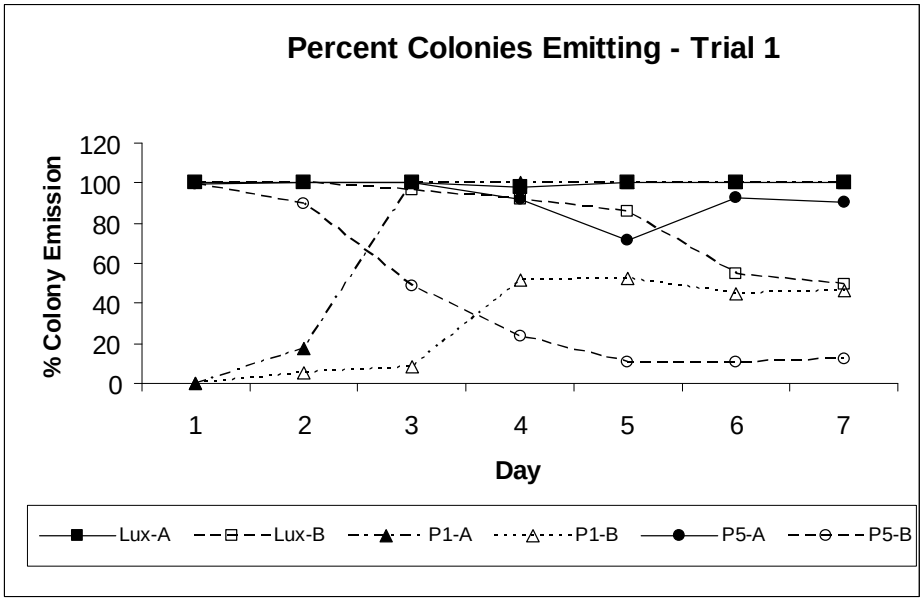

Figure 14. (Trial #1) Biophotonic stability of *Escherichia coli* MM294 transformed with one of three plasmids, with and without antibiotic selection.

Plasmids = pAk1*lux*, pXen1, and pXen5; A= with respective antibiotic; B= without respective antibiotic (Ampicillin or Erythromycin).

Interestingly, bacteria transformed with pXen5 showed poor stability of photonic emission (% colony basis), but intensity of emission increased (Figure 13; Figure 14). On d 7, 4 of the 6 plasmids had increased photonic emission. Bacterial densities are shown in Table 9, and cannot adequately explain the numerical increase in photonic emission, at least not for every plasmid type. All but P5 showed marked increase in viable cell densities. It is also likely that the increase is due to a shift in length of image acquisition (Table 9). Samples imaged for 15 sec yielded an RLU greater than samples imaged for 30 sec, indicating the camera may have become saturated at 30 sec, limiting the computer's ability to distinguish between pixels of photonic emission. When taking

timed images, the software stacks the images, intensifying the color and brightness of the computer-adapted image. In the future, images should be taken in real-time (which do not consist of stacked images) as opposed to timed-acquisition.

Polynomial models, order 1 through 4, adequately (P<0.0001) represented photonic emission data for cultures grown with and without antibiotics (Figure 15; Figure 16). However, less consistent patterns were noted for the percentage of emitting colonies. Among cultures grown with antibiotics, the number of emitting colonies by P1A (P<0.0001), but not LuxA or P5A, was adequately represented by polynomial models orders 1 through 4 (Figure 17). For those grown without antibiotics, P1B, P5B, and LuxB, were adequately represented by the 4th order model (P<0.0001).

Table 9. (Trial # 1) Biophotonic emission, viable cell densities, and photonic stability over time of *Escherichia coli* MM294 transformed with one of three plasmids, with and without antibiotic selection.[1]

	LuxA				LuxB			
Day	Acquisition Time, sec	Mean RLU/sec	Avg. VCD, cfu/mL	Avg. % Colonies Emitting	Acquisition Time, sec	Mean RLU/sec	Avg. VCD, cfu/mL	Avg. % Colonies Emitting
1	600	109.23	1.15E+08	100.00	600	109.23	1.14E+08	100.00
2	30	2180.56	1.77E+08	100.00	30	2183.43	2.05E+08	100.00
3	30	2124.89	1.5E+08	100.00	30	2151.70	1.42E+08	96.35
4	30	2133.46	1.4E+08	97.79	30	2140.35	1.54E+08	92.01
5	30	2159.79	1.7E+08	100.00	30	2115.77	1.66E+08	85.68
6	30	2175.28	1.84E+08	100.00	30	2018.77	1.84E+08	54.90
7	15	4040.62	1.87E+08	100.00	15	3178.40	1.08E+08	49.07
	P1-A				P1-B			
Day	Acquisition Time, sec	Mean RLU/sec	Avg. VCD, cfu/mL	Avg. % Colonies Emitting	Acquisition Time, sec	Mean RLU/sec	Avg. VCD, cfu/mL	Avg. % Colonies Emitting
1	600	109.05	9.72E+06	0	600	109.19	7.30E+06	0
2	30	2106.18	2.11E+07	17.65	30	2133.05	5.08E+08	5.27
3	30	2097.16	6.36E+07	100.00	30	1565.65	1.57E+07	8.56
4	30	2143.88	1.66E+08	100.00	30	1899.07	6.82E+07	51.61
5	30	2131.50	1.90E+08	100.00	30	1743.53	1.17E+08	52.54
6	30	2147.33	1.62E+08	100.00	30	1741.79	1.38E+08	44.93
7	15	3917.13	1.76E+08	100.00	15	3087.19	1.24E+08	46.06
	P5-A				P5-B			
Day	Acquisition Time, sec	Mean RLU/sec	Avg. VCD, cfu/mL	Avg. % Colonies Emitting	Acquisition Time, sec	Mean RLU/sec	Avg. VCD, cfu/mL	Avg. % Colonies Emitting
1	600	109.22	3.79E+07	99.66	600	109.20	5.18E+07	99.74
2	30	1922.69	8.07E+07	100.00	30	838.89	8.11E+07	89.32
3	30	1055.28	7.44E+07	100.00	30	288.54	1.22E+08	48.36
4	30	1473.29	8.84E+07	92.11	30	708.01	1.10E+08	23.58
5	30	1311.95	8.22E+07	71.14	30	1797.57	1.32E+08	10.56
6	30	946.18	7.89E+07	92.97	30	1963.19	1.33E+08	10.31
7	30	1389.82	7.67E+07	90.22	30	1909.16	1.28E+08	12.20

[1] Plasmids: pAk1*lux*, pXen1, and pXen5

[2] A= with respective antibiotic; B= without respective antibiotic (Ampicillin or Erythromycin).

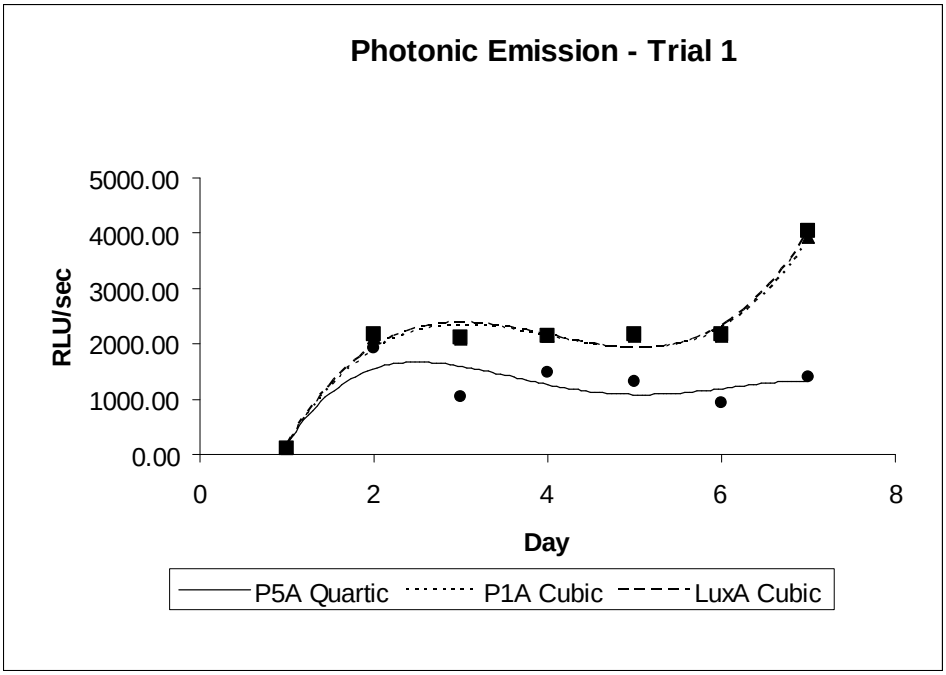

Figure 15. (Trial 1) Polynomial modeling of biophotonic emission when *Escherichia coli* MM294 is transformed with one of three plasmids (pAk1*lux*; pXen1; pXen5) with antibiotic pressure (Ampicillin or Erythromycin).

P1A: $P < 0.0001$; Adj-$R^2 = 0.7397$; $Y = 77.87x^3-1002.69x^2-2915.28$;

P5A: $P < 0.001$; Adj-$R^2 = 0.6743$; $Y = -16.55x^4+320.94x^3-2170.27x^2-3815.43$;

LuxA: $P< 0.0001$; Adj-$R^2 = 0.9713$; $Y = 108.75x^3-1311.69x^2-35555.30$;

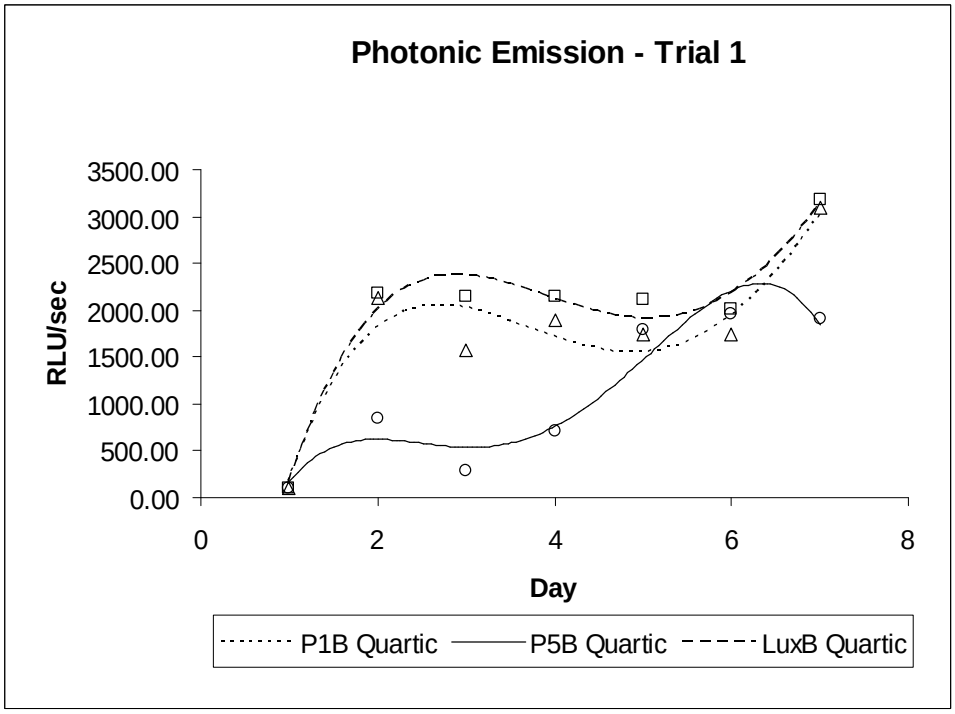

Figure 16. (Trial 1) Polynomial modeling of biophotonic emission when *Escherichia coli* MM294 is transformed with one of three plasmids (pAk1*lux*; pXen1; pXen5) without antibiotic pressure.

P1B: $P < 0.01$; Adj-$R^2 = 0.7335$; $Y = -17.79x^4+355.68x^3-2445.96x^2-4497.42$;

P5B: $P < 0.0001$; Adj-$R^2 = 0.9196$; $Y = -26.79x^4+405.14x^3-2022.09x^2-2273.52$;

LuxB: $P = 0.04$; Adj-$R^2 = 0.8639$; $Y = -10.1448x^4+247.30x^3-1947.32x^2-4154.65$;

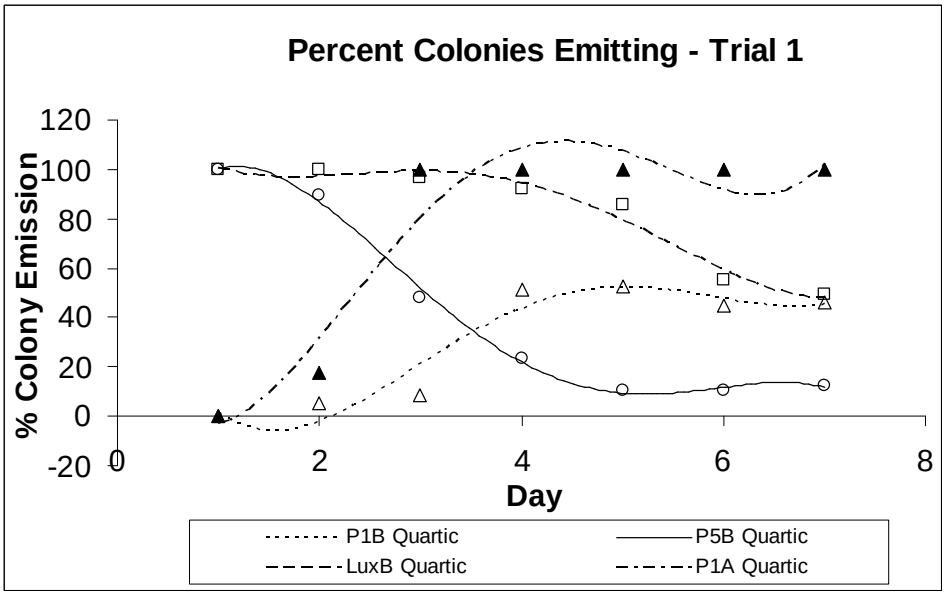

Figure 17. (Trial #1) Fit of polynomial models by biophotonic stability when *Escherichia coli* MM294 is transformed with one of 3 plasmids (pAk1*lux*; pXen1; pXen5) and cultured without antibiotic pressure (Ampicillin or Erythromycin).

LuxA and P5A did not adequately fit polynomial models orders 1 through 4 ($P<0.05$) and are not shown.

P1A: $P < 0.0001$; Adj-$R^2 = 0.9253$; $Y = 1.07x^4-16.63x^3+80.61x^2+39.14$.

P1B: $P < 0.01$; Adj-$R^2 = 0.6901$; $Y = 0.60x^4-10.64x^3+62.12x^2+73.75$;

P5B: $P < 0.001$; Adj-$R^2 = 0.9251$; $Y = -0.60x^4+10.44x^3-58.24x^2+51.65$;

LuxB: $P < 0.01$; Adj-$R^2 = 0.8369$; $Y = 0.39x^4-6.10x^3+30.20x^2+134.24$;

Trial 2. In the second trial, acquisition times were uniform throughout (2 sec acquisition), curtailing any negative effects from stacked images observed from longer acquisition times of trial 1. However, bacterial densities where much greater than in trial 1 resulting in greater RLUs and an inconsistent ability to obtain countable-plates Table 9; Table 10. As a result, an inability to determine stability of photonic emission over time (i.e., colonies emitting in absence of antibiotics) could not be fully evaluated (Figure 18). Finally, aside from a spike in photonic emission on d 3 by LuxA and LuxB, the general trend for photonic emission was consistent across the 7-d trial among treatments (Figure 19).

Polynomial models order 1 through 4 adequately fit photonic emission data by LuxA, P1A, P5A, LuxB, P1B, and P5B, with the best fit of data by LuxA and P5B, Adj-R^2 = 0.9713 and 0.9196, respectively (Figure 20; Figure 21). It should be noted, however, that the lack of countable plates (Table 10) may be affecting degree of fit to polynomial models. Polynomial models order 1 through 4 were also evaluated for photonic stability. LuxA, LuxB, and P5A were not adequately represented by polynomial models (Figure 22). The best fit to models was by P1B and P5B (Adj-R^2 = 0.9221 and 0.8251, respectively).

As shown in Figure 12, the plasmids contain either the ABCDE or the CDABE gene cassette. The CDABE cassette, as found in pAK1*lux*, is the naturally occurring form (Engebrecht et al., 1985). To aide transformation of gram-positive bacteria, the genes were rearranged to ABCDE, with appropriate ribosomal binding sites added (Winson et al., 1998). Both pXen1 and pXen5 contain the ABCDE construct, but can be used to transform both gram-negative as well as gram-positive bacteria. pXen5 is unique

in that it contains a transposon element, for a stable chromosomal insertion, eliminating the need for antibiotic selection. Attempts to transform *Streptococcus bovis* with pXen1 and pXen5 were unsuccessful do to the thick cell wall of this gram-positive bacterium (data not shown). As a result, *E. coli* was used to evaluate these plasmids. The plasmids can be cultivated in both gram-negative as well as gram-positive bacteria, however, pXen1 and pXen5 are optimized for gram-positive physiology, intended to only be cultivated or multiplied in gram-negaives. This may explain the variable results for cultures transformed with pXen 1 and pXen5.

It is yet unclear whether using pXen5 to transform ruminal bacteria would adequately bypass the need for ruminal antibiotic infusion. Table 9 and Figure 13 indicated a rapid loss in photonic emission upon removal of antibiotics. Though bacteria were successfully transformed using pXen5, stable chromosomal insert was not achieved for reasons not yet understood. One other drawback to using pXen5 is that photonic emission tended to be numerically less for pXen5 than other plasmids evaluated (Figure 13; Table 9).

Table 10. (Trial # 2) Biophotonic emission, viable cell densities, and photonic stability over time of *Escherichia coli* MM294 transformed with one of three plasmids, with and without antibiotic selection.[1,2,3]

	LuxA				LuxB			
Day	Acquisition Time, sec	Mean RLU/sec	Avg. VCD, cfu/mL	Avg. % Colonies Emitting	Acquisition Time, sec	Mean RLU/sec	Avg. VCD, cfu/mL	Avg. % Colonies Emitting
1	2	24678.40	1.21E+05	100	2	25570.62	TMTC	100.00
2	2	8870.00	TMTC	100	2	8539.79	TMTC	100.00
3	2	22286.91	1.21E+06	100	2	20577.51	1.30E+06	91.42
4	2	8209.24	1.82E+06	100	2	7692.15	2.37E+06	92.60
5	2	15705.91	1.19E+06	100	2	12855.12	1.75E+06	80.48
6	2	14041.26	1.95E+06	100	2	10483.16	5.52E+06	70.97
7	2	15720.83	3.01E+06	100	2	7155.70	5.02E+06	59.41
	P1-A [1]				P1-B [1]			
Day	Acquisition Time, sec	Mean RLU/sec	Avg. VCD, cfu/mL	Avg. % Colonies Emitting	Acquisition Time, sec	Mean RLU/sec	Avg. VCD, cfu/mL	Avg. % Colonies Emitting
1	2	1148.95	TMTC	TMTC	2	1614.94	TMTC	TMTC
2	2	3965.10	5.06E+04	39.75	2	6450.15	9.49E+04	45.24
3	2	7186.79	6.01E+05	96.51	2	7952.47	9.04E+05	91.96
4	2	16965.59	TMTC	ND	2	16670.58	TMTC	ND
5	2	18316.96	4.72E+06	99.91	2	15660.85	5.04E+06	86.20
6	2	14471.77	7.92E+06	97.21	2	7452.81	5.01E+06	13.65
7	2	13182.47	9.16E+06	95.58	2	1780.05	6.29E+06	9.50
	P5-A [1]				P5-B [1]			
Day	Acquisition Time, sec	Mean RLU/sec	Avg. VCD, cfu/mL	Avg. % Colonies Emitting	Acquisition Time, sec	Mean RLU/sec	Avg. VCD, cfu/mL	Avg. % Colonies Emitting
1	2	12030.18	TMTC	100.00	2	15249.97	TMTC	100.00
2	2	3738.00	TMTC	ND	2	3514.71	TMTC	ND
3	2	7339.24	6.39E+05	93.88	2	5901.07	9.08E+05	4.77
4	2	10778.25	8.34E+05	100.00	2	9467.15	9.98E+05	23.58
5	2	8462.00	5.07E+05	99.79	2	12437.18	1.74E+06	10.30
6	2	14672.16	2.14E+05	95.40	2	19828.34	3.07E+06	70.22
7	2	9124.13	6.34E+05	93.67	2	15220.46	2.29E+06	57.55

[1] ND = Not determined; TMTC = Too many to count

[2] Plasmids: pAk1*lux*, pXen1, and pXen5

[3] A= with respective antibiotic; B= without respective antibiotic (Ampicillin or Erythromycin)

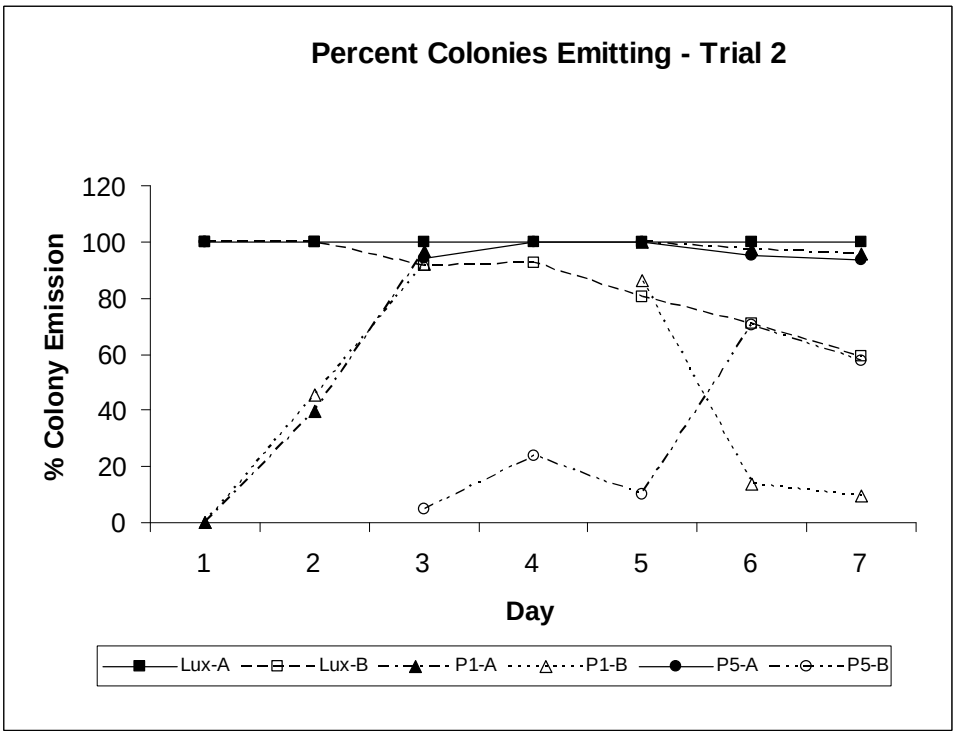

Figure 18. (Trial #2) Biophotonic stability of *Escherichia coli* MM294 transformed with one of three plasmids (pAk1*lux*; pXen1; pXen5), with and without antibiotic selection (Ampicillin or Erythromycin).

Plasmids = pAk1*lux*, pXen1, and pXen5; A= with respective antibiotic; B= without respective antibiotic (Ampicillin or Erythromycin).

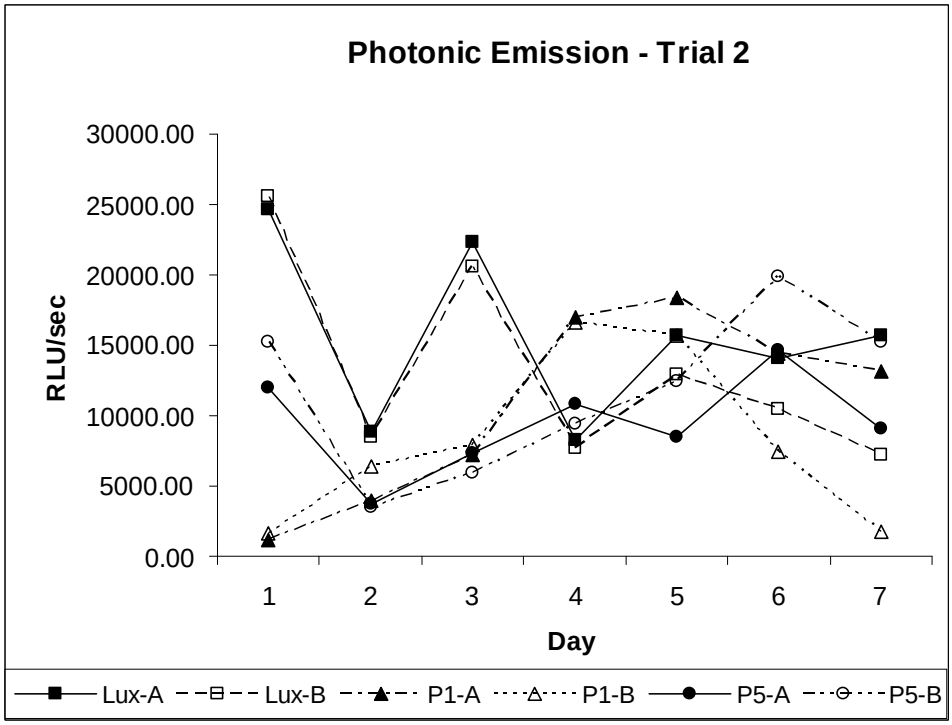

Figure 19. (Trial # 2) Biophotonic emission of *Escherichia coli* MM294 transformed with one of three plasmids (pAk1*lux*; pXen1; pXen5), with and without antibiotic selection (Ampicillin or Erythromycin).

Plasmids = pAk1lux, pXen1, and pXen5; A= with respective antibiotic; B= without respective antibiotic (Ampicillin or Erythromycin).

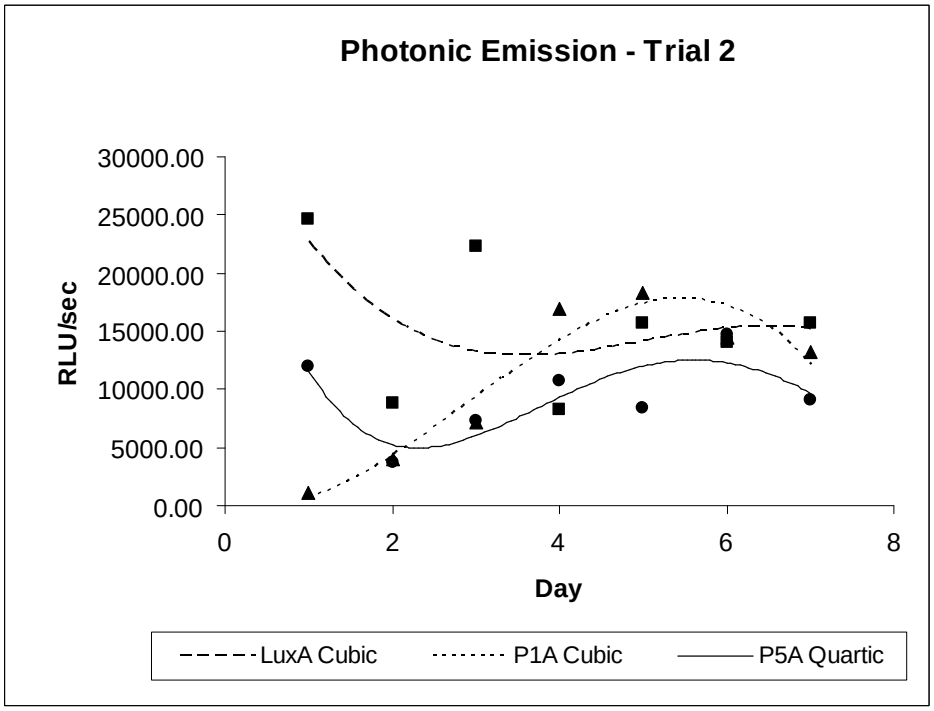

Figure 20. (Trial 2) Polynomial modeling of biophotonic emission when *Escherichia coli* MM294 is transformed with one of three plasmids (pAk1*lux*; pXen1; pXen5) with antibiotic pressure (Ampicillin or Erythromycin).

P1A: $P < 0.0001$; Adj-$R^2 = 0.7397$; $Y = 77.87x^3-10002.69x^2-2915.28$;

P5A: $P < 0.001$; Adj-$R^2 = 0.6743$; $Y = -16.55x^4+320.94x^3-2170.27x^2-3815.43$;

LuxA: $P < 0.0001$; Adj-$R^2 = 0.9713$; $Y = 108.75x^3-1311.69x^2-3555.30$;

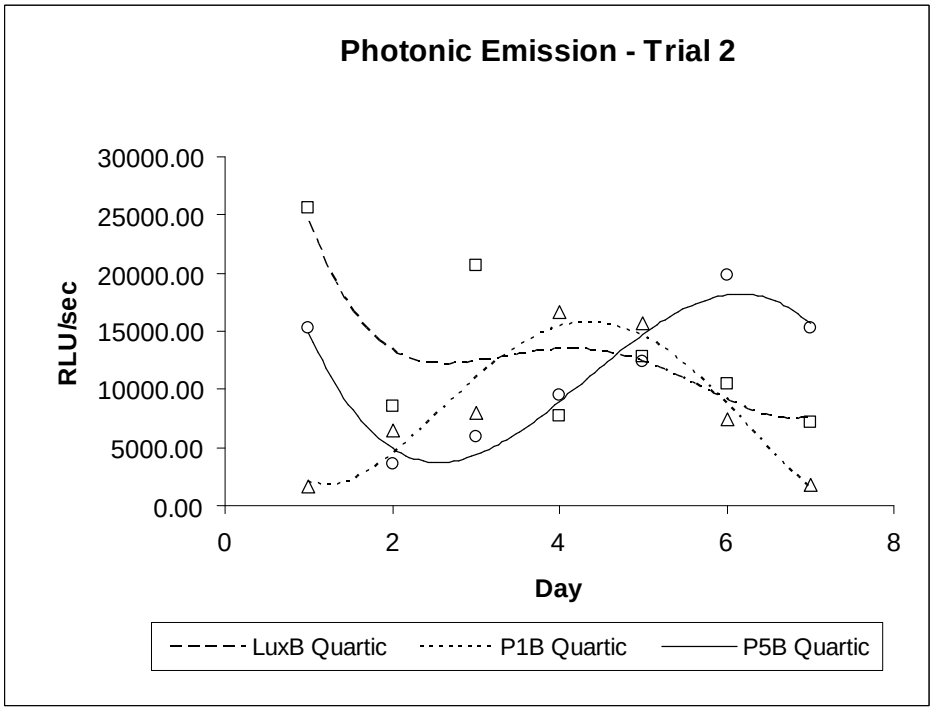

Figure 21. (Trial 2) Polynomial modeling of biophotonic emission when *Escherichia coli* MM294 is transformed with one of three plasmids (pAk1*lux*; pXen1; pXen5) without antibiotic pressure.

P1B: $P < 0.01$; Adj-$R^2 = 0.7335$; $Y = -17.79x^4+355.68x^3-2445.96x^2-4497.42$;

P5B: $P < 0.001$; Adj-$R^2 = 0.9196$; $Y = -26.79x^4+405.14x^3-2022.09x^2-2273.52$;

LuxB: $P = 0.04$; Adj-$R^2 = 0.8639$; $Y = -10.14x^4+247.30x^3-1947.32x^2-4154.65$;

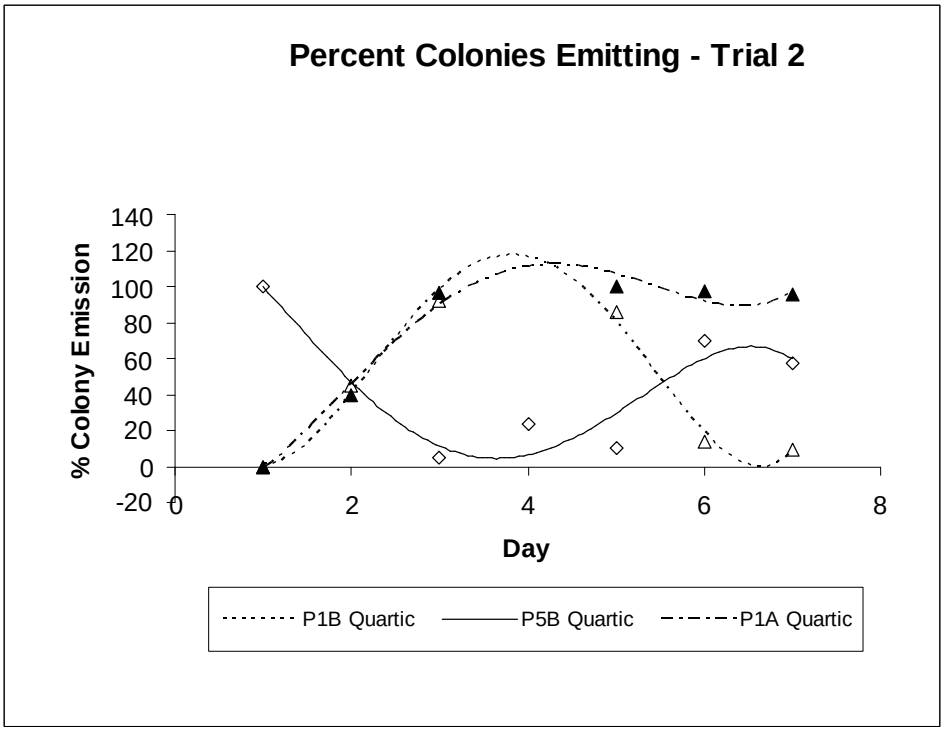

Figure 22. (Trial #2) Fit of polynomial models by biophotonic stability when *Escherichia coli* MM294 is transformed with pXen1 or pXen5 and cultured with antibiotic pressure (Ampicillin or Erythromycin).

LuxA, LuxB, and P5A did not adequately fit polynomial models orders 1 through 4 ($P<0.05$), and are not shown.

P1A: $P<0.0001$; Adj-$R^2 = 0.7641$; $Y=1.98x^4-33.42x^3+191.55x^2+354.99$

P1B: $P<0.0001$; Adj-$R^2 = 0.9221$; $Y=3.11x^4-51.31x^3+281.32x^2+453.70$

P5B: $P<0.0001$; Adj-$R^2 = 0.8251$; $Y=-2.20x^4+34.75x^3-176.51x^2-58.87$

Experiment 2

Data evaluating rudctions in photonic detection by ruminal fluid was conducted. All cultures contained an equal number of bacteria (P = 0.16; Figure 23; Table 11), indicating that nothing inherently in the ruminal fluid inhibited growth. However, there was a tendency toward a reduction in cell density among cultures containing ruminal fluid. It should be noted that *E. coli* has been isolated from natural ruminal fluid, but always in very miniscule concentrations (Wolin, 1969). Previously, *E. coli* has been shown to be incapable of persisting in ruminal fluid. Hollowell and Wolin (1965) conducted a systematic series of experiments involving filtering, sterilizing, and fractioning ruminal fluid in comparison to the standard growth media, Antibiotic Medium 3. Their findings indicated that inhibitors of *E. coli* growth was of unknown origin and was not related to 1) a lack of available substrate within growth media, 2) composition of gas phase (N_2 vs CO_2), 3) a heat-labile compound, 4) due to volatile fatty acids, nor 5) due to anything that could be removed by filtration.

One variable evaluated by Hollowell and Wolin (1965) was the impact of volatile fatty acids on viability of *E. coli*. Using Antibiotic Medium 3 (pH 7) as the basal media, the following was added: 60 m*M* acetate, 20 m*M* propionate, 15 m*M* butyrate, 3 m*M* valerate, and 2 m*M* isovalerate, which were considered common concentrations within the rumen. No adverse affects were noted. In a follow-up experiment, Wolin (1969) revisited the effect volatile fatty acids have on the growth of *E. coli* as a function of pH. In this experiment, VFA concentrations were adjusted to: 15 m*M* acetic, 10 m*M* propionic, 11 m*M* butyric, 10 m*M* valeric, 20 m*M* isobutyric, 20 m*M* isovaleric. Using

Antibiotic Medium 3, after VFA addition, pH was adjusted to 6.0, 6.5, and 7.0. A strong interaction between VFA and pH (6.0, 6.5, and 7.0) was observed, resulting in 96, 69, and 2% inhibition of growth, respectively.

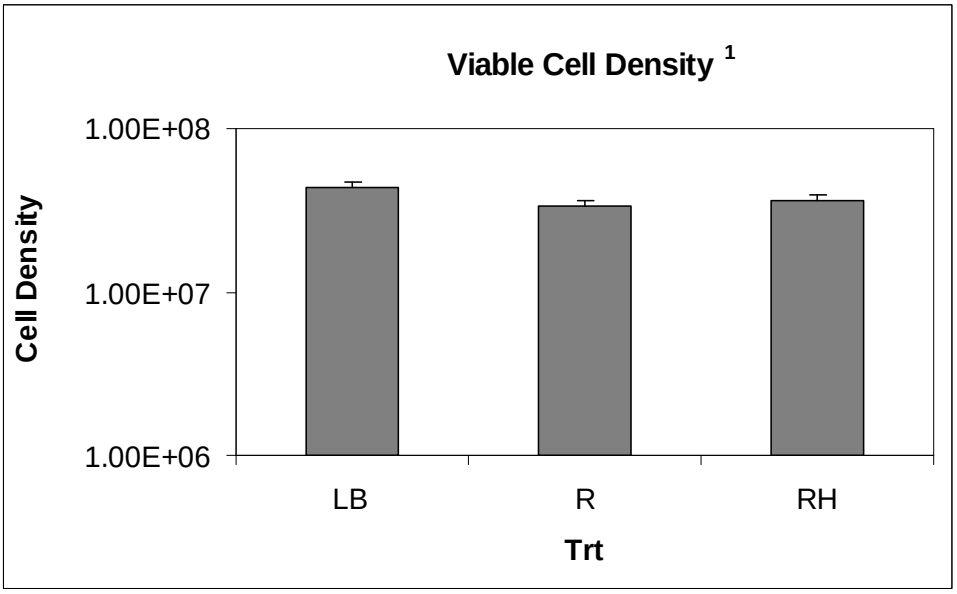

Figure 23. Viable cell density of *lux*-transformed *Escherichia coli* MM294 grown in LB or ruminal fluid.

LB = Luria-Bertaini broth, Rumen = Ruminal fluid + LB powder, and Rumen+Hay = Ruminal fluid + LB powder + Ground hay.

Table 11. Evaluation of redced photonic detection and growth of *E. coli* MM294 in ruminal fluid or LB.

Trt [1]	RLU, Real Time [2]	CFU/mL [3]
LB	1.1601 [b]	4.35E+07
R	0.3855 [a]	3.34E+07
RH	0.129 [a]	3.67E+07
P-value	< 0.0001	0.1598
SE	0.2619	2.98E+06

[1] LB = Luria-Bertaini broth, Rumen = Ruminal fluid + LB powder, and Rumen+Hay = Ruminal fluid + LB powder + Ground hay

[2] RLU = Relative light unit measured in real time

[3] CFU = Colony forming unit

[abc] Means within colume without common superscript differ ($P<0.05$).

In the current study, *E. coli* cell density was slightly less, numerically, when grown in R or RH than LB (Figure 23). The reason greater reductions in growth were not observed, as in Wolin (1969), is likely due to the fact that media pH was adjusted to 7.0 prior to inoculation. For the current objective, this is not a problem. Normal ruminal pH for a forage-based diet is approximately 6.5 and ruminal bacteria can easily persist between a pH of 6 and 7. At this state, *E. coli* was merely used as a model for evaluating the emission and detection of biophotonic light.

Absorbance loss of photonic emission due to the opaque nature of ruminal fluid and the obstruction of photonic light by feed particles was evaluated (Figure 24). There was over 50% reduction ($P<0.0001$) in photonic detection between LB and R with only a

trend toward reduction between R and RH. Given traditional parameters governing spectroscopy that also apply to luminescence (i.e., absorbance, reflectance, obstruction, etc), the results are not surprising but serve as a bech mark of understanding needed prior to proceeding.

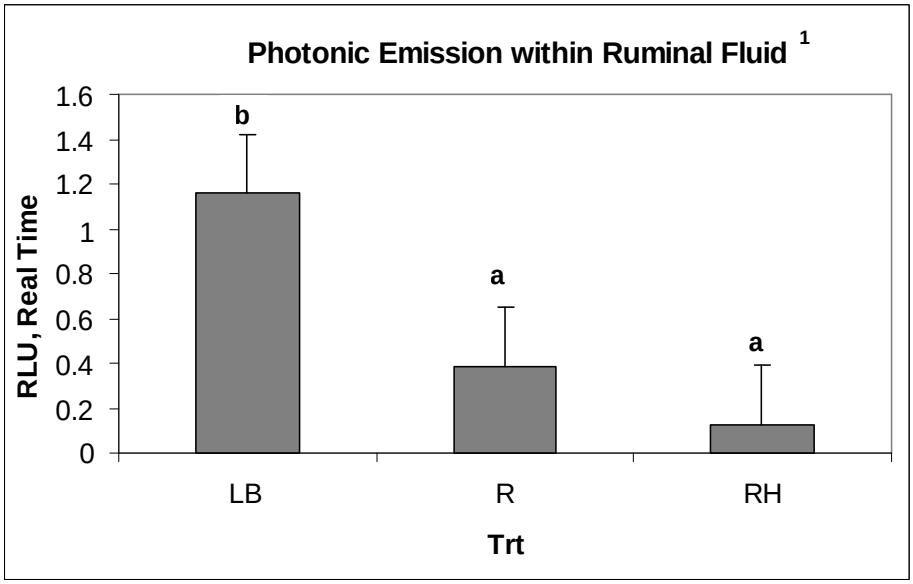

Figure 24. Effect of ruminal fluid and feed particles on photonic emission of *lux*-transformed *Escherichia coli* MM294.

[1] RLU = relative light unit; LB = Luria-Bertaini broth, Rumen = Rumininal fluid + LB powder, and Rumen+Hay = Ruminal fluid + LB powder + Ground hay.
[abc] Means without common superscript differ ($P<0.05$).

Luciferases begin loosing functionality at 30ºC, with complete loss reported at 44ºC (Mackey et al., 1994). Vessels in this experiment were maintained at 39ºC with no

apparent loss in photonic emission. Lastly, anaerobic fermentation of ruminal bacteria yields relatively little ATP in comparison to oxidative metabolism. This may be a limiting factor in using firefly luciferins that requires ATP, because as much as 20% of bacterial energy is expended in luminescence (Meighen, 1991; Węgrzyn and Czyż).

Conclusions

Currently, intrinsic characteristics of the rumen limit the use of biophotonics in mircofloral evaluation, namely, the opaque nature of ruminal fluid, the presence of feed/plant particuclate matter, and the limitations of using antimicrobial agents in a complex microbial ecosystem. As such, a number of factors still need to be investigated to completely assess the value of ruminal bioluminescence and to explore potential means of bypassing current constraints.

Given the anaerobic nature of the rumen, photonic emission may be interrupted due to the rate of ATP production under anaerobic conditions. Literature is conflicting on the role of oxygen in luminescence (Meighen, 1991; Mary, 1993; Hastings, 1996). Traditionally, the reaction equation is written to require molecular oxygen, however, conflicting results have immerged. Therefore, research should be directed at further exploration before live-animal applications are sought.

A number of prominent bacteria in the rumen are gram-positive and are difficult to transform, potentially limiting assessment of the 3 *lux* plasmids evaluated herein. Beyond this obstacle, currently, only one, possibly two, bacteria can be transformed to allow differential luminescent imaging in mixed culture, limiting the exploratory scope of this technique. The addition of fluorescent proteins may increase the investigative

capacity. In addition, data indicates that the opaqueness of ruminal fluid along with the presence of feed particulates would be mitigating factors in accurately detecting rumen photonics. Quantity of feed used in the current trial did not parallel that of *in vivo* ruminal fill, meaning greater obstruction is possible. Lastly, photonic emission is known to decrease upon removal of antibiotic pressure, and to infuse the rumen with antibiotics would likely be detrimental to native flora, confounding any results from a digestibility trial. The best option would be to culture strains with a stable chromosomal construct, eliminating photonic instability over time. However, the indiscriminant chromosomal insertion may have variable consequences leading to the disruption of open reading frames or individual genes, which may significantly alter bacterial physiology and efficiency, and should therefore be investigated.

Collectively, these factors need to be investigated prior to implementing biophotonics as a means of monitoring ruminal microflora. It would seem that time would be best invested by trying to improve usefulness of photonic data. Current applications are largely qualitative, such as in giving clear evidence to the general location of bacteria and their movement. However, strength of emission will vary with growth curves. To date, no one has successfully, and consistently, correlated photonics with cell densities. If this can be achieved either through further genetic manipulation or technological development, the value and use of biophotonic applications would greatly increase.

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APPENDIX

ANAEROBIC MICROBIOLOGICAL GROWTH MEDIA

Growth media for ruminal bacteria are typically not commercially available, with the exception of *Streptoccus bovis.* S. bovis was grown using the commercial available medium: Brain-Heart Infusion Broth and grown on Blood Agar with Tryptic soy as its base.

All remaining bacterial strains cultivated were obtained from American Type Culture Collection (ATCC®). Also lited at depository are recommended growth media developed by those depositing original cell lines. Recipes shown herein are as they appear at ATCC®. In a few cases, revisions to the original recipe were made. In such cases, both the original recipe and the modified recipe (designated as "Revised" in the title) are shown.

ATCC Medium 1102: (for growth of *Ruminobacter amylophilus*)

Chopped meat carbohydrate medium with 0.1% cellobiose, 0.1% maltose, 0.1% starch, and 0.1% Tween 80.

Ingredient:

Item	Quantity
Ground Beef	500 g
Distilled water	1.0 L
NaOH	25 mL
Peptone	30 g
Yeast Extract	5 g
Cellobiose	0.1%
Maltose	0.1%
Starch	0.1%
Tween 80	0.1%
K_2HPO_4	5 g
0.025% Resazurin	4 mL
L-cystein · HCl	0.5 g

Instructions:

Using lean beef or horse meat, remove fat and connective tissue and grind. Mix meat, water, and NaOH and bring to a boil while stirring. Cool to room temperature, skim fat off surface, and filter, retaining filtrate to restore 1 L original volume.

To filtrate add peptone, yeast extract, cellobiose, maltose, starch, and Tween 80, K2PO4, and resazurin solution. Boil, cool, and add 0.5 g L-cystein · HCl; adjust pH to 7.0. Under 97% nitrogen, 3% hydrogen, dispense 7 mL over 1 part meat particles to 4 to 5 parts fluid per test tube. Cap with butyl rubber stoppers under N_2, H_2, and autoclave in press for 15 min under fast exhaust.

ATCC Medium 1102 – Revised: (for growth of *Ruminobacter amylophilus*)

Ingredient:

Item	Quantity
Beef Extract *	18 g
Cellobiose	0.0625 g
Maltose	0.0625 g
Starch	0.0625 g
Tween 80	0.0625 g
Peptone	30 g
Yeast Extract	5 g
K_2HPO_4	5 g
0.025% Resazurin solution	4 mL
L-Cysteine · HCl	0.5 g
Distilled Water	1 L

* Commercially available from MP Biomedicals, LLC

Instructions:

Bring to a boil under 98% CO_2, and 2% H_2. Cool slightly and add L-Cysteine · HCl. Dispense medium using anaerobic techniques and the same gas phase. Autoclave medium at 121°C for 15 min. Adjust final pH to 7.0.

ATCC Medium 1734: (for growth of *Fibrobacter succinogenes*)

Ingredient:

Item	Quantity
Basal Medium:	
Ruminal Fluid	400 mL
K_2HPO_4	0.45 g
KH_2PO_4	0.45 g
NaCl	0.9 g
$(NH_4)_2SO_4$	0.9 g
$CaCl_2$	0.09 g
$MgSO_4 \cdot 7\ H_2O$	0.18 g
$NaHCO_3$	6.37 g
Cellobiose	5 g
Casitone	5 g
Resazurin	1 mg
Distilled Water	575 mL
Agar (if needed)	15 g
Soluble Starch	0.1755 g
Alkaline solution:	
L-Cysteine · HCl	0.5 g
$Na_2S \cdot 9H_2O$	0.25 g
Distilled Water	25 mL

Instructions:

Bring basal medium to boil under 98% CO_2, and 2% H_2. Cool slightly and add freshly prepared alkaline solution. Dispense medium using anaerobic techniques and the same gas phase. Autoclave medium at 121°C for 15 min. Adjust final pH to 6.8 ± 0.2.

ATCC Medium 602: (for growth of Butyrivibrio fibrisolvens and Selenomonas ruminantium)

Ingredients:

Item	Quantity
Basal Medium:	
Ruminal Fluid	30 mL
Glucose	0.05
Maltose	0.05
Soluble Starch	0.05
Peptone	0.05
Yeast Extract	0.05
$(NH_4)_2SO_4$	0.05
Resazurin Solution (see below)	0.4 mL
Salt solution (see below)	50 mL
L-Cysteine · HCl · H_2O	0.05 g
Distilled Water	20 mL
Salt Solution:	
$CaCl_2$ (anhydrous)	0.2 g
$MgSO_4$	0.2 g
K_2HPO_4	1.0 g
KH_2PO_4	1.0 g
$NaHCO_3$	10.0 g
NaCl	2.0 g
Distilled Water	1 L
Resazurin Solution:	
Resazurin	25 mg
Distilled Water	100 mL

Instructions:

Basal Medium: Mix all ingredients and boil 10 to 20 min or until color changes fromo pink to a yellow tinge. Cool in ice water bath under O_2-free CO_2. Flow of gas should cause gentle bubbling sufficient enough to exclude air. Remove from ice bath and add L-Cystein. Adjust pH to 7.0 with 8N NaOH or 5*N* HCl. Dispense medium anaerobically into tubes flushed with 100% N_2, to prevent pH change during storage. Secure anaerobic cap and autoclave for 12-15 min at 121°C.

Ruminal Fluid: Filter ruminal contents through cheese cloth. Store under CO2 in glass bottles in the refrigerator. Allow particulate matter to settle and use only supernatant.

Salt Solution: Dissolve $CaCl_2$ and $MgSO_4$ in 300 mL of distilled water. Add 500 mL water and the remaining salts while swirling slowly. Add 200 mL of distilled water, mix and store at 4°C.

ATCC Medium 158: (for growth of *Ruminococcus flavefaciens* and *Ruminococcus albus*)

Ingredients:

Item	Quantity
Solution 1:	
K_2HPO_4	1.5 g
Distilled Water	500 mL
Solution 2:	
KH_2PO_4	1.5 g
$(NH_4)_2SO_4$	3 g
NaCl	3 g
$MgSO_4$	0.3 g
$CaCl_2$	0.3 g
Distilled Water	500 mL
Solution 3:	
Resazurin	0.05 g
Distilled Water	50 mL
Solution 4:	
Glucose	3 g
Cellobiose	3 g
Distilled Water	325 mL
Solution 5:	
Cystein · HCl	0.75 g
Distilled Water	25 mL
Solution 6:	
Na_2CO_3	6 g
Distilled Water	100 mL

Instructions:

Combine 150 mL solution 1, 150 mL of solution 2, 1 mL of solution 3, 220 mL solution 4, and 400 mL of ruminal fluid. Bring to a boil under CO_2 gas. Autoclave at 121°C for 20 min. While stirring, allow to cool under CO_2 gas addition. Aseptically add 17 mL solution 5 and 66 mL solution 6. Adjust pH to 6.6.