An exploration of ecological concepts in the context of antimicrobial resistance and the use of phytochemical compounds within the ruminant gut microbiome

by

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DEDICATION

I dedicate this thesis to my parents.

You are the reason I've made it this far!

Thank you.

ABSTRACT

Secondary plant metabolites have recently been gaining interest in livestock production systems following the ban of in-feed antibiotics within the European Union. The rise in antimicrobial resistance found in pathogenic and non-pathogenic bacteria has lead to increased interest in the research community regarding the use of phythochemicals as an alternative to antibiotics. The purpose of this research was to evaluate the impact of including phytochemicals in a livestock production system. Specifically, a high tannin-containing forage, sainfoin (*Onobrychis viciifolia*), was evaluated *in vitro* for its antimicrobial effect on *Escherichia coli*. We determined that phytochemicals alone are not as inhibitory as synthetic antibiotics. Thus, the use of combination therapy to deter the development of antimicrobial resistance was evaluated. A myriad of plant compounds were screened for their synergistic interactions with ciprofloxacin. Geraniol, an essential oil, was identified to possess good antimicrobial activity and synergistic interactions with ciprofloxacin. Therefore the effect of long term exposure to both ciprofloxacin and geraniol were examined. Results demonstrated that once an antimicrobial concentration threshold was reached, resistance to ciprofloxacin increased markedly in the presence of both geraniol and ciprofloxacin. Finally, an *in vivo* trial was conducted in which forty steers were fed sainfoin or alfalfa over a 9-week period to evaluate its ability to reduce *E. coli* shedding and its impact on gut microbiota in the context of popular theoretical ecology concepts. Results from the *in vivo* study indicate that sainfoin was able to promote a slight decrease in generic *E. coli* shedding which could be maintained throughout the trial. Using high-throughput sequencing, the effect of sainfoin on the microbial ecosystem of the ruminant gut was evaluated. Sainfoin induced a significant shift in the microbial community structure of the rumen and to a lesser

extent in the hindgut. Using ecology theories, a hypothesis was formulated regarding the mechanisms that mediate the development of tolerance and the fundamental ecological processes controlling microbial population shifts. Understanding how the gut ecosystem functions and predicting its behaviour in the presence of various fluctuating environmental conditions will enable more efficient manipulation of the rumen and promote best management practices in livestock production.

FOREWARD

This thesis was prepared following a manuscript format. The thesis is comprised of five manuscripts each corresponding to a chapter. Manuscript I and II have been published in the Canadian Journal of Plant Science and the Journal of Applied and Environmental Microbiology, respectively. Manuscript III, IV, and V will be submitted to the Journal of Applied and Environmental Microbiology. All manuscripts are formatted in accordance with the guidelines of the Canadian Journal of Plant Science.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS..**ii**

DEDICATION...**iv**

ABSTRACT..**v**

FOREWARD..**vii**

TABLE OF CONTENTS..**viii**

LIST OF TABLES..**xii**

LIST OF FIGURES..**xiv**

LIST OF COPYRIGHTED MATERIAL FOR WHICH PERMISSION WAS OBTAINED..**xvii**

LIST OF ABBREVIATIONS...**xix**

CHAPTER ONE: GENERAL INTRODUCTION...**1**

CHAPTER TWO: LITERATURE REVIEW..**3**

2.1 Microbial ecosystem of the ruminant gut...**3**

2.2 How to study microbial ecosystems?...**7**

2.3 Next-generation sequencing technology..**8**

2.4 Introduction to antimicrobial resistance...**9**

2.5 Microbial mechanisms of antimicrobial resistance...**9**

2.6 Agriculture and antimicrobial resistance..**12**

2.7 Combination therapy and methodologies to assess antimicrobial synergism, additivity, and antagonism...**16**

2.8 Phytochemicals...**26**

2.9 Tannins..**33**

2.10 Ecology theories...**54**

2.11 **Research hypothesis and objectives**..**60**

CHAPTER THREE: MANUSCRIPT I...**61**

Condensed tannin concentrations found in vegetative and mature forage legumes grown in western Canada…..**61**

3.1 Abstract...**62**

3.2 Introduction...**63**

3.3 Material and methods...**64**

3.4 Results and discussion..**68**

CHAPTER BRIDGE TO CHAPTER FOUR...**80**

CHAPTER FOUR: MANUSCRIPT II...**81**

Potential to reduce *Escherichia coli* shedding in cattle feces using sainfoin *Onobrychis viciifolia*) forage: *in vitro* and *in vivo* comparisons...81

4.1 Abstract...**82**

4.2 Introduction...**83**

4.3 Materials and methods..**85**

4.4 Results...**92**

4.5 Discussion..**100**

CHAPTER BRIDGE TO CHAPTER FIVE..**105**

CHAPTER FIVE: MANUSCRIPT III...**106**

Bet hedging response increases antimicrobial resistance in combination therapy...**106**

5.1 Abstract..**107**

5.2 Introduction...**108**

5.3 Materials and methods...**111**

5.4 Results...**118**

5.5 Discussion..**126**

CHAPTER BRIDGE TO CHAPTER SIX..**132**

CHAPTER SIX: MANUSCRIPT IV...**133**

Microbial community dynamics of the ruminant gut in response to feeding a tanninferous diet...**133**

6.1 Abstract..**134**

6.2 Introduction...**135**

6.3 Materials and methods...**138**

6.4 Results...**142**

6.5 Discussion..**157**

CHAPTER BRIDGE TO CHAPTER SEVEN...**163**

CHAPTER SEVEN: MANUSCRIPT V..**164**

Heterogeneity of the rumen and hindgut microbial ecosystem..............................**164**

7.1 Abstract..**165**

7.2 Introduction...**166**

7.3 Materials and methods...**169**

7.4 Results...**174**

7.5 Discussion..**187**

CHAPTER EIGHT: GENERAL DISCUSSION..**194**

CHAPTER NINE: CONCLUSIONS AND FUTURE DIRECTIONS......................**203**

9.1 General conclusions...**203**

9.2 Future directions...**204**

CHAPTER TEN: REFERENCES...**205**

APPENDICES...**225**

APPENDIX I..**225**

COPYRIGHT AGREEMENT FOR CHAPTER TWO: Literature Review

Figure 2.2...**226**

APPENDIX II..**230**

COPYRIGHT AGREEMENT FOR CHAPTER TWO: Literature Review

Figure 2.13...**231**

APPENDIX III...**234**

COPYRIGHT AGREEMENT FOR CHAPTER THREE: Manuscript I.................**234**

APPENDIX IV...**235**

COPYRIGHT AGREEMENT FOR CHAPTER FOUR: Manuscript II..................**236**

LIST OF TABLES

Table 2.1 Antimicrobial interactions as defined by the fractional inhibitory concentration (FIC) index……………….……….…...………………...**18**

Table 2.2 Design of a time-kill assay for compound A and B in combination and alone……………………………………………………………..…..**23**

Table 3.1 Summary of condensed tannin (CT) concentration of forages, region grown, analytical method, and plant part utilized………………………………...**69**

Table 3.2 Determination of extractable CT concentrations of whole plant forage legume species using the butanol-HCl technique……………….........................**71**

Table 3.3 Determination of average extractable CT concentration in whole plant forage legume species at different stages of maturity using the butanol-HCl technique……………………………………….……………….……………...**75**

Table 3.4 Effect of growing season on extractable CT concentration using butanol-HCl technique in whole plant forage legume species grown in western Canada………………………………………………….………………...**78**

Table 4.1 Minimum inhibitory concentration (MIC) of purified phenolic compounds, sainfoin and alfalfa extracts against various *Escherichia coli* isolates……………………………………………………….….................**93**

Table 4.2 Animal trial: Correlations between animal intake, outdoor temperature, and fecal *E. coli* numbers………………………....…………………...**98**

Table 5.1 Concentrations of geraniol and ciprofloxacin used in the cell viability and yield assay for *E. coli* O157:H7……………………......……………….**115**

Table 5.2 Rapid screening for inhibitory activity of various phytochemicals against *E. coli* O157:H7……………………………………………………………...**119**

Table 5.3 Rapid screening for inhibitory activity of various essential oils against *E. coli* O157:H7……………………………………………………………...**120**

Table 5.4 FIC indices of various phytochemicals in combination with ciprofloxacin using the checkerboard method…………………………………………………**120**

Table 6.1 Summary of pyrosequencing data and richness and diversity estimates for rumen fluid and fecal samples collected from four steers fed an alfalfa or sainfoin hay diet across three time periods………….……………………………………..**144**

Table 7.1 Distribution of sequences, coverage, richness and diversity indices for rumen fluid and fecal samples collected from four steers fed an alfalfa or sainfoin hay diet across three time periods……………………………………………………..**175**

Table 7.2 Phylum composition of samples collected from the rumen fluid and feces of four steers fed alfalfa-based diets……..……………………………………..**181**

Table 7.3 Composition of core genera (>1% relative abundance) in rumen fluid and fecal samples collected from four steers fed alfalfa-based diets ……….........**182**

Supplementary Table 7.1 Average genera (<1% relative abundance) in rumen fluid and fecal samples collected four steers fed alfalfa-based diets……………………**185**

LIST OF FIGURES

Figure 2.1 Example of a checkerboard array for compound A and B using two-fold dilutions……………………………………………………….................**20**

Figure 2.2 Placement of E-test strips for synergy testing……………………………**25**

Figure 2.3 Structure of cinnamic acid and caffeic acid…………………...………....**29**

Figure 2.4 Structure of catechol and pyrogallol……………………………………..**29**

Figure 2.5 Structure of quinones……………………………………………………....**30**

Figure 2.6 Pyran, flavone and flavonol structure……………………………………**31**

Figure 2.7 Basic structure of coumarin……………………………………………...**32**

Figure 2.8 Structure of isoprene and eugenol……………………………...….......**33**

Figure 2.9 Hydrolyzable tannins: gallotannin and ellagitannin. Hydrolysis of these compounds will yield a carbohydrate molecule and a phenolic acid such as gallic acid and ellagic acid……………………………..................................**35**

Figure 2.10 Structure of catechin (flavan-3-ol)……………………………………….**35**

Figure 2.11 The chemical structure of gallic acid and ellagic acid. They are the phenolic acids esterified with hydroxyl groups of a carbohydrate to form hydrolysable tannins……………………..…………………………………………..**36**

Figure 2.12 Condensed tannin structure containing three catechin moieties…………**37**

Figure 2.13 Biosynthesis pathway of anthocyanidins and proanthocyanidins………..**39**

Figure 4.1 Death rates of *E. coli* O157:H7 cip^r cells inoculated in cattle feces with the addition of either 0.5 % (w/w) ground sainfoin silage or 0.5 % (w/w) *p*-coumaric acid and incubated at different temperatures (-20, 37, or

5°C)………...………………………………………………………..........**95**

Figure 4.2 Effect of outdoor temperature (A) and diet (B) on the viability of fecal *E. coli* spp. over time……………………………………….…………………....**96**

Figure 4.3 Average fecal *E. coli* numbers from feces of cattle fed either a sainfoin (silage or hay) or alfalfa (silage or hay) diet……………...………………………...**99**

Figure 5.1 *E. coli* O157:H7 resistance development profile of ciprofloxacin in combination with geraniol………………………………….……………………**123**

Figure 5.2 *E. coli* O157:H7 resistance against three antimicrobial treatments: ciprofloxacin alone, geraniol alone, or ciprofloxacin + geraniol…..............................**124**

Figure 5.3 Morphological differences in *E. coli* O157:H7 after prolonged exposure to various antimicrobial treatments: no antimicrobial, ciprofloxacin, geraniol oil, ciprofloxacin + geraniol…………………………………………….....**125**

Figure 6.1 Rarefaction curves for rumen and hindgut samples at a 97% sequence similarity level……………………………………………………………..**145**

Figure 6.2 Statistical representation of true diversity in the rumen and hindgut……………………………………………….............**146**

Figure 6.3 Relative abundance of microbial phyla within the rumen and hindgut for each treatment group and time period……………………………………...**149**

Figure 6.4 Jaccard coefficient tree (at a 0.05 genetic distance) as an estimate of similarity and distance between samples……………………………………..**150**

Figure 6.5 Effect of tannin intake on the most abundant phyla in the rumen and hindgut……………………………………………………………..**151**

Figure 6.6 Effect of tannin intake on the relative abundance of genera in the rumen and

hindgut……………………………………………………………………..**153**

Supplementary figure 6.7 Relative abundance of the top 20 bacterial genera within the (A) rumen and (B) hindgut for each treatment group and time period..........**156**

Figure 7.1 Rarefaction analysis at a 95% similarity cut-off for each Operational Taxonomic Unit (OTU)..……………………………………..……...**176**

Figure 7.2 Species richness and diversity plots of rumen (filled markers) and hindgut (open markers) samples over time. (A) Chao (o) and ACE (□) richness indices. (B) Conversion of diversity indices to effective species: Simpson's reciprocal index (o) and the exponential of Shannon's index (□)……………………......**177**

Figure 7.3 Rumen and hindgut bacterial communities clustered using principal coordinates analysis (PCoA) of the unweighted UniFrac metric matrix……………………………………………………….........**180**

Figure 7.4 The species-area power relationship for the (A) rumen and (B) hindgut microbial community over time…………………………………………………**184**

LIST OF COPYRIGHTED MATERIAL FOR WHICH PERMISSION WAS OBTAINED

CHAPTER TWO: Literature Review

Figure 2.2 Placement of E-test strips for synergy testing taken from White et al. (1996) published in:

White, R. L., Burgess, D. S., Manduru, M. and Bosso, J. A. 1996. Comparison of three different *in vitro* methods of detecting synergy: time-kill, checkerboard, and E-test. Antimicrob Agents Chemother **40**: 1914-8.

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Figure 2.13 Biosynthesis pathway of anthocyanidins and proanthocyanidins taken from Aerts et al. (1999) published in:

Aerts, R. J., Barry, T. N. and McNabb, W. C. 1999. Polyphenols and agriculture: beneficial effects of proanthocyanidins in forages. Agric Ecosyst Environ **75**: 1-12.

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CHAPTER THREE: Manuscript I

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CHAPTER FOUR: Manuscript II

Potential to reduce *Escherichia coli* shedding in cattle feces using sainfoin (*Onobrychis viciifolia*) forage: *in vitro* and *in vivo* comparisons

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LIST OF ABBREVIATIONS

AAFC	Agriculture and Agri-Food Canada
ACE	abundance-based coverage estimator
ADF	acid detergent fibre
bp	base pair(s)
CFIA	Canadian Food Inspection Agency
CMIB	Compendium of Medicating Ingredient Brochures
CT	condensed tannin(s)
D	Daltons
DM	dry matter
DNA	deoxyribonucleic acid
EMBL	European Molecular Biology Laboratory
FIC	fractional inhibitory concentration
HGT	horizontal gene transfer
ICE	integrated conjugative element
Kb	kilo bases
Mb	mega bases
MIC	minimum inhibitory concentration
NDF	neutral detergent fibre
rRNA	ribosomal RNA
rDNA	ribosomal DNA
RNA	ribonucleic acid

nt	nucleotide(s)
OTC	over-the-counter
OTU	operational taxonomic unit
PCR	polymerase chain reaction
VDD	veterinary drug directorate
VFA	volatile fatty acids

CHAPTER ONE

GENERAL INTRODUCTION

Therapeutic and sub-therapeutic use of antibiotics in livestock production is an integral component for animal health and welfare. However, increasingly studies have speculated a link between antimicrobial resistance in human pathogens to antibiotic use in the agriculture sector. With this, the European Union banned the addition of in-feed antibiotics in 2006 as a precautionary measure (Millet and Maertens 2011). This in-feed antibiotic ban has also inflicted more stringent regulations upon foods exported to countries which prohibit in-feed antibiotics use. Additionally, many fear this policy will soon be imposed in the North American market especially as the demand for organically grown foods increases (Hayes et al. 2002).

The ban on antibiotic growth promoters in the European Union has resulted in an increased research interest in secondary plant metabolites as an alternative to antibiotics. Many secondary plant metabolites have been found to exert antimicrobial activity against a wide range of pathogenic and non-pathogenic bacteria (Duncan et al. 1998; Friedman et al. 2003; Luciano and Holley 2009; O'Donovan and Brooker 2001). Of the numerous plant compounds currently being studied, condensed tannins (CT) have been one of the most well-researched classes of plant compounds (Aerts et al. 1999; McSweeney et al. 2001b). Condensed tannins are well known for their numerous benefits in ruminant production systems including anti-bloat (Li et al. 1996), anti-helmintic (Min and Hart 2003), rumen protein escape effects (Aerts et al. 1999; Waghorn et al. 1987),

antimicrobial activity (Berard et al. 2009), in addition to reducing enteric methane emissions (Waghorn et al. 2002). Therefore, the use of CTs in livestock production systems should appeal to livestock producers, consumers, and environmentalists when administered at levels known to exert these beneficial effects.

Although the use of phytochemicals appears to be an appealing alternative to antibiotics, few have explored the impact of these compounds on antimicrobial resistance and whether bacteria can develop resistance mechanisms to these compounds. This first aim of this study was to identify a high-quality phytochemical/tannin-containing forage which may be used in livestock production systems to support animal health while reducing fecal shedding of potential pathogens. Second, the ruminant microbial ecosystem was characterized in this study to determine the effects that feeding a tannin-containing forage would have on the bacterial community dynamics of the gut. This study also tested the suitability of using natural plant compounds in combination with synthetic antibiotics to deter the development of resistance. Third, the use of popular ecological models were used in an attempt to provide mechanistic explanations for bacterial processes occurring in various experiments. Applying these theoretical ecology models to bacterial communities will allow improved predictive power of natural ecosystems especially in host gut health.

CHAPTER TWO

LITERATURE REVIEW

2.1 Microbial ecosystems of the ruminant gut

The bovine digestive tract can be dissected into major digestive functions: pre-gastric fermentation (reticulo-rumen) and post-rumen. Collectively, the various segments of the ruminant digestive tract represent complex microbial ecosystems with varying biodiversity and functional roles including intricate symbiotic relationships within the gut (Stewart 1997). Additionally, an important symbiotic relationship exists between the host and its microbial ecosystem. The host provides rumen microorganism with an optimal growth environment (temperature, pH, and nutrients) and the microbial consortium produces micronutrients, VFAs, and microbial proteins essential for growth and health of the host (Hungate 1966).

The rumen is a large pre-gastric fermentation chamber with a holding capacity of over 100 L in mature cattle (Hobson and Stewart 1997) and housing a highly specialized and dense microbial community capable of digesting feed particles and providing nutrients to its host (Hobson and Stewart 1997; Krause et al. 2003). Having been studied for over 50 years, the rumen is one of the most researched and well understood microbial ecosystems and is comprised of prokaryotes (10^{10} CFU mL^{-1}), eukaryotes including protozoa (10^{6} CFU mL^{-1}) and fungi (10^{3}-10^{7} CFU mL^{-1}), and methane-producing archaea (10^{9} CFU mL^{-1}) (Hespell et al. 1997). However, the rumen microbial ecosystem is not

static but rather is in a perpetual fluctuating state as microorganisms are washed out of the rumen. In addition, the rumen microbes are sensitive to changes in environment, diet, host health, and ambient temperature (Callaway et al. 2010; Romero-Perez et al. 2011; Russell and Rychlik 2001; Tajima et al. 2001). This fluctuating state has led to an increased interest in identifying microbial community dynamics in the presence of varying ruminal environments and using this knowledge to enhance manipulation of rumen fermentation.

Carbohydrates are the most abundant substrate for rumen bacteria (Hungate 1975). Feed carbohydrate degradation in ruminants revolves around rumen microbial function which can be divided into three major processes: cellulolytic (structural carbohydrate), non-cellulolytic (non-structural carbohydrate), and methanogenic processes. These functions form intertwined pathways and an intricate balance between these three functions is essential to the host and rumen microbes. Cellulolytic rumen microorganisms such as the Gram-positive *Ruminococcus albus*, *R. flavefaciens*, and Gram-negative *Fibrobacter succinogenes* utilize cellulose and hemicelluloses (Flint 1997). Due to the inherentent structure of plant cell walls, cellulotlytic rumen microorganisms must work as a consortium to produce the enzymatic activity to degrade xylans, mannans, and pectins to access the cellulose fibrils embedded within the plant cell wall (Coen and Dehority 1970; Flint et al. 2008). By convention, cellulolytics typically attach directly to fiber particles in a highly ordered fashion and form biofilms (Flint et al. 2008; Krause et al. 2003). They are also known to be strict anaerobes, have a narrow optimal pH range between 6.5 to 7, and require branched chain VFAs produced by other bacteria to grow (Hobson and Stewart 1997).

In addition, plant cell walls are characterized by the presence of other complex polysaccharides such as hemicelluloses and pectin. The predominant hemicellulose fermenting rumen bacterial species include: *Butyrivibrio fibrisolvens*, *Prevotella ruminocola*, *R. flavefaciens*, and *R. albus* (Dehority 2003). Alternatively, *B. fibrisolvens*, *P. ruminicola*, *Lachnospira multiparus* and *Peptostreptococcus* spp. have been identified as the predominant pectinolytic ruminal species (Dehority 2003). Non-cellulolytic and amylolytic rumen microorganisms include *Streptococcus bovis*, *Selenomonas ruminantium*, *P. ruminicola*, and *Ruminobacter amylophilus,* however, *B. fibrisolvens* and *P. ruminicola* are also amylolytic (Dehority 2003; Krause et al. 2003).

Another important class of rumen bacteria are the proteolytics such as *R. amylophilus*, *B. fibrisolvens*, *S. bovis,* and *P. ruminicola* (Hobson and Stewart 1997; Hungate et al. 1964). These bacteria won't only breakdown proteins to amino acids, but also breakdown VFAs, ammonia and some also hydrolyze urea. The ammonia produced as a byproduct from these organisms is integral to most rumen organisms as ammonia is considered to be the main source of nitrogen for microbial amino acid synthesis (Bryant 1974; Hobson and Stewart 1997). This is yet another example of co-dependence which exists within the rumen microbial ecosystem.

The archaea, specifically the methanogens, are thought to represent approximately 0.3-3% of the rumen biomass (Sharp et al. 1998; Ziemer et al. 2000). The methanogenic rumen bacteria are responsible for the production of methane from carbon dioxide and hydrogen (Flint 1997). These organisms play an integral role in preserving homeostasis within the rumen ecosystem as they help maintain low hydrogen levels, a requirement for optimal growth of microbes in the rumen. However, methane is a greenhouse gas and

represents an energy loss for the host (Hobson and Stewart 1997).

There are two major classes of protozoa within the rumen: ciliates and flagellates (i.e. *Monocercomonas* spp. and *Trichomonas* spp.). The majority of the ruminal protozoa population consists of ciliates which exist primarily within the reticulo-rumen and can represent up to half of the rumen microbiome biomass (Ozutsumi et al. 2005; Williams 1986). Within the rumen ciliates, two major groups exist: entodiniomorphid (oligotrich) and holotrich. The holotrich are known to preferentially utilize soluble carbohydrates while entodiniomorphid ingest and ferment particulate material (Williams 1986; Williams and Coleman 1992). Additionally, protozoa derive their main nitrogen source through ingestion of rumen bacteria (Gutierrez et al. 1962; Ozutsumi et al. 2012) thus affecting bacterial counts within the rumen. Studies have demonstrated a close symbiotic relationship between protozoa and methanogens through interspecies hydrogen transfer. Hydrogen generated from fermentation is transferred from protozoa to methanogens for removal as high levels of hydrogen are inhibitory to protozoa (Sharp et al. 1998).

Anaerobic fungi have been found to exist in the rumen, hindgut and feces of ruminants (Liggenstoffer et al. 2010). They are well known for their high fibrolytic activity due to production of cell-bound and cell-free cellulolytic, hemicellulolytic, glycolytic, and proteolytic enzymes (Lowe et al. 1987; Orpin 1975; Williams and Orpin 1987). Currently, six genera (*Cyllamyces, Necallimastix, Piromyces, Orpinomyces, Anaeromyces, and Caecomyces*) containing 18 species are currently known to exist within the rumen environment (Orpin 1994; Ozkose et al. 2001).

2.2 How to study microbial ecosystems?

Until recently, characterizing microbial ecosystems has been a daunting task. Traditionally, identifying bacteria in ecosystems was dependent on successful cultivation of each organism. Most rumen organisms are obligate anaerobes with only a small proportion being facultative anaerobes (Hobson and Stewart 1997). Given that anaerobic culture techniques are cumbersome, culturing organisms from the rumen ecosystem has been one of the main challenges faced by rumen microbiologists (Krause and Russell 1996). In addition to being labour intensive, it has been presumed that only a minute fraction (<1%) of the microbes within most ecosystems can be recovered by cultivation-based techniques (Amann et al. 1995). Recent developments in molecular biological methods have allowed researchers to further characterize the uncultivable microorganisms in various ecosystems via culture-independent techniques (Whitford et al. 1998). Nowadays, genomic DNA can be extracted from biological samples where genes of interest (i.e. 16S rRNA gene) are amplified using polymerase chain reaction (PCR), and thus sequenced for taxonomic identification.

The small subunit ribosomal RNA (rRNA) gene is a universal gene present in all prokaryotes (16S) and eukaryotes (18S). The ubiquity, genetic stability, and high-copy number of the small subunit rRNA gene makes it an ideal molecular marker to characterize microbial communities (Wright et al. 2005). The 16S rRNA gene is comprised of highly conserved regions interspersed with hypervariable regions. The variability in the hypervariable regions is used to identify phylogenetic relationships and taxonomically characterize organisms (Wright et al. 2005). In order to amplify regions of

the 16S rRNA gene, universal primers complimentary to highly conserved regions are designed and used to amplify the hypervariable regions of most bacteria present in a given sample (Amann et al. 1995).

2.3 Next-generation sequencing technology

Since 2005, the advent of parallelized high-throughput sequencing technologies has revolutionized research in the area of genomics and metagenomics (Ansorge 2009; Zhou et al. 2010). In comparison to the chain-termination Sanger sequencing method which is laborious and time consuming, next-generation sequencing technologies have automated and parallelized the sequencing process through amplification and sequencing of millions of short DNA sequences within multiple samples (Zhou et al. 2010). The rapid development of new sequencing platforms and decreased sequencing costs combined with increased computational power and tools has drastically advanced our understanding of both cultivable and un-cultivable micro-organisms (Caporaso et al. 2011; Zhou et al. 2010). The Roche 454 GS FLX platform is currently one of the most widely used next-generation sequencing platforms for 16S rRNA based studies (Zhou et al. 2010). Using this sequencing platform, it is possible to generate 750 Mb of DNA sequence per day with an average length of 400 nucleotide (nt) versus 6 Mb of DNA sequence per day with sequence lengths between 600-1000 nt with Sanger sequencing (Caporaso et al. 2011; Zhou et al. 2010).

2.4 Introduction to antimicrobial resistance

Discovery of the first antibiotic, penicillin, by Alexander Fleming in 1928 was the beginning of a new era. Many believed that infectious diseases would be eradicated with the use of antibiotics. During this era, new classes of antibiotics were rapidly developed and mass production of antibiotics began (Blondeau and Vaughan 2000). The first documented cases of antibiotic resistance was published in 1940, where *Escherichia coli* was found to be resistant to penicillin (Abraham and Chain 1988), followed by penicillin resistant *Staphylococcus aureus* in 1944 (Kirby 1944). Despite the increasing number of resistant bacteria, people were reluctant to acknowledge that this may develop into a serious issue. In his Nobel Prize acceptance lecture, even Alexander Fleming himself had warned that bacteria could develop resistance to penicillin (Fleming 1945). Currently, pharmaceutical companies have shifted their efforts away from antibiotic research and development owing to wide spread bacterial resistance and short antibiotic life spans (Bax 1997).

2.5 Microbial mechanisms of antimicrobial resistance

Antimicrobial resistance can develop in a variety of environments such as waste water treatment systems, soil, agriculture systems, and within the human body (Soulsby 2007). There are two main ways for bacteria to acquire resistance: through horizontal transfer of genetic material or expression of a previously latent resistant gene (Soulsby 2007). In environments where a complex microbial community exists, the potential for genetic material coding for resistance or resistance genes to be exchanged between bacteria is

likely if selective antimicrobial pressure exists. Selective antimicrobial pressure exists when a bacterial population is subjected to continuous low doses of an antimicrobial agent (Kolar et al. 2001; Witte 2000). Bacteria which have acquired mechanisms of resistance will survive and proliferate while susceptible bacteria will be eliminated. The application of manure onto soil which are both bacteria-rich environments exposed to antimicrobials and agricultural chemicals, may promote the exchange of genetic material as a result of selective pressure (Chander et al. 2006; Witte 2000). In a report by the World Health Organization (WHO), they report on several studies conducted using a variety of antimicrobials approved for use in livestock to examine the extent of antimicrobial breakdown following passage through the gastrointestinal tract. In two different studies where amoxicillin was administered to pre-ruminant and veal calves, 50-60% of the amoxicillin dose was recovered in the urine (World Health Organization 2012). Also, synthetic antimicrobials and chemicals can withstand natural degradation for long periods of time, thereby allowing these molecules to accumulate in the soil over time (Kümmerer 2004). Consequently, this mixture of bacteria, antimicrobials, and chemicals incorporates all the elements which promote selective pressure and development of antimicrobial resistance.

Acquisition of antimicrobial resistance genetic material in bacteria

As described above, for bacteria to express antimicrobial resistance, they must first acquire the genetic material coding for resistance from either vertical inheritance or horizontal gene transfer (HGT). Horizontally transferred genetic material may be acquired as a direct transfer from another bacterium (using naked DNA) or from a mobile DNA element such as a plasmid, transposon, or bacteriophage (McDermott et al. 2003).

Bacteria may also become progressively resistant through chromosome mutations. Such is the case for fluoroquinolone resistance seen in some strains of *E. coli* whereby mutations in the target site (topoisomerase genes) lends the bacterium resistant to fluoroquinolones (Yoshida et al. 1988).

Mobile DNA elements play an integral role in the acquisition of antimicrobial resistance. By definition, mobile DNA elements are segments of DNA that contain genes which code for enzymes or proteins that confer the mobility of DNA within a genome and among bacteria (Frost et al. 2005). Among bacteria, mobility of DNA segments can occur via transformation, conjugation, and transduction. Transformation was the first mechanism of HGT to be discovered. Mechanistically, transformation is the process of naked DNA uptake from closely related bacteria (Frost et al. 2005). In comparison, conjugation requires direct cell-to-cell contact and the DNA segment must be in the form of a self-replicating mobile element such as a plasmid or chromosomally integrated conjugative element (ICE) (Frost et al. 2005). Specifically, plasmids are extra-chromosomal segments of DNA with the ability to self-replicate independently from the chromosome. They may be transferred among bacteria via a conjugative bridge (also known as a pilus appendage) following mating between a bacterium which contains the resistance material and one which does not (Conly 2002; Karczmarczyk et al. 2011). Whereas, ICEs are mobile DNA segments that encompass all the genes required for its excision, transfer by conjugation, and integration within another replicon (i.e. plasmid, phage, or another cell) (Bellanger et al. 2009). Finally, transduction is the transfer of DNA segments via bacteriophage (also known as phages), a self-replicating bacterial virus (Frost et al. 2005).

Within a cell, genetic material, termed transposons, may also ‘jump’ within and between chromosomal DNA, phages or plasmids thus allowing them to be transferred to other cells (Frost et al. 2005). The mobility of this type of DNA segment is dependent on transposase genes contained within the transposon sequence (McDermott et al. 2003).

The genetic material containing resistance components may code for various mechanisms of resistance. Efflux pumps can pump the antimicrobials out of the cell before it can reach its target within the cell and cause changes to cell permeability (Chander et al. 2006). Modifying enzymes are another way in which bacteria can degrade or inactivate antimicrobials by altering the antimicrobial’s structure (Khachatourians 1998). Resistance genes may also enable bacteria to alter their antimicrobial target site, whereby the antimicrobial can reach the target site within the cell but is unable to interact with the altered target site (Soulsby 2007). Additionally, bacteria may produce a secondary target site which is resistant to the antimicrobial’s inhibitory activity while the sensitive target sites remain intact (Soulsby 2007).

2.6 Agriculture and antimicrobial resistance

Regulations and Policies

In the agriculture sector, Health Canada regulates the sale and distribution of all antimicrobials in Canada through the *Food and Drug Acts and Regulations* and *Controlled Drug and Substance Act* (Government of Canada 1985; Government of Canada 1997). In veterinary medicine, the legislation is overseen by the Veterinary Drugs Directorate (VDD). The Canadian Food Inspection Agency (CFIA), which reports to the

Minister of Agriculture and Agriculture and Agri-Food Canada (AAFC), is responsible for the regulation of medicated feeds under the *Feeds Act* and *Regulations* (Government of Canada 1983; Government of Canada 1985). The CFIA's responsibility in the animal feed industry is to ensure the safety and quality of ingredients (excluding veterinary drugs) for animal feed. In addition, the Compendium of Medicated Ingredients Brochure (CMIB) which is regulated by Health Canada, contains a list of all the antimicrobials and their combinations which are approved for use in animal feed. Antimicrobials listed in the CMIB do not require a prescription from a veterinarian to be employed in livestock (CFIA 2008).

At the provincial level, each province has the capability of introducing their own regulations as long as they are at least as stringent as the federal regulations (Health Canada 2009). In contrast, veterinary medicine is a provincially governed body therefore, in Manitoba, the *Veterinary Medical Act of Manitoba* and the *Pharmaceutical Act of Manitoba* enables veterinarians to prescribe drugs (Government of Manitoba 1999; Government of Manitoba 2010). In Manitoba, antimicrobials listed in the CMIB do not require a prescription from the veterinarian and are therefore used without veterinary supervision. Over-the-counter (OTC) antimicrobials are available for purchase from approved vendors such as feed mills, pharmaceutical companies, and veterinary clinics. The OTC antimicrobials available include injectable antimicrobials and antimicrobials which are added to feed and water (Government of Manitoba 1999; Government of Manitoba 2010). This type of practice is seen as a high risk factor with regard to antimicrobial resistance as producers may administer antimicrobials for a disease which is not treatable by antimicrobials (i.e. viral infections) in addition to administering

antimicrobials at doses which promote antimicrobial resistance. Furthermore, the length of time on antimicrobial and route of administration may not be appropriate. Without direct oversight from a veterinarian, the onus is on the producer to treat their livestock as per label directives. For economic reasons, producers may only call upon a veterinarian if their livestock are experiencing a medical emergency, potentially leading to the misuse of antimicrobials.

Antimicrobial use in agriculture

Animals are a reservoir of pathogens such as *E. coli* O157:H7, *Campylobacter jejuni*, *Salmonella enterica* and many other bacteria, hence, antimicrobial use and antimicrobial resistance in food producing animals is of great concern (Health Canada 2002). The use of antimicrobials in agriculture has gained much attention due to fears of transmission of antimicrobial resistant bacteria to humans through the food chain (Soulsby 2007). Of utmost importance is the fact that many antibiotics used in livestock are also used in human medicine such as bacitracin, tetracyclines, sulphonamides, lincosamides, penicillin, and aminoglycosides (Conly 2002). In agriculture, Conly (2002) reports that only 10% of antimicrobials are used for therapeutic purposes leaving the remainder of antimicrobial usage to prophylactic use and to promote growth. As global food demands have increased, producers have moved towards intensive farming practices which include the use of growth promoters to ensure maximum production in an economically sustainable fashion. With intensive production practices, other problems may arise such as excessive animal waste leading to environmental contamination, increased threat of disease, and antimicrobial resistance (Tilman et al. 2002).

Sub-therapeutic levels of antimicrobials may be administered to animals as a prophylactic or as a growth promoter (Khachatourians 1998). In high-density farming, disease outbreaks can produce disastrous outcomes; therefore producers may opt to administer sub-therapeutic levels of antimicrobials in feed or water if symptoms are present in a few animals. Prophylactic use of antimicrobials in animals should not be used as a substitute for good animal husbandry practices (good sanitation and hygiene in animal housing units), which can become an issue in high-density farming (Khachatourians 1998).

The use of growth promoters in animals is a controversial issue with regards to antimicrobial resistance. The practice of in-feed antimicrobials to promote growth goes against antimicrobial resistance prevention guidelines (i.e. low doses for a long period of time) as it provides continuous selective pressure (Chander et al. 2006). Additionally, this practice attracts negative attention as the fundamental basis for this practice is due to economic gains. The purpose of growth promoters is to maximize the ratio of weight gain to feed intake in order to minimize feed costs and to shorten the "time to market" (Health Canada 2002). Growth promoters are most commonly used in the swine and poultry industry. Most feedlot cattle productions are now using hormone implants as growth promoters with the exception of monensin, which is an ionophore commonly used in cattle feed as a growth promoter. Ionophores are compounds synthesized by microbes which have antimicrobial activity. Ionophore resistance has been shown although is not considered a threat to human health due to a lack of structural similarity to antimicrobials used in human medicine (Health Canada 2002).

2.7 Combination therapy and methods to assess antimicrobial synergy, additivity and antagonism

Some antimicrobial combinations have been shown to exhibit synergistic interactions against target organisms. With the advent of increasing antimicrobial resistance, combination drug therapies are becoming more common. The use of antimicrobial combinations in the clinical setting is most often used in patients who are critically ill and possibly septic (Pillai et al. 2001; Saiman 2007). Antimicrobial combination therapy may also reduce the antimicrobial effective dose thereby reducing side effects and toxicity in addition to deterring the development of antimicrobial resistance (Pillai et al. 2001; Saiman 2007). The use of combination therapy with phytochemicals and antimicrobials may prove to be beneficial in the agriculture sector by potentially reducing the amount of antimicrobials used livestock and decreasing the likelihood of resistance development.

Synergy, antagonism, additive, and indifferent interactions

The nature of the drug interaction is variable in antimicrobial combinations. Depending on the mechanism of action for each antimicrobial, the drug interaction may be synergistic, antagonistic, additive, or indifferent. Synergism is defined as the positive interaction of antimicrobials where the activity of the antimicrobial combination is significantly greater than the sum of the individual activity for each compound (Cuenca-Estrella 2004). On the contrary, antagonism occurs when the inhibitory activity of the combined antimicrobials is markedly reduced in comparison to the activity of the

antimicrobials used separately (Cuenca-Estrella 2004; Pillai et al. 2001). Additivity in an antimicrobial combination is equivalent to the sum of the inhibitory activity of the individual antimicrobials (Cuenca-Estrella 2004). Conversely, indifference in antimicrobial combination therapy is representative of the inhibitory activity of the most potent antimicrobial when used on its own (Cuenca-Estrella 2004). In this regard, combinations resulting in additivity or indifference cannot be considered synergistic, meaning that the mechanisms for antimicrobial activity are either similar in both antimicrobials or only one mechanism can be inhibitory at a given time (Pillai et al. 2001).

FIC index

The fractional inhibitory concentration (FIC) index is a value which compares the minimum inhibitory concentration (MIC) from the antimicrobial combination assay to the MIC of the antimicrobial on its own. It is calculated using the following formula (Pillai et al. 2001) and the antimicrobial interactions defined using the FIC values in Table 2.1:

$$\text{FIC for compound A} = \frac{\text{MIC of A (in combination assay)}}{\text{MIC of A (alone)}}$$

$$\text{FIC for compound B} = \frac{\text{MIC of B (in combination assay)}}{\text{MIC of B (alone)}}$$

$$\text{FIC}_A + \text{FIC}_B = \text{FIC index}$$

Table 2.1 Antimicrobial interactions as defined by the fractional inhibitory concentration (FIC) index

FIC index	Antimicrobial interaction
≤ 0.5	Synergistic
> 0.5 – 1.0	Additive
1-4	Indifferent
> 4	Antagonistic

(Pillai et al. 2005)

Alternatively, a synergistic interaction may also be considered when the bacterial concentration is reduced by more than 90% of its initial concentration, akin to a difference in population (DP) <0.1 (log DP < -1). The log DP can be calculated using the following formula (Fang and Lin 1995):

$$\text{Log DP} = \text{Log}\,(N/N_0) = (\text{Log}\,N) - (\text{Log}\,N_0)$$

where N is the inoculum concentration after a 24 hour incubation period and N_0 is the initial inoculum concentration.

The checkerboard method

The checkerboard method is the most widely used method to test for synergy. It is relatively easy to set up and the laboratory equipment needed for this method is often readily accessible in a standard microbiology laboratory. In addition, the mathematical calculations (FIC index) used in this method are simple (Pillai et al. 2001).

Prior to setting up a checkerboard array, the MIC of the two antimicrobials to be evaluated should be known as the MIC should fall approximately in the middle of the

concentration range to be tested. The recommended maximum concentration to be tested should be two dilutions above than the MIC and the lowest concentration being four or five dilutions below the MIC (Pillai et al. 2001).

The checkerboard method is commonly done in a microtitre plate. Along the x-axis of the plate, each row will have the same dilution pattern (i.e. two-fold dilutions) for compound A. On the y-axis, all the columns will have the same dilution pattern for compound B (Figure 2.1). Therefore, all wells in the same column or row will have the same concentration for that compound (Hemaiswarya et al. 2008; Pillai et al. 2001). In addition, there should be one column and one row with each antimicrobial alone (Pillai et al. 2001). This column/row will act as an internal control to determine the MIC of each compound within the assay. This assay is typically performed in Mueller-Hinton broth as it is a drug-free media. The antimicrobial may also be diluted using this broth (Pillai et al. 2001). Overall, the researcher may tailor the design to their specific needs (i.e. change dilution factor, concentration range; perform assay in test tubes; use different media).

Example:

MIC for compound A: 256 μg mL^{-1}

At two-fold dilutions, the maximum concentration for compound A should be at least 1024 μg mL^{-1} (two dilutions above the MIC) and the minimum should be at least 16 μg mL^{-1} (4 dilutions below the MIC).

MIC for compound B: 64 μg mL^{-1}

At two-fold dilutions, the maximum concentration for compound B should be at least 256 μg mL^{-1} and the minimum concentration should be at least 4 μg mL^{-1}.

A → B ↓	**1** 1024 mg/mL	**2** 512 µg/mL	**3*** 256 µg/mL	**4** 128 µg/mL	**5** 64 µg/mL	**6** 32 µg/mL	**7** 16 µg/mL	**8** 8 µg/mL	**9** 4µg/mL	**10** 0 µg/mL
A – 256 µg/mL										
B – 128 µg/mL										
C* – 64 µg/mL										
D – 32 µg/mL										
E - 16 µg/mL										
F – 8 µg/mL										
G – 4 µg/mL										
H – 0 µg/mL										

* MIC

Figure 2.1 Example of a checkerboard array for compound A and B using two-fold dilutions

The design of a checkerboard assay allows a different combination of compound A and B in each well (Pillai et al. 2001). This allows for the determination of the optimum concentration combination which will inhibit growth synergistically.

Once the checkerboard array is designed, the antimicrobials are added to the wells at their appropriate concentrations and an aliquot of the inoculum is added to each well for a final concentration of 5×10^5 CFU mL^{-1}. Plates are subsequently incubated overnight (approximately 18 hours). The MIC is defined as the lowest antimicrobial concentration which will inhibit growth as detected by the unaided eye (White et al. 1996). The MIC must be determined for the antimicrobial combination as well as the MIC for each antimicrobial alone in order to calculate the FIC index.

One of the major drawbacks with regard to the checkerboard method is that the assay only evaluates bacteristatic activity as opposed to bactericidal activity. This is an important point to consider especially in the clinical setting as synergistic antimicrobial combinations are often used to treat critically ill patients. For the aforementioned reason, the checkerboard method is not validated for use in clinical testing (Saiman 2007).

The time-kill method

The time-kill method is another commonly used assay to evaluate synergy in antimicrobial combinations, although it is more laborious and time-consuming (White et al. 1996). In this particular method, the bactericidal effect of the different antimicrobial combinations is evaluated as a function of time.

The assay is performed in tubes which contain the individual compounds at various concentrations and other tubes which contain a range of different antimicrobial

combinations (Hemaiswarya et al. 2008; National Committee for Clinical Laboratory Standards 2002). The concentration range used should be from one-quarter of the MIC (lowest concentration) to twice the MIC (maximum concentration) for the bacterial strain being evaluated (Table 2.2) (Hemaiswarya et al. 2008; National Committee for Clinical Laboratory Standards 2002). In the final step, the inoculum is added at a concentration which will yield a final tube concentration of 5×10^5 CFU mL^{-1}. Aliquots are taken at 0 h to determine the initial bacterial count in the assay and another aliquot is taken at 24 h, although, more aliquots could be taken between this time (Hemaiswarya et al. 2008). The aliquots are diluted in a 10-fold dilution series (if necessary) and plated onto agar plates and the colonies counted (White et al. 1996).

In this assay, synergy is present when a ≥100-fold or 2-$\log_{10}$ decrease in colony count is observed for a particular antimicrobial combination at 24 h in comparison to the colony count for the most potent individual antimicrobial at 24 h and the colony count of that antimicrobial combination at 0 h (Pillai et al. 2001; White et al. 1996). Furthermore, a decrease in colony count which is less than 10-fold at 24 h for any antimicrobial combination in comparison to the most potent individual antimicrobial at 24 h is defined as additivity or indifference. A ≥100-fold increase in colony count for an antimicrobial combination at 24 h in comparison to the most potent individual antimicrobial at 24 h and to the colony count at 0 h for that combination is defined as antagonism (White et al. 1996).

Table 2.2 Design of a time-kill assay for compound A and B in combination and alone

Tube 1		Tube 2		Tube 3		Tube 4		Tube 5		Tube 6		Tube 7		Tube 8	
[A]	[B]	[A]	[B]	[A]	[B]	[A]	[B]	[A]	[B]	[A]	[B]	[A]	[B]	[A]	[B]
2 x MIC	2 x MIC	2 x MIC	¼ x MIC	¼ x MIC	2 x MIC	¼ x MIC	¼ x MIC	¼ x MIC	0	0	¼ x MIC	2 x MIC	0	0	2 x MIC

While the checkerboard assay measures the bacteristatic activity of the compounds in combination, the time-kill method measures the bactericidal activity. This difference is meaningful in the clinical setting in which the drug therapy needs to be bactericidal (Pillai et al. 2001). The time-kill method is an approved (Committee on Clinical Laboratory Standards) method to evaluate synergy in a clinical setting (Pillai et al. 2001; Saiman 2007). In addition, the time-kill method may be more desired than the checkerboard method as it is an assay which is done in real-time as opposed to the checkerboard method which is only measured once (Pillai et al. 2001). Consequently, the main disadvantage to the time-kill method is that it is laborious, time-consuming, and limited in the number of concentration combinations and antimicrobial agents which can be tested (Pillai et al. 2001). Also, due to the nature of the sampling method for colony counting, there is a higher risk of contamination necessitating extra caution when doing the time-kill method (Pillai et al. 2001).

The E-test method

The epsilometer test (E-test), is a newer antimicrobial susceptibility testing method. In this assay, plastic strips are covered with an antimicrobial of interest on one side and a concentration scale for the antimicrobial on the other side. A drug-free media such as Mueller-Hinton agar is spread with the bacterial strain of interest and the strips are placed in a cross formation at a 90° angle on the agar plate (Figure 2.2). The strips are positioned in such a way that the MIC of each compound is at the intersection of the cross. The plates are incubated for approximately 18 hours (White et al. 1996).

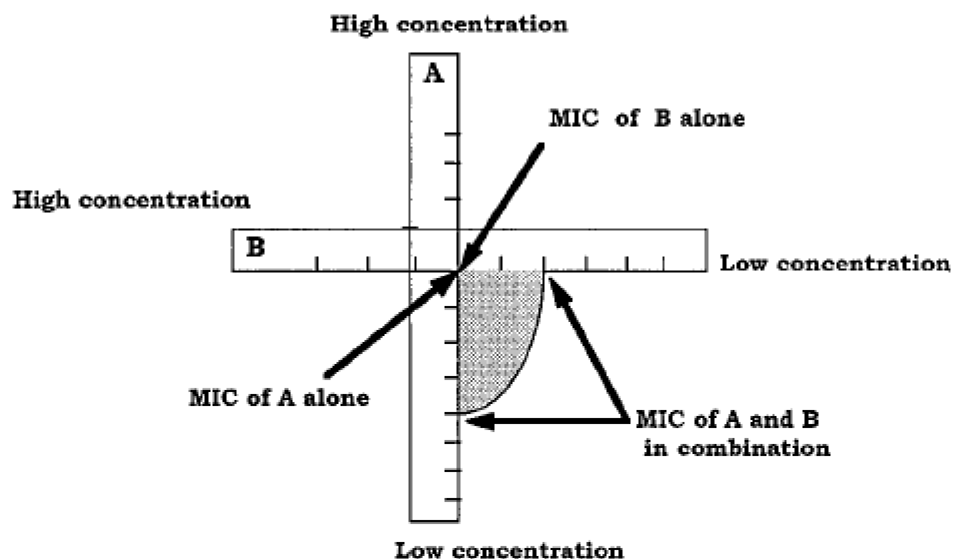


Figure 2.2 Placement of E-test strips for synergy testing taken from White et al. (1996)

To determine the presence of synergy, the zones of inhibition are evaluated to determine the MIC of the antimicrobial combination. The MIC is defined as the concentration at which the inhibition zone intersects the strips as seen in Figure 2.2 (White et al. 1996). The MIC values for the antimicrobial combinations are then used to calculate the FIC index, thereby determining the type of antimicrobial interaction (White et al. 1996).

The E-test is a fast and easy method to evaluate synergy. In addition, a variety of antimicrobial strips are commercially available. Similarly to the checkerboard method, this method only evaluates bacteristatic activity. Another disadvantage to this method is that although many strips are commercially available, testing synergy of non-commercially available compounds is not possible.

Concluding remarks

Each method has its advantages and disadvantages, therefore, the nature of the experiment will dictate which method is optimal to use. Within each method there are a multitude of variations which can be done to tailor the assay to the researcher's needs. On the contrary, the multitude of variations which can be done makes it difficult to compare results with other studies. The analysis of synergy in all the methods discussed have some degree of subjectivity therefore, caution should be taken when interpreting results from the literature. Additionally, margins of error can be large due to factors such as pipetting errors, solubility problems, and media used. In conclusion, the rise in antibiotic resistance and the decline in drug development have made synergy testing an important area of research in the clinical setting. However, combination therapy should also be seen as a viable option in the agriculture sector to help mitigate antimicrobial resistance. More research is needed in this area to fine tune current methods and/or to develop new methods which would be more accurate in evaluating synergy. Currently, there is a need for a method to evaluate synergy which is fast, easy, reliable, accurate, and precise.

2.8 Phytochemicals

What are phytochemicals?

Plants produce a variety of compounds which are termed primary and secondary metabolites. The primary metabolites are compounds which are part of the plant's metabolic pathways and the remainders are secondary metabolites. Secondary

metabolites are thought to be involved in the plant's defence mechanism (Pichersky and Gang 2000). As the human body has an immune system to attack foreign bodies, plants produce secondary metabolites to protect themselves against insects, herbivores, pathogens (bacteria and fungi), or to suppress the growth of other competing plant species in the vicinity (Bennett and Wallsgrove 1994; Mitchell-Olds et al. 1998; Pichersky and Gang 2000). On the contrary, some of these secondary metabolites can also be aromatic, colourful, and flavourful with the purpose of attracting pollinators or herbivores to assist in spreading their seeds (Pichersky and Gang 2000). Secondary compounds vastly differ among plant species (Bennett and Wallsgrove 1994).

Biosynthesis of these compounds in plants is commonly influenced by the growth environment. Some compounds, for example, are produced to help the plant grow in low-nutrient environments by synthesizing compounds which will inhibit the growth of neighbouring plants (Mitchell-Olds et al. 1998; Singh and Bhat 2003). Secondary metabolite synthesis can be very restrictive such that they are often only synthesized at certain stages of development and in certain tissues (Pichersky and Gang 2000). Condensed tannin (CT) concentration in legumes, for example, will generally be more concentrated in the flower tissue rather than in the leaves or stems in order to protect the reproductive tissue from insects during pollination (Pichersky and Gang 2000).

Although secondary metabolites are beneficial to the plant, some of these compounds are toxic to herbivores. These compounds can be genotoxic, hepatotoxic, pneumotoxic, neurotoxic, or cytotoxic in nature (Singh and Bhat 2003). On the contrary, secondary metabolites can have beneficial properties which may be useful in human medicine and agriculture including antimicrobial, antioxidant, antimutagenic,

anticarcinogenic, and anti-inflammatory activities (Singh and Bhat 2003). These effects further protect the plant from disease. Many of these metabolites are found in fruits, vegetables, and wine.

Secondary metabolites are subdivided into several different classes according to their structure. The major compounds to be discussed in this review are phenolics/polyphenols and terpenoids/essential oils. Within the phenolics/polyphenols group there are smaller categories which include simple phenols/phenolic acids, quinones, flavones/flavonoids/flavonols, tannins, and coumarins (Cowan 1999).

Classes of phytochemicals

Phenolics/polyphenols

Simple phenols

The phenolics/polyphenols group has a common aromatic ring structure with a varying number of hydroxyl groups. The simple phenols and phenolic acids are the simplest unit in this group and are the building blocks for more structurally complex compounds. The structure of these compounds generally consists of an aromatic hydrocarbon with a single substitution (Cowan 1999). The structure of these compounds is produced from the condensation of acetate molecules such as terpenoids or from the modification of an aromatic amino acid (Bennett and Wallsgrove 1994). Some examples of simple phenols include cinnamic acid and caffeic acid (Fig. 2.3).

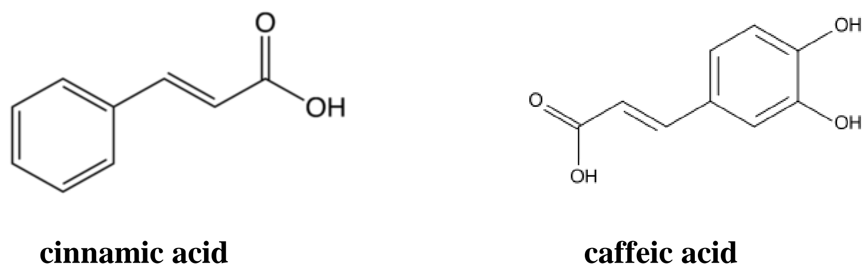


Figure 2.3 Structure of cinnamic acid and caffeic acid

Compounds such as catechol and pyrogallol (Fig. 2.3) are also considered as simple phenols, but their structure consists of an aromatic hydrocarbon ring with hydroxyl substitutions (Cowan 1999). The degree of hydroxylation is thought to be responsible for the increased inhibitory activity of a compound (Cowan 1999; Friedman et al. 2003). In addition, some research has shown that the degree of oxidation is also responsible for the increased toxicity of phenols such that oxidized phenols can inhibit enzymes by reacting with their sulfhydryl groups and forming covalent bonds (Cowan 1999; Scalbert 1991).

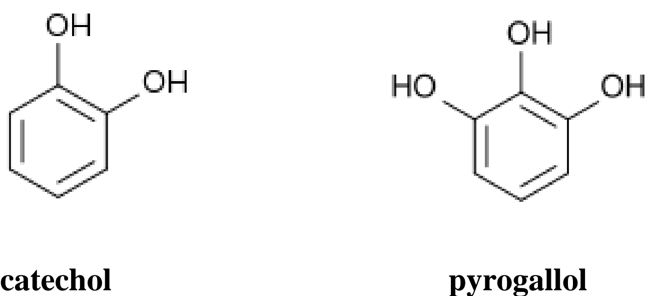
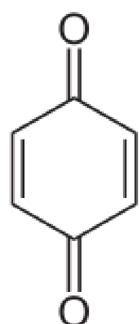


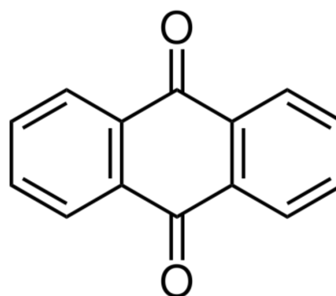
Figure 2.4 Structure of catechol and pyrogallol

Quinones

Similarly to simple phenols, quinones have a conjugated hydrocarbon ring with

two carbonyl groups (Fig. 2.5). The ketones present in their structure make quinones highly reactive compounds. Quinones are coloured compounds which occur quite frequently in nature (Cowan 1999). Some examples of quinones include coenzyme Q (ubiquinone) and vitamin K (naphthoquinone) (Cowan 1999). They are responsible for the browning reaction in fruits and vegetables (Vamos-Vigyazo 1981) as well as melanin synthesis in skin pigmentation (Land et al. 2004). Quinones are involved in redox cycling which produces superoxide molecules, thus the inhibitory activity of quinones may be elicited through its production of free radicals (Cape et al. 2006).





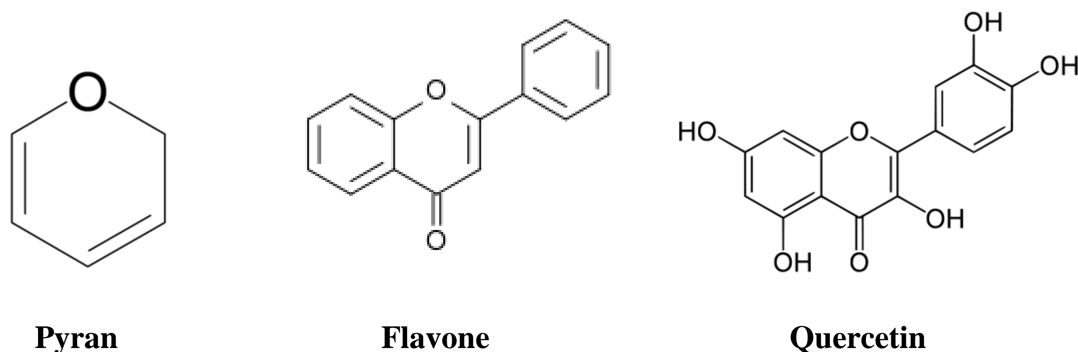
1,4-benzoquinone **anthraquinone**

Figure 2.5 Examples of quinone structures

Flavanoids

Flavonoids are a large group of polyphenols which represent the flavones and flavonoles. The flavanoids consist of two benzene rings linked by a pyran ring. Flavones are similar to quinones except that they have only one carbonyl group instead of two and become a flavonol with the addition of a hydroxyl group (Fig. 2.6) (Cowan 1999). As flavonoids are produced in plants in response to microbial infections, they are considered to be phytoalexins. By definition, phytoalexins are plant secondary metabolites produced

following an infection by a plant pathogen (Bennett and Wallsgrove 1994). Phytoalexins are known as the plant's "antibiotics" therefore, they are also good antimicrobial compounds *in vitro* (Cowan 1999). To date, the mode of action of flavonoids has not been fully elucidated. There are some findings which suggest that they elicit toxicity to bacteria by altering the cell membrane, although exact mechanisms have not been fully elucidated. Some have stated that similarly to simple phenols, increased hydroxylation will yield a higher toxicity, although others have found that their data does not support this theory (Chabot et al. 1992; Cowan 1999; Sato et al. 1996).



Pyran **Flavone** **Quercetin**

Figure 2.6 Pyran ring linking two benzene rings in flavonoid structures. Flavone, a subclass of flavonoids. Quercetin, a flavonol (subclass of flavonoids).

Coumarins

The basic structure of coumarin (Fig. 2.7) consists of a benzene ring fused to a pyran ring with a carbonyl group. There are a variety of coumarins which have been discovered. Their popularity stems from their antithrombotic (Thastrup et al. 1985), anti-inflammatory (Piller 1975), and vasodilatory activities (Cowan 1999; Namba et al. 1988). The specific antimicrobial mode of action of coumarins is not completely understood

(Cowan 1999).

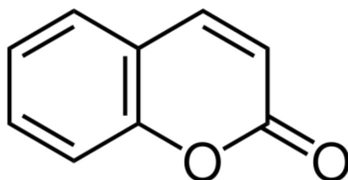
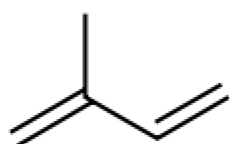
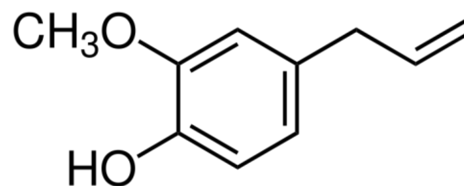


Figure 2.7 Basic structure of coumarin

Terpenoids/Essential oils

Essential oils are well known for their fragrance and flavour properties. Some examples of essential oils are eugenol, oregano oil, peppermint oil, thymol, camphor, methanol, and carvacrol (Cowan 1999; Wallace 2004). They are often used in the food industry as additives to enhance flavour, aroma, and act as preservatives (Cowan 1999; Wan et al. 1998). The basic unit of essential oils is an isoprene unit (Fig. 2.8). Essential oils are also termed terpenoids as their structure consists of monoterpenes (C10), diterpenes (C20), triterpenes (C30), and tetraterpenes (C40), hemiterpenes (C5), and sesquiterpenes (C15). The isoprene units join together and form a cyclic hydrocarbon structure with alcohol, aldehyde or ester derivatives (Wallace 2004). The inhibitory mode of action of these compounds has not been completely elucidated, although it is thought to be associated with membrane disruption leading to increased cell membrane permeability due to their lipophilic property (Cowan 1999; Di Pasqua et al. 2006).





isoprene **eugenol**

Figure 2.8 Structure of isoprene and eugenol

2.9 Tannins

Tannins are water soluble secondary metabolites of higher plants which act as a defense mechanism for the plants against insects, pathogens, and herbivores (Cowan 1999).

Types and structures

Haslam (1966) states that tannins are present in various sizes as they can have a molecular weight ranging from 300 to 3,000 daltons (D). More recently, Khanbabaee & Ree (2001) have described tannins with molecular weights of up to 20,000 D. These researchers concluded that molecular weight should not be included as a defining factor when describing and identifying tannins. Molecular weight can, however, affect the properties of the tannin. Tannins with a large molar mass will exert a higher anti-nutritional effect and lower levels of biological activity (Chung et al. 1998). However, there are some compounds which have structures similar to that of tannins which are not water soluble (Bate-Smith 1975; Stafford and Lester 1981). Therefore, Horvath (1980) developed a broader definition which included all tannins as: “any phenolic compound of sufficiently high molecular weight containing sufficient phenolic hydroxyls and other

suitable groups (i.e. carboxyls) to form effectively strong complexes with protein and other macromolecules under the particular environmental conditions being studied". They can be found in virtually all parts of the plant including the bark, wood, leaves, fruits, and roots (Scalbert 1991). Tannins are well known for their ability to bind and complex with proteins, but can also bind to polysaccharides and minerals.

Tannins can be classified into three major groups: hydrolysable tannins, proanthocyanidins (PA) also known as condensed tannins, and complex tannins which all differ in their chemical structures. The hydrolysable tannins (Fig. 2.9) consist of a polyol carbohydrate such as β-D-glucose at the center of a molecule in which some or all of its hydroxyl groups are esterified with phenolic acids such as catechin (Fig. 2.10) (Reed 1995). The hydrolysable tannins can be further subdivided into gallotannins and ellagitannins where the phenolic groups are gallic and ellagic acid (Fig. 2.11), respectively (Cowan 1999; Hagerman et al. 1992; Reed 1995; Singh and Bhat 2003). More specifically, gallotannins are compounds which have galloyl units (monomers of gallic acid) esterified to D-glucose (Li et al. 2006). The structure of ellagitannin is comprised of a polyol unit such as D-glucose esterified to hexahydroxydiphenic acid units (phenol moiety). Upon hydrolysis, the hexahydroxydiphenic acid units which are two galloyl units bound together by a C-C bond will undergo spontaneous dehydration to yield a carbohydrate molecule and a phenolic acid such as ellagic acid (Haslam 1966). In most cases, the structure of hydrolysable tannins will have D-glucose as the polyol core, but some hydrolysable tannins will have quinic acid as the core of the structure forming tara gallotannins (Haslam 1966).

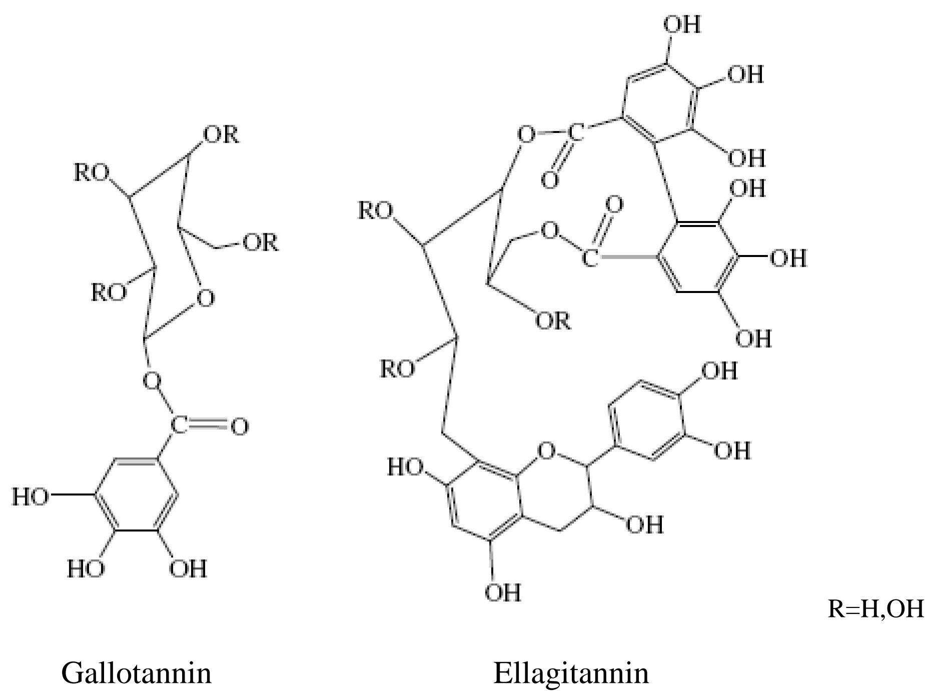


Figure 2.9. Structure of hydrolyzable tannins: gallotannin and ellagitannin.

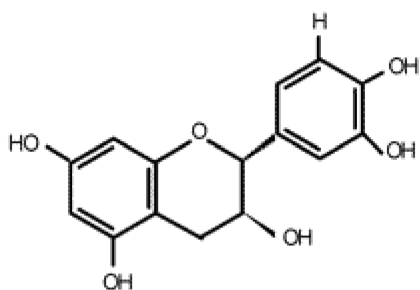


Figure 2.10 Catechin (flavan-3-ol) structure

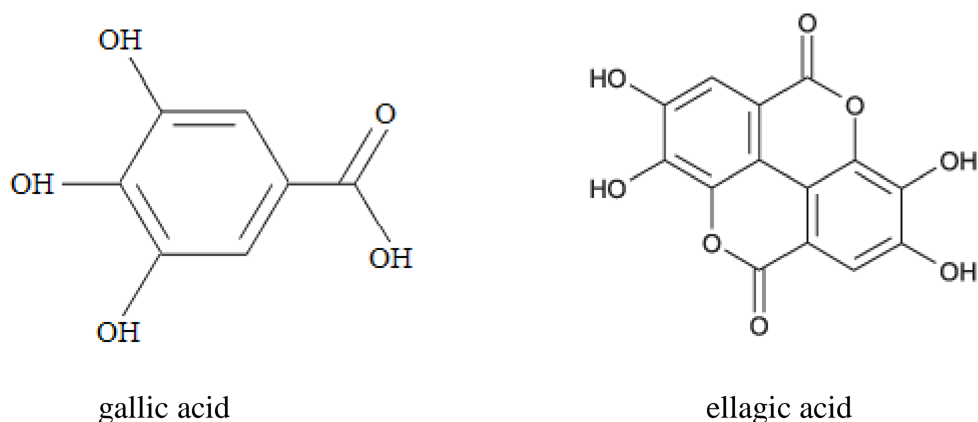


Figure 2.11. The chemical structure of phenolic acids: gallic acid and ellagic acid.

In comparison to CTs, hydrolysable tannins are more prone to enzymatic (tannase) and non-enzymatic (by weak acid/base and hot water) hydrolysis leading to the production of phenolic acids (ellagic acid or gallic acid) and carbohydrate (glucose) (Haslam 1966; Reed 1995).

Structurally, proanthocyanidins, are polymers of flavan-3-ols (Fig 2.10) held together by an interflavan carbon bond (Fig. 2.12). There are multiple catechin moieties (Fig. 2.10) which are attached in a specific manner. The C-4 of one catechin is linked to the C-6 or C-8 of the second catechin (Fig. 2.12) (Clifford and Scalbert 2000). Unlike the hydrolysable tannins, CTs do not readily undergo hydrolysis due to the presence of these interflavan carbon bonds (Reed 1995).

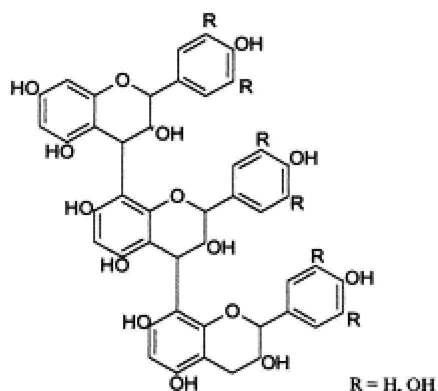


Figure 2.12 Condensed tannin containing three catechin moieties

In addition to hydrolysable tannins and CTs, there is another group of tannins entitled complex tannins. The tannins in this group possess attributes belonging to both hydrolyzable and CTs and are therefore classified in a different group (Li et al. 2006). Their structure is comprised of a catechin or epicatechin unit glycosidically linked to an ellagitannin or gallotannin. Subsquently, when hydrolysis of a complex tannin occurs a catechin or epicatechin unit and ellagic acid or gallic acid will be produced (Bhat et al. 1998).

Location of CTs in the plant

Condensed tannins may be found in various parts of the plant such as the cell wall, flower, bark, stem, and seed. The localization of CTs in the plant can vary greatly depending on the plant, however, they are usually more concentrated in the leaves and stems (Aerts et al. 1999; McMahon et al. 2000). In *L. corniculatus* var. *japonicus* the CTs are found in the stems, petioles, and flowers (Morris et al. 1993), whereas in white clover, they are found primarily in the flowers (Jones et al. 1976), and in alfalfa they are only present in the seed coat (Koupai-Abyazani et al. 1993). The CTs found in sainfoin seem

to be distributed to all the organs of the plant except in the cotyledons and roots. The organ containing the highest concentration of CTs in the sainfoin plant is the leaf with concentrations ranging from 27.2 to 75.3 mg g^{-1} DM (Koupai-Abyazani et al. 1993; Lees 1993). A high CT concentration in the leaves is not unusual as this is where the plant will be most vulnerable to pathogens, diseases, herbivores, and insects. In contrast, Terril *et al*. (1992) states that the CT concentration in legumes will generally be more concentrated in the flower tissue rather than in the leaves or stems. In their study, they measured the CT concentration in various parts of sulla forage and found the following distribution: 1.3, 3.6, and 6.9 % in the stems, leaves, and flowers, respectively (Terrill et al. 1992). The occurrence of higher concentrations of CTs in the flower tissue is speculated to be part of the plant's defense mechanism to protect its reproductive organs (Iason et al. 1995). However, a review by McMahon et al. (2000) indicates that other studies have shown that tannin localization is quite variable depending on plant species. Within the plant tissue, CTs are more specifically located in the plant vacuoles and remain unreactive until cell disruption occurs thereby preventing tannins from complexing with plant cellular enzymes. Upon cell rupture, the CTs become reactive and complex with various proteins originating from the plant, animal or from microbes (McMahon et al. 2000; Waghorn and McNabb 2003), as described above.

Factors that influence plant tannin concentration

Factors that contribute to plant tannin concentrations include a genetic predisposition within a species, environmental conditions, and maturity (McMahon et al. 2000). In the latter case, the plant will have a decreased CT concentration in some leaves

as they senesce. The reduced amount of CTs in the leaves is a result of decreased activity of the enzymes (dihydroflavonol reductase, leucocyanidin reductase, and chalcone synthase) responsible for the biosynthesis of proanthocyanidins as seen in Figure 2.15. The aforementioned enzymes are most active in the first growth stages of the leaflets and decline in activity as the leaf matures (Joseph et al. 1998; McMahon et al. 2000; Singh et al. 1997). Consequently, as the plant matures, the beneficial effects of the plant exhibited by the CTs will decrease making the plant less potent.

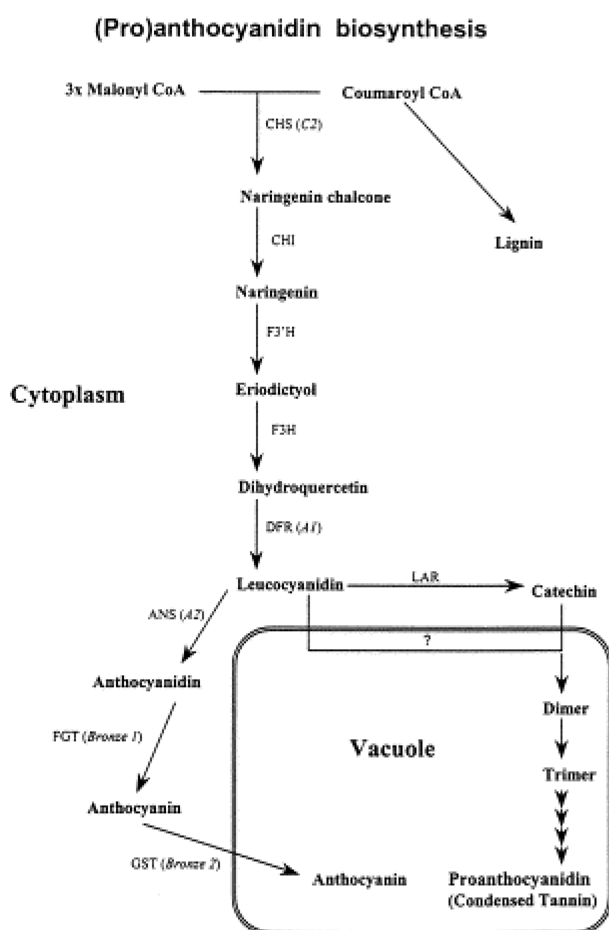


Fig. 2.13 Biosynthesis pathway of anthocyanidins and proanthocyanidins. Enzymes in the pathway: chalcone synthase (CHS), chalcone isomerase (CHI), flavanone-3’-hydroxylase

(F3'H), flavanone-3-hydroxylase (F3H), dihydroflavonol reductase (DFR), leucoanthocyanidin reductase (LAR), PA condensing enzymes (?), anthocyanidin synthase (ANS), flavanol-UDP-glucosyl transferase (FGT), glutathione-S-transferase (GST) (Aerts et al. 1999).

Other factors which can influence CT concentration in plants include growth temperature and soil fertility. Lees et al. (1994) observed a significant decrease in CT concentration in clones of big trefoil when the plant was grown at 30°C as opposed to 20°C. In another study by Barry and Forss (1983), they observed a four-fold decrease in CT concentration when big trefoil was grown in high-fertility soils in contrast to low-fertility soils. Fertilizer increases plant DM yield which in turn decreases the concentration of tannins (McMahon et al. 2000). In addition, plant species will affect the tannin concentration due to the varying structures of CTs (i.e. degree of polymerization and number of hydroxyl groups) in plants (Aerts et al. 1999).

What plants contain CTs?

CTs have been found in a number of forage species such as *Onobrychis viciifolia*, *Lotus corniculatus*, and *Trifolium pretense*. Temperate legumes are known to contain higher concentrations of CTs as compared to grasses which have a relatively low concentration of CTs. In addition, plants containing CTs will often contain other phenolic compounds (Waghorn and McNabb 2003). In the past, it was thought that only tropical forages contained CTs. The butanol-HCl extraction method has recently been improved to allow for the extraction of fiber bound and protein bound proanthocyanidins (Waghorn

and McNabb 2003).

Extraction techniques

Over the years, many analytical methods have been developed to identify and quantify CTs in plants, although, the complexity of CT structures has made their analysis rather difficult (Schofield et al. 2001). Recently, tannin extraction methodologies have been refined and as a result have shown that only 70-80% of total CTs in plants are extractable (Barry and McNabb 1999; Terrill et al. 1992; Waghorn and McNabb 2003). A percentage of tannins in plants are unavailable as they are bound to fiber or protein, making it more difficult to extract as compared to free tannins. Therefore, additional steps must be used to extract protein- and fiber-bound CTs (Barry and McNabb 1999; Terrill et al. 1992). Barry and Manely (1986) define free tannins as those which are not precipitated following high speed centrifugation of plant macerates, whereas bound tannins will precipitate with proteins and carbohydrates. As extraction methods have improved, it is now possible to extract bound tannins. Currently, the acid-butanol and vanillin assays which are both colorimetric assays are the most commonly used methods to identify and quantify CTs in plant tissues. The acid-butanol method is one which can be used to isolate and quantify free, fiber- and protein-bound CTs (Porter et al. 1986). In this reaction, CTs are subjected to a hot acid treatment which causes oxidative depolymerization of the interflavan bond to yield red anthocyanidins of which the absorbance is measured (Hagerman et al. 1992; Schofield et al. 2001). This method is currently the most widely used method to quantify CTs (Schofield et al. 2001). The vanillin assay is another method used to quantify CTs by reacting vanillin with CTs and

forming colored complexes of which the absorbance is measured (Broadhurst and Jones 1978). This method can yield inaccurate CT values due to its lack of specificity for CTs (Schofield et al. 2001). As a consequence of the heterogeneity of CTs in a given sample and reactions with non-CT compounds, slightly different color intensities may be obtained. This challenge exists for both the acid butanol and vanillin assay (Schofield et al. 2001).

Impact on ruminants:

Impact of hydrolysable tannins on ruminants

It is important to note that hydrolysable tannins are toxic to ruminants (Shi 1988). It is believed that microbial tannases and other enzymes produced in the rumen breakdown hydrolysable tannins to low-molecular weight phenols such as gallic acid, pyrogallol, phloroglucinol, and subsequently to acetate and butyrate (Murdiati et al. 1992). These phenols are subsequently absorbed by the rumen and can be further metabolized to other metabolites. Currently, no specific metabolite has been identified as the perpetrator of toxicity and it may be possible that toxic metabolite(s) differ among plant species (Reed 1995). The symptoms of hydrolysable tannin toxicity include necrosis of the liver, kidney damage, and hemorrhagic gastroenteritis (Filippich et al. 1991). Mortality is likely to occur in cattle and sheep when the diet contains 20% or more hydrolysable tannins (Reed 1995).

Impact of CTs on ruminants

Ruminants or their microbes are unable to degrade CTs, therefore, their

mechanism of toxicity is different from that of hydrolysable tannins. The toxicity of CTs however, is difficult to diagnose because the symptoms are similar to those of malnutrition. Toxicity can occur when animals are fed a diet low in protein with CT concentrations above 70g kg^{-1} DM (Barry and McNabb 1999; Mangan 1959; Reed 1995). Increasing the tannin concentration to 5-9% can be dangerous to the animal as this level of tannins may exhibit serious antinutritional effects including decreased fiber digestibility (Reed et al. 1985), inhibition of some rumen bacteria (Chesson et al. 1982) and anaerobic fungi (Aikin and Rigsby 1985) as well as a marked decrease in feed intake (Marten and Ehle 1984).

Nutrient availability - CT and proteins

As mentioned previously, tannins are well known for their protein complexing properties. Protein complexing can be beneficial to ruminants when the plant contains lower tannin concentrations or it can be deadly at higher concentrations. In order to produce beneficial effects, the concentration of tannins being consumed by the ruminant must be well monitored. It is recommended that the concentration of CT consumed be between 2-4% of dry matter (DM) (Barry and Forss 1983). At this concentration the tannins will bind with feed protein upon chewing thereby protecting proteins from microbial degradation, as well as inhibiting protease in the rumen (Barry and Forss 1983). Subsequently, the tannin-protein complex will be degraded at the more acidic pH of the lower digestive tract making the amino acids available for absorption in the small intestine, thus improving the efficiency of amino acid utilization (Mueller-Harvey 2006). However, tannins do not preferentially bind to specific proteins and may bind with proteins from various sources such as plant proteins, saliva proteins, or proteins already

present in the rumen (i.e. digestive enzymes) (Mitaru et al. 1984; Waghorn and McNabb 2003). It is also imperative to mention that when ruminants consume a higher concentration of tannins (above 70g kg^{-1} DM) or their diet lacks an adequate supply of proteins, tannins will bind to all the proteins available and potentially lead to protein deficiency (Barry and McNabb 1999; Waghorn and McNabb 2003).

Furthermore, research has shown that some browsing animals such as deer have developed an ability to produce proline-rich salivary proteins which can bind to tannins being consumed, such that they are no longer available to protect feed proteins from microbial degradation in the rumen, However, production of proline-rich salivary proteins will reduce the anti-nutritional effect associated with browsing high CT forage (Austin et al. 1989). In addition, it is possible for some animals to produce salivary proteins which are so specific that they will only bind to one type of CT, usually the most prevalent CT present in the diet of the animal. This situation exists in moose where they produce salivary proteins which only bind to one specific type of CT; therefore, all other CTs will not bind to these salivary proteins and will be available to bind to proteins in the feed or rumen (Hagerman et al. 1992). Fortunately, domesticated cattle and sheep do not possess this ability; hence, tannins can be used to assist feed proteins bypass the rumen (Austin et al. 1989).

The tannin-protein bond is very dependent on the pH of its environment. At a pH between 3.5 and 7.5 stable complexes of CTs and proteins will form whereas a pH below 3.5 or above 8.5 will not allow these complexes to form. Therefore, complexes will form in the rumen which has a pH of 6.0-7.0 and dissociate in the abomasum which has a decreased pH of 2.5-3.5 (Aerts et al. 1999). The type and strength of the bond between

the CT and protein will be dictated by characteristics of the proteins and tannins. The number of free hydroxyl groups present in the tannin structure will affect the strength of the bond since hydrogen bonds can be formed with proteins and carbohydrates (Haslam 1989). Other types of bonds may be formed between tannins and proteins such as hydrophobic bonds since tannins are thought to have hydrophobic sites (Aerts et al. 1999; Oh et al. 1980) or covalent bonds may form by means of an oxidative polymerization reaction in the presence of UV radiation, polyphenol oxidase, or heating (Reed 1995).

Microbial protein synthesis

In addition to its protein binding property, tannins may also increase the efficiency of microbial protein synthesis. Research has shown that when ruminants consume forage-containing tannins, there is an increase of non-ammonia nitrogen flow to the duodenum which is greater than the amount of nitrogen consumed (Reed 1995; Waghorn et al. 1987). This is an indication that increased production of microbial proteins maybe occurring since nitrogen is not freely produced in the rumen (Reed 1995). There are a few reasons that may explain the increased microbial mass in the presence of tannins. Research has shown that tannins may possess inhibitory effects against bacteriophages and protozoa in the rumen thereby giving rumen bacteria more opportunity to thrive (Klieve et al. 1996; Makkar et al. 1995). Getachew et al. (2000) also believe that CTs act as an antilytic agent for ruminal bacteria and improve the partitioning of nutrients which are released in the rumen thus improving the efficiency of feed utilization as well as increasing microbial mass.

Nutrient availability - CT and fibre

As noted earlier, tannins may complex with proteins, polysaccharides, and minerals consequently affecting their digestion in the rumen. Tannins tend to bind more specifically to some polysaccharides such as cellulose, hemicellulose, and pectin (McSweeney et al. 2001a). Tannins may also affect fiber digestion by inhibiting rumen cellulolytic bacteria. Some examples of rumen cellulolytic bacteria which may be inhibited by tannins include *F. succinogenes* and *Ruminococcus* spp. (McSweeney et al. 2001a). However, research has shown that the level of cellulolytic inhibition by tannins present in forage will vary depending on the forage species (Lowry et al. 1996). *Calliandra calothyrsus* with a CT concentration of 9.5% CT of DM (Palmer et al. 2000), *L. pedunculatus* at 6.3% CT of DM (Barry and Duncan 1984), and the addition of an *Acacia mearnsii* extract containing 2.5% CT of DM (Carulla et al. 2005) all showed a decrease of approximately 0.05 to 0.04 % in apparent fiber digestibility when fed to ruminants (Barry and Manley 1986). In a study where quebracho tannin extract was added to the diet up to 2 % DM, apparent fiber digestibility was not affected (Beauchemin et al. 2007). Similarly, Waghorn et al. (1987) fed sheep *L. corniculatus* L. with a CT concentration of 2.2 % DM and observed that NDF digestibility was not affected by the presence of CT in the diet. Although tannins have been shown to inhibit rumen cellulolytic bacteria, microbial protein synthesis does not seem to be affected by this (McSweeney et al. 2001b). Moreover, rumen fiber degrading fungi were not inhibited by the presence of tannins in the rumen (McSweeney et al. 2001a).

Nutrient availability - CT and minerals

The mucosa of the digestive tract can be damaged as a result of feeding of CT-rich forages, which in turn affects the absorption of other nutrients such as minerals and essential amino acids. In the case of methionine, a deficiency can be crucial as it is required for the detoxification of some plant toxins such as cyanogenic glycosides (Reed 1995). In one study, two groups of sheep were both fed *L. corniculatus* L. with a tannin concentration of 22 g kg^{-1} DM. One group also received an intraruminal infusion of PEG (polyethylene glycol) which selectively binds to tannins and removes any effects caused by tannins. Minerals which had decreased absorption in the presence of tannins were sulphur, potassium, and magnesium where apparent absorption values decreased by 0.21, 5.7, and 0.12 g d^{-1}, respectively, in the group not receiving intraruminal PEG (Waghorn et al. 1987).

CTs and microbial activity

When tannins form complexes with minerals, mineral availability to microorganisms is reduced. Scalbert (1991) has reported that the ability of tannins to bind to iron making it unavailable to microbes is a property that characterizes tannins as antimicrobial compounds. Other minerals that may be bound by tannins include copper and cobalt. A lack of available iron and other minerals may compromise the metabolism of bacteria as they may not be able to bind to substrates and secrete active enzymes due to the absence of required co-factors (McDonald et al. 1996).

Reduced microbial activity in the presence of tannins can be attributed to many factors including decreased nutrient availability. Studies have also shown that at high

tannin concentrations, rumen bacteria may undergo morphological changes. Some of these morphological changes can lead to cell lysis, depressed growth, and abnormal cell division (O'Donovan and Brooker 2001). In other cases, rumen bacteria begin producing glycocalyx and glycoproteins in the presence of high tannin concentrations as a possible defense mechanism (Chiquette et al. 1988). Knowledge regarding the mechanism by which tannins inhibit certain rumen bacteria is not extensive. Tannins interact with rumen microbes by binding to their cell wall resulting in the excretion of extracellular enzymes. It is possible that these two reactions may interfere with microbial nutrient utilization and growth (McSweeney et al. 2001b).

Although tannins inhibit many rumen microbes, some microbial species have been found to be tolerant of tannins. Such species include *S. gallolyticus* (formerly known as *S. caprinus)*, *Clostridium* spp., species in close relation to *S. bovis,* and an unidentified species of the *Proteobacteria* group (Brooker et al. 1994; Nelson et al. 1998). *S. gallolyticus* was found in the rumen of feral goats which consumed different species of *Acacia* containing high concentrations of tannins. This bacterium was not inhibited by a significant concentration of hydrolysable tannins and CTs at a concentration of 3%, although it was not capable of utilizing these phenolics as an energy source (Brooker et al. 2000). In another study, *S. galloyticus* synthesized an extracellular carbohydrate matrix composed mainly of glucose in response to exposure to CTs (O'Donovan and Brooker 2001). This extracellular matrix served to protect this bacterium and resulted in the development of a tannin tolerance which allowed it to proliferate at tannin concentrations 10-fold greater than what *S. bovis* could tolerate.

It has been found that certain microbial enzymes are more resistant to tannins than

others. Such is the case with some microbial cell wall bound enzymes which are much more resistant to tannins versus extracellular enzymes (Bae et al. 1993; Reed 1995). Some of the bacterial enzymes that are more prone to inhibition by tannins include urease, carboxymethylcellulase, protease, glutamate dehydrogenase, alanine aminotransferase, pectinases, cellulases, β-galactosidases, and proteases (Bell et al. 1965; Makkar et al. 1988; Smart et al. 1961). More specifically, studies have shown that CTs from sainfoin (*O. viciifolia*) are capable of inhibiting microbial proteases from *Butyrovibrio fibrisolvens* and *S. bovis* (Jones et al. 1994). It has also been postulated that tannins have a low affinity for lipases (Horigome et al. 1988). Pell et al. (2000) have reported that certain bacteria are capable of rearranging the lipids in their cell membrane to protect proteins in their structure that may be susceptible to tannins.

Environmental benefits associated with CTs

As there is increasing concern that greenhouse gas emissions should be decreased, CTs have captured the attention of researchers through their potential to reduce methane and nitrous oxide emissions from ruminants. Waghorn et al. (2002) have shown in their studies that there was a 16% reduction in methane production per unit of intake when sheep were fed *L. pedunculatus* at a tannin concentration of 8g kg^{-1} of dry matter in comparison to animals fed the same diet with the addition of PEG to remove the effects of CTs. These trials suggest that tannins may reduce methanogenesis and alternatively redirect energy towards animal growth (Beauchemin et al. 2007). It was hypothesized that the hydrogen from methane could be used for acetate synthesis, although future research is required to elucidate this finding. Condensed tannins may also reduce nitrous

oxide emissions through its protective protein effect. Inefficient nitrogen metabolism within ruminants is associated with an increase in urinary urea excretion. Through nitrification and denitrification processes naturally occurring in the soil and groundwater, some urinary urea can be converted to ammonia and nitrous oxide, a greenhouse gas (Oenema et al. 2005). Through its protective-protein effect, CTs have been shown to improve nitrogen use within the ruminant leading to decreased urinary urea concentrations (Waghorn and McNabb 2003).

As described above, CTs have an affinity for urease and therefore CTs present in the forage may manipulate the nitrogen utilization in ruminants. Tannins have been shown to decrease ammonia emissions from manure by inhibiting microbial urease activity and thereby decreasing the amount of ammonia produced (Hussain and Cheeke 1995; Makkar 1993).

Production benefits - bloat

One of the most beneficial effects that tannins can exert in livestock is their ability to prevent pasture bloat. This common disorder in ruminants occurs during the fermentation of legumes which are rich in highly digestible proteins such as white clover (*T. repens*) and alfalfa (Aerts et al. 1999; McMahon et al. 2000; Waghorn and McNabb 2003). The bloat is a result of rumen fermentation gases being trapped in a stable proteineaceous foam (Mangan 1988; Waghorn and McNabb 2003). The foam will encapsulate the gas preventing its escape which leads to the rumen becoming bloated (Mangan 1959). With fermentation ongoing, more gas is produced, eventually building more pressure in the rumen to the extent where death occurs as a result of suffocation

(Aerts et al. 1999). Furthermore, bloating in the animal will result in decreased feed intake, lack of weight gain, and reduced milk production in dairy cattle (Lees 1992). Conventionally, bloat was controlled by means of administering two oral doses of detergents daily (Aerts et al. 1999). These detergents act as surfactants thereby reducing the surface tension which breaks the foam bubbles and releases the gas. Administering these detergents can be costly and labor intensive as it is difficult to get the cattle to ingest the detergents, especially when they are already bloated (Lees 1992). As an alternative, tannin-containing forages may be consumed as a preventative measure to reduce the risk bloat. Condensed tannins from forage bind to the proteins making them unavailable to form foam in the rumen. To be rendered bloat-safe, the cattle must ingest a CT concentration of 5g kg^{-1} of dry matter (Li et al. 1996; Waghorn and McNabb 2003). Unfortunately, most tannin containing plants in temperate regions have a tannin concentration below this level (Aerts et al. 1999). McMahon et al. (1999) conducted a study over a four year period in which steers were fed alfalfa with the inclusion of sainfoin at 0, 10, 20% DM intake to reduce the occurrence of bloat. In three of the four years, the inclusion of sainfoin at levels as low as 10% reduced the incidence of bloat by 45 to 93%. In order to reduce the incidence of bloat in grazing systems, McMahon et al. (2000) conducted a two year study to determine the survivability of sainfoin in a mixed pasture system. The ratio of alfalfa to sainfoin seeded was 75:25 where alfalfa and sainfoin were seeded in alternate rows. The pasture stand was 36% sainfoin prior to grazing and remained at 33% following the grazing season in the first year. In the past, this mixed pasture system has not been practiced because sainfoin is not a persistent crop. In the aforementioned study, sainfoin had difficulty surviving in the following year due to

invasion of weeds and grasses as well as winter kill (McMahon et al. 2000). In the second year, sainfoin represented 20% of the pasture in the spring and 14% at the end of the grazing season with 45% of the pasture being weeds. The number of animal deaths due to bloat was two and seven in each year. Of the seven deaths in the second year, six of the deaths were of animals in paddocks which contained less than 10% sainfoin. Nevertheless, this grazing strategy may be effective in reducing the occurrence of bloat if sainfoin can be maintained at a minimum of 15% of the pasture (McMahon et al. 2000).

Resulting production benefits – wool/milk

Research has shown that an increase in wool growth and milk production in sheep (McNabb et al. 1996) and dairy cows can be seen when fed legume forage containing 20-40 g of CT kg^{-1} of DM (Woodward et al. 1999). Condensed tannins enhance the availability of amino acids to the small intestine which can be utilized to increase the production of milk, wool, and growth of the animal (Aerts et al. 1999). Woodward et al. (Woodward et al. 1999) observed that feeding *L. corniculatus* to dairy cattle led to a 60% increase in milk yield and 10% increase in milk protein as compared to cattle fed perennial ryegrass. The milk did not have a significant difference in the concentration of whey, casein, milk fat, and lactose. Similarly, wool growth in sheep increased when animals were fed a diet that contained a low concentration of CTs (1 g CT/kg DM) (Barry et al. 1999).

Production benefits - parasites

Antihelminthic properties of tannins are currently being investigated for their effects in ruminants. With the ever-increasing resistance of gastro-intestinal parasites to chemical antihelminthics and a considerable increase in organic production systems, there is a growing need to find an alternative to prevent economic losses (Waller 1997). Research has shown that CTs can have an inhibitory activity against adult parasites (*in vivo*) and larval stages (*in vitro*) (Luscher et al. 2005). Subsequently, many studies have looked at legume forages containing CTs to evaluate their ability to inhibit gastro-intestinal nematodes (Min and Hart 2003; Min et al. 2005; Min et al. 2004; Niezen et al. 1998). Condensed tannins may inhibit parasites in a direct manner whereby the tannins complex with the parasites thereby making them unable to thrive (Min and Hart 2003). CTs may directly affect nematode infections by decreasing fecal egg counts. In a study by Niezen et al. (1998) lambs were grazed on one of six forages containing CTs (*Hedysarum coronarium*, *Lotus corniculatus* and *L. pedunculatus*) or without CTs (*Medicago sativa, Plantago lanceolata, Lolium perenne, Trifolium repens*) to evaluate the effect of CT containing forage on fecal egg count. Their results indicate that the CT containing forages significantly ($p<0.001$) reduced fecal egg count with the most drastic reduction seen in animals grazing *Hedysarum coronarium*. It is also possible that CTs act in an indirect manner to inhibit internal parasites. It has been speculated that the increased protein availability to the animal caused by CTs may enhance the immune system thereby increasing the animal's resistance to parasitic infections (Bown et al. 1991). Nevertheless, the exact mechanism in which CTs act against intestinal parasites still needs to be further elucidated.

Concluding remarks

Tannins are a vast group of compounds and cannot be grouped as one. As such, different plant species will act differently in ruminants. Improved tannin extraction methodologies are continuously being developed or refined in order to accurately measure tannin concentration in plants. Currently, no uniform standard exists to measure CT concentration in plants, therefore, care must be taken when making comparisons with other studies. As mentioned previously, it was only recently that temperate region legumes, plants, and grasses were discovered to contain low concentrations of CTs. In addition, it is difficult to compare CT concentrations in plants among studies because so many factors such as growing conditions and extraction methodologies affect the reported tannin concentrations. Therefore, caution must be used when comparing earlier studies to current research. However, as more research is conducted on plant phenolics, farmers and nutritionists will eventually be able to utilize this knowledge to improve animal health and farming practices. Feeding CTs to ruminants has been associated with many benefits such as reducing methane emissions, bloat, and improving protein availability in ruminants. Plant tannin concentrations from New Zealand and Australia are considerably higher than those observed in western Canada and may in fact produce negative effects in animals. To date very little data has been reported on the CT concentration of native plant species in Western Canada.

2.10 Ecology theories

The advent of high throughput sequencing has revolutionized the study of microbial ecology. Since the emergence of next-generation high throughput sequencing

technology, microbial ecologists have realized that culture techniques have drastically underestimated the bacterial diversity within ecosystems (Pedrós-Alió 2007). Thus, as deep sequencing efforts continue to be applied, microbial ecologists will be able to study the diversity within ecosystems with higher resolving power and thus with new perspective. However, with a plethora of sequence data being produced, microbial ecologists will face new challenges including putting sequencing data into a biologically meaningful context. In addition, high-throughput sequencing of natural ecosystems under changing environmental conditions will allow researchers to acquire increased predictive power to understand microbial ecosystems. Applying fundamental ecological theories and developing pragmatic models in order to further understand microbial community structures and their behavior in natural environments will be a key outcome of this technology.

Island biogeography theory

By definition, biogeography is the study of taxa distribution over space and time. Originally applied to macro-ecosystems (eukaryotes), MacArthur and Wilson's (1967) island biogeography theory describes how several biogeographical processes can be used to predict a community structure. First, the theory dictates that species richness (number of species) of an island is dictated by the balance between rate of immigration of new species from a distant habitat and extinction rate of species on the island. Second, a distance-decay law which implies that as the distance between an island and its mainland increases, community similarities will decrease (MacArthur and Wilson 1967). Lastly, a taxa-area relationship will predict species richness of an island. An increase in island

size, for example will allow for an increase in species richness. This relationship can be defined by the following equation: $S = cA^z$ where S is the number of species, c is a taxon- and location-specific constant, A is the area size, and z is the slope of the logarithmic species-area relationship. A steeper slope (higher z value) is indicative of a strong positive relationship between area and species richness, whereas a low z value represents little spatial specialization. This relationship was originally defined in macro-ecosystems (MacArthur and Wilson 1967), but the advent of high-throughput sequencing has resulted in increasing research applying this theoretical concept to micro-ecosystems. The z value for species within an insular island has been reported to be approximately 0.26 for micro-organisms and 0.3 for macro-organisms (Bell et al. 2005). In an extensive review of microbial seed banks, Lennon and Stuart (2011) determined the frequency distribution of z-values for micro- and macro-organisms and concluded that macro-organisms had a significantly ($P<0.0001$) greater z-values than micro-organisms. Baas Becking's (1934) vision of "everything is everywhere, but the environment selects" has often been used to elucidate the greater z-values seen in macro-organisms as compared to micro-organisms. The minute size of micro-organisms is believed to allow them to disperse easily and without limitations (Lennon and Jones 2011).

Bet hedging, dormancy, and toxin anti-toxin modules

Traditionally, bacterial cell division is perceived as a single celled bacterium dividing into two identical progeny bacteria. However, studies have shown that despite being genetically identical, clones within an isogenic population may express a range of phenotypes. Recent studies suggest that bacteria can switch between phenotypic states in

response to fluctuations in environmental conditions (Beaumont et al. 2009; Jones and Lennon 2010; Rotem et al. 2010). In addition, bacteria may also stochastically switch between phenotypic states as a bet hedging strategy to anticipate changes in environmental conditions. Variations in phenotypic states within an isogenic population are used as an evolutionary strategy to increase the likelihood for survival of a subpopulation.

Bacteria often face adverse conditions especially in natural habitats including unpredictable fluctuations in temperature, pH, nutrient availability, salinity, and the presence of toxins and antimicrobials (Dubnau and Losick 2006). In order to anticipate and cope with these naturally occurring stressors, a random subpopulation of bacteria in a clonal population can epigenetically (no mutations required) switch on gene expression of various fitness traits to increase their chances of survival. For example, it’s been shown that individual organisms in a genetically identical population of bacteria can randomly differentiate into different phenotypes whereby random variations in gene expression can confer specific advantages to some cells (Adam et al. 2008). This notion suggests that when difficult conditions arise, a subpopulation from the clonal population will be selected for, persist and contribute to the overall survival of the original population. Furthermore, numerous studies have found that these phenotypic changes are transient and thus reversible upon removal of the stressor (Adam et al. 2008; Lioy et al. 2010; Rotem et al. 2010).

Bacteria have a variety of bet-hedging phenotypic states they can change into in order to overcome fluctuating environmental stressors including spore formation/cannibalism, genetic competence, swimming/chaining, and persistence. Of

interest, cell persistence is a well-studied mechanism bacteria can utilize to effectively overcome antimicrobial exposure. In a clonal population, phenotypic differentiation of a subpopulation to a persister/dormant state, which is functionally analogous to spores, can protect these cells from the lethal effects of antimicrobials and ensure the survival of their genetic lineage (Dubnau and Losick 2006). Several studies with *Staphylococcus aureus* (Bigger 1944) and *Escherichia coli* (Balaban et al. 2004) have shown that in a clonal population of these species, it is possible to isolate cells which are in a state of growth arrest. Upon exposure to antimicrobials, these phenotypic mutants are insensitive to the drug challenge. Furthermore, once the antimicrobial is retracted, these cells return to an active growth state (Adam et al. 2008).

It has been recognized that one way in which these cells switch between an actively growing and persister phenotype is through the use of toxin-antitoxin modules (Keren et al. 2004). Typically, toxin-antitoxin modules are comprised of a pair of genes, within the same operon, coding for the expression of a toxin and anti-toxin. In this tightly regulated system, overexpression of the toxin leads to a state of growth arrest which can subsequently be reversed simply by expression of the anti-toxin. The ubiquity of toxin-antitoxin modules in bacteria has led many to believe that these modules must have additional functions which have yet to be elucidated (Rotem et al. 2010).

Concluding remarks

The high microbiota density and dependency on a wide range of complex metabolic interactions makes the rumen a naturally competitive environment and thus the addition of antibiotics and/or phytochemical rich diets will inevitably exert some

selective pressures on the microbial population. The potential repercussions from selective pressures have not been well studied within the ruminant gut. Previous gut microbiome research has suggest the following potential impacts of selective pressure on a gut microbiome: a) can provide a fitness advantage to a particular group of organisms (i.e. commensal or pathogenic bacteria) (Looft et al. 2012) b) epigenetic and/or mutational changes resulting in phenotypic variability within a clonal population to increase fitness (Adam et al. 2008) c) extinction/immigration of microbial population as a result of disturbed metabolic interactions (Costello et al. 2012). Applying popular macro-ecology theoretical concepts may increase our knowledge on how these selective pressures can affect the ruminant gut microbiome throughout each section of the gut and will enhance our understanding of the overall gut function and animal health. In addition, bacterial persistence versus true antimicrobial resistance may have great implications in how we mitigate antimicrobial resistance in the future.

2.11 Research hypothesis and objectives

1. Identify a high-quality phytochemical/tannin-containing forage which may be used in livestock production systems to support animal health while reducing fecal shedding of potential pathogens such as E. coli O157:H7.
2. Characterize the ruminant gut microbial ecosystem to determine the effects feeding a tannin-containing forage will have on the bacterial community dynamics of the gut.
3. Evaluate the suitability of using natural plant compounds in combination with synthetic antibiotics to deter the development of resistance.
4. Evaluate the applicability of popular ecological models to ruminant gut microbial populations to help elucidate microbial community dynamics.

CHAPTER THREE

MANUSCRIPT I

Condensed tannin concentrations found in vegetative and mature forage legumes grown in western Canada

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3.1 ABSTRACT

There has been limited effort to examine condensed tannin (CT) concentrations of forage legume species grown in western Canada. Using the butanol-HCl technique, extractable CT concentrations were measured in *Trifolium hybridum* L.*, T. ambiguum* M. Bieb*, T. pratense* L.*, T. repens* L.*, Dalea purpurea* Vent., *Onobrychis viciifolia* Scop.*, Lotus corniculatus* L., *Medicago sativa* L., and *Astragalus cicer* L. collected from research and variety trial plots across the Canadian prairies. Above ground plant biomass was harvested at the vegetative and mature physiological stages for two growing seasons. *Dalea purpurea*, a native legume, had the highest mean CT concentration of 68.6 ± 22.6 g kg^{-1} DM, with minimum and maximum values ranging from 37.9 to 92.9 g kg^{-1} DM. *Onobrychis viciifolia* had the second highest mean CT concentration (46.0 g kg^{-1} DM) with a range of 16.3 to 94.4 g kg^{-1} DM. The third highest mean CT concentration of 15.1 g kg^{-1} DM was found in *L. corniculatus* with a range from 0.0 to 25.7 g kg^{-1} DM. Forage biomass had higher CT concentrations ($P<0.05$) when harvested at the mature stage for all species except *O. viciifolia* which had higher CT concentrations in the vegetative state. The potential benefit of inclusion of these species in ruminant diets at the observed concentrations requires further exploration.

3.2 INTRODUCTION

When included in ruminant diets at concentrations between 5 and 55 g kg^{-1} DM, condensed tannins (CT) have been shown to provide numerous benefits to ruminant production such as bloat prevention (Li et al. 1996), reductions in fecal egg count (Min and Hart 2003), protection of plant protein from microbial degradation (Aerts et al. 1999; Waghorn et al. 1987) and improved liveweight gain, milk yield (Wang et al. 1996), wool growth and ovulation in sheep (Min et al. 2000). In addition, Waghorn et al. (2002) reported a 16% reduction in methane production in animals fed with *L. pedunculatus* with a CT concentration of 53 g kg^{-1}. Most studies on plant tannins have measured CT concentrations in plants grown in a semi-arid or tropical region (Jackson et al. 1996; Terrill et al. 1992), but only limited data, as indicated in Table 3.1 has been collected on CT concentration of forage legumes grown in a temperate climate such as that of western Canada (McMahon et al. 2000). As shown in Table 3.1, most studies examining CT concentrations in temperate climates have been done in New Zealand which may not reflect growth conditions in Canada. Plant tannin concentrations from semi-arid or tropical regions are considerably higher than those observed in western Canada (temperate region). High CT concentrations (above 80 g kg^{-1} DM) can lower feed intake due to decreased palatability of tannin-containing feeds (Marten and Ehle 1984) and lower feed digestibility by inhibiting ruminant microorganisms (Chesson et al. 1982; Reed 1995). Furthermore, a CT concentration above 9% can be fatal to the animal, particularly when there is no alternative source of feed (Kumar 1983; Kumar and Singh 1984).

In addition to an observed relationship between CT concentration and temperature at which the legume is grown (Anuraga et al. 1993), many other factors have been shown to influence CT concentration including species (Terrill et al. 1992), soil fertility (Barry and Forss 1983), tissue damage by insects, bacteria, fungi, and herbivores (Haring et al. 2008), stage of plant maturity (McMahon et al. 2000; Schreurs et al. 2007), and methodology used for analyses (Terrill et al. 1992). Knowledge of CT concentrations in forage legumes and identifying potential inclusion levels in ruminant diets may improve animal productivity while potentially improving environmental sustainability through decreased nitrogen excretion (McMahon et al. 1999), methane emissions (Waghorn et al. 2002) and manure nitrous oxide emissions (Waghorn and McNabb 2003). The objectives of this study were to determine extractable CT concentrations in forage legumes grown at eight sites across the prairie region of western Canada and to evaluate the effect of plant maturity and growing season on CT concentration.

3.3 MATERIALS AND METHODS

Growth of plants

Medicago sativa L. (alfalfa; var. Beaver, Runner, Geneva, Ameristand 201+Z, Spredor 3, Spredor 4, Algonquin, HPS brand premium alfalfa blend), *Trifolium hybridum* L. (alsike clover; var. Aurora), *T. ambiguum* M. Bieb (kura clover), *T. pratense* L. (red clover), *T. repens* L. (white clover), *Dalea purpurea* Vent. (purple prairie clover), *Onobrychis viciifolia* Scop. (sainfoin; var. Melrose, Nova, Perly, Emyr, Splendid, SL 895), *Lotus corniculatus* L. (birdsfoot trefoil; var. AC Languille, Leo), and *Astragalus*

cicer L. (cicer milkvetch; var. Oxley II, Windsor) were grown at different sites across western Canada over two growing seasons (2005 and 2006). Sampling sites were located in: Alberta (Neapolis, Barrhead), Saskatchewan (Saskatoon), and Manitoba (Arnaud, Beulah, Roblin, Rosebank, and Virden). Above ground plant biomass was harvested at two physiological stages: flowering/full bloom (mature stage) and after regrowth vegetative stage for each of two growing seasons. Due to unfavourable weather conditions and poor regrowth, some plots were not sampled at both stages of growth and/or in both growing seasons at the same site.

Sample collection

To minimize weed contamination, whole plants were selected and clipped individually to a level 3.75 cm above the soil surface. Whole plant samples were subsequently placed in Delnet bags (Delstar Technologies, Middleton, DE), stored on ice during transport to the laboratory, and stored at -20°C until further analysis.

Whole plants were freeze-dried and ground through a 1mm screen using a sample mill (Tecator, Cyclotec 1093, Hoganas, Sweden). Ground plant material was stored at room temperature until samples were analyzed for CT.

Determination of extractable condensed tannin concentrations from whole plants

Purification of CT from O. viciifolia and D. purpurea for use as reference standards.

Purified CT from *O. viciifolia* and *D. purpurea* in vegetative growth stage were used as reference standards in this study. Condensed tannins were extracted from fresh-frozen *O. viciifolia* and *D. purpurea* using a 7:3 (v/v) acetone/water solution containing 0.1% ascorbic acid whereby the acetone was subsequently removed by rotary-evaporation. Impurities such as pigments, lipids, and other debris were removed by

washing the solution three times with diethyl ether followed by centrifugation at 27000 x *g* for 15 min. Samples were freeze-dried and resolubilized in a 1:1 (v/v) methanol/water mixture. Each mixture was purified chromatographically on a Sephadex LH-20 as described in Terrill et al. (1992). Purified CT were freeze-dried and stored in a desiccator at -20°C in the dark until required. Standard solutions for *O. viciifolia* and *D. purpurea* were prepared by dissolving 1 g of previously purified CT from *O. viciifolia* and *D. purpurea* in 1 L of water.

Quantification of extractable CT from plant samples

Extractable CT were measured in duplicate for each plant sample as described in Terrill et al. (1992). It is important to note that this technique extracts free CT only and does not extract protein- and fibre-bound CT. For each replicate, 500 mg of freeze-dried ground plant material was extracted three times with a 20 mL solution of 7:3 (v/v) acetone/water containing 0.1% ascorbic acid and 10 mL diethyl ether (4.7:2.0:3.3 acetone:water:diethyl ether (v/v)). The mixture was vortexed and centrifuged at 27000 x *g* for 15 min to precipitate any solid material. The supernatant from duplicate samples was combined and allowed to settle until the aqueous and organic phase were visibly separate. The upper, organic phase was removed and discarded. The aqueous phase was centrifuged at 27000 x *g* to remove solvent and debris and dried by rotary evaporation at 40°C. The supernatant was brought to a volume of 100 mL using distilled water.

A mixture of butanol/HCl (95% butan-1-ol: 5% HCl (36%) (v/v); 6 mL) was added to either 1 mL of the standard solution (*O. viciifolia* or *D. purpurea*) or an unknown sample CT solution, vortexed and incubated in a boiling water bath at 100°C for 75 min. Once color development occurred, the tubes were cooled on ice and

immediately read at 550 nm using a spectrophotometer (UltraSpec Plus 4054, Pharmacia, Baie d'Urfe', QC). The butanol/HCl solution without added CT was used as a blank. Standard curves were developed (0, 0.25, 0.5, 0.75, 1.0 mg CT mL^{-1}) with freshly prepared *O. viciifolia* and *D. purpurea* CT standards using the procedure as described above. The *O. viciifolia* CT standard was used to determine CT concentrations from all samples except for the *D. purpurea* samples for which the *D. purpurea* CT standard was used as it had a higher CT concentration than *O. viciifolia*.

In order to account for variations in the rate of spectrophotometric color development between the plant material and CT, the *M. sativa* CT solution was used to calibrate for background color as it does not contain significant quantities of CT. To precipitate any CT present in the extract, 200 mg of polyvinylpolypyrrolidone (PVPP) was added to 10 mL of *M. sativa* CT solution. This mixture was held at room temperature for 30 min followed by centrifugation at 300 x *g* for 10 min. The supernatant was collected and subjected to the butanol/HCl method above. Absorbance measurements were subsequently subtracted from all sample absorbance measurements.

Statistical analyses

All statistical analyses were performed using the statistical software SAS (SAS 9.1; SAS, Cary, NC). Arithmetic means, standard deviations, coefficients of variations (CV), and min/max were calculated for all species using PROC means. Number of sites for each forage legume species was calculated as the total number of sample sites over both years. The data was tested for normality using PROC Univariate with CT concentration as the variable and PROC Mixed was performed to analyze fixed effects (stage of maturity and growing season). Fixed effect interactions could not be tested as

we were unable to harvest both mature and vegetative samples for each species in both years as a result of poor weather conditions at some sites. Tukey-Kramer's multiple comparisons test was used to determine significant differences ($P<0.05$) in CT concentrations between growth stages (mature vs. vegetative) and growing seasons within each species. The LS means, standard errors of the means, standard deviations, and CV for CT concentrations were calculated using PROC Mixed for both stages of maturity within a species (all sites and years combined) and for each growing season within each species (all sites and growth stages combined) were reported.

3.4 RESULTS AND DISCUSSION

There is a wealth of published data on CT concentration in several forage species although little information is available for CT concentration in forage species grown in western Canada (Table 3.1). The range of CT concentrations as determined by minimum and maximum values and the CV for each species is rather large (Table 3.2). The discrepancies between the minimum and maximum values and those reported elsewhere may be attributed to differences in climate (Anuraga et al. 1993; Lees et al. 1994), differing CT extraction methodologies (Terrill et al. 1992), soil fertility (Barry and Forss 1983), plant maturity (McMahon et al. 2000), and plant tissue used (Aerts et al. 1999; McMahon et al. 2000). Large variations in values obtained can be partially explained by the number of sites as increased variability in environmental conditions (climate and soil fertility) over sites and between years will result in wider ranges of CT concentrations in the forages sampled (McMahon et al. 2000).

Table 3.1. Summary of condensed tannin concentration of forages, region grown, analytical method, and plant part utilized

Forages	**CT concentration ($g\ kg^{-1}$ of DM)**	**Region grown**	**Analytical method**	**Plant part used for CT analysis**	**CT measured**	**Reference**
Legumes						
Temperate						
Onobrychis viciifolia (sainfoin)	38.5	Canada	butanol-HCl	leaves	extractable	(McMahon et al. 1999)
Lotus corniculatus (birdsfoot trefoil)	7.0	New Zealand	butanol-HCl	whole plant	extractable	(Terrill et al. 1992)
	35.8	New Zealand	butanol-HCl	leaves	extractable	(Jackson et al. 1996)
	35.4	New Zealand	butanol-HCl	leaves	total CT*	(Schreurs et al. 2007)
Lotus pedunculatus (big trefoil)	61.0	New Zealand	butanol-HCl	whole plant	extractable	(Terrill et al. 1992)
	98.5	New Zealand	butanol-HCl	leaves	total CT	(Schreurs et al. 2007)
Lotus tenuis (narrow leaf birdsfoot trefoil)	2.0	New Zealand	butanol-HCl	whole plant	extractable	(Terrill et al. 1992)
Trifolium pratense (red clover)	0.4	New Zealand	butanol-HCl	leaves	extractable	(Jackson et al. 1996)
Trifolium repens (white clover)	1.3	New Zealand	butanol-HCl	leaves	total CT	(Schreurs et al. 2007)
Hedysarum coronarium (sulla)	33.0	New Zealand	butanol-HCl	whole plant	extractable	(Terrill et al. 1992)
	37.7	New Zealand	butanol-HCl	leaves	extractable	(Jackson et al. 1996)
Medicago sativa (lucerne)	0.0	New Zealand	butanol-HCl	leaves	extractable	(Jackson et al. 1996)
Coronilla varia (crownvetch)	16.0	New Zealand	butanol-HCl	whole plant	extractable	(Terrill et al. 1992)
Ornithopus sativus (serradella)	4.0	New Zealand	butanol-HCl	whole plant	extractable	(Terrill et al. 1992)

Lupinus polyphyllus (perennial lupin)	1.1	New Zealand	butanol-HCl	whole plant	extractable	(Terrill et al. 1992)
Astragalus cicer (cicer milkvetch)	0.4	New Zealand	butanol-HCl	whole plant	extractable	(Terrill et al. 1992)
Tropical						
Acacia angustissima	33.0	Zimbabwe	butanol-HCl	leaves	extractable	(Hove et al. 2001)
Desmodium ovalifolium	94.0	Columbia	butanol-HCl	leaves	extractable	(Barahona et al. 1997)
Flemingia macrophylla	90.0	Columbia	butanol-HCl	leaves	extractable	(Barahona et al. 1997)
Calliandra calothyrsus	196.0	Zimbabwe	butanol-HCl	leaves	extractable	(Hove et al. 2001)
Leucaena leucocephala	134.0	Zimbabwe	butanol-HCl	leaves	extractable	(Hove et al. 2001)
Grasses (temperate)						
Lolium perenne (perennial ryegrass)	1.1	New Zealand	butanol-HCl	whole plant	extractable	(Terrill et al. 1992)
	0.5	New Zealand	butanol-HCl	leaves	total CT	(Schreurs et al. 2007)
Herbs (temperate)						
Chichorium intybus (chicory)	1.4	New Zealand	butanol-HCl	whole plant	extractable	(Terrill et al. 1992)
	1.0	New Zealand	butanol-HCl	leaves	extractable	(Jackson et al. 1996)
Holcus lanatus (Yorkshire fog)	1.4	New Zealand	butanol-HCl	whole plant	extractable	(Terrill et al. 1992)
	2.3	New Zealand	butanol-HCl	leaves	extractable	(Jackson et al. 1996)
Sanguisorba minor (sheeps burnet)	1.0	New Zealand	butanol-HCl	whole plant	extractable	(Terrill et al. 1992)

*Total CT: extractable + fiber and protein bound

Table 3.2. Determination of extractable CT concentrations of whole plant forage legumes species using the butanol-HCl technique

Species	Number of sites[1]	Total no. of varieties	Mean (g kg^{-1} DM)	Standard deviation	CV (%)	Minimum (g kg^{-1} DM)	Maximum (g kg^{-1} DM)
Medicago sativa (alfalfa)	7	9	0.0	0.0	453.3	0.0	0.1
Trifolium hybridum (alsike clover)	5	1	9.6	7.4	76.9	0.0	19.5
Astragalus cicer (cicer milkvetch)	3	2	0.0	0.0	315.0	0.0	0.0
Trifolium ambiguum (kura clover)	5	1	2.4	3.1	128.6	0.0	9.6
Dalea purpurea (purple prairie clover)	5	1	68.7	22.6	32.9	37.9	92.9
Trifolium pratense (red clover)	6	1	3.3	4.4	132.3	0.0	15.3
Onobrychis viciifolia (sainfoin)	3	6	46.0	19.3	42.0	16.3	94.4
Lotus corniculatus (birdsfoot trefoil)	8	2	15.1	6.3	41.6	0.0	25.7
Trifolium repens (white clover)	5	1	2.3	4.6	201.5	0.0	11.9

[1] Total number of sites sampled over both years

Dalea purpurea, a native legume, had the highest CT concentrations of all the species examined in this survey, with a CT concentration ranging from 37.9 to 92.9 g kg^{-1} DM (Table 3.2). *Onobrychis viciifolia* had the second highest mean CT concentration at 46.0 g kg^{-1} DM with a range of 16.3 to 94.4 g kg^{-1} DM between six different varieties (Table 3.2). To our knowledge, only one other study (McMahon et al. 1999) has reported CT concentrations of *O. viciifolia* grown in western Canada. The concentration reported in that study (38.5 g kg^{-1} DM) was comparable to those observed in the current study. The species with the third highest CT concentration was *L. corniculatus* with a mean concentration of 15.1 g kg^{-1} DM and a range of 0.0 to 25.7 g kg^{-1} DM between two varieties (Table 3.2). Terrill et al. (1992) reported an extractable CT concentration in *L. corniculatus* (*cv*. unknown) of 7 g kg^{-1} DM whereas Waghorn and Shelton (1997) reported an extractable CT concentration of 31.7 g kg^{-1} DM (*cv*. Grasslands Goldie).

Previous studies have shown that the *Trifolium* species contains little to no tannins. Jackson et al. (1996) reported an extractable CT concentration of 0.40 g kg^{-1} DM in *T. pratense* leaves (vegetative) and Schreurs et al. (2007) found that leaves harvested at the vegetative stage from *T. repens* had a total CT concentration of 1.3 g kg^{-1} DM. In comparison, our results show that mean CT concentrations from whole plant *T. ambiguum*, *T. pratense*, and *T. repens* harvested in the vegetative and mature state averaged 2.4, 3.3, 2.3 g kg^{-1} DM, respectively (Table 3.2). The differences in the CT concentrations between this study and the aforementioned studies may be associated with type of plant tissue measured (i.e. leaf vs. whole plant) and plant maturity. Further, *M. sativa* and *A. cicer* collected in the current study did not contain any CT (Table 3.2) which is in agreement with other studies (Goplen et al. 1980; Terrill et al. 1992).

Plant maturity has been shown to be one of the factors that can influence the CT concentration in plants (McMahon et al. 2000). Condensed tannin concentration in all species was higher in the mature samples than the vegetative samples except for *O. viciifolia* where the vegetative samples had a higher CT concentration (58.7 g kg^{-1} DM) as compared to the mature samples (33.7 g kg^{-1} DM, Table 3.3). The higher CT concentration obtained can be explained by the fact that CT in *O. viciifolia* are more concentrated in the leaf tissue therefore, as the plant matures the stem to leaf ratio will increase thereby reducing the CT concentration (Lees 1993; Lees et al. 1995; Taylor and Quesenberry 1996). In addition, enzymes responsible for the production of CT in *O. viciifolia* have been shown to decline in activity as the plant matures (Joseph et al. 1998; Singh et al. 1997).

Unlike *O. viciifolia*, plant maturity tended to increase CT concentration in the clover species. Mature *T. repens*, for example, had a higher CT concentration as compared to the vegetative samples (6.9 and 0.0 g kg^{-1} DM, respectively). Previous studies have shown that CT are only present in the flowers of this species (Jones et al. 1976). Similarly, the CT concentration in *L. corniculatus* was greater in the mature sample, compared to that of the vegetative samples (18.6 and 9.4 g kg^{-1} DM, respectively, as indicated in Table 3.3. Morris et al. (1993) measured CT concentration in various parts of *L. corniculatus* (var. *japonicus*) plants and found the highest CT concentration to be in the flowers (15 g kg^{-1} fresh weight) and petals (8 g kg^{-1} fresh weight) followed by petioles, stem, roots (all under 5 g kg^{-1} fresh weight), with leaves containing no CT. However, Poli et al. (2006) reported extractable CT concentrations in *L. corniculatus* leaves and stem of 1.69 and 0.05 g kg^{-1} DM. From the aforementioned data and the data

reported by Terrill et al. (1992), it appears that with the exception of *O. viciifolia*, most legume species have a higher CT concentration in their flowers as compared to their leaves. It has been speculated by Barry (1989) that the higher CT concentration in the flowers is to protect the reproductive organ of the plant.

Table 3.3. Determination of average extractable CT concentration in whole plant forage legume species at different stages of maturity using the butanol-HCl technique

Specie	n	Vegetative† ($g\ kg^{-1}$ DM)	Mature† ($g\ kg^{-1}$ DM)
Trifolium hybridum (alsike clover)	12	0.0 ± 4.4*a*	11.6 ± 2.0*b*
Trifolium ambiguum (kura clover)	12	0.1 ± 1.1*a*	4.1 ± 0.9*b*
Dalea purpurea (purple prairie clover)	6	N/A	68.7 ± 9.2
Trifolium pratense (red clover)	20	0.2 ± 0.6*a*	8.1 ± 0.7*b*
Onobrychis viciifolia (sainfoin)	49	58.7 ± 3.0*a*	33.7 ± 3.0*b*
Lotus corniculatus (birdsfoot trefoil)	12	9.4 ±2.7*a*	18.6 ± 1.2*b*
Trifolium repens (white clover)	7	0.0 ± 2.0	6.9 ± 2.3

LS mean ± standard error of the mean. Statistical significance ($P<0.05$) is denoted by different letters within a species.
N/A: not available
†mature: flowering/full bloom; vegetative: regrowth (after harvest)

A significant time effect over the two-year collection period was not apparent in any of the species examined (Table 3.4). Although no significant time effect was detected, some species had lower mean values in the first year of sampling (*T. ambiguum*, *T. repens, and D. purpurea*) in comparison to year two of sampling, whereas other species had lower mean values in year two (*T. hybridum*, *T. pratense*, and *L. corniculatus*) indicating that numerical differences did not follow a yearly trend. Plant species used for this study were not all grown in the same sites for both years; therefore, we can speculate that variations in climate (temperature and precipitation) and soil conditions between years and within each site likely contributed to the larger standard errors seen in some species (Table 3.4). Anuraga et al. (1993) were able to show that variations in soil moisture and growing temperature can significantly increase or decrease CT concentration in *L. pedunculatus* and *L. corniculatus.*

This study provides much needed information regarding extractable CT concentrations of cultivated and native forage legumes grown in western Canada. To our knowledge, this is the first report of the presence of CT in *D. purpurea*. Further, the CT concentration of this native legume is greater than all other species examined. *Onobrychis viciifolia* persistence and CT concentration in a pasture environment has been studied by McMahon et al. (2000). These researchers seeded *O. viciifolia* and *M. sativa* at a ratio of 25:75. The resulting pasture, which was grazed rotationally over a two-year period, contained 36% *O. viciifolia* in year one and 14% in year two. McMahon et al. (2000) suggest that an intake of 10 kg DM per day of *O. viciifolia* with an assumed CT concentration of 40 g kg^{-1} DM would be sufficient to achieve the anti-bloat CT concentration of 5 g kg^{-1} DM as recommended by Li et al. (1996). As such, a 12.5% *O.*

viciifolia pasture content is required to produce CT concentrations above this threshold. Interestingly, nine deaths due to bloat were reported in the study by McMahon et al. (2000). Seven of the nine deaths occurred in year two where the overall *O. Viciifolia* was 14%. Forages with a higher CT concentration such as *Dalea purpurea* could therefore be minimally included to the diets to reduce the incidence of bloat and improve production efficiency. It should be noted that the widely-used HCl-butanol technique used in this current study and that of McMahon et al. (2000) does not quantify bound CT. Thus it is difficult to equate quantitative analysis of extractable tannin with resulting biological activity in the animal. However, previous studies have shown that bound CT are inactive and do not affect microbial fermentation provided they remain in the bound form (Makkar 2003; Makkar et al. 1995).

Table 3.4. Effect of growing season on extractable CT concentration using butanol-HCl technique in whole plant forage legume species grown in western Canada

Species	n	Growing season	
		Year 1 (g kg^{-1} DM)	Year 2 (g kg^{-1} DM)
Trifolium hybridum (alsike clover)	12	9.8 ±3.2	9.5 ± 3.2
Trifolium ambiguum (kura clover)	12	2.1 ±1.1	3.3 ± 1.9
Dalea purpurea (purple prairie clover)	6	63.0 ± 14.0	74.4 ± 14.0
Trifolium pratense (red clover)	20	4.0 ±1.2	2.2 ± 1.7
Lotus corniculatus (birdsfoot trefoil)	12	17.2 ± 1.7	16.5 ± 3.8
Trifolium repens (white clover)	7	2.4 ± 2.5	4.3 ± 3.9

LS mean ± standard error of the mean. Data were not significantly ($P>0.05$) different within a species.

The CT concentrations of forage legume species measured in this study can potentially be used in best management practices on pasture. Establishment of native or tame pastures which contain *O. viciifolia* or *D. purpurea* at the concentrations described previously by McMahon et al. (2000) has the potential to achieve the desired benefits previously mentioned in terms of animal productivity and animal health. It is not necessary to grow *O. viciifolia* and *D. purpurea* in pure stands but rather include them with other forage legume species as part of a mixed pasture system as described by McMahon et al. (2000). The two year data also suggest that annual variation of CT concentration is not significant (Table 3.4). We suggest that these species can be included in pasture systems with some degree of confidence that plant CT concentration will not change considerably hence CT concentration of diet will be determined by plant persistence in the stand. Furthermore, to enhance growth productivity of *O. viciifolia* and *D. purpurea* in a mixed pasture system, additional breeding studies to improve their agronomic properties are warranted.

BRIDGE TO CHAPTER FOUR

The benefits of CT-containing forage in livestock production systems have been well established in the literature. As such, the study in chapter three sought to identify forages which grow well in a western Canadian environment with CT concentrations in the range required to elicit these animal health and production benefits. As revealed in our results, *Dalea purpurea* (purple prairie clover), followed by *Onobrychis viciifolia* (sainfoin), and *L. corniculatus* had the highest mean CT concentrations.

Research has shown that sainfoin, a nutrient dense forage, can support high levels of animal production. Additionally, phenolics such as CTs found in sainfoin have been shown to have antimicrobial activity. Fecal shedding of pathogenic bacteria in livestock production is a concern therefore practices which may help reduce pathogen shedding are of particular interest to the agriculture sector. Thus in the following study, we hypothesize that phenolic compounds found in sainfoin will reduce the survival of pathogenic *E. coli in vitro*. We also postulate that in an *in vivo* sainfoin feeding trial of feedlot cattle, fecal shedding of generic *E. coli* will also be reduced.

CHAPTER FOUR

MANUSCRIPT II

Potential to reduce *Escherichia coli* shedding in cattle feces using sainfoin (*Onobrychis viciifolia*) forage: *in vitro* and *in vivo* comparisons

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4.1 ABSTRACT

There is a growing concern about the presence of pathogens in cattle manure and its implications on human and environmental health. The phytochemical rich forage, sainfoin (*Onobrychis viciifolia*) and purified phenolics (*trans*-cinnamic acid, *p*-coumaric acid, and ferulic acid) were evaluated for their ability to reduce the viability of pathogenic *Escherichia coli* strains including *E. coli* O157:H7. Minimum inhibitory concentrations (MIC) were determined using purified phenolics and acetone extracts of sainfoin and alfalfa (*Medicago sativa*), a non-tannin containing legume. Ground sainfoin or pure phenolics were mixed with fresh cattle feces and inoculated with a ciprofloxacin-resistant strain of *E. coli* O157:H7 cip^{r} to assess its viability at -20°C, 5°C, or 37°C over 14 d. Forty steers were fed either a sainfoin (hay or silage) or alfalfa (hay or silage) diet over a 9 wk period. In the *in vitro* study, the MIC values for coumaric (1.2 mg mL^{-1}) and cinnamic (1.4 mg mL^{-1}) acid were 10 to 20-fold lower than the sainfoin and alfalfa extracts. In the inoculated feces, the -20°C treatment had death rates which were at least twice as high as the 5°C treatment irrespective of the additive used. Sainfoin was less effective than coumaric acid in reducing *E. coli* O157:H7 cip^{r} in inoculated feces. During the animal trial, fecal *E. coli* numbers declined marginally in the presence of sainfoin (silage and hay) and alfalfa silage but not hay indicating the presence of other inhibitory secondary compounds in alfalfa. In conclusion, phenolic-containing forages can be used as a means of minimally reducing *E. coli* shedding in cattle without affecting animal production.

4.2 INTRODUCTION

Fecal shedding of *Escherichia coli* O157:H7 is a concern in beef and dairy cattle production (Hutchison et al. 2005; Kudva et al. 1998). Feeding strategies that include high levels of forage reduce the abundance of *E. coli* excreted but is generally regarded as an uneconomical practice because it results in decreased growth rates and milk production by animals (Callaway et al. 2003). Alfalfa (*Medicago sativa*) is a high quality forage that can support high levels of production in beef and dairy cattle (Benchaar et al. 2007; Hermann et al. 2002; Plaizier 2004), but is not as effective as grain-based diets in achieving increased gain (Moody et al. 2007). In many countries, niche markets for forage-fed beef and dairy products have developed (Tilman et al. 2002), and phytochemical containing forages might have a role. One such forage is sainfoin (*Onobrychis viciifolia*), which is a perennial temperate forage legume. Sainfoin is drought resistant, high in protein, has good agronomic properties, and contains moderate condensed tannin levels (3-5 % of dry matter) (McMahon et al. 1999; Morrill et al. 1998; Mowrey and Volesky 1993).

In western Canada the production environment is unique with respect to the extreme low temperatures during the winter (Frauenfeld et al. 2007). The soil is frozen during the winter and urine and feces do not penetrate below the surface. During spring-thaw, the soil is still frozen, and accumulated manure mixes with snow-melt and contaminates surface water with phosphorus and zoonotic pathogens (e.g. *E. coli*) via run-off which is a public health concern (Gessel et al. 2004; Pell 1997).

Phytochemicals/tannins have antimicrobial properties against *E. coli* (Duncan et al.

1998; Friedman et al. 2003; Olasupo et al. 2003; Wells et al. 2005). The use of high quality phytochemical/tannin-containing forages may have the dual benefit of being good quality forage, as well as reducing *E. coli* shedding. We are not aware of any studies that have assessed the use of the tannin containing forage sainfoin (*Onobrychis viciifolia*) for its ability to reduce fecal shedding of *E. coli* under western Canadian winter conditions. Sainfoin can support high levels of animal production (Cavallarin et al. 2007; Wang et al. 2007), but the long-term effects of feeding a phytochemical/tannin containing forage on *E. coli* shedding are not known. This may be a concern because there is now evidence that tannin resistant strains of *E. coli* have been isolated from the digestive tract of ruminants (Smith et al. 2005; Zoetendal et al. 2008). In this research, we hypothesized that phenolic compounds and sainfoin extracts would reduce the survival of pathogenic and non-pathogenic *E. coli in vitro.* Additionally, we hypothesized that feeding sainfoin to feedlot cattle during a Canadian winter would reduce generic *E. coli* shedding in feces.

4.3 MATERIALS AND METHODS

Bacterial cultures. *E. coli* O157:H7 (human isolate, isolated in 2006) was obtained from Dr. Karlowsky (Clinical Microbiology, Health Sciences Centre, Winnipeg, Manitoba). *Escherichia coli* 12 and 13 (human isolates, isolated in March 2005) are from our own laboratory collection (Department of Animal Science, University of Manitoba). Both *E. coli* strains were isolated from the digestive tract of ulcerative colitis patients. A ciprofloxacin resistant strain of *E. coli* O157:H7 (*E. coli* O157:H7 cip^r) was isolated by daily transfers of 500 µL of an overnight culture of *E. coli* O157:H7 to 9.5 mL of fresh Mueller-Hinton broth (Becton Dickinson, Sparks, MD, USA) with a ciprofloxacin (Fluka, Buchs, Switzerland, UK) concentration starting at 0.005 µg mL^{-1}. The ciprofloxacin concentration increased daily by 2-fold up to a final concentration of 2.6 µg mL^{-1}.

MIC of phenolic acids. Ferulic acid (Fluka, Milwaukee, WI, USA), *para*-coumaric acid (Sigma-Aldrich, St-Louis, MO, USA), and *trans*-cinnamic acid (Sigma-Aldrich, St-Louis, MO, USA) were each solubilized in 100 % acetone to a concentration of 15 mg mL^{-1} and further diluted with sterile 0.9 % NaCl to a final concentration of 4 mg mL^{-1}. Sterile 96-well plastic plates with lids (Corning, NY, USA) were used to perform the assay. To each well, with the exception of the first well in each column (top row), 50 µL of Mueller-Hinton broth (Becton Dickinson) was added. To the first well in each column, 150 µL of a test compound solution was added and serially diluted by 1.5-fold increments for the remaining wells in the column. The acetone concentration in the starting well for each compound (first well in each column) was no more than 25 %. To ensure that acetone concentrations did not

inhibit bacterial growth, an acetone control with an initial concentration of 25 % was run simultaneously in the same plate using the same dilutions as used in the compound assays.

Each culture was prepared by selecting four, well separated colonies from a one day-old Eosin Methylene Blue (EMB, Becton Dickinson) agar plate into Mueller-Hinton broth and incubated overnight at 37 °C. The cultures were adjusted to achieve a turbidity equal to that of a 0.5 McFarland Standard (National Committee for Clinical Laboratory Standards 2002) using Mueller-Hinton broth. The cultures were further diluted and added to each well to obtain a final concentration of 5×10^5 CFU mL^{-1} (National Committee for Clinical Laboratory Standards 2002). In the same plate, a standardization control was also performed using ciprofloxacin (Fluka) at a starting concentration of 0.05 µg mL^{-1}. Only one bacterial strain (*E. coli* O157:H7, *E. coli* 12, and *E. coli* 13) was assayed per plate but all test compound solutions were assayed against each strain. Each plate assay was done in duplicate. The plates were covered, sealed with Parafilm to prevent evaporation, and incubated at 37 °C for 16-20 h. Following the incubation period, 40 µL of a 0.2 mg mL^{-1} colourless solution of *p*-iodonitrotetrazolium violet (p-INT, Sigma-Aldrich) was added in each well as an indicator of viability (Eloff 1998a). The plates were incubated at 37 °C for 1 h. A color change to violet/red (dye reduction) indicated the presence of viable cells, while a lack of color change represented no bacterial activity. The MIC value was determined from the well with the lowest concentration of antimicrobial with no color change.

Growth of forage. Sainfoin (*Onobrychis viciifolia*) and alfalfa (*Medicago sativa*) were grown at the Brandon Research Station (Brandon, Manitoba, Canada). The forage crops were harvested at the late bud-first flower growth stage and a second cut (regrowth)

was also harvested at the late bud-first flower growth stage and made into hay or silage bales. Silage bales were slipped into a plastic silage tube wrapping, and hay was made by sun drying the forage before baling.

Forage preparation, extraction, and condensed tannin measurements. Forage samples (sainfoin silage 1st and 2nd cut, sainfoin hay 1st and 2nd cut, and alfalfa silage 2nd cut) were freeze-dried and ground through a 0.5mm screen using a sample mill (Tecator, Cyclotec 1093, Hoganas, Sweden), A crude extract was prepared by shaking the ground plant material (60 g) with 600 mL of 70 % acetone for 2 h at room temperature in the dark followed by centrifugation at 210 × *g* for 10 min to remove all plant material (Eloff 1998b). Extracts were then dried by rotary evaporation, weighed, re-solubilized with 100 % acetone, and diluted by 50 % with sterile water. Extractable condensed tannins in the plants were extracted, purified, and measured using the butanol/HCl method as outlined in Terrill et al. (1992) and Berard et al. (2011).

MIC of sainfoin and alfalfa extracts. Each extract was serially diluted with Mueller-Hinton broth in 96-well plates using the same method as previously described for the phenolic MIC assay. The starting dried extract concentration for each extract was 100 mg mL^{-1} in the first well of the column and was serially diluted by 1.2-fold increments for the remaining wells in the column.

Fecal incubation assay. Rectal fecal samples were collected from 10 steers fed an alfalfa hay diet and pooled together. A subsample (1g) of feces was taken, diluted 10-fold in buffered peptone water, and assayed on a Tryptone Soya Agar (Oxoid, Hampshire, England) plate containing 1.5 μg mL^{-1} ciprofloxacin to test for the presence of ciprofloxacin resistance

before adding *E. coli* O157:H7 cip^r to the samples. Three subsamples (2 g) of feces were taken to determine dry matter content and pH. The pooled sample was subdivided into four portions weighing 1,245 g each and placed into large zip-lock plastic bags. Each sample was assigned to a treatment: coumaric acid + *E. coli* O157:H7 cip^r, ground sainfoin 1st cut + *E. coli* O157:H7 cip^r, no additive + *E. coli* O157:H7 cip^r, or a negative control (no *E. coli* O157:H7 cip^r or additive added). Two samples were assigned to each treatment.

Bacterial inoculation. *E. coli* O157:H7 cip^r was isolated from a one-day old EMB agar plate containing 1.5 µg mL^{-1} of ciprofloxacin and inoculated in six tubes of Tryptone Soya Broth (10 mL) containing 1.5 µg mL^{-1} of ciprofloxacin. Cultures were held in a shaking incubator overnight at 37 °C. Enumeration of all cultures was performed on EMB agar plates containing 1.5 µg mL^{-1} ciprofloxacin. The cultures were centrifuged at 3,300 × *g* for 10 min and the supernatant was removed. The bacterial pellet was resuspended in sterile water and the slurry from each tube was added to three of the four fecal samples (the fourth sample was a negative control). Each bag received the slurry from two tubes to ensure that the bacterial concentration would reach approximately 10^9 cells g^{-1} of feces. The bags were hand massaged for approximately 5 min to ensure thorough mixing. A subsample (1 g) from each bag was taken to quantify *E. coli* O157:H7 cip^r added using EMB agar plates containing 1.5 µg mL^{-1} ciprofloxacin.

Formulation of additives in fecal samples. The pH of all fecal samples was measured after the addition of compounds or extracts. Two sample bags received 1 g (0.5 % w/w) of either *p*-coumaric acid or ground sainfoin (silage 1st cut) and hand massaged for approximately 5 min. The pH of all the samples after the addition of additives was measured

and three subsamples (1 g) from each sample were taken to measure dry matter. Another subsample (1 g) was taken and used to quantify *E. coli* O157:H7 cip^{r} after the addition of the additives. The four sample bags were further subdivided into 6-200 g samples. Each treatment was also subjected to one of three temperatures (in duplicate): 5 °C, 37 °C, and freeze (-20 °C)/thaw (5 °C) for 14 d and sampled at 48 h, 1 wk, and 2 wk post-inoculation. In the freeze/thaw treatment, samples were immediately frozen at -20 °C. Duplicate subsamples were removed from the frozen samples at each sampling time and thawed for 16 h at 5 °C to simulate a spring thaw.

***E. coli* O157:H7 cip^{r} quantification.** Two 0.5 g subsamples were taken from each bag and mixed with 4.5 mL of sterile buffered peptone water (BPW; Becton Dickinson) and serially diluted 10-fold in buffered peptone water. Plate counts were performed in duplicate for each sample on EMB containing 1.5 μg mL^{-1} ciprofloxacin. Concurrently, pH measurements were taken of all samples. When *E. coli* O157:H7 cip^{r} could no longer be recovered, a resuscitation step was performed, where samples were diluted in BPW (0.5 g in 4.5 mL) and were incubated at 5 °C overnight and re-plated on EMB agar with and without 1.5 μg mL^{-1} ciprofloxacin in an attempt to allow damaged cells, if any were present, to recover.

Animal trial. Animals were cared for in accordance with the guidelines of the Canadian Council of Animal Care (1993). The beef cattle trial took place from January to March 2007 with a 2-wk adaptation period and a 9-wk experimental period using 40 beef steers (ten animals per pen). Steers were housed in outdoor pens and fed one of four dietary treatments: sainfoin silage or hay, alfalfa silage or hay. The 1st cut forages were mixed with

the 2nd cut forages to obtain more consistent nutrient profiles and CT concentrations throughout the feeding period. Animals were blocked by weight into dietary treatments and fed an alfalfa silage (pen 1 and 3) or hay (pen 2 and 4) diet for a two week adaptation period. Following the alfalfa adaptation period, two pens of cattle were adapted to the sainfoin diets (pen 1: silage and 2: hay) using the following sainfoin to alfalfa ratio: 25:75 for 2 d, 50:50 for 2 d, 75:25 for 2 d, and 100 % sainfoin thereafter. Animals were fed twice daily *ad libitum* and water was available *ad libitum* throughout the entire trial period. Feed intake was measured with feeders equipped with calibrated automatic weigh scales (GrowSafe Systems, Alberta, Canada) so that accurate individual animal feed intake measurements could be obtained throughout the experiment.

Fecal grab samples (approximately 500 g) were taken once per week during weeks 1-2, weeks 4-7, and biweekly thereafter over an 11-week period. A subsample (approximately 3 g) of feces from each animal was pooled by pen following sampling. Pooled samples from each pen were subjected to culture-based quantification of generic *E. coli* in triplicate. More specifically, three 1 g subsamples were taken from each pooled sample and separately mixed with 9 mL of sterile buffered peptone water (BPW; Becton Dickinson) and serially diluted 10-fold in buffered peptone water and plated on chromogenic *E. coli* plates (Hardy Diagnostics, HardyCHROM Coliform EC, Santa Maria, CA, USA).

Fecal dry matter. Three subsamples (3 g) were taken from each sample, weighed, dried in an oven at 60 °C overnight, and re-weighed to determine fecal dry matter.

Statistics. All the statistical analyses were performed using the statistical software SAS (SAS 9.1; SAS, Cary, NC). All MIC (for pure phenolic compounds and extracts) and

pH (from the feces incubation assay) data were analyzed using PROC Means. The Student's paired-t test ($P < 0.05$) was used to determine statistical significance between treatment means. Bacterial numbers from the fecal incubation experiment as well as from the animal trial were transformed to $\log_{10}$ equivalents and subjected to a repeated measures analysis of variance using PROC Mixed. Tukey–Kramer's multiple comparisons test was used to determine significant differences ($P < 0.05$) within a treatment (between time points) and between treatment means. Death rates of *E. coli* O157:H7 cip^r in the feces incubation experiment were determined by using the slope value from the linear regressions which were fit using least-square means of *E. coli* O157:H7 cip^r for each treatment.

4.4 RESULTS

MIC of phenolic compounds. The MIC values for ferulic, cinnamic, and coumaric acids were approximately 10^5-fold less inhibitory than ciprofloxacin (Table 4.1) and there was no significant differences among *E. coli* strains. Coumaric (1.2 mg mL^{-1}) and cinnamic acids (1.4 mg mL^{-1}) were the most, and ferulic acid (2.8 mg mL^{-1}) the least inhibitory. The MIC values for ferulic, cinnamic, and coumaric acids were 10 to 20-fold more inhibitory than for sainfoin and alfalfa extracts (Table 4.1) and the greatest inhibitory effect was with the least mature forage (1st cut). Sainfoin silage and hay 1st cut were the most inhibitory extracts, but we chose to use the silage in subsequent experiments because silage is the preferred forage preservation technique in Canada (Table 4.1). Condensed tannin concentrations in the forage were (mg g^{-1} DM): sainfoin silage 1st cut, 9.02; sainfoin silage 2nd cut, 15.58; sainfoin hay 1st cut, 8.39; sainfoin hay 2nd cut, 23.38.

Table 4.1. Minimum inhibitory concentration (MIC) of purified phenolic compounds, sainfoin and alfalfa extracts against various *Escherichia coli* isolates

Antimicrobial compounds	MIC ($mg\ mL^{-1}$)[4]		
	E. coli O157:H7[1,3]	*E. coli* 12[1,3]	*E. coli* 13[2,3]
Ciprofloxacin[a]	$2.1 \times 10^{-5} \pm 0.005$	$1.0 \times 10^{-5} \pm 0.001$	$1.3 \times 10^{-5} \pm 0.003$
Coumaric acid[b]	1.1±0.21	1.3±0.01	1.3±0.01
Cinnamic acid[b]	0.99±0.20	1.49±0.30	1.78±0.01
Ferulic acid[c]	2.60±1.41	2.89±1.11	2.89±1.11
Sainfoin silage 1st cut[d]	25.60±2.3		
Sainfoin silage 2nd cut[de]	33.50±0.2		
Sainfoin hay 1st cut[d]	25.60±2.3		
Sainfoin hay 2nd cut[e]	36.85±3.35		
Alfalfa silage 2nd cut[e]	40.85±7.35		

[1] *E. coli* isolate from phylogenetic group B2 fimbria type H10.

[2] Ulcerative colitis *E. coli* isolate from phylogenetic group D fimbria type H6.

[3] *E. coli* spp. belonging to phylogenetic group B2 and D are extra intestinal pathogenic where B2 is the more virulent group.

[4] MIC units for forage extracts are mg of extract mL^{-1}

[a-e] Means within a row were tested for significance using the Student's t test. Different lettered superscripts are significant at $P<0.05$.

Fecal incubation assay. Ciprofloxacin resistant *E. coli* O157:H7 (*E. coli* O157:H7 cip^r) was not isolated from the cattle feces and *E. coli* O157:H7 cip^r seeded into feces could be recovered without background bias. There was no difference in growth rate between *E. coli* cip^r and wild type *E. coli* O157:H7 (unpublished data). *Escherichia coli* O157:H7 cip^r was recovered when seeded into cattle feces at approximately 10^9 cells mL^{-1} at time zero and declined in all experiments thereafter, but the rate of decrease depended on the treatment (Fig. 4.1). At d 14, neither *E. coli* O157:H7 cip^r *nor* generic *E. coli* could be recovered in any of the treatments. *Escherichia coli* O157:H7 cip^r declined the least within the 5 °C treatment and had the greatest decline at -20 °C treatment. These results were independent of the inclusion of coumaric acid or ground sainfoin silage (Fig. 4.1). Dry matter and pH did not vary significantly between treatments ($P < 0.05$).

Animal trial. The animal trial took place during the winter, and ambient temperatures ranged from -27 to -6 ˚C (Fig. 4.2A) with a wind chill effect as low as -40 ˚C on some days. Feed intake varied significantly ($P < 0.05$) between treatment groups and weeks. Animal performance did not vary significantly during the trial ($P < 0.05$).

In addition, feed intake and temperature were significantly ($P < 0.05$) correlated in the silage-fed (sainfoin and alfalfa) animals but not in the hay-fed (sainfoin and alfalfa) animals (Table 4.2). Generic *E. coli* numbers varied approximately 2 logs during the experiment (Fig. 4.2B) and the sainfoin-fed (silage and hay) cattle had the lowest generic *E. coli* numbers overall (Fig. 4.3). The CT concentration (mg g^{-1} DM) for each diet following mixing of 1st and 2nd cut forage is as follows: alfalfa silage, 1.75; alfalfa hay, 1.11; sainfoin silage, 11.92; and sainfoin hay, 10.54.

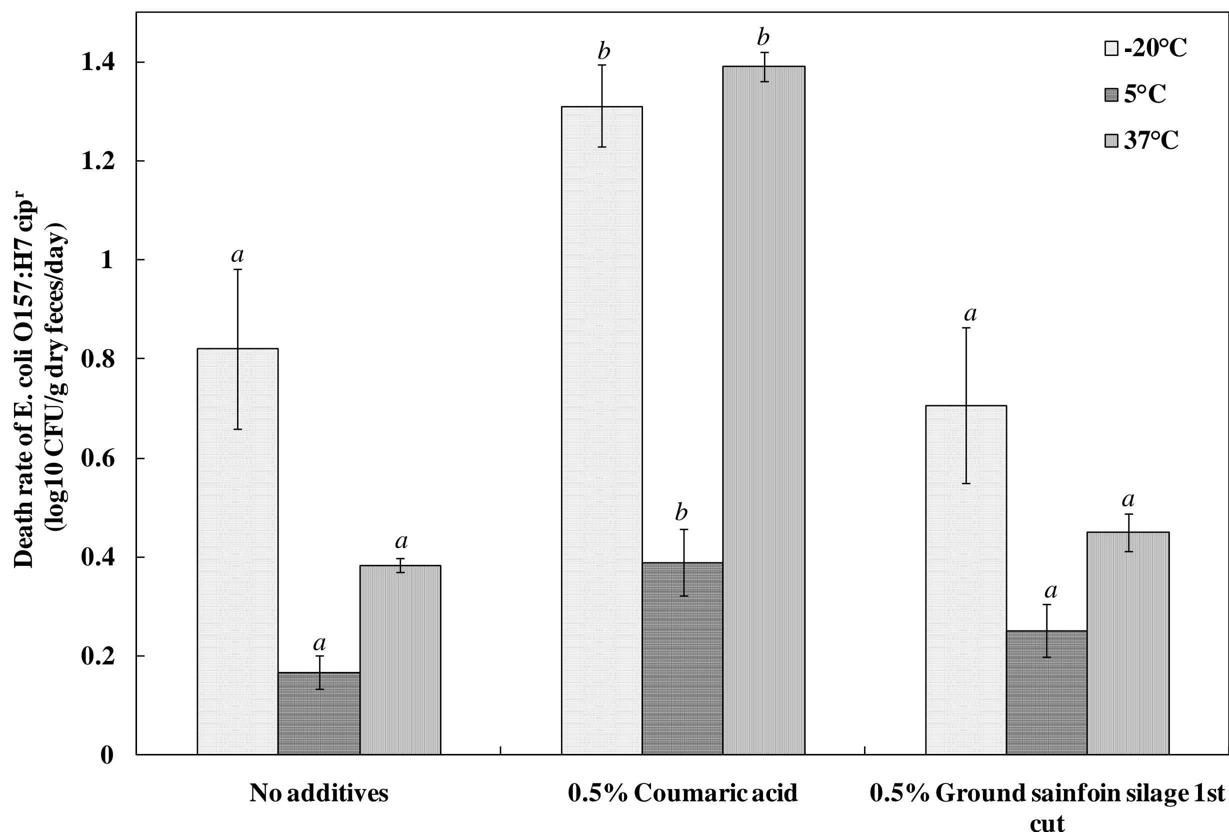
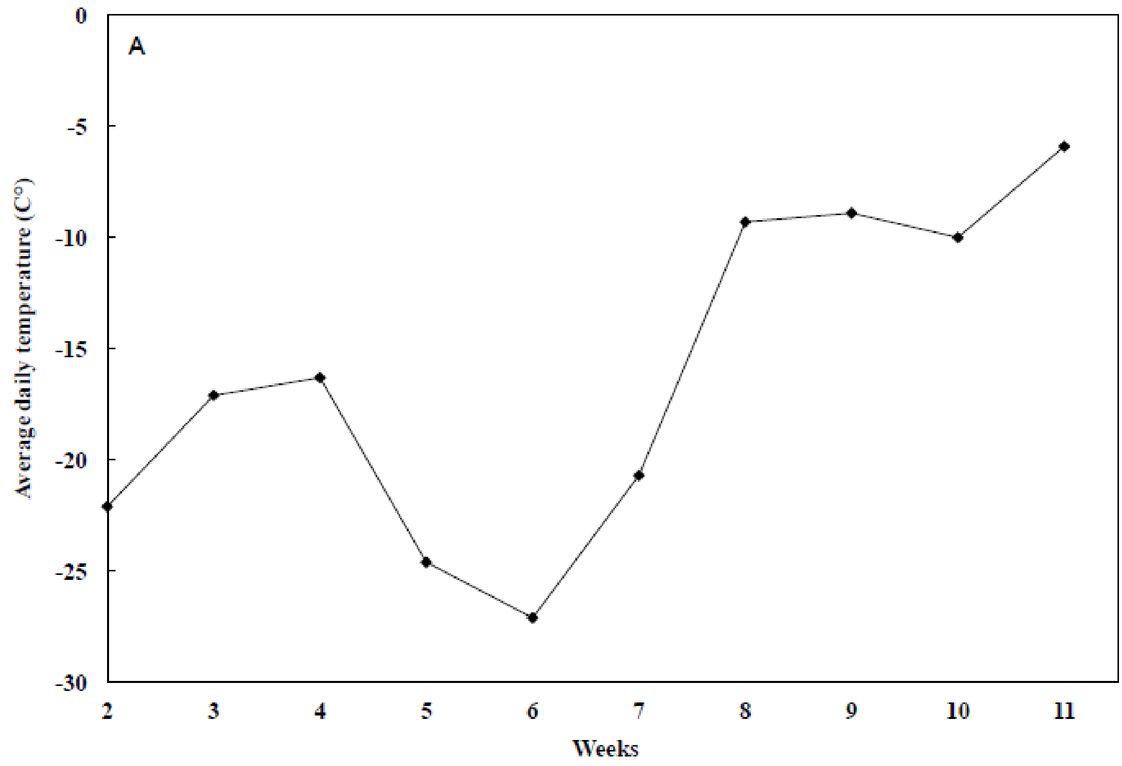


Figure 4.1. Death rates of *E. coli* O157:H7 cip^r cells inoculated in cattle feces with the addition of either 0.5 % (w/w) ground sainfoin silage or 0.5 % (w/w) *p*-coumaric acid and incubated at different temperatures (-20, 37, or 5 °C). Death rates (log_{10} CFU g^{-1} dry feces day^{-1}) were calculated as the slope from the average linear regression of each additive and temperature treatment (Fig. 4.2A and B). Results shown are death rate averages with respective standard errors of the mean.


A
Average daily temperature (C°)
0
-5
-10
-15
-20
-25
-30
2
3
4
5
6
7
8
9
10
11
Weeks

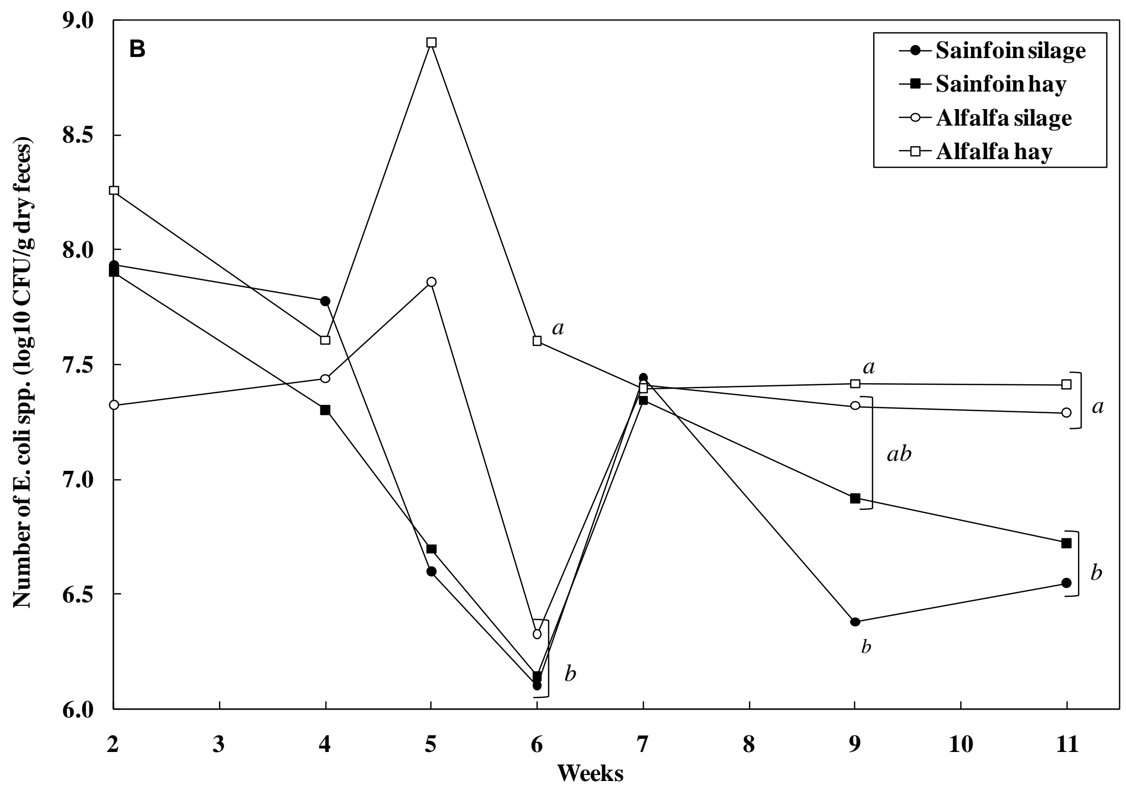

B
Sainfoin silage
Sainfoin hay
Alfalfa silage
Alfalfa hay
Number of E. coli spp. (log10 CFU/g dry feces)
9.0
8.5
8.0
7.5
7.0
6.5
6.0
a
a
a
ab
b
b
b
2
3
4
5
6
7
8
9
10
11
Weeks

Figure 4.2. Effect of outdoor temperature (A) and diet (B) on the viability of fecal *E. coli* spp. over time. Outdoor temperature data (°C) during the trial (A) are presented to show the apparent relationship between outdoor temperature and fecal *E. coli* numbers ($\log_{10}$ CFU g^{-1} dry feces) (B). Temperature data were from Environment Canada using the Winnipeg-Forks location (http://www.climate.weatheroffice.ec.gc.ca/climateData/canada_e.html) (A) and are presented as mean weekly temperature. Fecal samples were collected from animals fed one of 4 diets: sainfoin silage (-◆-), sainfoin hay (-■-), alfalfa silage (-◇-), and alfalfa hay (-□-). Week 2 is representative of the control period where all animals were fed an alfalfa (hay or silage) diet prior to the start of the experimental diets. Fecal *E. coli* spp. numbers are a pooled average for each treatment. Statistical significance ($P < 0.05$) between means is denoted by different letters. Bracketed points have the same letter(s).

Table 4.2. Animal trial: Correlations between animal intake, outdoor temperature, and fecal *E. coli* numbers

Diets	Intake vs. mean daily temperature		Intake vs. *E. coli* spp. numbers		Mean daily temperature vs. *E. coli* spp. numbers	
	Correlation	P-value	Correlation	P-value	Correlation	P-value
Sainfoin silage	0.63734	0.0001	0.19484	0.3974	-0.11526	0.6188
Sainfoin hay	0.07155	0.5561	-0.00879	0.9707	0.09696	0.6842
Alfalfa silage	0.32828	0.0055	0.31131	0.1815	0.33435	0.1496
Alfalfa hay	0.01088	0.9288	-0.5195	0.0189	-0.14728	0.5355

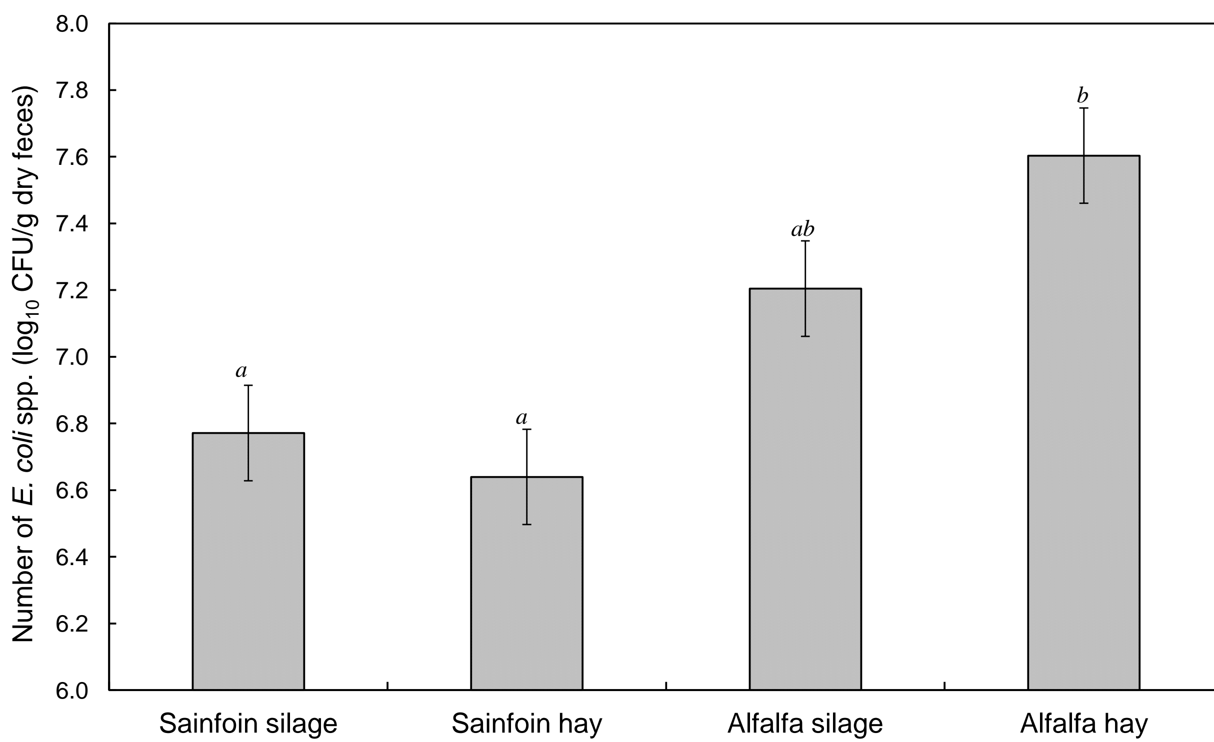


Figure. 4.3. Average fecal *E. coli* spp. numbers from feces of cattle fed either a sainfoin (silage or hay) or alfalfa (silage or hay) diet. Average *E. coli* spp. numbers (log_{10} CFU g^{-1} dry feces) were measured in fecal samples pooled by treatment (diet). Fecal *E. coli* spp. numbers were subsequently averaged per treatment during the experimental period (wk 4 to 11). Data presented are averages with their respective standard error of the mean shown as vertical lines. Statistical significance ($P < 0.05$) between means is denoted by different letters.

4.5 DISCUSSION

We could demonstrate that coumaric and cinnamic acid were more efficacious than ferulic acid at inhibiting three strains of *E. coli* (Table 4.1). Duncan et al. (1998) demonstrated that *E. coli* O157:H7 was inhibited by the coumarins esculetin, umbelliferone, and scopoletin. Olasupo et al. (2003) found that carvacrol, cinnamic acid, diacetyl, eugenol, and thymol were effective at inhibiting *Salmonella typhimurium* and *E. coli*.

Friedman et al. (2003) evaluated compounds with hydroxy and methoxy benzenoid ring substitutions against *E. coli*, *Listeria monocytogenes*, *Campylobacter jejuni*, and *S. enterica*. Increased hydroxylation of a compound enhanced its inhibitory activity while an added methoxy group either enhanced or had no effect on bactericidal activity. Grbic-Galic (1986) demonstrated that *E. coli* could degrade ferulic acid under anaerobic conditions, thus potentially explaining the reduced MIC value of ferulic acid (Table 4.1).

Our *in vitro* results (Fig. 4.1) indicate that freeze-thaw (-20 °C treatment) had a significant effect in reducing *E. coli* O157:H7 cip^r numbers and could be accelerated by the presence of phytochemicals. In contrast, the incubation at 5 and 37 °C resulted in almost no reduction in *E. coli* O157:H7 cip^r numbers in the no additive treatments (Fig. 4.1). The aforementioned suggests that defecation during spring (5 °C) would result in extended *E. coli* survival (Fig. 4.1). In contrast, Kudva et al. (1998) found that *E. coli* O157:H7 inoculated in bovine feces survived the longest at -20, 4, and 23 °C and that at 37 °C, a rapid decline in *E. coli* O157:H7 numbers could be seen. Viability of *E. coli*

O157:H7 inoculated in untreated bovine manure slurry increased at temperatures below 23 °C. In their bovine fecal samples stored at -20 °C, *E. coli* O157:H7 could still be detected after 100 d but not in the bovine untreated slurry samples. This potentially indicates that dry matter content and the type of effluent waste such as fresh feces (solid, no urine) has a different physiological effect on *E. coli* compared to a manure slurry which has a low solid content and contains urine.

Our results (Fig. 4.1) on increased death of *E. coli* O157:H7 cip^r during freezing in the presence of phytochemicals are supported by the literature. Freezing is commonly used in the food industry to reduce the microbial contamination of food (Ingham et al. 2006; Sage and Ingham 1998; Yamamoto and Harris 2001) and can even be used to reduce *E. coli* survival in wastewater (Gao et al. 2006). Cressy et al. (2003) demonstrated that combining the essential oil, isoeugenol with freeze-thawing increased the death of *Listeria monocytogenes*. Pérez-Conesa et al. (2006) demonstrated that carvacrol and eugenol reduced the survival of both *L. monocytogenes* and *E. coli* in biofilms.

The ciprofloxacin resistant strain used is a good model for pathogenic *E. coli*. We base this on the following facts: 1) In Table 4.1, the *E. coli* O157:H7 was the same strain used to develop the *E. coli* O157:H7 cip^r strain and there were no differences ($P < 0.05$) in antimicrobial responses among strains (Table 4.1). 2) When *E. coli* cip^r could not be cultured at the end of the fecal incubation experiment, we attempted to culture generic *E. coli* to ensure that the *E. coli* cip^r strain was in fact an accurate representation of generic *E. coli*; we could not grow generic *E. coli* either. 3) There was no difference in growth rate between *E. coli* cip^r and the wild type. The main reason for using a ciprofloxacin

resistant strain (which is a natural mutant generated in our laboratory) was to make it is easier to isolate and differentiate from the rest of the bacterial population.

Our *in vivo* results are in agreement with the work of Wells et al. (2005) who demonstrated that phenolic-containing forages inhibit the growth of *E. coli* O157:H7 *in vitro*. In our *in vivo* experiment, we quantified generic *E. coli* as opposed to *E. coli* O157:H7. Generic *E. coli* was used in our *in vivo* experiment in order to better assess a ruminant gut adapted population and to eliminate inoculation effects. The inhibitory effects could be maintained for 9 wks in an outdoor feedlot trial (Fig. 4.2B and 4.3) in a Canadian winter even when the effects of extreme wind chill were included (Fig. 4.2A). Apparently, the adaptation observed in generic *E. coli* to tannins (Smith et al. 2005; Zoetendal et al. 2008) did not occur (Fig. 4.2B). The numbers of generic *E. coli* when plotted on a weekly basis are highly variable and resulted in a reduction of statistical power. However, if the values are averaged across the experiment, the numbers are numerically lower for sainfoin versus alfalfa although not statistically different from alfalfa silage. In general, the numbers of generic *E. coli* we obtained were similar to that of other investigators who have fed forage-based diets to cattle (Callaway et al. 2003).

The decline in generic *E. coli* numbers at low temperatures (Fig. 4.2) is difficult to explain. Barboza et al. (2006) studied muskoxen and rumen function adaptation in cold temperatures. They found that the passage rate of the fluid phase during cold temperatures increased. The rational for this is that because the animals are cold they need more energy, which in ruminants comes primarily from VFA absorption in the rumen (Flint et al. 2008; Russell and Rychlik 2001). As discussed in a review by Brosh (2007), during extreme cold temperatures, the body must expend more energy to maintain

body temperature, therefore, the animal's metabolism increases as does feed intake.

In our study, we found that feed intake varied significantly ($P < 0.05$) between weeks and diet treatments. Upon statistical analysis, we found that temperature and feed intake were significantly ($P < 0.05$) correlated for both silage diets but not for the hay diets (Table 4.2 and Fig. 4.2A and B). We speculate that feed intake declined during extreme cold temperatures in the silage-fed animals as a result of the feed being frozen (higher moisture content in silage). In contrast, feed intake in the hay-fed cattle actually increased in response to the extreme cold temperatures.

We suspect that the reason that there was no *E. coli* adaptation to the sainfoin was because of the presence of multiple compounds in sainfoin. Barrau et al. (2005) demonstrated that most of the antilarval activity in sainfoin was in the acetone-water extract which contained condensed tannins and flavonol glycosides rutin, nicotiflorin, and narcissin. In addition, Lu et al. (Lu et al. 2000) characterized seven cinnamic acid derivatives and nine flavonoid glycosides among the low molecular weight extracts in sainfoin.

Although the above discussion emphasized the direct interaction of tannins or phytochemicals with *E. coli*, an alternative mode of action can be hypothesized. Studies have shown that condensed tannins are inhibitory to structural carbohydrate fermenting bacteria (e.g. *Ruminococcus albus*, *R. flavefaciens*, and *Fibrobacter succinogenes*) (Flint et al. 2008; McSweeney et al. 2001b). Tannins may inhibit these bacteria and consequently reduce the release of soluble sugars from the plant cell wall matrix which could serve as a carbon source for *E. coli* (Flint et al. 2008). Thus, we suspect that sainfoin could have a dual effect in that it serves to release inhibitory compounds, but

also reduces the availability of soluble sugars to *E. coli*by inhibiting cellulolytic bacteria. In comparison, alfalfa has very low concentrations of CT and was thus expected to have a lower inhibitory activity against *E. coli*.

We concluded from this study that sainfoin when added to cattle diets and fed for several months reduced generic *E. coli* numbers in fresh feces and that no adaptation occurred as the reduction in numbers persisted over the course of the 11-wk trial. The proposed mode of action is likely via the combined action of phenolic and flavonol glycosides which have antimicrobial activity (Lu et al. 2000; Wells et al. 2005). By virtue of their different chemical structures, the modes of antimicrobial action are probably different. Consequently, resistance in a relatively short period of time is not likely to develop. One of the most interesting observations was that phytochemicals could enhance the death of *E. coli* O157:H7 when present in the -20°C freeze-thaw treatment an observation that could potentially have significant implications for manure management under spring and winter conditions in western Canada. Further studies are required to confirm whether pathogenic *E. coli* O157:H7 shedding could be reduced in a ruminant production system as was seen in our *in vivo* study where generic fecal *E. coli* shedding was reduced.

BRIDGE TO CHAPTER FIVE

In the previous chapter, the aim was to determine whether phenolic compounds found in sainfoin, sainfoin extracts, and whole plant sainfoin when fed to cattle could inhibit and reduce fecal *E. coli* shedding. The results indicate that when fed to cattle, sainfoin induced a slight decrease in generic fecal *E. coli* shedding as compared to alfalfa. However, in the *in vitro* portion of the study, secondary plant compounds whether in the form of a purified formulation or plant extract were considerably less inhibitory than ciprofloxacin, a synthetic antimicrobial.

When antimicrobial resistance emerges, it is a common practice in clinical settings to utilize combination therapy in an attempt to overcome resistance mechanisms in bacteria. Given that secondary plant compounds are not as effective as synthetic antimicrobials, the next chapter explores the use of combination therapy as a mean of reducing the MIC and deterring the development of resistance to synthetic antimicrobials in the presence of phytochemicals.

CHAPTER FIVE

MANUSCRIPT III

Bet hedging response increases antimicrobial resistance in combination therapy

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5.1 ABSTRACT

The rise in antimicrobial resistance has led to an increased interest in secondary plant compounds as an alternative to synthetic antimicrobials. However, previous work shows that many secondary plant compounds are not as inhibitory as synthetic antimicrobials. Thus, a common clinical practice has been to use combination therapy as a means to deter antimicrobial resistance mechanisms from developing. Importantly, bacteria have a remarkable ability of adapting to adverse environmental conditions such as exposure to therapeutics, thus it is likely that resistance will also ensue in combination therapy. In this study, we explored the implications of using secondary plant compounds and ciprofloxacin in combination therapy to retard the development of resistance. A total of 12 plant compounds and seven essential oils were screened for their inhibitory activity against *E. coli* O157:H7. Of the surveyed plant compounds, geraniol oil, was deemed to have the lowest toxicity and displayed synergistic interactions with ciprofloxacin. In a long-term resistance development assay with geraniol and ciprofloxacin, geraniol did not reduce the development of ciprofloxacin resistance in *E. coli* O157:H7, but rather increased its level of resistance against ciprofloxacin as measured by a 63-fold increase in the MIC vs. a 32-fold increase in the MIC when resistance developed to ciprofloxacin alone. Herein, we propose that this highly fit phenotype arose as a byproduct of a “bet-hedging” strategy in a clonal population.

5.2 INTRODUCTION

Secondary plant metabolites harboring antimicrobial properties have been increasing in popularity in the wake of increasing antimicrobial resistance in several emerging pathogens and a decline in the development of novel antimicrobial agents within the pharmaceutical industry (Overbye and Barrett 2005). In addition, the agriculture sector has been under harsh scrutiny due to the potential for food-producing animals to act as reservoirs for antimicrobial resistance genes and as vectors for transmission of these genes to humans (Aarestrup et al. 2008). In light of these concerns, the World Health Organization has categorized antimicrobials based on their importance in human medicine as a means to mitigate their use in animals. Macrolides and quinolones, for example, have been categorized as “critically important antimicrobials”. However, the macrolide tylosin is one of the most commonly used antimicrobial growth promoters in the North American swine industry (Kalmokoff et al. 2011). The quinolones are of high clinical importance in human medicine due to their broad-spectrum activity against both Gram-negative and -positive organisms. For this reason, quinolones are generally not used as dietary growth promoters however, they are approved for therapeutic use in veterinary medicine (World Health Organization 2007).

One strategy to overcome antimicrobial resistance is the use of combination drug therapy. A widely used combination in human medicine includes the use of a ß-lactam with a ß-lactamase inhibitor to suppress the action of ß-lactam degrading enzymes produced by bacteria (Lee et al. 2003). However, frequent use of ß-lactamase inhibitors has allowed bacteria to adapt and overcome their effects (Chaibi et al. 1999). In this

regard, it has become important that new strategies are developed to extend the "shelf life" of current antimicrobials. In this study, the aim was to explore the effectiveness of combined natural plant antimicrobials in an effort to curb bacterial resistance.

From previous work, we demonstrated that an ethanol extract from sainfoin (*Onobrychis viciifolia*), a tannin-rich plant, and structurally related pure compounds (cinnamic, coumaric, and ferulic acid) had antimicrobial properties. However, their potency was significantly less than that of ciprofloxacin, a fluoroquinolone (Berard et al. 2009). As a result of their relatively low potency, these natural plant compounds cannot be used as standalone antimicrobial agents. It is for this reason that combination therapy with synthetic antimicrobials has been explored by a number of researchers (Braga et al. 2005; Hu et al. 2001).

As consumers become increasingly aware of the issues surrounding antimicrobial resistance, demand for organic and "natural" food products has progressively risen (Jacob et al. 2008). In an effort to increase food safety, antimicrobial plant compounds, whether in the form of plants or as semi-purified preparations, could be fed to food-producing animals to reduce pathogen shedding, such as *E. coli* O157:H7. However, the use of plant compounds in animal production systems may promote resistance development to antimicrobial agents of critical importance to human health such as ciprofloxacin. It is recognized that while quinolones such as ciprofloxacin are not approved for use as growth promotants in Canadian ruminant livestock production, the low levels of quinolone resistance seen in cattle (Pereira et al. 2011) and the quinolones' critical importance to human medicine makes ciprofloxacin a good model for these types of studies. In this research we explored the potential of plant phenolic compounds to reduce

the development of bacterial resistance to ciprofloxacin.

5.3 MATERIALS AND METHODS

Plant compounds. A total of twelve plant compounds were screened in this study. These compounds included thymol (Fisher Scientific Canada, Ottawa, ON), catechol (Fisher Scientific), pyrogallol (Fisher Scientific), piperine (Sigma-Aldrich, St. Louis, MO), 1-aminocyclopropane-1 carboxylic acid (ACAA) (Sigma-Aldrich), anthraquinone (Fisher Scientific), quercetin (Fisher Scientific), paclitaxel (Sigma-Aldrich), hypericin (Sigma-Aldrich), artemisinin (Sigma-Aldrich), galanthamine hydrobromide (Sigma-Aldrich) and caffeic acid (Sigma-Aldrich). The following essential oils were also screened for inhibitory activity: cinnamon bark oil (Sigma-Aldrich), citral (Sigma-Aldrich), bay oil (Sigma-Aldrich), geraniol (Sigma-Aldrich), clove bud oil (Sigma-Aldrich), thyme oil (white) (Sigma-Aldrich), and origanum oil (Sigma-Aldrich).

Bacterial strain. *E. coli* O157:H7 (human isolate; isolated in 2006) was obtained from Dr. James Karlowsky (Clinical Microbiology, Health Sciences Centre, Winnipeg, Manitoba, Canada).

Rapid screening assay. The twelve plant compounds were screened for their inhibitory activity against *E. coli* O157:H7 at three different concentrations (0.5, 2, and 5 mg mL^{-1}) with the exception of pyrogallol where concentrations of 0.125 and 0.25 mg mL^{-1} were used as no growth could be detected at 0.5 mg mL^{-1}. Stock solutions (50 mg mL^{-1}) were made for all plant compounds using appropriate solvents to enhance solubility. The *E. coli* O157:H7 culture used for inoculation was prepared by picking four well-separated colonies from a 1-day-old eosin-methylene blue (EMB; Becton Dickinson, Sparks, MD) agar plate, suspending the colonies into Mueller-Hinton broth (MHB; Oxoid, Cambridge,

UK) and incubating the suspension overnight at 37˚C. The *E.coli* suspension was adjusted to achieve turbidity equal to that of a 0.5 McFarland standard (~10^8 CFU mL^{-1}) using MHB (National Committee for Clinical Laboratory Standards 2002). The diluted bacterial suspension was further diluted to 5 x 10^7 CFU mL^{-1} with MHB to create a stock solution. For each phytochemical, a 0.1 mL aliquot of the bacterial stock (5 x 10^7 CFU mL^{-1}) was added to a tube containing 9.9 mL of a phytochemical diluted with MHB to achieve a final concentration of 5 x 10^5 CFU mL^{-1} (National Committee for Clinical Laboratory Standards 2002). The antimicrobial activity of each solvent was also studied in separate tubes. All tubes were incubated at 37˚C with shaking at 150 rpm for 24 h. Tubes were visually observed for growth (turbidity) and the MIC was recorded at the lowest concentration where no visual growth/turbidity could be observed . All assays were performed in duplicate.

Checkerboard assay. From the rapid screening assay, ACAA, caffeic acid, catechol, cinnamon bark oil, clove bud oil, geraniol, pyrogallol, and thymol were identified as the most inhibitory plant compounds. The aforementioned compounds were subsequently used to examine their interaction effect in combination with ciprofloxacin using the checkerboard method (Pillai et al. 2005).

The checkerboard assays were carried out in 96-well microtitre plates as outlined by Pillai et. al (2005). The MIC value for each antimicrobial compound was used to determine the appropriate concentration range for each checkerboard assay. The maximum concentration for each antimicrobial was two dilutions above their MIC and the minimum at four dilutions below their MIC. The stock solutions for each antimicrobial compound were prepared to achieve a concentration two dilutions above

the maximum concentration. For each microtitre plate, 100 µL of MHB (Oxoid) was added to each well. To each well in the first column, 100 µL of the ciprofloxacin stock solution was added and diluted in serial double dilutions across the x-axis except in the last column where no ciprofloxacin was added. To each well in the top row, 100 µL of a plant compound stock solution was added and diluted in serial double dilutions across the y-axis except for the last row where no plant compound was added. As the ciprofloxacin control row was not further diluted by the addition of the inhibitory plant compound, it was further diluted with 100 µL of MHB (Oxoid) to achieve the same ciprofloxacin dilution pattern as the preceding rows in the plate. The last column and row in the plate contained one compound and acted as an internal positive control for each antimicrobial compound to estimate its MIC. The last well in the last column/row acted as a growth control (no antimicrobial) in the presence of solvents used for each compound.

All wells were then inoculated with 5 µL of an *E. coli* O157:H7 (5 x 10^6 CFU mL^{-1}) suspension, prepared similarly to the rapid screening assay, in order to achieve a final concentration of 5 x 10^5 CFU mL^{-1} in each well. Plates were covered, sealed with paraffin wax to prevent evaporation and incubated at 37˚C for 18 h. The MIC was defined as the well with the lowest concentration of antimicrobials without growth (turbidity). The MIC was determined for the antimicrobial combination, as well as the MIC for each antimicrobial alone, to calculate the Fractional Inhibitory Concentration (FIC) index (Pillai et al. 2005).

The fractional inhibitory concentration (FIC) index is a value comparing the MIC from the antimicrobial combination assay to the MIC of the antimicrobial on its own. It is calculated from the following formula (Pillai et al. 2005):

$$\text{FIC for compound A} = \frac{\text{MIC of A (in combination assay)}}{\text{MIC of A (alone)}}$$

$$\text{FIC for compound B} = \frac{\text{MIC of B (in combination assay)}}{\text{MIC of B (alone)}}$$

$$FIC_A + FIC_B = \text{FIC index}$$

Antimicrobial interactions are defined as follows: FIC index: ≤ 0.5, synergistic; >0.5 to 4.0, indifferent; >4.0, antagonistic (Pillai et al. 2005). The checkerboard assays were completed in triplicate microtitre plates for each antimicrobial combination.

Time to ciprofloxacin resistance assay. From the checkerboard method, a synergistic interaction between geraniol (a low toxicity compound) and ciprofloxacin was identified and used in the following experiments. Growth assays were performed to determine the sub-inhibitory concentration (SIC) of geraniol on *E. coli* O157:H7. Geraniol was two-fold serially diluted, starting at a concentration of 0.1024 to 0.0001 µL mL^{-1}, in 96-well microtitre plates. In each well, 50 µL of MHB (Oxoid) was added and 100 µL of the geraniol stock solution (0.3072 µL mL^{-1}) was added to the first well in the row. The geraniol stock was subsequently serially diluted across all wells within the row, except for the last well which acted as the growth control (no geraniol). A bacterial suspension of *E. coli* O157:H7 was prepared, as previously described, and diluted to a bacterial density of 2 x 10^6 CFU mL^{-1}. In each well, 50 µL of the bacterial suspension was added to achieve a final bacterial density of 1 x 10^6 CFU mL^{-1}. The microtitre plate was covered, sealed with paraffin wax and the absorbance was read at 625 nm. The plate was incubated at 37˚C for 3 hours (previous growth curve experiments with and without geraniol showed that at 3 hours, bacteria would be within the exponential growth phase –

data not shown). Following incubation, another absorbance reading at 625 nm was taken. Each assay was completed in triplicate. The initial absorbance reading from each well was subtracted from the post-incubation values. The highest geraniol concentration, which did not affect growth (subinhibitory concentration; SIC) was used in the following assay.

***E. coli* O157:H7 cell viability and yield.** Cell viability and yield of *E. coli* O157:H7 was tested in the presence of 6 different antimicrobial treatments over 9 days in duplicate (Table 1). A suspension of *E. coli* O157:H7 was prepared, as previously described, and diluted to 5 x 10^5 CFU mL^{-1}. To each 50 mL tube, 37 mL of MHB (Oxoid), 1 mL of an *E. coli* O157:H7 suspension and 1 mL of each antimicrobial stock solution (geraniol and/or ciprofloxacin) was added. Tubes were incubated at 37˚C in a shaking incubator at 150 rpm for 9 days.

Table 5.1. Concentrations of geraniol and ciprofloxacin used in cell viability and yield assay for *E. coli* O157:H7

Treatment	Geraniol added (µL mL^{-1})	Ciprofloxacin added (µg mL^{-1})
ger+cip (0.0004)	0.0004	0.016
ger+cip (0.16)	0.16	0.016
ger+cip (0.32)	0.32	0.016
cip	NA	0.016
ger	0.32	NA
growth control	NA	NA

NA: Not applicable; none added

Cell yield. A 96-well microtitre plate was filled daily with 900 µL of MHB (Oxoid) in each well, except for wells in the first row, which were filled with 990 µL of MHB (Oxoid). To each well in the first row, 10 µL from each treatment tube (Table 5.1) was

added, 10-fold serially diluted and then absorbance measured at 625 nm for each well.

Cell viability. After absorbance was measured, serial dilutions of each treatment were plated on EMB (Becton Dickinson) agar plates with and without ciprofloxacin (0.016 µg mL^{-1}). Colonies were counted following a 10-hour incubation period.

***E. coli* O157:H7 resistance challenge**. An isogenic population of *E. coli* O157:H7 was continuously challenged to gradually increasing concentrations of one of three antimicrobial treatments. These treatments consisted of: ciprofloxacin, geraniol and ciprofloxacin+geraniol. A suspension of *E. coli* O157:H7 was prepared, as described previously, but only a single colony was picked and grown to create an isogenic population. The bacterial suspension was diluted to a final density of 2×10^7 CFU mL^{-1}. In each 15 mL tube, antimicrobial agents were added at sub-MIC concentrations, which were low enough to allow bacterial growth (ciprofloxacin: 0.016 µg mL^{-1}; geraniol: 0.0004 µL mL^{-1}; ciprofloxacin+geraniol: 0.016 µg mL^{-1} + 0.0004 µL mL^{-1}). Bacterial suspensions were subsequently incubated at 37˚C in a shaking incubator (150 rpm) overnight. Following incubation, the bacterial suspensions were observed for turbidity. The presence of turbidity was used as a growth indicator to passage the culture to fresh media with an increased antimicrobial concentration; therefore, 1 mL of the previous bacterial suspension was added to fresh media containing a slightly higher antimicrobial concentration than the previous passage to a final volume of 15 mL. This process was repeated daily for two weeks. All bacterial suspensions were stored at -20˚C with 20% glycerol for re-use in the event that no turbidity was detected following increased antimicrobial concentrations. After three incremental increases in concentrations, an MIC for ciprofloxacin was measured using the E-test method (following manufacturer

instructions; bioMérieux, Marcy l'Etoile, France). Bacterial cell morphology was observed in the most antimicrobial resistant cultures using differential interference contrast microscopy (Axioimager, Zeiss, Thornwood, NY).

5.4 RESULTS

A total of 12 plant compounds were screened at increasing concentrations (0.125 to 5 mg mL^{-1}) and growth of *E. coli* O157:H7 was recorded (Table 5.2). Pyrogallol had the highest inhibitory activity and no growth was observed at 0.5 mg mL^{-1} or higher concentrations. Seven essential oils were also screened at concentrations ranging between 0.5 and 10 µL mL^{-1}. Geraniol, clove bud oil and cinnamon bark oil were the most inhibitory with no growth detected at 5 µL mL^{-1} or higher (Table 5.3).

Interactions between ciprofloxacin and the most potent compounds, from the previous screening test (Tables 5.2 and 5.3) were evaluated. Using the checkerboard assay, additive interactions were detected with ACAA, catechol, cinnamon bark oil, clove bud oil and thymol. Synergistic interactions were detected between ciprofloxacin and caffeic acid, in addition to ciprofloxacin and geraniol (Table 5.4). Due to the lower animal toxicity of geraniol (World Health Organization 2003), we focused further studies on this compound.

To further evaluate the effects of geraniol with ciprofloxacin, the highest sub-inhibitory concentration (SIC) of geraniol on *E. coli* O157:H7 was determined (SIC=0.0004 µL mL^{-1}). Growth assays of *E. coli* O157:H7 in the presence of geraniol (0.0004 µL mL^{-1}) at increasing concentrations of ciprofloxacin (up to 1.024 µg mL^{-1}) were completed to further characterize assay conditions (data not shown). Growth rate and growth yield were inversely proportional to ciprofloxacin concentration (data not shown). The ideal assay range for geraniol was 0.0004 to 0.32 µL mL^{-1} and 0.016 µg mL^{-1} for ciprofloxacin.

Table 5.2. Rapid screening for inhibitory activity of various phytochemicals against *E. coli* O157:H7

Compound name	Concentration (mg mL^{-1})				
	0.125	0.25	0.5	2	5
Thymol	NA	NA[1]	growth	no growth	no growth
Catechol	NA	NA	growth	no growth	no growth
Pyrogallol	growth	growth	no growth	no growth	no growth
Piperine	NA	NA	growth	growth	growth
ACAA[2]	NA	NA	growth	growth	no growth
Anthraquinone	NA	NA	growth	growth	growth
Quercetin	NA	NA	growth	growth	growth
Paclitaxel	NA	NA	growth	growth	growth
Hypericin	NA	NA	growth	growth	growth
Artemisinin	NA	NA	growth	growth	growth
Galanthamine hydrobromide	NA	NA	growth	growth	no growth
Caffeic acid	NA	NA	growth	growth	no growth

[1] NA: not applicable

[2] ACAA: 1-aminocyclopropane-1 carboxylic acid

Table 5.3. Rapid screening for inhibitory activity of various essential oils against *E. coli* O157:H7

Essential oil	Concentration (μL mL^{-1})		
	0.5	5.0	10.0
Cinnamon bark oil	growth	no growth	no growth
Citral	growth	growth	no growth
Bay oil	growth	growth	no growth
Geraniol	growth	no growth	no growth
Clove bud oil	growth	no growth	no growth
Thyme oil (white)	growth	growth	growth
Origanum oil	growth	growth	growth

Table 5.4. FIC[1] indices[2] of various phytochemicals in combination with ciprofloxacin using the checkerboard assay

Compound name	Ciprofloxacin	
	FIC	Interaction
ACAA[3]	0.52	additive
Caffeic acid	0.32	synergistic
Catechol	0.63	additive
Cinnamon bark oil	1.00	additive
Clove bud oil	0.56	additive
Geraniol	0.50	synergistic
Pyrogallol	1.06	indifferent
Thymol	0.63	additive

[1] FIC: Fractional Inhibitory Index
[2] Synergy: ≤0.5, additive: >0.5-1.0, indifferent: 1-4, antagonistic >4
[3] ACAA: 1-aminocyclopropane-1 carboxylic acid

Assays at these concentrations demonstrated an inverse relationship between geraniol concentration and growth rate and between geraniol concentration and cell yield, when ciprofloxacin (0.016 µg mL^{-1}) was kept constant (Fig. 5.1). This pattern was particularly evident when only optical density was measured (Fig. 5.1A). However, when cells were harvested daily and plated, recovery of viable cells for all treatments declined rapidly after two days with the exception of the ger+cip (0.0004 µL/mL) treatment, and cell numbers were generally lower as geraniol concentration increased (Fig. 5.1B).

A synergistic interaction was also observed between ciprofloxacin and geraniol using the checkerboard assay (Table 5.4). Cultures were transferred daily for two weeks in the presence of geraniol and ciprofloxacin to determine whether geraniol would hinder the development of ciprofloxacin resistance. Over the two-week period, the geraniol and ciprofloxacin concentration were gradually raised to levels above their respective MICs. Geraniol did not limit development of resistance to ciprofloxacin, but rather increased it 63-fold above the MIC (Fig. 5.2). When *E. coli* was grown only in the presence of ciprofloxacin there was only a 32-fold increase in the MIC to ciprofloxacin. To verify that these surprising results were not the consequence of contamination, the experiment was repeated with nearly identical results.

To evaluate the long-term effects (two weeks) of cell growth in the presence of geraniol and ciprofloxacin, cell morphology was observed using differential interference contrast microscopy (Fig. 5.3). Ciprofloxacin resistant cells were smaller (2-3 µm) (Fig. 3B) as compared to the control (no antimicrobials; 3-4 µm) (Fig. 5.3A). Cells grown in the presence of geraniol (Fig. 5.3C) or in combination with ciprofloxacin (Fig. D) were

elongated.

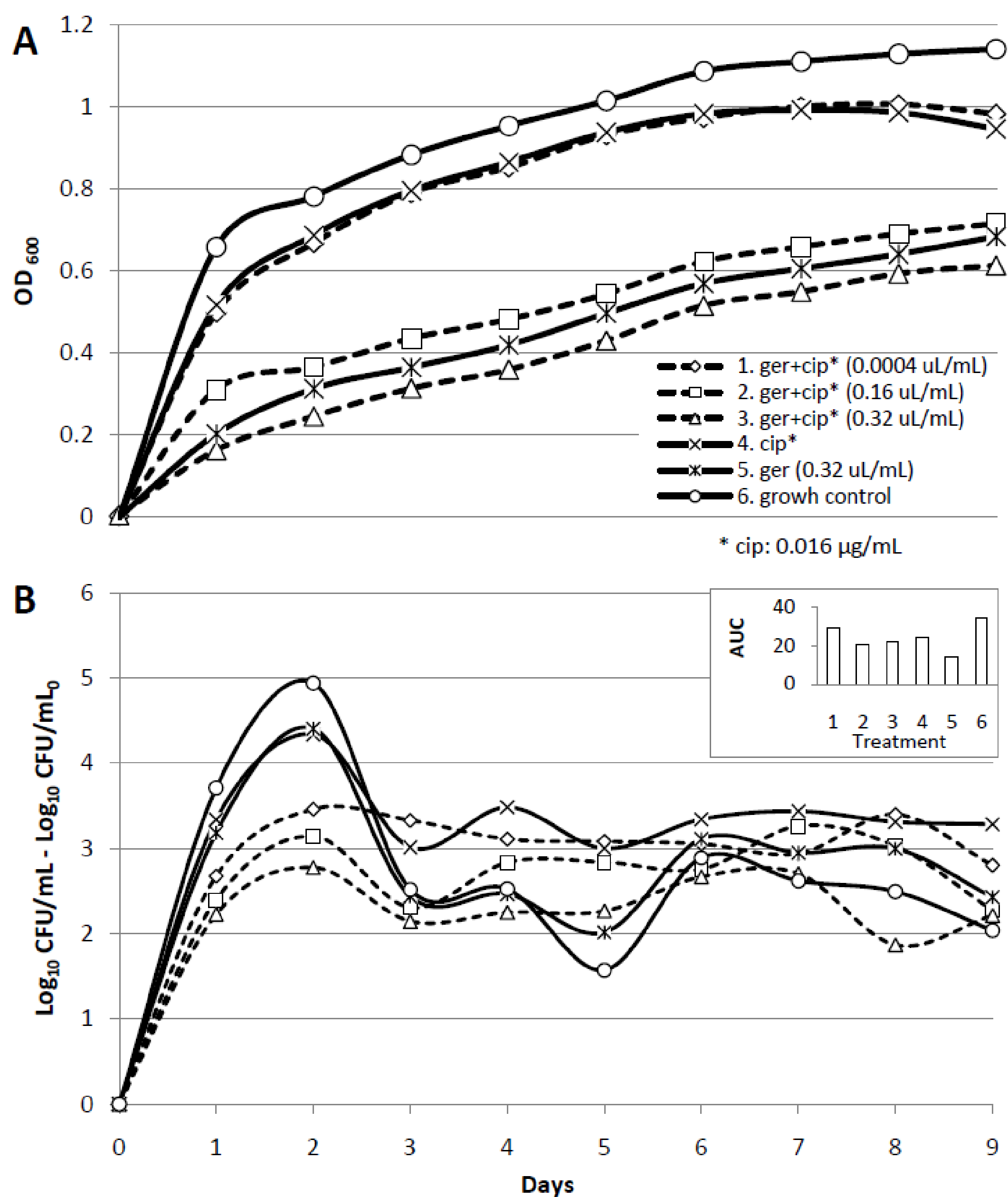

Figure 5.1. *E. coli* O157:H7 resistance development profile of ciprofloxacin (0.016 µg mL^{-1}) in combination with geraniol at three different concentrations (0.0004, 0.16, or 0.32 µL mL^{-1}), ciprofloxacin alone (0.016 µg mL^{-1}), geraniol alone (0.32 µL mL^{-1}), and a

growth control (no antimicrobials). Resistance was evaluated by measuring (A) cell yield: optical density of growth and (B) cell viability: plate counts. Area under the curve (($\Delta \log_{10}$ CFU/mL) days) was also plotted (inset) for each treatment.

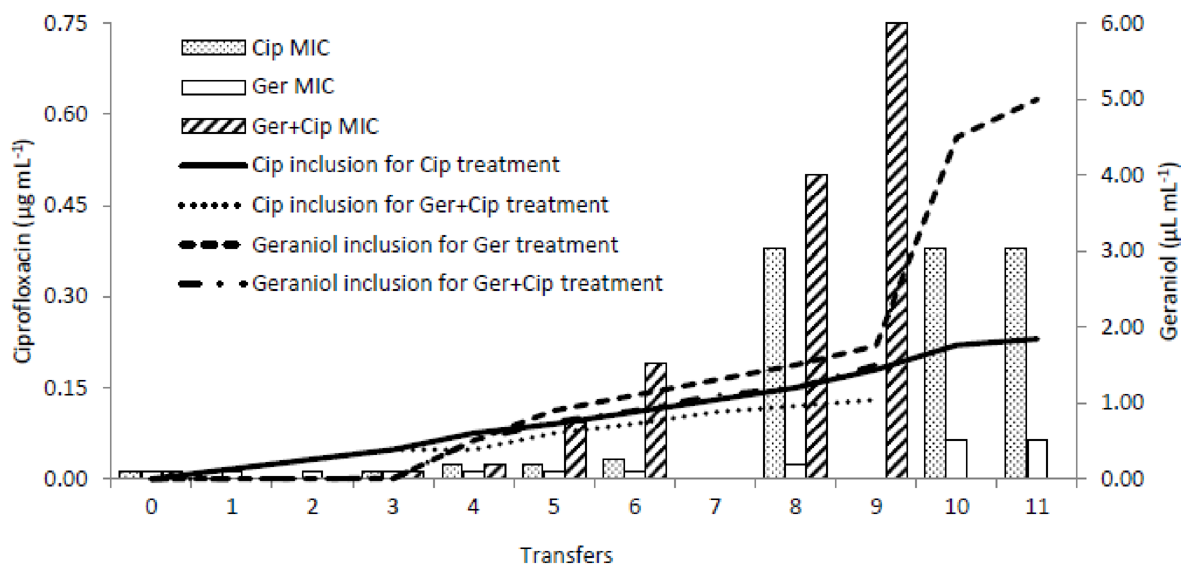

Figure 5.2. *E. coli* O157:H7 resistance against three antimicrobial treatments: ciprofloxacin alone (Cip), geraniol alone (Ger), or ciprofloxacin + geraniol (Ger+Cip). Results shown are MIC values taken after *E. coli* O157:H7 growth at increasing antimicrobial concentrations.

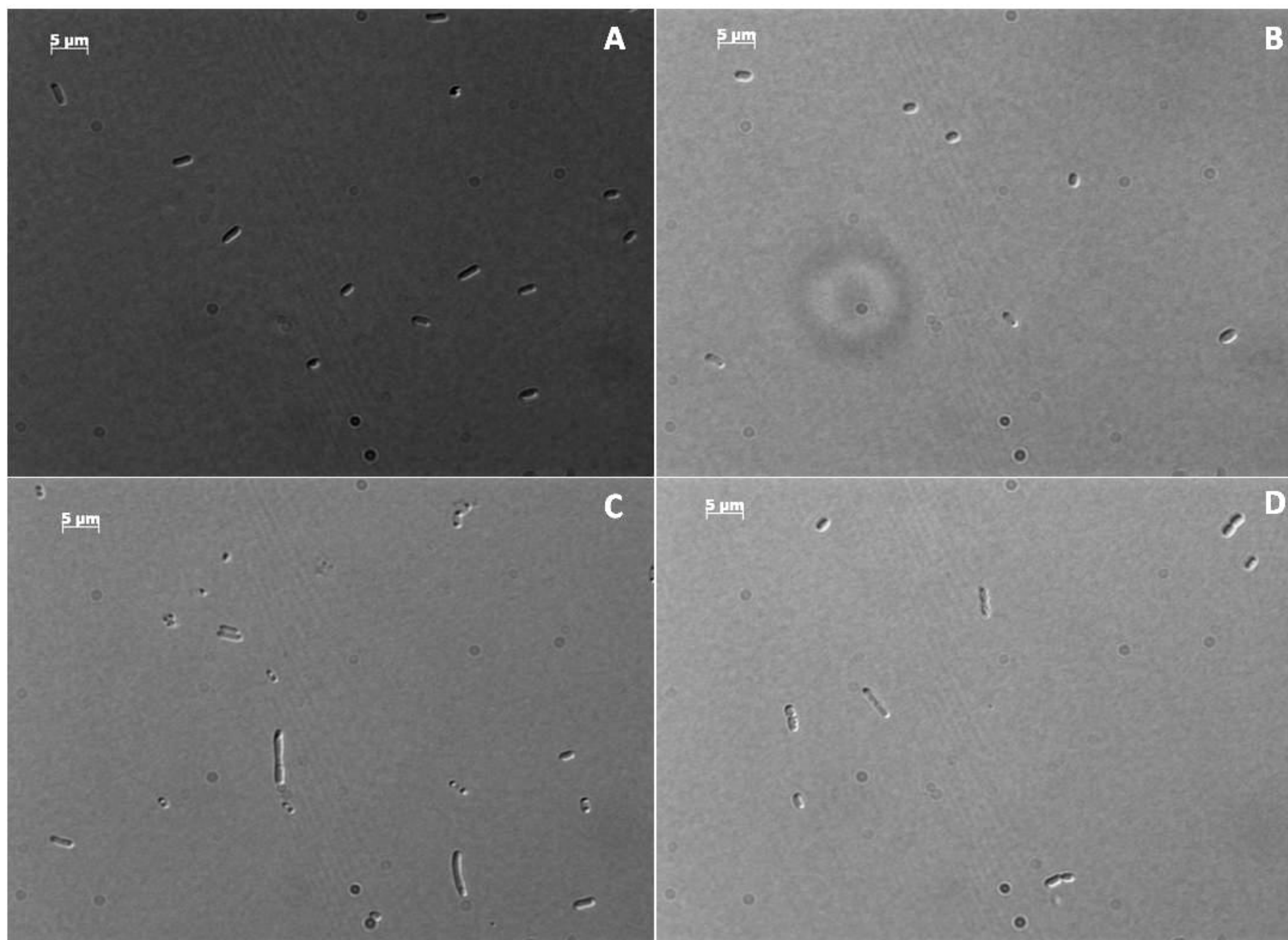

Figure 5.3. Morphological differences in *E. coli* O157:H7 after prolonged exposure to various antimicrobials: control (no antimicrobial) (A); ciprofloxacin (B); geraniol oil (C); ciprofloxacin and geraniol (D).

5.5 DISCUSSION

Nineteen natural antimicrobial products were screened for their antimicrobial activity and tested to determine whether the most inhibitory compounds would exhibit an additive, synergistic, or antagonistic interaction with ciprofloxacin (Table 5.2, 5.3, and 5.4). The fractional inhibitory concentration (FIC) index was used to determine the interaction between antimicrobial combinations. An FIC index of ≤ 0.5, >0.5 to 4.0, and >4.0 indicated a synergistic, indifferent, and antagonistic response, respectively (Pillai et al. 2005). Results indicated that only caffeic acid and geraniol had synergistic activity with ciprofloxacin, while most other natural compounds exhibited an additive interaction and none were antagonistic (Table 5.4).

These results are congruent with those of Lorenzi et al. (2009) which demonstrated that geraniol was able to increase the antimicrobial activity of quinolones, β-lactams, and chloramphenicol against multi-drug resistant (MDR) strains of *Enterobacter aerogenes*, *E. coli*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*. In the above study, the bacteria strains were deemed to be MDR due to overexpression of an efflux pump (AcrAB). When geraniol was tested against mutants with an *acrAB* operon deletion, no synergistic activity could be detected, therefore, the authors concluded that geraniol's mode of action was in part attributable to its efflux pump inhibitor activity (Lorenzi et al. 2009).

Thus, geraniol was examined in further detail here as it is considered to be less toxic than caffeic acid and is approved for use as a flavoring agent and generally considered safe by the Joint FAO/WHO Expert Committee on Food Additives (JECFA)

(World Health Organization 2003).

In batch mode, bacterial growth rates and yield were reduced when exposed to a sub-MIC concentration of ciprofloxacin alone; however, the addition of geraniol further decreased growth rates and yield (Fig. 5.1A). The geraniol alone treatment (0.32 μL mL^{-1}) had the lowest area under the curve (AUC) value for cell viability which suggest that the combination of ciprofloxacin at sub-MIC concentrations and geraniol may act antagonistically by forfeiting growth rates and cell yield as a fitness cost in order to maintain cell viability (Fig. 5.1B). This type of compensatory mechanism in resistant phenotypes has been widely documented for several bacteria exposed to a variety of antimicrobials (Björkholm et al. 2001; Nagaev et al. 2001; Nilsson et al. 2003).

Cells were passaged 12 times while progressively increasing the concentration of ciprofloxacin or geraniol or a combination of the two. Resistant phenotypes developed at higher concentrations of geraniol and ciprofloxacin (Fig 5.2). However, the most surprising result was that resistance to ciprofloxacin dramatically increased in the presence of geraniol. It thus appears that resistance to ciprofloxacin only increased once a ciprofloxacin threshold level (approx. 0.09 μg mL^{-1}) was exceeded and the presence of geraniol lowered the ciprofloxacin threshold level by half (approx. 0.048 μL mL^{-1}). This result suggests that if the ciprofloxacin threshold level is not exceeded, resistance is not as likely to develop.

These resistant phenotypes exhibited changes in cell morphologies: ciprofloxacin – small cells; geraniol – elongated; and combination – intermediate (Fig. 5.3). There were also cell morphological variations within each of these treatments. Some cells exposed to geraniol (Fig. 5.3C) became elongated without cell wall invaginations (no

septa) mixed with normal sized cells while cells exposed to the combination treatment (ciprofloxacin + geraniol) appeared elongated with cell wall invaginations. These findings suggest that geraniol may affect cellular machinery during cell division. Studies have shown that essential oils increase cell permeability and membrane fluidity through its accumulation between the fatty acyl chains of the membrane lipid bilayer thereby disrupting cell membrane integrity (Bard et al. 1988; Hammer et al. 2004). In addition, an increase in cell membrane permeability allows for higher proton and ion permeability resulting in a disruption of the intracellular pH (Sikkema et al. 1995). In one study, the use of geraniol against yeast (*Candida albicans* and *Saccharomyces cerevisiae*) resulted in an increased rate of potassium leakage out of the cells in addition to increased membrane fluidity and permeability (Bard et al. 1988).

The question of how these phenotypes arose mechanistically should be addressed. It is likely that this experiment selected for persistence and not resistance. Resistance is genetically acquired, while persistence is a transient phenotype switched on or off by epigenetic mechanisms. Bacterial persistence has been shown to be the result of bimodal growth rates within a genetically identical bacterial population (Dubnau and Losick 2006; Veening et al. 2008). As a “bet hedging” strategy, a small fraction of cells from an isogenic population will express slow growth and low cell yield which protects them from the lethal effects of antimicrobials. However, these slow growing cells are not considered resistant because as they return to an active growth state, their sensitivity to antimicrobials is comparable to that of normal cells (Balaban et al. 2004). In the present study, bimodal growth was clearly observed in batch culture. As the antimicrobial concentration of compounds increased, growth rate and cell yield declined (Fig 5.1A).

This bimodal response was also observed in viable cell counts but only for the first 48 h of batch growth, thereafter, cell viability stabilized and remained fairly stable for the following 7 days (Fig 5.1B).

This persistence via epigenetic modulation has been well documented in other studies (Adam et al. 2008; Balaban et al. 2004; Lee et al. 2010; Rotem et al. 2010). One of the key features of this persistence model is the lack of genetic mutation. In the present experiments this feature was evident in several ways. Following each passage, the preceding transfer culture was stored in a glycerol solution and frozen at -20˚C. In order to verify results, some cultures were re-grown with the addition of antimicrobials at the highest concentration previously exposed to. In most cases, to obtain growth, antimicrobial concentrations had to be decreased, as cultures were no longer as "resistant" as previously shown. One putative explanation for this loss in "resistance" is that it was a function of an epigenetic change rather than a permanent genetic mutation. Another observation which supported the persistence model was the presence of a secondary inhibitory zone surrounding the E-test strip. These observations suggested that within an isogenic population of *E. coli* O157:H7, not all cells expressed the "persister" phenotype. In addition, when antimicrobial concentrations were high and near the MIC upper limit breakpoint, cultures appeared to grow poorly as no turbidity could be seen. However, the theory of epigenetic persistence rather than development of resistance via genetic mutations needs to be validated with further studies. Primarily, DNA sequencing of the quinolone resistance-determining regions in the target genes gyrA and parC gene should be carried out to confirm the lack of genetic mutations within these regions. Secondly, relative expression level for efflux pump genes should also be tested to further

characterize the mechanism of increased resistance seen in this study.

Some studies have also shown evidence of cross-resistance between different classes of antimicrobials. Braoudaki and Hilton (2004) investigated the ability of *E. coli* O157, *S. enterica* serovar Typhimurium, *S. enteric* serovar Virchow to develop cross-resistance to a variety of antimicrobials after adaptation to common household biocides such as triclosan, benzalkonium chloride, and chlorhexidine. Their results showed that *E. coli* O157 frequently developed cross-resistance between antimicrobials and biocides. Specifically, *E. coli* O157 isolates adapted to benzalkonium chloride expressed increased resistance to amoxicillin, amoxicillin-clavulanic acid, chloramphenicol, imipenem, tetracycline, and trimethoprim whereas triclosan adapted isolates showed increased resistance to all previously mentioned antimicrobials in addition to erythromycin and ciprofloxacin. Braoudaki and Hilton (2004) were also able to demonstrate cross-resistance to other antimicrobials such as ciprofloxacin, tetracycline, trimethoprim, and chloramphenicol in addition to the biocide triclosan following adaptation to erythromycin.These findings of cross-resistance between antimicrobials support our hypothesis that adaptation to geraniol, a natural antimicrobial, in *E. coli* O157:H7 may have conferred increased resistance to ciprofloxacin.

Increasing trends in antimicrobial resistance and consumer demand for natural and/or organically grown food has forced the agriculture industry to put a greater focus on reducing antimicrobial use, potentially with the increased use of natural antimicrobials. However, results from this study suggest that excessive use of antimicrobials in combination with natural alternatives may only increase the potential for persistence. Bacteria are fascinating organisms with the remarkable ability to adapt to

adverse conditions. Thus, reducing survival of unwanted microorganisms continues to be a complex and daunting task. With that said, it is likely that antimicrobial resistance can only be mitigated by increasing antimicrobial stewardship amongst producers, veterinarians, physicians and the general public as judicious use of antimicrobials appears to be the most successful way of mitigating the development of antibiotic resistance.

BRIDGE TO CHAPTER SIX

The previous chapter examined the use of phytochemicals and synthetic antimicrobials in combination tests to reduce the MIC of synthetic antimicrobials and retard the development of antimicrobial resistance. Results from this study were not as expected. Rather than decreasing the MIC of ciprofloxacin and delaying the onset of resistance, combination tests using geraniol, a non-toxic essential oil, increased the ciprofloxacin MIC by two-fold in comparison to ciprofloxacin monotherapy.

The previous study suggests that the resistance developed was not based on genetic mutations but arose as an epigenetic bet hedging strategy utilized by clonal bacterial populations to produce tolerant phenotypic variants. Previous research has shown that clonal bacteria can hedge their bets towards different phenotypic variants. This evolutionary model allows for an increased likelihood that a subpopulation of clones will survive unpredictable and fluctuating environmental conditions. In the previous chapter, it was speculated that the highly resistant phenotype was in a dormant/reduced metabolic state which allowed it to be less susceptible to the inhibitory effects of antimicrobials.

The purpose of the following study was to determine whether similar concepts of dormancy and bet-hedging could be observed at an ecosystem level. It was hypothesized that within ruminant gut ecosystems, feeding a tanniniferous diet would inhibit some microbial taxa while others would thrive due to better fitness traits and ensure the maintenance of microbial diversity of the ecosystem.

CHAPTER SIX

MANUSCRIPT IV

Microbial community dynamics of the ruminant gut in response to feeding a tanniniferous diet

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6.1 ABSTRACT

Secondary plant compounds have been gaining popularity following the European Union's ban on all in-feed antimicrobials as of 2006. Specifically, condensed tannins (CTs) have been researched extensively in ruminants due to their wide range of beneficial effects in terms of animal health and production. In this study, the microbial community dynamics of the ruminant gut in response to feeding a tanniniferous diet was characterized. Forty steers were fed either a sainfoin hay (*Onobrychis viciifolia*; a CT containing forage) or alfalfa hay (non-CT containing forage) diet over an 11-week period. Microbial community composition in the rumen and hindgut was determined using high throughput sequencing of the 16S rRNA gene. An operational taxonomic unit (OTU)-based cluster analysis revealed that both rumen fluid and fecal samples originating from animals fed the same diet consistently clustered closely to each other. Results also showed that rumen samples of animals fed a CT-containing diet had a decrease in Bacteroidetes which was replaced by an increase in the less abundant Fibrobacters, whereas no marked diet effect was seen in the hindgut at the phylum level. Feeding a tanniniferous diet was correlated with an increase in cellulolytic organisms which were initially present in low abundance, while markedly decreasing the number of proteolytic *Prevotella* spp. in the rumen. In summary, feeding a tanniniferous diet resulted in an increase of the "rare biosphere" (rarer species), in the rumen microbial ecosystem while yielding little effect on hindgut microbial populations.

6.2 INTRODUCTION

The administration of sub-therapeutic levels of antimicrobials to food producing animals has been associated with an increase in antimicrobial resistance of gut bacteria (Alexander et al. 2011; Berge et al. 2006). In 2006, the European Union banned all antibiotic growth promoters in animal feed as a precautionary measure (Millet and Maertens 2011). As such, there has been an increased interest towards using secondary plant compounds as alternatives to synthetic antibiotics. Condensed tannins (CT) are natural plant phenolic compounds well known for their antimicrobial activity and ability to interact with proteins (Berard et al. 2009; Jones and Mangan 1977). More specifically, tannin-protein complexes are formed in the rumen where the pH is near neutral (pH 6-7) and are subsequently degraded post-ruminally where the pH decreases to less than 3.5 in the abomasum (true stomach) before returning to near 7 in the small intestine; where released proteins are absorbed by the host (Mueller-Harvey 2006). Structurally, CTs are polymers of flavan-3-ol connected by interflavan carbon bonds. The interflavan carbon bonds prevent CTs from readily being hydrolyzed in the gut (Barry and McNabb 1999). Waghorn et al. (1987) explored the impact of CT on ruminal protein metabolism by feeding sheep *Lotus corniculatus* and *L. pedunculatus*, containing 22 and 55 g CT kg^{-1} DM, respectively, with and without supplementation of polyethylene glycol (PEG). Inclusion of PEG serves to bind and inactivate CT without affecting microbial digestive enzymes (Barry and Manley 1986; Jones and Mangan 1977). Animals fed the lower CT-containing forage *L. corniculatus* had increased apparent absorption of essential amino acids from the small intestine when not supplemented with PEG, whereas amino acid

absorption in sheep consuming the high CT forage *L. pedunculatus* were not affected by PEG supplementation. It is presumed that when fed to ruminants, tanniniferous forages can decrease solubilization and microbial degradation of plant proteins (Barry and McNabb 1999; McNabb et al. 1996).

The meat and milk produced from ruminants is an important source of food for many worldwide and the symbiotic relationship which exists between the rumen gut microbes and their host is an important economical feature for producers as it allows feeding of low-quality feeds. Rumen microorganisms can degrade indigestible cellulose and hemi-cellulose to produce volatile fatty acids absorbed by the host as a source of energy (Hobson and Stewart 1997). Furthermore, microbial protein outflow is considered to be an important source of protein for the animal (Broderick 1994). However, inefficient use of feedstuffs can lead to excessive N excretion and increased methane emissions (Patra 2011). Studies have shown that feeding CT containing diets to ruminants is associated with reduced methane emissions (McMahon et al. 1999; Woodward et al. 2002; Woodward et al. 2001). Thus efficient manipulation of rumen metabolism is an important consideration for both livestock producers and environmentalists.

In order to manipulate rumen metabolism, a complete understanding of the rumen ecosystem is required. Although the rumen is one of the most well studied ecosystems, many questions remain unanswered such as (i) which microorganisms are part of this ecosystem? (ii) how do these microorganisms contribute to the functioning of the rumen? and (iii) how are these organisms affected by environmental factors? To gain a better understanding of this integral ecosystem, one may apply popular macro-ecology theories

to help elucidate fundamental processes which occur in microbial ecosystems in response to environmental factors. This study attempts to contribute knowledge and insight which will be valuable in answering these questions. Thus, the objectives of this study were to characterize the microbial community dynamics of the ruminant gut in response to feeding a tanniniferous diet and to determine whether the ecosystem response follows theorical population models.

6.3 MATERIALS AND METHODS

Animal trial and samples. Animals were cared for in accordance with the guidelines of the Canadian Council on Animal Care (1993). Rumen fluid and fecal grab samples were collected from eight beef steers during an 11-week outdoor feeding trial (2-week dietary adaptation period and a 9-week experimental period), also described in Berard et al. (2009). Animals were fed one of two dietary treatments: sainfoin hay or alfalfa hay. First- and second-cut forages were mixed to obtain a consistent nutrient profile throughout the trial. A 2-week dietary adaptation period consisted of all animals being fed the alfalfa hay diet. During the 9-week experimental period, four animals were progressively introduced to the sainfoin hay diet with the following sainfoin-to-alfalfa ratios: 25:75 for two days, 50:50 for two days, 75:25 for two days, and strictly sainfoin hay thereafter. Animals were fed twice a day ad libitum with free access to water. Feeders with automatic weight scales (GrowSafe Systems, Airdrie, AB, Canada) were used to record feed intake for each animal.

Rumen fluid samples collected using the oro-ruminal (Geishauser) probe method where a weighted probe with small holes attached to a tube and suction is inserted through the animal's oral cavity and guided down into the rumen (Geishauser 1993). Once the probe reaches the rumen, suctioning is applied and rumen fluid collected. The initial 100 mL is discarded due to potential saliva contamination. Both rumen fluid and fecal grab samples were taken once per week during the dietary adaptation period, weeks 4 to 7, and biweekly thereafter over an 11-week period. Following collection, both rumen fluid and fecal samples were stored at -20˚C until further analysis.

DNA and pyrosequencing. Genomic DNA was extracted using ZR Fecal MiniPrep Kits (Zymo Research, Orange, CA, USA) from rumen fluid and fecal grab samples for weeks 1, 2, 5, 6, 9, and 11. DNA from each sample was quantified by measuring the OD_{260} using a spectrophotometer (Beckman Coulter, Brea, CA, USA). To assess the PCR efficiency, each DNA sample was amplified using primers (27f: 5'-GAAGAGTTTGATCATGGCTCAG; 342r: 5'- CTGCTGCCTCCCGTAG) targeting the V1 to V2 hypervariable regions of the bacterial 16S rRNA gene. Polymerase chain reactions were conducted using 25 µL reaction volumes comprised of 2.5 µL Econo Taq 10X reaction buffer (Lucigen, Middleton, WI, USA), 0.1 µL Econo Taq DNA Polymerase with Mg++ (Lucigen), 1 µL $MgCl_2$ (Bio-Rad Laboratories Inc, Hercules, CA, USA; 50 mM), 0.5 µL each of the forward and reverse primers (25 µM), 0.5 µL dNTP Mix (Bio-Rad Laboratories Inc), 18.9 µL nuclease-free water (Promega, Madison, WI, USA), and 1 µL genomic DNA. Reactions were carried out in a thermo cycler (Bio-Rad Laboratories Inc) using the following conditions: initial denaturation at 94˚C for 3 min, 36 cycles of denaturation at 94˚C for 60 sec, annealing at 60˚C for 60 sec, elongation at 72˚C for 60 sec, followed by a final extension at 72˚C for 5 min. PCR efficiency was assessed by visualisation of the amplicon using electrophoresis. DNA samples yielding an inadequate amplicon were diluted until a good amplicon could be detected. Subsequently, genomic DNA samples were all diluted to a concentration of 50 µg mL^{-1} and a final volume of 50 µL. Three composite samples for each animal and sample type (rumen fluid and feces) were made where 25 µL of diluted genomic DNA (50 µg mL^{-1}) from weeks 1 and 2 (time period 1), 5 and 6 (time period 2), and 9 and 11 (time period 3). A total of 48 composite genomic DNA samples (24 rumen fluid, 24 fecal

grab) were sent to Research and Testing Laboratory (Lubbock, TX, USA) for pyrosequencing using a 454 GS FLX Titanium Sequencing System (454 Life Sciences, a Roche company, Branford, CT, USA) targeting the V1 to V3 hypervariable region of the 16S rRNA gene. Further details of this method are described in Dowd (2008).

Tannin analysis. Each week, a feed sample was taken and analyzed for CT concentration using the butanol-HCl method with modifications as described in Berard et al. (2011) and Terril et al. (1992).

Data analysis. Pyrosequencing data was analyzed using the Mothur package tools (Schloss et al. 2009). A series of quality/trimming steps were taken to remove low-quality reads; i.e., sequences below an average quality score of 35, with homopolymers greater than eight bases, and one or more ambiguous base call ("N") were removed from the dataset. Sequences were aligned using kmer searching (kmer size=8) against the SILVA alignment database for 16S rRNA gene and the Needleman algorithm to make the pairwise alignments between the query and template (de-gapped SILVA alignment database). Gaps were re-inserted in both the query and template (SILVA alignment database) sequences using the nearest alignment space termination algorithm (DeSantis et al. 2006a). Sequences which did not span the longest alignment region were removed from the dataset. Pyrosequencing noise due to pyrosequencing base call errors was minimized in the dataset using the pre-cluster algorithm by Huse et al. (2010), whereby rare sequences highly similar to abundant sequences are re-classified as their abundant homologue.

Pairwise distances between aligned sequences were calculated and used to cluster sequences into operational taxonomic units (OTUs) at a 95% similarity cutoff using the

complete linkage (furthest neighbour) algorithm. The most abundant sequence from each OTU was extracted and classified using the Bayesian classifier with a minimum bootstrap cutoff of 80% against the SILVA (from Latin *silva*, forest) reference non-redundant database (SILVA RefNR).

Mothur was used to calculate species richness (Chao1 and abundance-based coverage estimation (ACE)), diversity (non-parametric Shannon and Simpson) indices, rarefaction curves, and dendogram using the Jaccard index (Schloss et al. 2009).

Statistical analysis. Comparisons between diets and time periods were carried out for all diversity indices and relative abundance data at the phylum, family, and genus ranks. A Box-Cox power transformation (Box and Cox 1964) was used to transform non-normally distributed diversity and richness indices using a Box-Cox SAS macro (http://www.datavis.ca/sasmac/boxcox.html). Richness and diversity indices were analyzed using the mixed procedure in SAS v.9.2 (SAS Institute, Cary, NC, USA). The Generalized Linear Mixed Model procedure (GLIMMIX) in SAS using either a Poisson distribution (log link) or negative binomial (logit link) was used to analyze abundance data at all rank levels. The Pearson Chi-Square/DF and -2 Log likelihood were used as indicators to assess model fit.

A simple linear regression analysis was performed to determine correlations between tannin intake and relative abundances of important phyla and families in both rumen and hindgut ecosystems using JMP, version 8 (SAS Institute Inc., Cary, NC, USA).

6.4 RESULTS

Rumen fluid and fecal samples were collected from 8 steers throughout an 11-week feeding trial. A total of 48 samples (24 rumen fluid and 24 fecal samples) were pyrosequenced (V1-3 hypervariable region) yielding 70,680 reads. Subsequent to quality filtering, 43,251 high-quality reads ranging from 350 to 625 bps were retained for downstream analysis. Using Mothur's pyrosequencing pipeline (Schloss et al. 2009) and SILVA's 16S rRNA database (Pruesse et al. 2007) reads were analyzed for richness and diversity and classified using the SILVA taxonomy. Rarefaction curves suggest that hindgut samples were much further from species saturation than rumen samples therefore, much deeper sequencing would be required to capture the full microbial diversity in hindgut samples. Therefore, the Chao1 estimate was used to compare true species richness between treatment groups (Fig. 6.1 and Table 6.1). There was an increase in species richness over time in animals fed either diet and in both rumen and hindgut samples ($P<0.05$, statistical results not shown; Table 6.1). Species richness (Chao1) in the rumen was comparable for both treatment groups (Table 6.1). In the hindgut, Chao1 estimates were slightly higher in sainfoin fed animals as compared to alfalfa fed animals although these differences were not statistically significant ($P>0.1$, statistical results not shown; Table 6.1). Transformation of diversity indices into effective species (Jost 2006) showed that the rumen species diversity only marginally increased over time in both treatment groups. Sainfoin fed animals showed a slightly higher increase in diversity according to the exponential of the non-parametric Shannon estimate and the lower Simpson diversity seen in these samples indicates an increase in rare

species (Fig. 6.2A). The hindgut samples showed a significant increase in diversity over time ($P<0.1$) with a less clearly defined diet effect (Fig. 6.2B).

Table 6.1. Summary of pyrosequencing data[a] and richness and diversity estimates for rumen fluid and fecal samples collected from four steers fed an alfalfa or sainfoin hay diet across three time periods

Experimental setup			Sequence library summary			Richness	Diversity	
Sample	Time	Diet	Average number of sequences	Average number of observed OTUs	Average sample coverage	Chao1 estimate OTUs	Shannon's diversity index	Simpson's index of diversity
Rumen	1	AH[b]	1331	485	71%	1273	5.340	0.118
		SH[c]	1159	539	66%	1429	5.959	0.019
	2	AH	720	379	58%	1342	5.830	0.024
		SH	1104	555	62%	1597	6.220	0.008
	3	AH	819	474	56%	1699	6.296	0.005
		SH	740	468	49%	1770	6.405	0.013
Hindgut	1	AH	874	514	53%	1902	6.334	0.010
		SH	797	522	47%	2161	6.602	0.005
	2	AH	847	550	48%	2072	6.639	0.006
		SH	861	609	43%	2355	6.968	0.002
	3	AH	751	568	37%	2422	7.110	0.002
		SH	813	604	37%	3054	7.085	0.002

[a] Sequences within an operational taxonomic unit (OTU) have a sequence divergence ≤ 3%
[b] AH: alfalfa hay
[c] SH: sainfoin hay; Period 1 was an adaptation period, all animals (AH and SH) were fed alfalfa hay

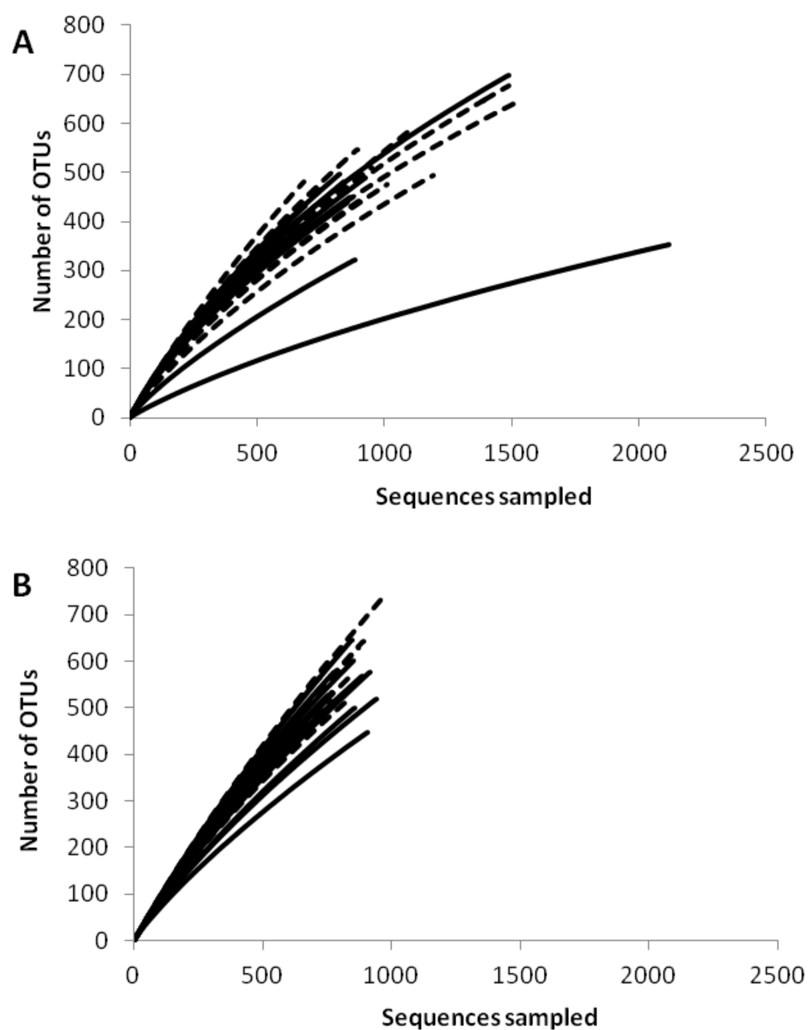


Figure 6.1. Rarefaction curves for (A) rumen and (B) hindgut samples at a 97 % sequence similarity level. Each line represents a sample. Sainfoin hay fed animal samples are represented by dashed lines while solid lines are samples from alfalfa fed animals.

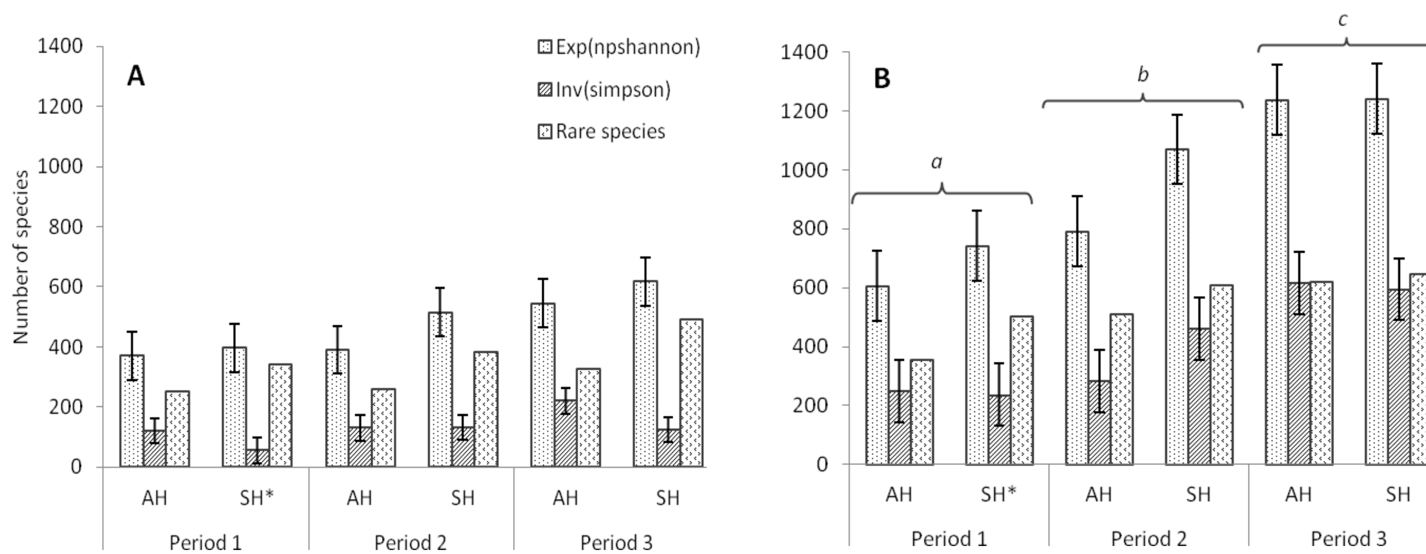


Figure 6.2. Statistical representation of true diversity. Diversity indices are transformed into effective number of species for (A) rumen and (B) hindgut. Effective number of species for each diversity index was calculated by taking the inverse of the Simpson diversity index and the exponential of the non-parametric Shannon index. Rare species were calculated as the difference between transformed indices. Statistical significance (P<0.05) is denoted by different italic letters.*Period 1 was an adaptaion period; all animals were fed an alfalfa hay diet.

Abbreviations: AH, alfalfa hay; SH, sainfoin hay; Exp(npshannon), exponential of the non-parametric Shannon index; Inv(simpson), inverse of the Simpson index.

A total of 17 and 13 phyla were identified in the rumen and hindgut, respectively. Within the rumen, the microbial composition was dominated by the Bacteroidetes followed by the Firmicutes, whereas the hindgut was dominated by Firmicutes (Fig. 6.3). At the genus level, *Prevotella* spp. and uncultured/unclassified organisms from the Prevotellaceae and RF16 family dominated the rumen microbial community. In the hindgut, uncultured organisms from the Ruminococccaceae, Lachnospiraceae, and Prevotellaceae families were the most prominent organisms (Suppl. Fig. 6.7).

Using Fast UniFrac (Hamady et al. 2010), a Jaccard coefficient tree (at a 0.05 genetic distance) showed that individual samples from the rumen and hindgut tended to cluster by diet (except for R.T3.SH.2 and F.T3.SH.2) however, hindgut samples additionally clustered by time period (Fig. 6.4).

The CT concentration was measured from feed samples taken weekly across the entire trial. Using a linear regression analysis, the effect of CT concentration on relative abundance of several different taxa was characterized. Regression plots at the phylum and genus level both showed positive and negative correlations with CT concentrations in the rumen (Fig 6.5A and B, respectively). Notably, there was a significant negative correlation ($P<0.001$) in the regression plot of Bacteroidetes in the rumen (Fig. 6.5A) which is supported by the same trend seen for the *Preveotella* and uncultured Prevotellaceae plots (Fig. 6.6A). In addition, a positive correlation ($P<0.0001$) with the Fibrobacteres and CT concentrations was seen (Fig. 6.5B). In the hindgut, strong negative correlations ($P<0.05$) were seen in various Firmicutes such as *Butyrivibrio*, *Mogibacterium* and *Acetitomaculum* with CT concentration (Fig. 6.6B).

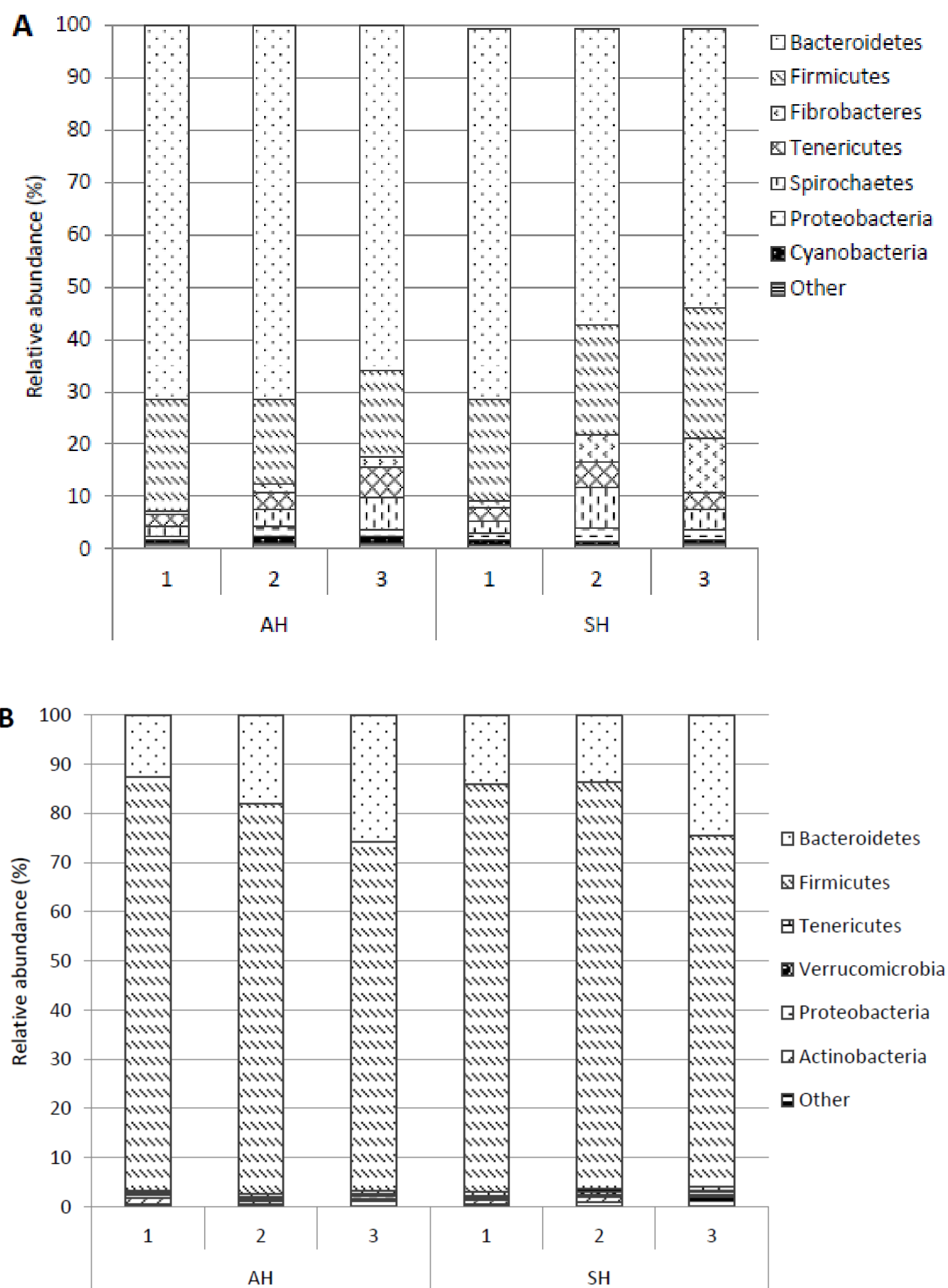


Figure 6.3. Relative abundance of microbial phyla within the (A) rumen and (B) hindgut for each treatment group and time period. Abbreviations: AH, alfalfa hay; SH, sainfoin hay.

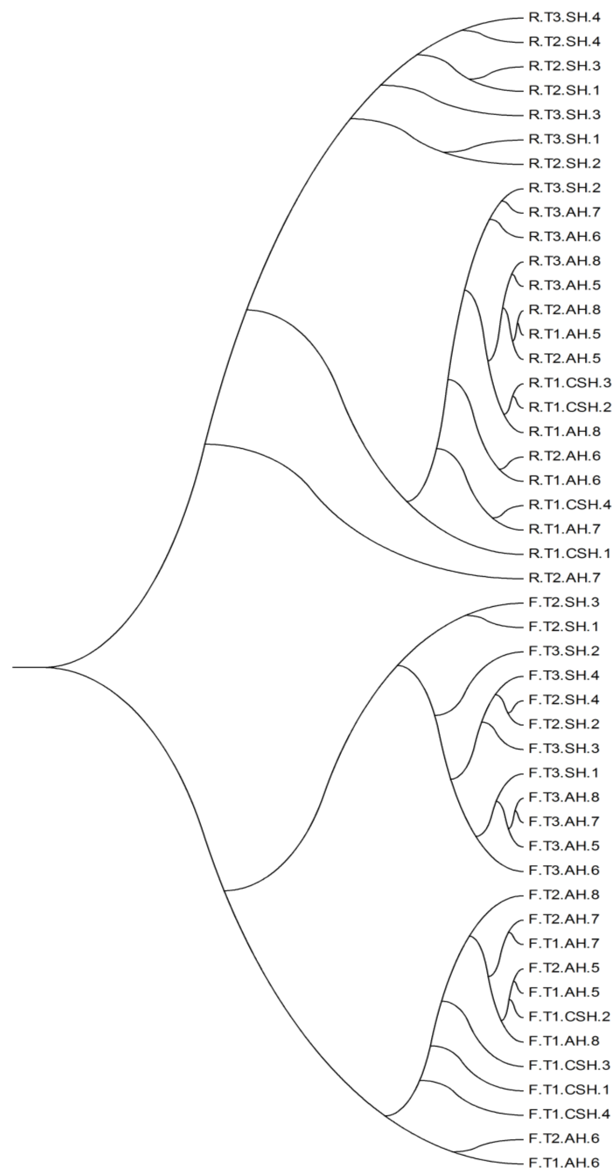
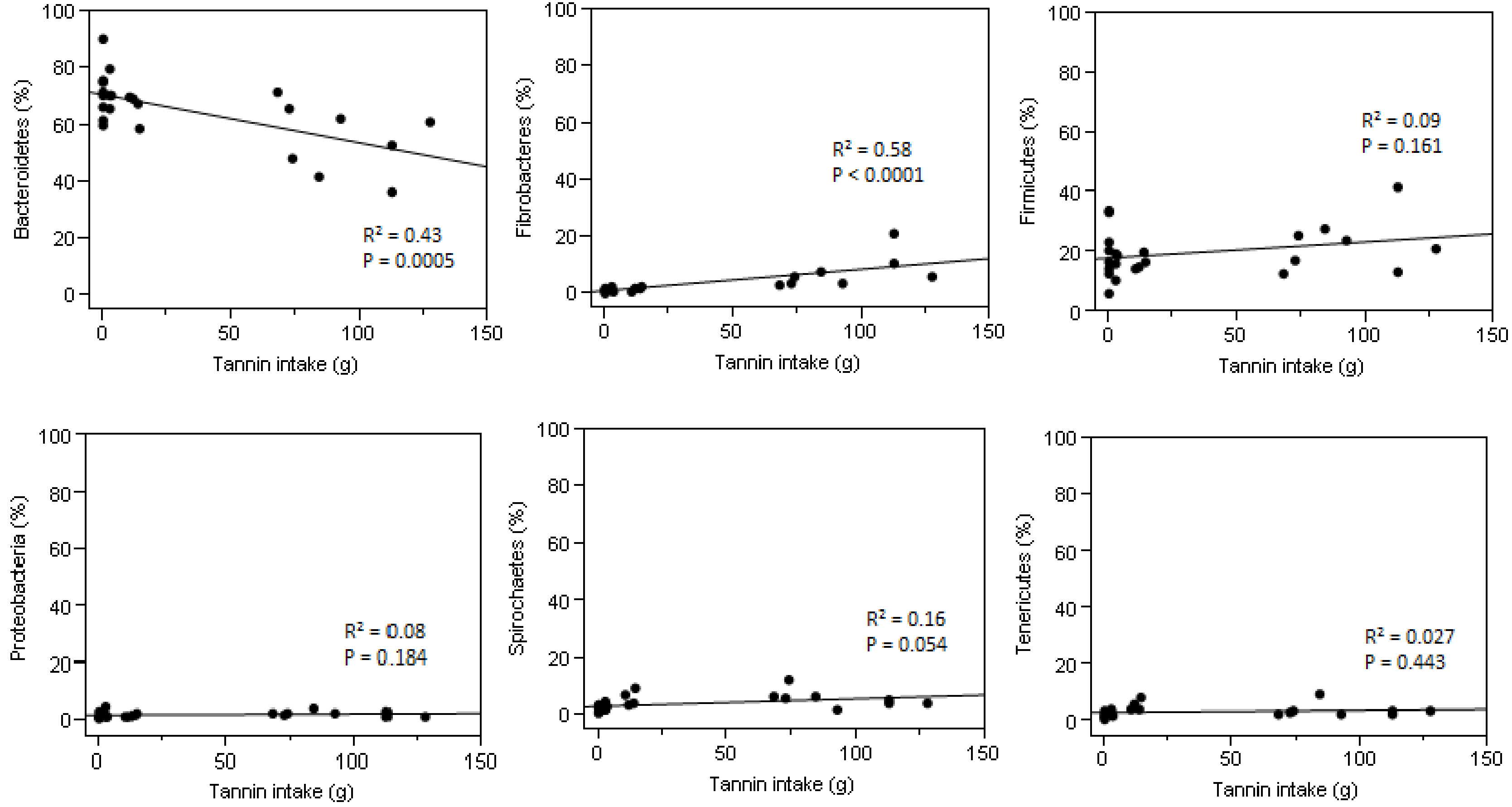


Figure 6.4. A Jaccard coefficient tree (at a 0.05 genetic distance) as an estimate of similarity and distance between samples. The first letter in the sample name (R or F) indicates whether the sample came from rumen fluid or fecal sample, the “T” followed by a number indicates the time period (T1, T2, or T3), and the remaining letters and number indicate diet (CSH: control sainfoin hay where animals were fed alfalfa hay in period one, SH: sainfoin hay, and AH: alfalfa hay) and animal number.


A
Bacteroidetes (%)
Tannin intake (g)
R² = 0.43
P = 0.0005
Fibrobacteres (%)
Tannin intake (g)
R² = 0.58
P < 0.0001
Firmicutes (%)
Tannin intake (g)
R² = 0.09
P = 0.161
Proteobacteria (%)
Tannin intake (g)
R² = 0.08
P = 0.184
Spirochaetes (%)
Tannin intake (g)
R² = 0.16
P = 0.054
Tenericutes (%)
Tannin intake (g)
R² = 0.027
P = 0.443

B

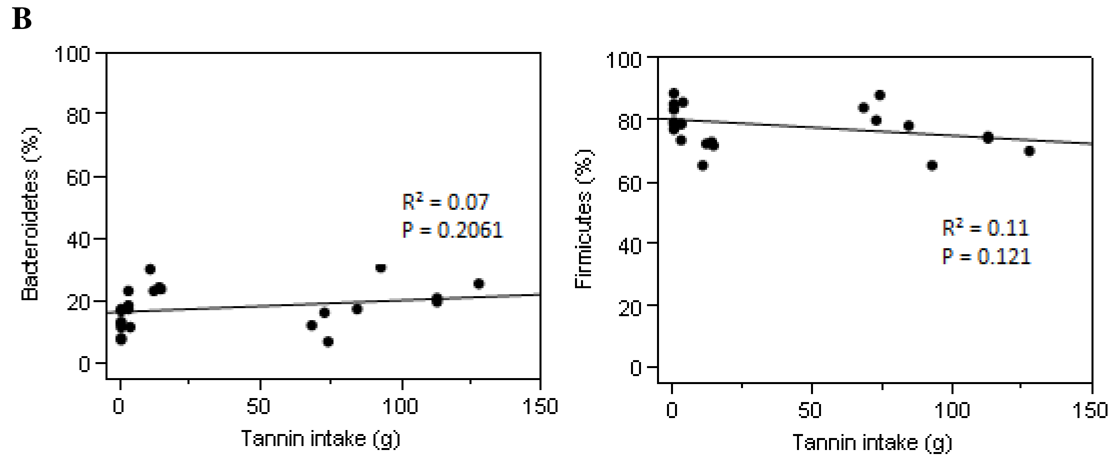
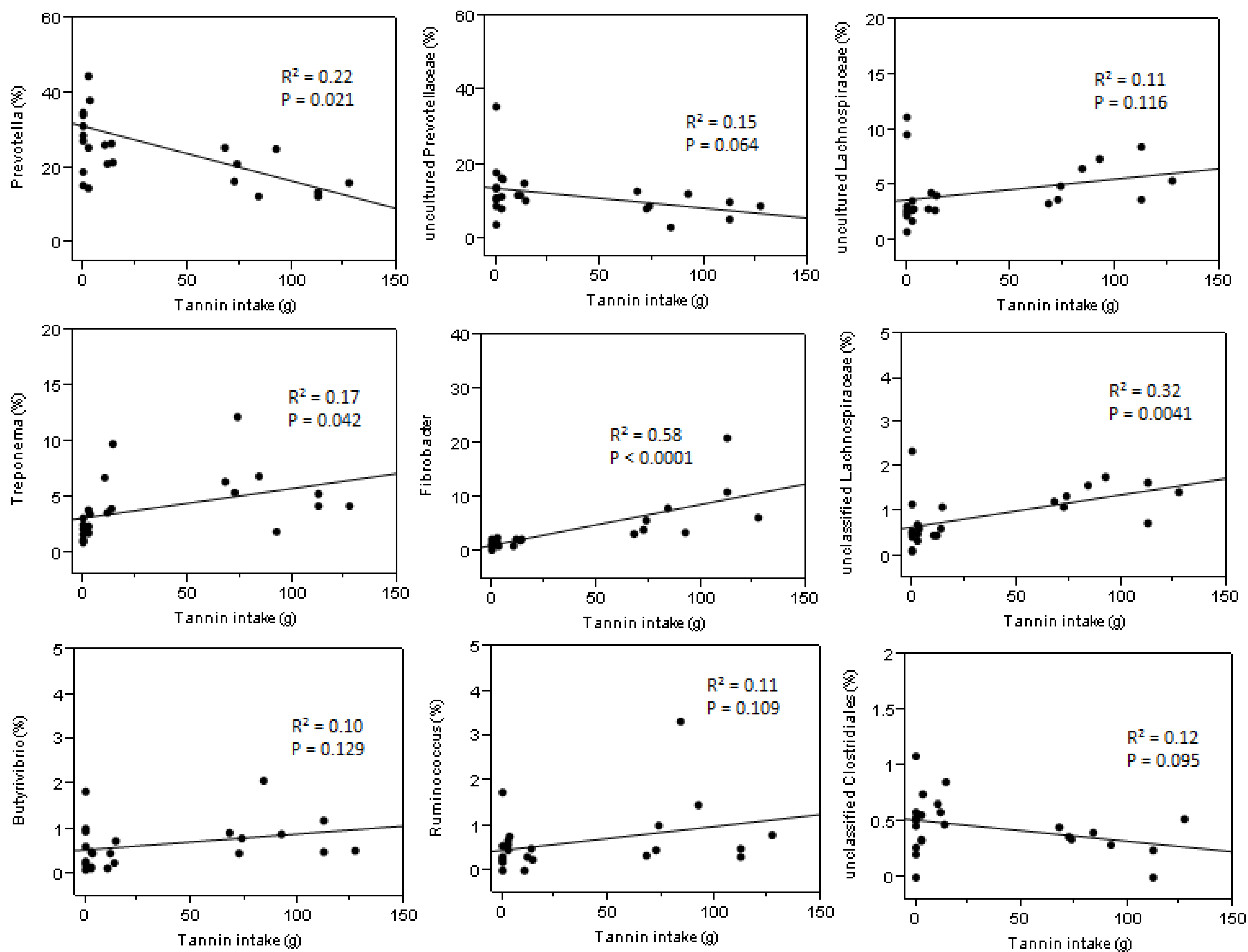
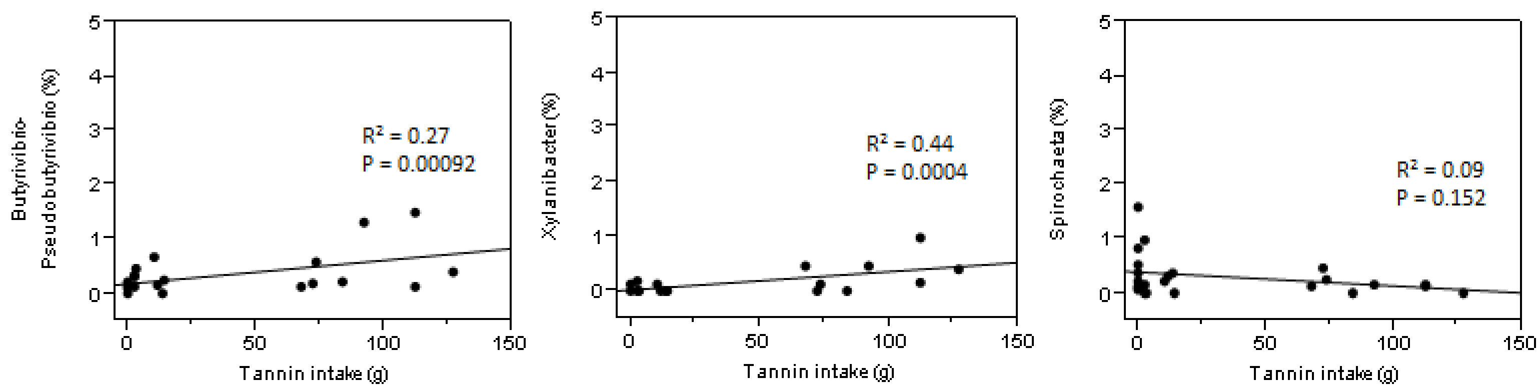


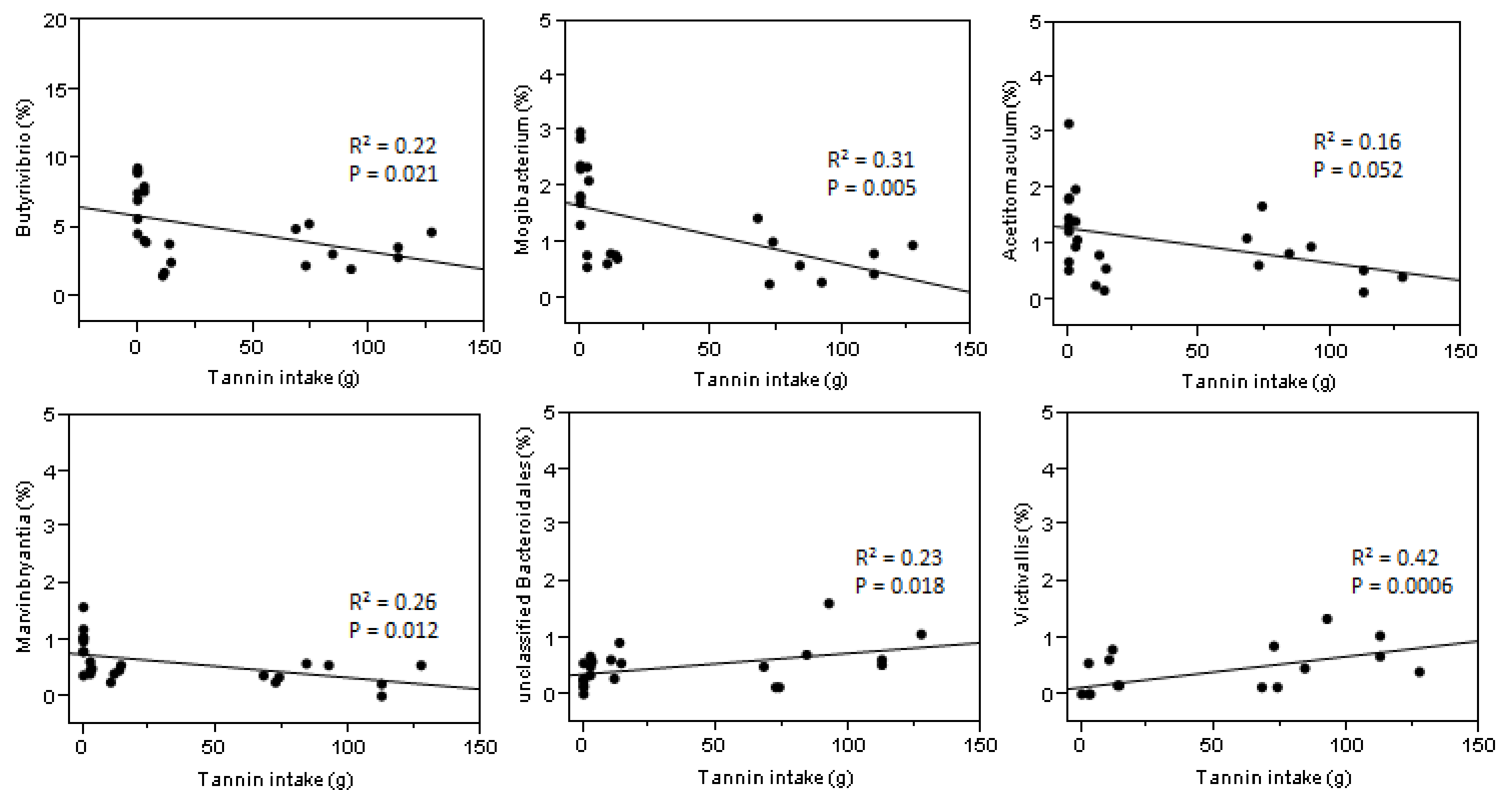
Figure 6.5. Effect of tannin intake on the most abundant phyla in the (A) rumen and (B) hindgut. Each scatter plot represents relative abundance of a phylum against tannin intake (g) for each animal. The R^2 and p-values are calculated from a simple linear regression.

A





B



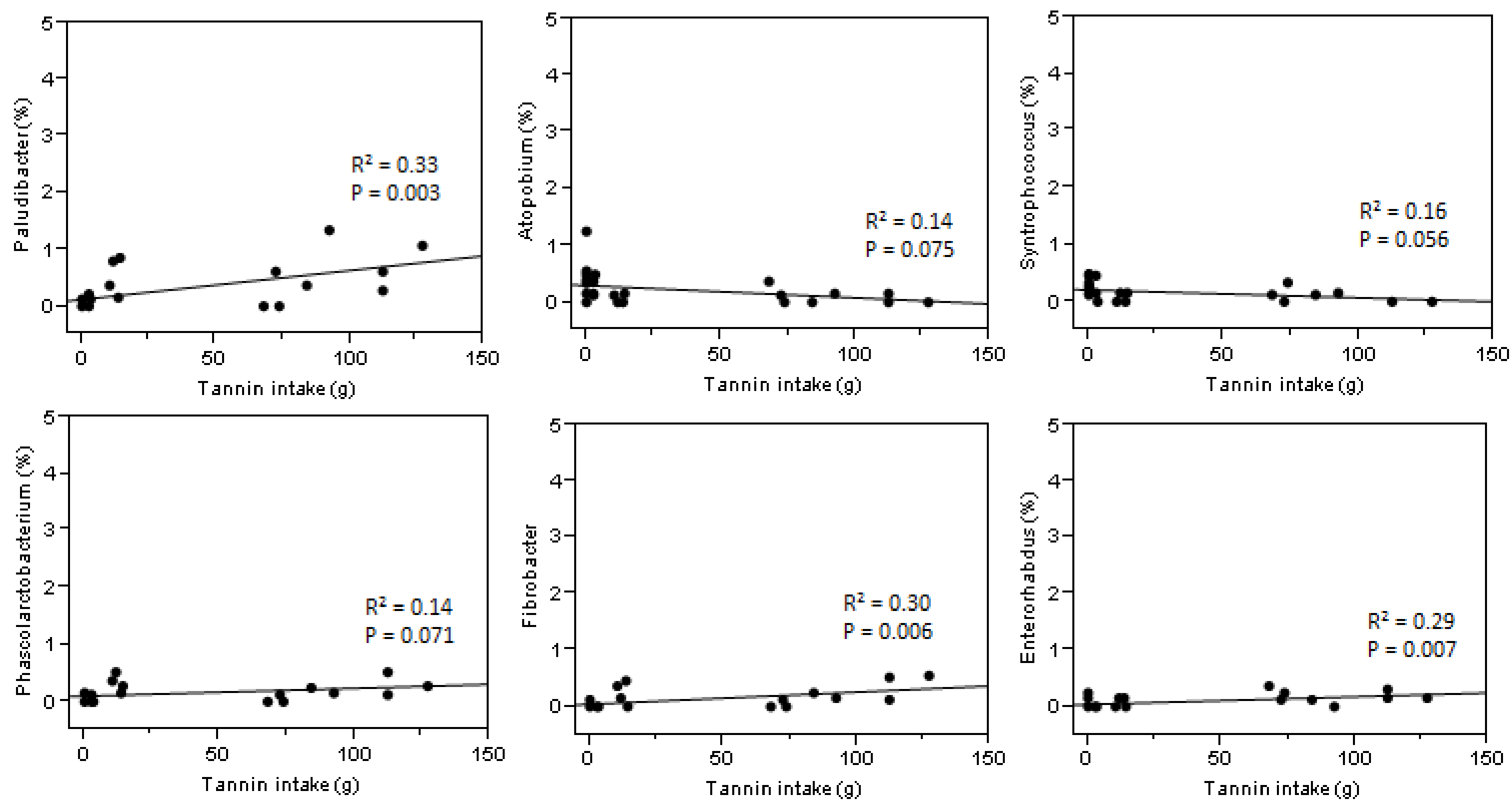
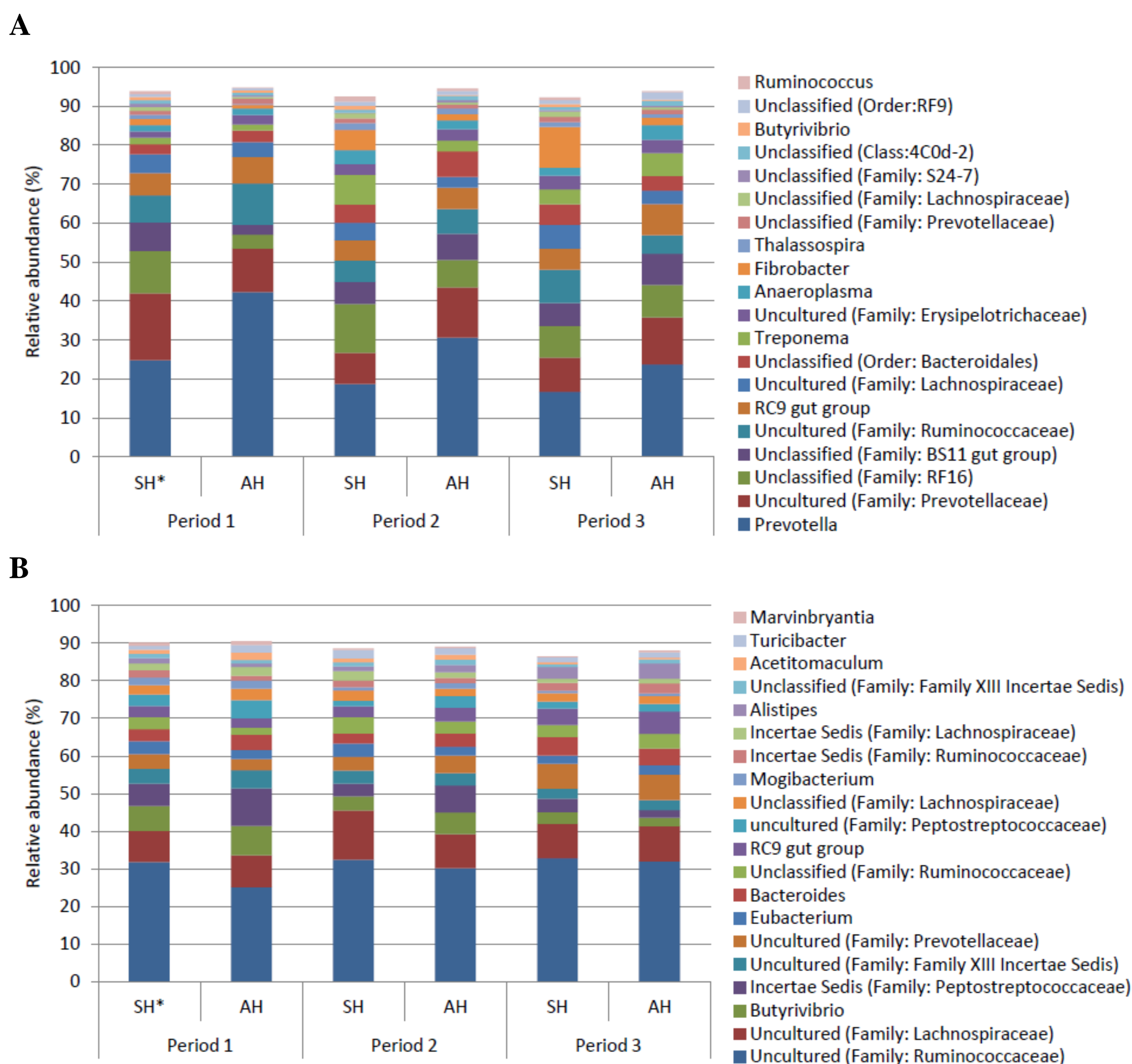


Figure 6.6. Effect of tannin intake on the relative abundance of genera in the (A) rumen, and (B) hindgut. Each scatter plot represents relative abundance of a genus against tannin intake (g) for each animal. The R^2 and P-values are calculated from a simple linear regression.



Supplementary Figure 6.7. Relative abundance of the top 20 microbial genera within the (A) rumen and (B) hindgut for each treatment group and time period. *All animal were fed an adaptation diet (alfalfa hay) during period 1. Abbreviations: SH, sainfoin hay; AH, alfalfa hay.

6.5 DISCUSSION

Microbial community dynamics in gut ecosystems remain poorly understood. Thus, predicting the response of a complex microbial community following environmental perturbations such as host stress, exposure to toxins and antimicrobials, becomes an even larger endeavour (Novak et al. 2010). Gut ecosystems house a large breadth of different bacteria which are tightly intertwined in a network-characterized by mutualistic, parasitic and commensalistic interactions (Chow et al. 2010), therefore it can be expected that a change in a single environmental factor will exert a “domino” type effect on the entire microbiome.

Historically, the rumen microbiome has been one of the most well studied ecosystems (Krause and Russell 1996). Ruminants depend on the rumen microbial community to digest their otherwise indigestible feedstuff and convert it to short-chain fatty acids and microbial proteins (Hobson and Stewart 1997). Research interest in optimization of rumen function by manipulation of the rumen ecosystem remains high with the goals of increasing feed conversion efficiency, decreasing methane emission, reducing nitrogen excretion and fecal pathogen shedding (Hobson and Stewart 1997; Kim et al. 2011). The aim of this study was to characterize the effects of feeding cattle a tanniniferous diet upon the rumen and hindgut microbiome.

The benefits of CTs in animal production systems have been documented. Most notably, CTs have been shown to improve protein utilization, prevent bloat, act as an anti-helmintic, and reduce methane emissions (Aerts et al. 1999; Li et al. 1996; Min and

Hart 2003; Waghorn et al. 2002). Its main mode of action in producing these benefits in animal production systems involves its interaction with microorganisms.

Jones et al. (1994) examined the effects of sainfoin on four rumen organisms: *Butyrivibrio fibrisolvens*, *Streptococcus bovis*, *Prevotella ruminicola*, and *Ruminobacter amylophilus*. Their study concluded that CTs at 100 and 25 μg mL^{-1} inhibited growth and protease activity, respectively in *B. fibriosolvens* and *S. bovis* whereas *P. ruminicola* and *R. amylophilus* were not affected (Jones et al. 1994). The authors attributed the inhibitory activity of CT to inhibition of extracellular enzymes which was more pronounced in organisms that were Gram-positive such as *B. fibrisolvens* and *S. bovis*. In the present study, positive correlations were detected between CT concentration and rumen cellulolytic organisms such as *Fibrobacter spp.* and *Ruminococcus spp.,* which indicated the potential for increased fibre digestion (Fig. 6.6A). The increase in cellulolytic organisms was also supported by an increase in *Treponema spp.*, which have been shown to have a strong symbiotic relationship with *Fibrobacter* and *Ruminococcus spp.* by fermenting soluble polysaccharides released as a by-product of cellulose breakdown (Stanton and Canale-Parola 1980). This finding is contradictory to other studies which have demonstrated that fibre digestion is reduced in animals fed a tanniniferous diet (Barry and Manley 1986; Palmer et al. 2000; Waghorn et al. 1987). In addition, this finding refutes our hypothesis in the discussion of chapter III where we attributed the decrease in *E. coli* spp. numbers to the inhibitory activity of CT against cellulolytic organisms resulting in lower levels of soluble polysaccharides available as substrate for *E. coli*. Although acid detergent fibre (ADF) values in sainfoin were significantly higher than alfalfa (Bouchard 2011), this likely resulted in increased substrate availability for

cellulolytic organisms. The increase in cellulolytic bacteria in the present study was concurrent with a marked decrease in the non-cellulolytic *Prevotella spp* (Fig. 6.6A). Owing to their high abundance in the rumen, *Prevotella spp.* can be asserted to play an important role in rumen function. The *Prevotella spp.* are well-known for their role in glucose homeostasis (via gluconeogenesis) in ruminants (Purushe et al. 2010). These non-cellulolytic organisms are also known to have proteolytic activity, and use peptides and ammonia as nitrogen sources (Purushe et al. 2010). In the present study, the large decline in *Prevotella spp.* may have stemmed from the protein protective effect of CTs limiting the amount of protein available as substrate for proteolytic bacteria such as *Prevotella spp*.

Unlike the rumen, the hindgut microbial community was dominated by the Firmicutes followed by Bacteroidetes (Fig. 6.3A and B). Similar to previous studies, it was found that organisms from the *Ruminococcaceae*, *Lachnospiraceae*, and *Prevotellaceae* family dominated the hindgut community (Callaway et al. 2010; Dowd et al. 2008; Shanks et al. 2011). A post-ruminal effect of tannins was not as evident at the phylum level as was seen in the rumen (Fig. 6.3B and Fig. 6.5B), although the cluster analysis showed that hindgut communities from animals fed the same diet were more similar to each other than those not fed the same diet (Fig. 6.4). Condensed tannins complex with proteins in the rumen and dissociate in the abomasum and lower gut, making proteins available for absorption by the host (Mueller-Harvey 2006). The supposition is that CTs are not generally absorbed by the host and remain largely undigested throughout the ruminant gastrointestinal tract and are subsequently excreted with little or no conformational modification (Terrill et al. 1994), therefore, the inhibitory

effect of CTs should carry over to the hindgut. At the phylum level, no correlations with condensed tannins could be detected in the hindgut, but at the genus level, many correlations were seen in organisms of low abundance (<1%) (Fig. 6.5B and 6.6B). Specifically, unclassified *Bacteroidales, Victivallis, Paludibacter, Phascolarctobacterium, Fibrobacter,* and *Enterohabdus* all appeared to have a significant positive correlation with CTs while *Butyrivibrio, Mogibacterium, Acetitomaculum, Marvinbryantia, Atopibium* had a negative correlation (Fig. 6.6B). Interestingly, genera showing a strong positive correlation in the hindgut were Gram-negative and genera with negative correlations were Gram-positive. These differences in Gram reaction correlation were also seen in a study by Smith and Mackie (2005) where they characterized the effect of CTs on the microbial population in the hindgut of rats. They reported a shift in the fecal bacterial population towards Gram-negative organisms such as *Enterobacteriaceae* and *Bacteroides* spp. due to the increased tolerance of these organisms to CTs. Smith and Mackie (2005) also reported a decrease in *Bacteroides*, *Prevotella*, and *Porphyromonas*, all low-G+C Gram-positive organisms. Also noteworthy is that all genera showing a negative correlation with CT concentration in the hindgut were, with the exception of *Atopibium*, all classified in the order Clostridiales (Gram positives). This finding is congruent with other studies which have also reported the inhibitory activity of CTs on clostridial species in the hindgut (Okubo et al. 1992; Smith et al. 2005). Jones et al. (1994) reported an inhibitory effect of CTs on the protease activity of *Butyrivibrio fibrisolvens* and *Streptococcus bovis*. The proposed modes of action include formation of CT and cell coat protease complexes resulting in inhibition of proteolytic activity and inhibition of cell wall synthesis (Jones et al. 1994).

The ruminant gut biodiversity dynamics seen in this study tend to agree with two prominent ecological models: niche theory and Hubbell's unified neutral model. The niche theory, a model branching from the island biogeography theory, states that species within an ecosystem are modulated by biotic and abiotic environmental parameters required for growth and symbiotic interactions (Chase and Leibold 2003). Hubbell's unified netural theory of biodiversity and biogeography states that the sum of species abundance changes must be equal to zero (Hubbell 2001). More specifically, a community harbours a fixed number of species, thus a decrease or extinction of a taxon will be replaced by an increase or immigration of another. Hubbell described this effect as the zero-sum dynamics of ecological drift, which is homologous to the process of genetic drift. In the present study, both theoretical models were applicable. Within the rumen, the most notable change seen between diets was a decrease in abundant organisms (Bacteroidetes, namely *Prevotella spp.*), which were replaced by an increase in less abundant organisms such as Fibrobacters and Spirochatetes (*Treponema spp.*), with richness remaining even between both treatment groups in time period three (Table 6.1, Fig. 6.3A). Additional evidence of the community shift can be seen in Fig 6.2A for time period three, where the exponent of the non-parametric Shannon diversity index was similar in both diets, however the inverse of the Simpson diversity index was much lower in the sainfoin fed group (Fig. 6.2A). The Shannon diversity index is more sensitive to rare species whereas the Simpson index has a bias towards more abundant species (Jost 2006), thus the calculated difference between these diversity indices can be used as an indicator of rare species. As seen in the present study, rare species in the rumen increased in animals fed a tanniniferous diet (Fig. 6.2A). As previously explained, the decrease in

Prevotella spp. and increase in cellulolytics was likely modulated by feeding a sainfoin diet higher in CTs and non-digestible fibre. It thus appears that both the niche theory and unified neutral model both hold true within the rumen.

In comparison to the rumen, the hindgut is a much less-well studied microbial ecosystem. As shown in the rarefaction curves (Fig. 6.1), it appeared the hindgut samples were much further from reaching the asymptote than rumen samples and thus deeper sequencing and more knowledge on the functional roles of members of this ecosystem would be required to deduce assertions based on ecological theories.

In conclusion, this study provides further evidence that even at low concentrations, CTs can exert a shift in the microbial community of the rumen and to a lesser extent in the hindgut without negatively affecting animal production. Nonetheless, it remains difficult to study the effect of environmental factors such as tanniniferous diets on microbial community dynamics without full characterization of the rumen and hindgut biodiversity and knowledge of their functional attributes. Future studies should focus on closing this knowledge gap by defining a benchmark for biodiversity within the ruminant gut.

BRIDGE TO CHAPTER SEVEN

Studies from chapter four, five, and six indicate that it is difficult to predict the behavior of both an individual member and the ecosystem as a whole in the presence of fluctuating environmental conditions. However, predictive power can be gained by applying theoretical macro-ecology concepts to micro-ecosystems. Garnering a greater understanding of the ruminant gut ecosystem and its behavior has positive implications for both ruminant and human health.

The following study examines and characterizes the microbial community structures of the rumen and hindgut by applying theoretical concepts from the well-defined island biogeography theory.

CHAPTER SEVEN

MANUSCRIPT V

Heterogeneity of the rumen and hindgut microbial ecosystem

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7.1 ABSTRACT

The applicability of island biogeography theory to the ruminant gut microbial community was examined as it is often assumed that the rumen and hindgut have similar microbiome composition but this supposition has never been tested. Garnering a greater understanding of ruminant microbial communities has significant implications not only for microbial ecology but also for veterinary and human medicine. The island biogeography theory, developed in the 1960s, has been a fundamental paradigm in the area of macro-ecology. Despite its popularity among ecologists, very few scientists have attempted to apply this theory to gut microbial ecosystems. One of the key concepts of this theory is the species-area power relationship stating that island size is directly proportional to species richness of the island. A perplexing question for ruminant microbiologists is whether microorganisms found in the rumen disperse to and colonize the hindgut. The applicability of the island biogeography theory to the heterogeneous environments that exist within the digestive tract was tested, specifically that of the ruminant animal. Microbial community composition from rumen fluid and fecal samples was determined using pyrosequencing. Results showed that microbial communities from the rumen and hindgut differed in relative abundance of specific organisms, but overall few organisms were exclusive to either location. It was also determined that rumen and hindgut z values, the power exponent from the species-area power law, were comparable to that seen in other microbial ecosystems. This is the first study that has applied the island biogeography theory to a mammalian gut microbial ecosystem.

7.2 INTRODUCTION

A global understanding of the heterogeneity of the gut microbiome is a fundamental question not only in humans but also for ruminants. The rumen is analogous in function to that of the human colon in that both chambers harbor microorganisms capable of fermenting undigested feedstuffs. Historically, the rumen, a pregastric fermentation chamber very adept at degrading plant cell wall material (Krause et al. 2003), has been one of the most well studied microbial ecosystems as a topic of investigation since the 1940's (Hobson and Stewart 1997; Hungate 1942). In contrast, the hindgut (collectively the cecum, colon, and rectum) of ruminants has been almost completely ignored, and studies on this microbiome have largely been dominated by research regarding zoonotic pathogens (Van Soest 1994).

The rumen and hindgut are linked anatomically by the omasum, abomasum (gastric stomach) and small intestine. As such, it is often assumed that the microbial populations of the rumen and hindgut are identical, or at least very similar (Lewis and Dehority 1985). But is this actually true? The same assumptions can be made with respect to the human digestive tract with anatomical links between the gastric stomach and colon. To our knowledge, only two studies have characterized and compared the rumen and hindgut microbiomes within the same animal (Callaway et al. 2010; Romero-Perez et al. 2011). Evaluation of the gut microbiome within the same animal over time is an important consideration as research has demonstrated that significant variation among individual animals occurs over time (Durso et al. 2010).

It was hypothesized that the microbial ecosystem of the ruminant digestive tract

may follow the paradigm of island biogeography. The island biogeography theory, first published by MacArthur and Wilson (1967), suggests that the taxonomic richness of an island is controlled by factors such as island size, balance between immigration and extinction, number of predators, and dispersal barriers. According to MacArthur and Wilson (1967), an island may be defined as any expanse of suitable habitat isolated from its mainland (a large habitat area having a stable population). As such, the island community structure is predominantly determined by the rates of immigration and extinction, which are influenced by the distance between the island and mainland and island size. As shown by MacArthur and Wilson (1967), an increase in the intervening distance results in a lower immigration rate, a concept known as the distance-decay law. Therefore, as the distance between an island and its mainland increases, the similarity in species composition between both communities will decrease. Another assumption made in the island biogeography theory states that as island size increases, a decrease in the rate of species extinction will be observed. MacArthur and Wilson (1967) then used the power function to suggest the "species-area relationship", which assumes that species richness will increase as island size increases.

Another facet to consider in the species-area relationship is habitat diversity. Invariably, as island size increases so will habitat diversity, therefore the heterogeneity of habitats within an island must be considered before deducing conclusions using the species-area relationship (Whittaker and Fernandez-Palacios 2007). Thus, it is possible to have higher species richness in a smaller area if habitat diversity is high. These theories have not been tested extensively in microbial ecosystems nor have they been studied within the mammalian gut.

Based on these theories, it is predicted here that the rumen and hindgut act as two vastly different microcosms each having a unique microbiome as a consequence of different functional attributes and dispersal barriers which exist between the rumen and hindgut. The rumen is larger in size than the hindgut, thus species richness is expected to be higher in the rumen according to the species-area relationship (MacArthur and Wilson 1967; Van Soest 1994). Despite a disparity in species richness due to the size differential, it was postulated that the rumen and hindgut would, nonetheless, be similar in species composition because they are anatomically linked; however, species abundance would differ as dictated by the distance-decay law. Overall, it was postulated that the ruminant gut as a whole would follow MacArthur and Wilson's (1967) island biogeography theory with the rumen functioning as the mainland and hindgut as an isolated island. To test these predications, the rumen and hindgut microbiome of four steers fed an alfalfa hay diet over an 11-week period using high throughput pyrosequencing of the V1 to V3 region of the 16S rRNA gene were compared. The sequence reads were converted to operational taxonomic units (OTUs) and analyzed using a standardized taxonomic vocabulary which was then used to interpret microbiome data within the context of the historical rumen microbiological literature.

7.3 MATERIALS AND METHODS

Animal trial and samples

Rumen fluid and fecal grab samples were taken from a beef cattle trial described in Berard *et al.* (2009). Animals were cared for in accordance with the guidelines of the Canadian Council on Animal Care (1993). Briefly, samples from four beef steers housed in the same outdoor pen and fed an alfalfa hay diet from January to March 2007 (2-week adaptation and 9-week experimental period). Fecal grab and rumen fluid samples collected using the oro-ruminal (Geishauser) probe method (Geishauser 1993), were taken once per week during weeks 1 to 2 (dietary adaptation period), weeks 4 to 7, and biweekly thereafter over an 11-week period. Animals were weighed weekly before feeding.

DNA extraction

For the purpose of this study, genomic DNA was extracted from rumen fluid and fecal samples taken during weeks 1, 2, 5, 6, 9, and 11 using the ZR Fecal DNA MiniPrep Kit (Zymo Research, Orange, CA, USA). The DNA was quantified by measuring absorbance at 260 nm using a spectrophotometer (Beckman Coulter, Brea, CA, USA). Each sample was amplified using 27f (5'-GAAGAGTTTGATCATGGCTCAG) and 342r (5'-CTGCTGCCTCCCGTAG) primers to specifically target the V1 to V2 hypervariable regions of the 16S rRNA bacterial gene to assess PCR efficiency. Each 25 µL PCR reaction contained 2.5 µL Econo Taq 10X reaction buffer (Lucigen, Middleton, WI, USA), 0.1 µL Econo Taq DNA Polymerase with Mg++ (Lucigen), 1 µL $MgCl_2$ (Bio-Rad Laboratories Inc, Hercules, CA, USA; 50 mM), 0.5 µL each of the forward and

reverse primers (25 µM), 0.5 µL dNTP Mix (Bio-Rad Laboratories Inc), 18.9 µL nuclease-free water (Promega, Madison, WI, USA), and 1 µL genomic DNA. Polymerase chain reactions were carried out in a thermo cycler (Bio-Rad Laboratories Inc) under the following conditions: initial denaturation at 94˚C for 3 min, 36 cycles of denaturation at 94˚C for 60 sec, annealing at 60˚C for 60 sec, elongation at 72˚C for 60 sec, followed by a final extension at 72˚C for 5 min. The amplicons were subsequently visualized using electrophoresis. If an amplicon could not be adequately visualized, the genomic DNA was diluted until a clear amplicon could be seen.

Bacterial tag-encoded GS FLX Titanium amplicon analysis

All genomic DNA samples were diluted to a concentration of 50 µg mL^{-1} and final volume of 50 µL. For each sample type (rumen fluid or fecal samples), composite samples for sequencing were created for each animal by combining equal amounts (25 µL) of diluted genomic DNA (50 µg mL^{-1}) from weeks 1 and 2 (time period 1), 5 and 6 (time period 2), and 9 and 11 (time period 3). A total of 24 genomic DNA samples (12 rumen, 12 fecal) were sent to the Research and Testing Laboratory (Lubbock, TX, USA) for pyrosequencing using a 454 GS FLX Titanium Sequencing System (454 Life Sciences, Branford, CT, USA) targeting the V1 to V3 hypervariable regions of the 16S rRNA gene, as described in Dowd et al. (2008).

Operational taxonomic units (OTU)-based data analysis

An OTU-based data analysis was conducted using Mothur, a comprehensive software package to analyze microbiome sequence data (Schloss et al. 2009). Low-quality sequences were removed from the dataset based on the following criteria: average quality score below 35, longest homopolymer greater than 8 nucleotides and presence of

an ambiguous base call ("N"). After removal of low-quality sequences, the dataset was comprised of 35 772 sequences. Sequences were aligned using kmer (kmer size=8) searching against the SILVA alignment database for 16S rRNA gene (http://www.arb-silva.de/). The Needleman algorithm was used to make pairwise alignments between the query and template (de-gapped SILVA alignment database) sequences. Subsequently, gaps were re-inserted in both query and template (SILVA alignment database) sequences using the NAST algorithm. Sequences which did not cover the longest alignment region were culled from the dataset. A pre-cluster step was performed using tools in the Mothur software package as suggested by Huse et. al (2010) to remove pyrosequencing noise due to pyrosequencing base call errors. The pre-cluster algorithm searches for rare sequences which are highly similar to abundant sequences. If the similarity between the rare sequence and abundant sequence is within the similarity threshold of the algorithm, the rare sequence will be clustered with the abundant sequence (i.e. rare sequence is an artifact of pyrosequencing base call error). Following completion of the pre-cluster analysis, the final dataset was comprised of 26 099 sequences.

Pairwise distances between aligned sequences were calculated and distance values were used to assign sequences to OTUs using a complete linkage (furthest neighbor) algorithm. A 95% similarity cutoff was used to cluster sequences into OTUs. Richness (Chao1 and ACE) and diversity (non-parametric Shannon and Simpson) indices were calculated using Mothur package tools (Schloss et al. 2009). The effective number of species for Simpson's reciprocal index was calculated by taking the inverse of the Simpson index while the effective number of species for the Shannon index was calculated by taking the exponential of the Shannon diversity index (Jost 2006).

The most abundant sequence from each OTU (95% similarity cutoff) was retrieved and classified using a Bayesian classifier with a minimum bootstrap cutoff of 80% against the SILVA reference non-redundant database (SILVA Ref NR). The SILVA Ref NR database follows the European Molecular Biology Laboratory (EMBL) taxonomy assignment and has been manually curated to include the most recent taxonomic information related to Bergey's Taxonomic Outline of the Prokaryotes (Garrity et al. 2004).

Phylogenetic analysis of sequenced data

Phylogenetic analysis was conducted using Fast UniFrac (Hamady et al. 2010). A megablast search (Altschul et al. 1990; Hamady et al. 2010) of the query sequences against the greengenes core set (DeSantis et al. 2006b) was performed. A sample identification mapping file was generated using a python script (http://128.138.212.43/fastunifrac/tutorial.psp) and the blast output file as input. The sample identification mapping file was used to link the query sequences' sample identification tags to sequence identification tags in the greengenes reference tree. A category mapping file was created to link the sample identification tags to group categories (i.e. sample type, time period, animal number). The sample identification tag and category mapping files were uploaded to the Fast UniFrac online server (http://128.138.212.43/fastunifrac/index.psp) and the greengenes core reference tree was selected. An unweighted principal coordinate analysis (PCoA) was selected.

Species-area relationship: calculation of *z* values, exponent in the power model

Number of OTUs (97% similarity) from Chao1 indices were plotted against either rumen (kg) or hindgut (kg) weight on a $\log_{10}$ scale for individual animals at each time period.

According to Parra (1978), total rumen and hindgut (colon and rectum) liquid content is estimated to represent approximately 9-13% and 0.8-1.5% of total body weight, respectively. In this study, median values for rumen (11%) and hindgut (1.15%) volumes were used. Power-law trendline, equation, and R^2 values between each gut compartment and species richness were also calculated and z slope values were retrieved for each animal for both the rumen and hindgut.

Statistical analysis

Statistical comparisons between sample types (rumen vs. hindgut), individual animals, and time periods were made using richness and diversity indices in addition to using abundance data at the phylum and genus level. Any organism identified in only one sample, was not included in the statistical analysis due to the possibility of misclassification or pyrosequencing error. A Box-Cox power transformation (Box and Cox 1964) was used to transform non-normally distributed diversity and richness indices using a Box-Cox SAS macro (http://www.datavis.ca/sasmac/boxcox.html). Richness and diversity indices were analyzed using the mixed procedure in SAS v.9.2 (SAS Institute, Cary, NC, USA). The Generalized Linear Mixed Model (GLIMMIX) procedure in SAS using either a Poisson distribution (log link) or negative binomial (logit link) was used to analyze abundance data at the phylum and genus level. The Pearson Chi-Square/DF and -2 log likelihood were used as indicators to assess model fit. Mean z slope values were calculated for rumen and hindgut and a statistical comparison of the means was made using a Student's t-test.

7.4 RESULTS

Table 7.1 outlines the basic sequence metrics of individual animals at each time point. The number of sequences ranged from 764 to 2 518 and the number of OTUs ranged from 360 to as high as 786. However, samples with the highest number of trimmed sequences were not necessarily the ones with the highest number of OTUs. Coverage ranged from 0.4 to 0.7 and was in general agreement with rarefaction curves (Fig. 7.1).

Sample richness and diversity indices for individual animals are given in Table 7.1 to provide an indication of variation. These indices were also statistically analyzed (Fig. 7.2A & B). The presence of an individual effect (all time points pooled for each individual) was tested, in both the rumen and hindgut. However, no significant differences ($P < 0.05$) in richness nor diversity were detected between individuals in either sample location. Conversely, when all time points for all individuals within each sample type were pooled, a significant ($P < 0.05$) sample location effect was observed for both richness and diversity (all indices higher in the hindgut). Time was tested within each sample location by pooling data for all animals within each time period. A statistically significant effect of time on richness (ACE: $P < 0.05$; Chao: $P < 0.1$) was observed in the rumen but not in the hindgut (Fig. 7.2A). Contrarily, diversity (transformed into number of effective species) was significantly different (Simpson's reciprocal index: $P < 0.05$; Shannon's exponential index: $P < 0.1$) by time in the hindgut but not in the rumen (Fig. 7.2B).

Table 7.1. Distribution of sequences, coverage, richness and diversity indices[1] for rumen fluid and fecal samples collected from four steers fed an alfalfa or sainfoin hay diet across three time periods

Parameters	Animal 1						Animal 2						Animal 3						Animal 4					
	Rumen			Fecal			Rumen			Fecal			Rumen			Fecal			Rumen			Fecal		
	T1	**T2**	**T3**	**T1**	**T2**	**T3**	**T1**	**T2**	**T3**	**T1**	**T2**	**T3**	**T1**	**T2**	**T3**	**T1**	**T2**	**T3**	**T1**	**T2**	**T3**	**T1**	**T2**	**T3**
Number of trimmed sequences	970	777	946	1147	1059	807	2518	764	939	1435	1184	886	1671	933	1011	957	916	1062	995	697	776	1393	1261	995
Number of OTUs	515	465	548	697	733	586	416	431	576	555	615	682	778	360	536	654	648	786	491	453	512	665	714	712
Coverage	0.6	0.5	0.6	0.6	0.5	0.4	0.9	0.6	0.6	0.7	0.6	0.4	0.7	0.7	0.6	0.5	0.4	0.4	0.7	0.5	0.5	0.6	0.6	0.4
Richness																								
Chao1	1260	1343	1564	2195	2538	2143	1121	1231	1734	1950	1928	2563	1562	1259	1464	1980	2181	2759	1319	1345	1888	2326	2077	2565
ACE	1960	2592	2884	3273	5581	4170	2141	2439	2728	3267	3114	5504	1601	2953	2190	3775	2365	5287	1986	2558	4047	5375	4030	5674
Diversity																								
Simpson's index of diversity	0.006	0.007	0.003	0.002	0.002	0.002	0.393	0.007	0.002	0.029	0.016	0.001	0.003	0.033	0.004	0.002	0.002	0.001	0.008	0.003	0.003	0.018	0.011	0.002
Shannon's diversity index	6.3	6.3	6.5	6.8	7.1	7.0	3.1	6.2	6.6	5.6	6.2	7.4	6.6	5.2	6.3	7.0	7.1	7.4	6.1	6.5	6.6	6.1	6.6	7.2
Effective Species[2]																								
Simpson's reciprocal index	158	141	342	401	600	566	3	135	453	34	64	792	319	30	258	436	527	836	123	381	379	54	90	422
Exponential of Shannon's index	529	558	646	877	1215	1119	22	470	756	267	496	1578	723	183	555	1069	1154	1592	437	672	760	443	704	1282
Rare species	371	417	304	476	615	554	20	335	303	233	433	787	405	153	297	633	626	756	314	291	381	389	613	860

[1] Clustered within a 0.05 genetic distance

[2] Conversion of diversity indices (Simpson and Shannon) to true diversity (Jost 2006)

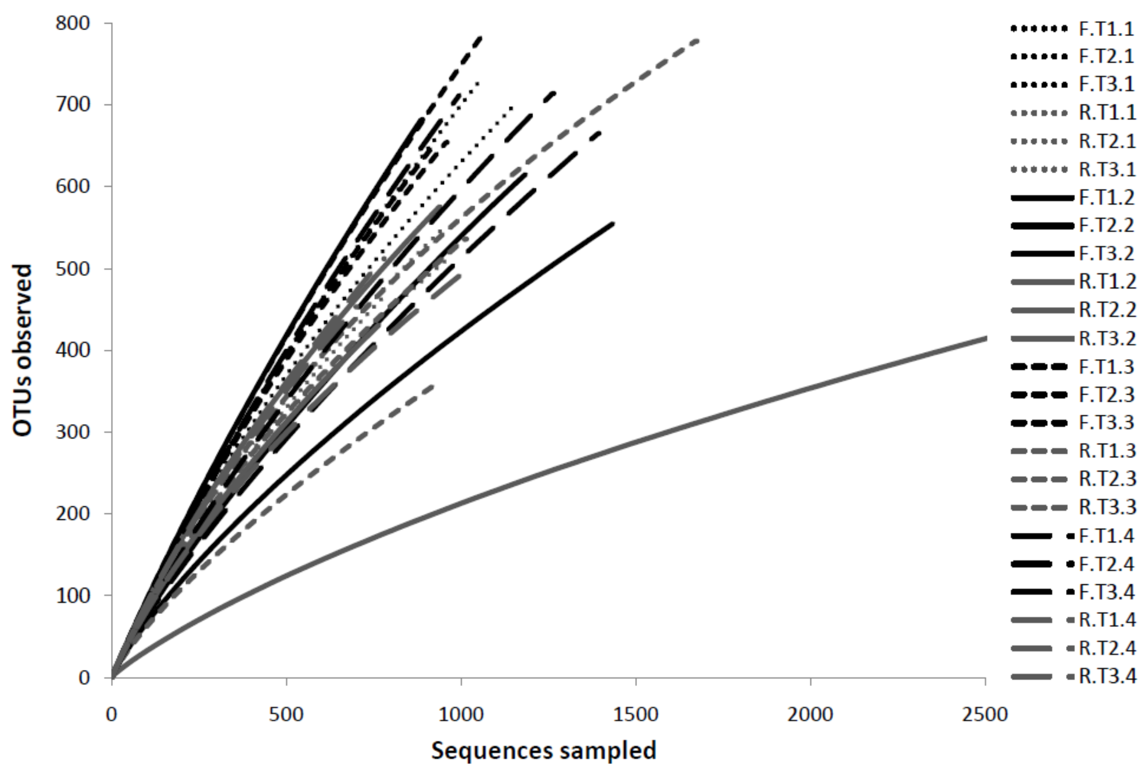

Figure 7.1. Rarefaction analysis at a 95% similarity cut-off for each OTU. Each line represents a sample and each line pattern represents a different animal. Sample names are assigned according to the following convention: first letter indicates a rumen or fecal sample, second letter ("T") followed by a number indicates time period, and the last number represents the animal number.

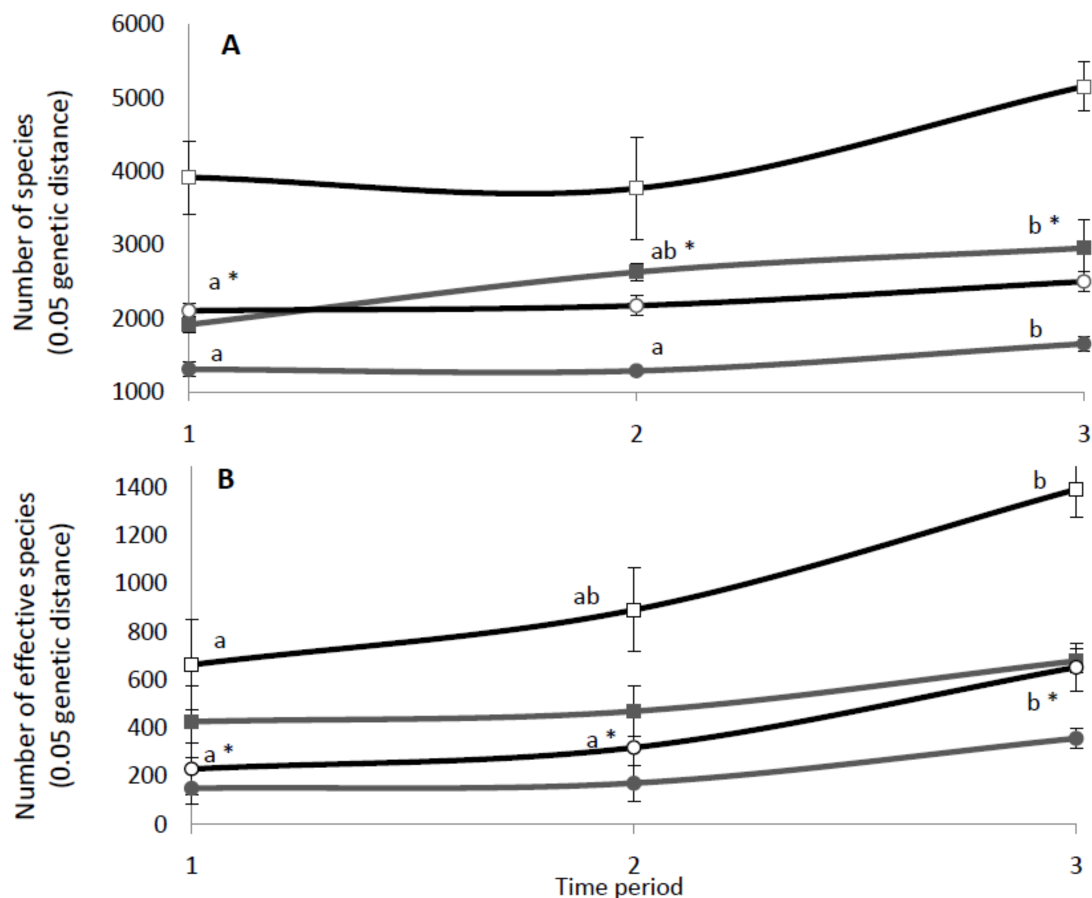

Figure 7.2. Species richness and diversity plots of rumen (filled markers) and hindgut (open markers) samples over time. (A) Chao (o) and ACE (□) richness indices. (B) Conversion of diversity indices to effective species: Simpson's reciprocal index (o) and the exponential of Shannon's index (□). Each data point represents animals averaged over time with standard errors of the mean for each index. Statistical significance of time period for each sample type was tested within each index. Statistical significance is denoted by different letters (P<0.05) or by a letter with an asterix (P<0.1). Each index was significantly different between sample type (P<0.05; significance not shown).

Examination of PCoA demonstrated that rumen and hindgut populations were vastly different (Fig. 7.3). The first axis explained 39.6% of the variation while the second axis only explained 3.6% of the population variation. Individual animals were also plotted and the degree of variation among animals was higher in the hindgut than in the rumen. The variation in the hindgut tended to be driven by variation over time. This trend was not as prominent in the rumen.

A total of 15 phyla were identified in the rumen and hindgut (Table 7.2). The Pearson square/DF criterion was used to test the statistical fit for phyla and the Poisson distribution was chosen as the best fit. The most dominant phyla in the rumen and hindgut were Bacteroidetes and Firmicutes, respectively. There was no difference in phyla composition between animals (within each sample location) and a time effect was only seen in the Bacteroidetes phyla within the hindgut.

Taxa at the family and genus level (Table 7.3) above a 1% relative abundance cut-off were divided into core rumen, core fecal or cosmopolitan (not confined to either gut compartments) taxa. This sample location core designation was based on the taxa being at least 1% of the population and the presence of a significant difference ($P < 0.1$) between rumen and hindgut. Using these criteria, 13 taxa were core to the rumen and 16 core to the hindgut. Taxa below 1% of the total appear in supplementary Table 7.1.

The species-area power law relationship was calculated for rumen and hindgut. Area of the rumen and hindgut were estimated from the weight of the animals. The z values were plotted individually by animal for rumen (Fig. 7.4A) and hindgut (Fig. 7.4B), respectively. The adjusted average z value for the rumen and hindgut was 0.89 and 0.64, respectively. One outlier was removed from each sample location due to low R^2 values

(Fig. 7.4A & B).

Figure 7.3. Rumen and hindgut bacterial communities clustered using principal coordinates analysis (PCoA) of the unweighted UniFrac metric matrix. Each point represents a sample. Time period is indicated for each point (T1, T2, or T3). The percent of variation described by the plotted PC axes is indicated on each axis.

Table 7.2. Phylum composition of samples collected from the rumen fluid and feces of four steers fed alfalfa-based diets

Phylum	Sample mean % (mean count)		P-value for parameter tested				
			Sample	Animal[1]		Time[1]	
	Rumen	Hindgut	Rumen vs. hindgut	Rumen	Hindgut	Rumen	Hindgut
Actinobacteria	0.25 (2.3)	0.84 (9.0)	NS	NS	NS	NS	NS
Bacteroidetes	59.57 (649.3)	14.93 (155.7)	<0.0001	NS	NS	NS	0.0094
Candidate division SR1(Sulphur River 1)	0.13 (1.3)	0.00 (0.0)	NS	NS	*	NS	*
Termite group 1 (TG-1)	0.10 (1.2)	0.01 (0.08)	NS	NS	NS	NS	NS
Candidate division TM7 (Torf, Mittlere Schicht)	0.07 (0.8)	0.02 (0.3)	NS	NS	NS	NS	NS
Chlamydiae	0.02 (0.3)	0.00 (0.0)	NS	NS	*	NS	*
Cyanobacteria	0.71 (7.6)	0.11 (1.2)	0.0639	NS	NS	NS	NS
Fibrobacteres	5.38 (54.2)	0.00 (0.0)	NS	NS	*	0.0396	*
Firmicutes	25.92 (287.3)	82.31 (871.4)	<0.0001	NS	NS	NS	NS
Lentisphaerae	0.57 (6.1)	0.34 (3.6)	NS	NS	NS	NS	NS
Planctomycetes	0.00 (0.0)	0.02 (0.2)	NS	*	NS	*	NS
Proteobacteria	1.94 (21.8)	0.40 (4.1)	0.0091	NS	NS	NS	NS
Spirochaetes	3.60 (38.6)	0.15 (1.6)	0.0004	NS	NS	0.0454	NS
Verrucomicrobia	0.02 (0.3)	0.36 (3.8)	NS	NS	NS	NS	NS
Unclassified	1.72 (18.2)	0.51 (5.4)	0.0303	NS	NS	NS	NS

Abbreviations: NS, not significant at $P < 0.1$

[1] Statistical significance tested between samples originating from same sample site (rumen or hindgut)

* no statistical analysis performed

Table 7.3. Composition of core genera (>1% relative abundance) in rumen fluid and fecal samples collected from four steers fed alfalfa-based diets

Taxa		Sample mean % (mean count)		P-value for parameter tested[1]		
				Sample	Time[2]	
Family: *Genus*	Phylum	Rumen	Hindgut	Rumen vs. Hindgut	Rumen	Hindgut
Prevotellaceae: *Prevotella*	Bacteroidetes	21.08 (230.8)	0.04 (0.4)	0.0011	NS	NS
RF16: *Unclassified*	Bacteroidetes	9.64 (108.5)	0.31 (3.3)	<0.0001	NS	NS
Prevotellaceae: *Uncultured*	Bacteroidetes	9.41 (102.8)	3.15 (32.7)	0.0001	NS	NS
BS11 gut group:*Unclassified*	Bacteroidetes	5.44 (58.6)	0.12 (1.3)	0.0002	NS	NS
Fibrobacteraceae: *Fibrobacter*	Fibrobacteres	5.39 (54.2)	0.00 (0.0)	NS	0.0278	*
Rikenellaceae: *RC9 gut group*	Bacteroidetes	5.04 (52.8)	2.30 (24)	0.0058	NS	NS
Prevotellaceae: *Unclassified*	Bacteroidetes	3.35 (36.3)	0.38 (3.8)	0.0011	NS	NS
Unclassified: *Unclassified*	Bacteroidetes	3.37 (34.8)	0.19 (2.0)	0.0014	NS	NS
Spirochaetaceae: *Treponema*	Spirochaetes	3.13 (33.3)	0.13 (1.3)	0.0066	0.0380	NS
Anaeroplasmataceae: *Anaeroplasma*	Tenericutes	2.30 (27.3)	0.05 (0.5)	0.0083	NS	NS
Erysipelotrichaceae: *Uncultured*	Firmicutes	2.35 (23.6)	0.36 (3.8)	0.0144	NS	NS
Unclassified: *Unclassified*	Unclassified	1.72 (18.2)	0.52 (5.4)	0.0302	NS	NS
Rhodospirillaceae: *Thalassospira*	Proteobacteria	1.14 (13.2)	0.09 (0.9)	0.0433	NS	NS
Ruminococcaceae: *Uncultured*	Firmicutes	5.49 (57.7)	24.33 (256.8)	<0.0001	NS	NS
Lachnospiraceae: *Unclassified*	Firmicutes	3.40 (37.8)	8.04 (85.3)	0.0035	NS	NS
Peptostreptococcaceae: *Uncultured*	Firmicutes	0.01 (0.08)	8.02 (84.3)	0.0462	NS	0.1338
Ruminococcaceae: *Unclassified*	Firmicutes	2.03 (22.5)	6.58 (69.8)	0.0007	NS	NS
Lachnospiraceae: *Uncultured*	Firmicutes	3.95 (45.0)	6.37 (67.6)	0.0482	NS	NS
Peptostreptococcaceae: *Unclassified*	Firmicutes	0.01 (0.08)	4.19 (44.3)	0.0003	NS	NS

Family_XIII_Incertae Sedis: *Unclassified*	Firmicutes	0.06 (0.7)	3.97 (41.8)	0.0034	NS	NS
Lachnospiraceae: *Butyrivibrio*	Firmicutes	0.84 (9.8)	3.73 (39.4)	0.0031	NS	NS
Clostridia(class): *Unclassified*	Firmicutes	0.95 (10.8)	3.19 (33.8)	0.0061	NS	NS
Bacteroidaceae: *Bacteroides*	Bacteroidetes	0.00 (0.0)	2.92 (30.3)	0.0031	*	NS
Family_XIII_Incertae Sedis: *Eubacterium*	Firmicutes	0.04 (0.4)	2.15 (22.8)	0.0310	NS	NS
Rikenellaceae: *Alistipes*	Bacteroidetes	0.00 (0.0)	1.83 (19.3)	0.0053	*	NS
Ruminococcaceae: *Incertae Sedis*	Firmicutes	0.07 (0.8)	1.52 (16.0)	0.0217	NS	NS
Family_XIII_Incertae Sedis: *Uncultured*	Firmicutes	0.11 (1.3)	1.47 (15.7)	0.0354	NS	NS
Erysipelotrichaceae: *Turicibacter*	Firmicutes	0.00 (0.0)	1.20 (12.7)	0.0985	*	NS
Family_XIII_Incertae Sedis: *Mogibacterium*	Firmicutes	0.12 (1.4)	1.02 (10.8)	0.0240	NS	NS
---Cosmopolitan genera---						
Bacteroidales (order): *Unclassified*	Bacteroidetes	1.79 (18.8)	1.78 (18.3)	NS	NS	NS

Abbreviations: NS, no significance at P < 0.1

[1] Effect of animal tested but not significant P < 0.1

[2] Statistical significance tested between samples originating from same sample site (rumen or hindgut)

* no statistical analysis performed

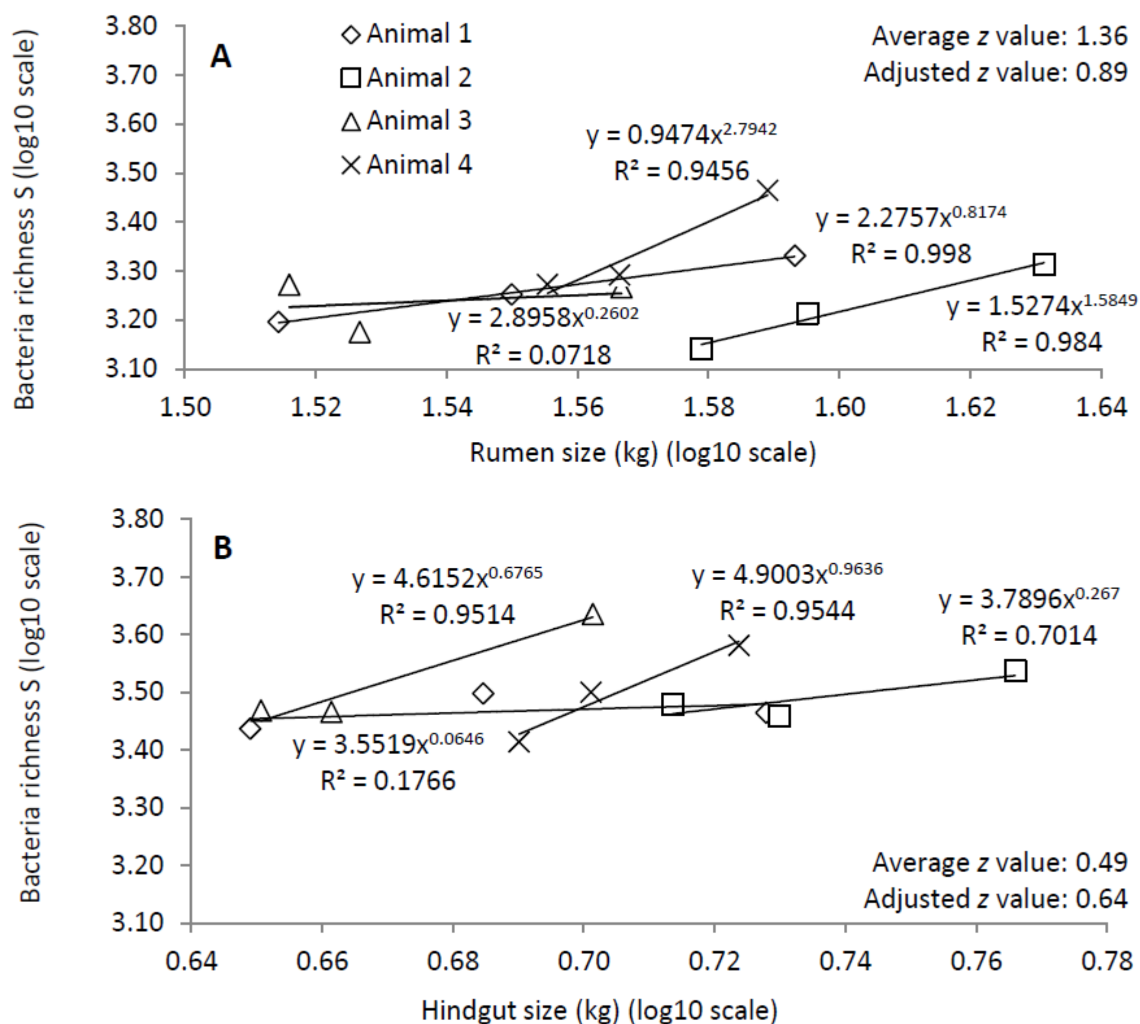

Figure 7.4. The species-area power relationship for the (A) rumen and (B) hindgut microbial community over time. A power-law trendline, equation, and R^2 value for each animal is included. Bacterial richness *S* (number of OTUs - 97% similarity using Chao1 richness indice) and rumen or hindgut size (kg) was plotted for each animal at all three time periods on a $\log_{10}$ scale. Statistical comparison of the means yielded a P-value of 0.1867.

Supplementary Table 7.1. Average genera (<1% relative abundance) in rumen fluid and fecal samples collected from four steers fed alfalfa based-diets

Family: *Genus*	Phylum	Sample mean %(mean count)	
		Rumen	Hindgut
Prevotellaceae: *Xylanibacter*	Bacteroidetes	0.36 (3.4)	0.00 (0.0)
Geodermatophilaceae: *Modestobacter*	Actinobacteria	0.02 (0.2)	0.00 (0.0)
Unclassified: *Unclassified*	SR1	0.13 (1.3)	0.00 (0.0)
Coriobacteriaceae: *Enterorhabdus*	Actinobacteria	0.00 (0.0)	0.08 (0.8)
Porphyromonadaceae: *Paludibacter*	Bacteroidetes	0.00 (0.0)	0.16 (1.7)
Porphyromonadaceae: *Parabacteroides*	Bacteroidetes	0.00(0.0)	0.07 (0.8)
Clostridiaceae: *Clostridium*	Firmicutes	0.06 (0.6)	0.18 (1.8)
Nocardiopsaceae: *Nocardiopsis*	Actinobacteria	0.00 (0.0)	0.02 (0.3)
Pseudonocardiaceae: *Saccharomonospora*	Actinobacteria	0.00 (0.0)	0.02 (0.3)
Pseudonocardiaceae: *Saccharopolyspora*	Actinobacteria	0.00 (0.0)	0.04 (0.4)
Nocardiopsaceae: *Thermobifida*	Actinobacteria	0.00 (0.0)	0.02 (0.3)
Corynebacteriaceae: *Corynebacterium*	Actinobacteria	0.00 (0.0)	0.02 (0.2)
Actinomycetales(order): *Unclassified*	Actinobacteria	0.00 (0.0)	0.02 (0.2)
M2PB4-65 termite group: *Unclassified*	Bacteroidetes	0.00 (0.0)	0.11 (1.1)
Porphyromonadaceae: *Barnesiella*	Bacteroidetes	0.00 (0.0)	0.10 (1.2)
Porphyromonadaceae: *Unclassified*	Bacteroidetes	0.00 (0.0)	0.03 (0.3)
Porphyromonadaceae: *Odoribacter*	Bacteroidetes	0.01 (0.1)	0.03 (0.3)
Bifidobacteriaceae: *Unclassified*	Actinobacteria	0.00 (0.0)	0.04 (0.4)
Coriobacteriacea: *Atopobium*	Actinobacteria	0.13 (1.1)	0.20 (2.1)
Coriobacteriacea: *Unclassified*	Actinobacteria	0.05 (0.4)	0.21 (2.3)
Coriobacteriacea: *Uncultured*	Actinobacteria	0.01 (0.1)	0.14 (1.5)
gir-aah93h0: *Unclassified*	Bacteroidetes	0.00 (0.0)	0.02 (0.3)
Porphyromonadaceae: *Candidatus Symbiothrix*	Bacteroidetes	0.00 (0.0)	0.02 (0.2)
p-2534-18B5 gut group: *Unclassified*	Bacteroidetes	0.00 (0.0)	0.31 (3.3)
Rikenellaceae: *dgA-11 gut group*	Bacteroidetes	0.00 (0.0)	0.21 (2.2)
Rikenellaceae: *Unclassified*	Bacteroidetes	0.01 (0.1)	0.73 (7.7)
S24-7: *Unclassified*	Bacteroidetes	0.17 (1.9)	0.13 (1.3)
Lineage III Elusimicrobium(class): *Unclassified*	Termite Group 1 (TG-1)	0.04 (0.4)	0.01 (0.1)
Lineage I Endomicrobia(class): *Unclassified*	TG-1	0.06 (0.8)	0.00 (0.0)
Unclassified: *Unclassified*	TM7	0.07 (0.8)	0.02 (0.3)
WA-aaa01f12: *Unclassified*	Chlamydiae	0.02 (0.3)	0.00 (0.0)
Chloroplast(class): *Unclassified*	Cyanobacteria	0.00 (0.0)	0.02 (0.3)
4C0d-29(class): *Unclassified*	Cyanobacteria	0.70 (7.5)	0.00 (0.0)
Bacillaceae: *Bacillus*	Firmicutes	0.02 (0.3)	0.02 (0.3)
Bacillaceae: *Terribacillus*	Firmicutes	0.00 (0.0)	0.02 (0.2)
Bacillaceae: *Unclassified*	Firmicutes	0.00 (0.0)	0.02 (0.2)
Clostridia(class): *Unclassified*	Firmicutes	0.02 (0.3)	0.05 (0.5)
Mollicutes(class): *Unclassified*	Firmicutes	0.07 (0.8)	0.01 (0.1)
RF3(class): *Unclassified*	Firmicutes	0.13 (1.3)	0.09 (0.9)
Erysipelotrichaceae: *Asteroleplasma*	Firmicutes	0.01 (0.2)	0.01 (0.1)
Erysipelotrichaceae: *Solobacterium*	Firmicutes	0.05 (0.5)	0.28 (3.0)
Erysipelotrichaceae: *Incertae Sedis*	Firmicutes	0.00 (0.0)	0.12 (1.3)
Erysipelotrichaceae: *Unclassified*	Firmicutes	0.01 (0.1)	0.17 (1.8)
Eubacteriaceae: *Eubacterium*	Firmicutes	0.00 (0.0)	0.02 (0.2)
Family XIII Incertae Sedis: *Anaerovorax*	Firmicutes	0.05 (0.6)	0.00 (0.0)
Lachnospiraceae: *Acetitomaculum*	Firmicutes	0.10 (1.0)	0.70 (7.4)
Lachnospiraceae: Blautia	Firmicutes	0.00 (0.0)	0.02 (0.3)
Lachnospiraceae: *Butyrivibrio-Pseudobutyrivibrio*	Firmicutes	0.41 (3.8)	0.00 (0.0)

Lachnospiraceae: *Catabacter*	Firmicutes	0.02 (0.3)	0.03 (0.3)
Lachnospiraceae: *Coprococcus*	Firmicutes	0.04 (0.6)	0.05 (0.6)
Lachnospiraceae: *Dorea*	Firmicutes	0.00 (0.0)	0.25 (2.6)
Lachnospiraceae: *Epulopiscium*	Firmicutes	0.00 (0.0)	0.04 (0.4)
Lachnospiraceae: *Howardella*	Firmicutes	0.04 (0.4)	0.06 (0.6)
Lachnospiraceae: *Incertae Sedis*	Firmicutes	0.18 (2.0)	0.90 (0.3)
Lachnospiraceae: *Lachnospira*	Firmicutes	0.00 (0.0)	0.03 (0.3)
Lachnospiraceae: *Marvinbryantia*	Firmicutes	0.04 (0.5)	0.49 (5.2)
Lachnospiraceae: *Oribacterium*	Firmicutes	0.09 (1.1)	0.00 (0.0)
Lachnospiraceae: *Roseburia*	Firmicutes	0.00 (0.0)	0.08 (0.8)
EMP-G18(order): *unclassified*	Firmicutes	0.02 (0.3)	0.05 (0.5)
RF9(order): *unclassified*	Firmicutes	0.84 (9.4)	0.55 (5.8)
Paenibacillaceae: *Brevibacillus*	Firmicutes	0.01 (0.1)	0.02 (0.2)
Paenibacillaceae: *Paenibacillus*	Firmicutes	0.00 (0.0)	0.02 (0.3)
Peptococcaceae: *uncultured*	Firmicutes	0.00 (0.0)	0.10 (1.1)
Peptostreptococcaceae: *Incertae Sedis*	Firmicutes	0.00 (0.0)	0.03 (0.3)
Unclassified: *Unclassified*	Firmicutes	0.62 (6.7)	0.70 (7.3)
Planococcaceae: *Lysinibacillus*	Firmicutes	0.02 (0.2)	0.01 (0.1)
Ruminococcaceae: *Anaerofilum*	Firmicutes	0.00 (0.0)	0.02 (0.3)
Ruminococcaceae: *Anaerotruncus*	Firmicutes	0.00 (0.0)	0.40 (4.2)
Ruminococcaceae: *Fastidiosipila*	Firmicutes	0.32 (4.1)	0.08 (0.9)
Ruminococcaceae: *Oscillibacter*	Firmicutes	0.00 (0.0)	0.02 (0.2)
Ruminococcaceae: *Ruminococcus*	Firmicutes	0.80 (10.2)	0.22 (2.3)
Staphylococcaceae: *Staphylococcus*	Firmicutes	0.01 (0.1)	0.01 (0.1)
Streptococcaceae: *Streptococcus*	Firmicutes	0.02 (0.2)	0.01 (0.1)
Lachnospiraceae: *Syntrophococcus*	Firmicutes	0.01 (0.1)	0.19 (2.0)
Thermoactinomycetaceae: *Thermoactinomyces*	Firmicutes	0.00 (0.0)	0.06 (0.7)
Veillonellaceae: *Phascolarctobacterium*	Firmicutes	0.00 (0.0)	0.10 (1.0)
Veillonellaceae: *Succiniclasticum*	Firmicutes	0.14 (1.8)	0.00 (0.0)
Clostridiales(order): *Unclassified*	Firmicutes	0.08 (0.8)	0.00 (0.0)
BS5(order): *Unclassified*	Lentisphaerae	0.03 (0.4)	0.00 (0.0)
RFP12(order): *Unclassified*	Lentisphaerae	0.14 (1.5)	0.02 (0.2)
Victivallaceae: *Uncultured*	Lentisphaerae	0.04 (0.3)	0.00 (0.0)
Victivallaceae: *Victivallis*	Lentisphaerae	0.36 (3.8)	0.32 (3.3)
Planctomycetaceae: *p-1088-a5 gut group*	Planctomycetes	0.00 (0.0)	0.02 (0.2)
Acetobacteraceae: *Unclassified*	Proteobacteria	0.00 (0.0)	0.10 (1.1)
Alcaligenaceae: *Sutterella*	Proteobacteria	0.37 (4.1)	0.02 (0.2)
Campylobacteraceae: *Campylobacter*	Proteobacteria	0.00 (0.0)	0.03 (0.3)
Candidatus_Captivus: *Unclassified*	Proteobacteria	0.01 (0.1)	0.01 (0.1)
Candidatus_Hepatincola: *Unclassified*	Proteobacteria	0.00 (0.0)	0.02 (0.2)
Class:Alphaproteobacteria: *Unclassified*	Proteobacteria	0.05 (0.6)	0.02 (0.2)
Class:Betaproteobacteria: *Unclassified*	Proteobacteria	0.02 (0.2)	0.00 (0.0)
Enterobacteriaceae: *Enteric Bacteria cluster*	Proteobacteria	0.00 (0.0)	0.02 (0.2)
Phylum:Proteobacteria: *Unclassified*	Proteobacteria	0.04 (0.5)	0.07 (0.8)
Rhodospirillaceae: *Uncultured*	Proteobacteria	0.07 (0.7)	0.00 (0.0)
Succinivibrionaceae: *Anaerobiospirillum*	Proteobacteria	0.05 (0.5)	0.00 (0.0)
Succinivibrionaceae: *Succinivibrio*	Proteobacteria	0.08 (0.8)	0.00 (0.0)
Succinivibrionaceae: *Uncultured*	Proteobacteria	0.02 (0.2)	0.00 (0.0)
Spirochaetaceae: *Spirochaeta*	Spirochaetes	0.13 (1.4)	0.02 (0.3)
Spirochaetaceae: *Unclassified*	Spirochaetes	0.34 (3.8)	0.00 (0.0)
Order:vadinHA64: *Unclassified*	Verrucomicrobia	0.02 (0.3)	0.04 (0.4)
Verrucomicrobiaceae: *Akkermansia*	Verrucomicrobia	0.00 (0.0)	0.32 (3.3)

7.5 DISCUSSION

The main goal of this study was to determine whether the island biogeography theory (MacArthur and Wilson 1967) can be applied to the microbial community structure of the ruminant gut ecosystem. Understanding factors which influence gut microbial communities will guide us towards a more complete understanding of the similarities and differences between the different gut ecosystems. Although taxonomically different, ruminant and human microbial gut functions have considerable overlap and there is a lack of appreciation for the extent of the similarities between these two gut ecosystems (Flint et al. 2008).

Principal coordinate analysis (Fig. 7.3) and categorical analysis (Table 7.2 and 7.3) demonstrated that the rumen and hindgut are distinct microbial ecosystems. Our results concur with that of Michelland et al. (2009) who used single-stranded conformational polymorphism analysis to evaluate the temporal and anatomical differences in the ruminant digestive tract. Although their techniques did not have the resolution to differentiate between taxa, they found that the rumen and hindgut had distinct microbiomes that varied over time. Callaway et al. (2010) were able to demonstrate microbiome differences between the rumen and hindgut of cattle fed grain-based diets via pyrosequencing of bacterial 16S rRNA genes. Romero-Perez et al. (2011) used terminal restriction fragment length polymorphism of 16S rRNA genes to examine differences between rumen and hindgut microbial communities of cattle fed forage diets and demonstrated that the microbiome between the two anatomical sites were also distinct. The analysis work conducted by both Callaway et al. (2010) and Romero-Perez

et al. (2011) was largely descriptive and did not examine the theoretical basis of observed differences that exist within microbial communities in the gut.

Although the rumen and hindgut have differing microbiome structures with Bacteroidetes dominating in the rumen and Firmicutes in the hindgut (Fig. 7.3, Table 7.2), it is largely a difference in taxa abundance not a difference in presence or absence of these species (Tables 7.1, 7.2 and 7.3). At the phylum level (Table 7.2), almost all phyla, with due exception of Fibrobacters, were found in both the rumen and hindgut. Taxa were arbitrarily divided into core and non-core species, based on whether their relative abundance was greater than 1% and whether there was a statistically significant difference between the rumen and hindgut. If a taxon was not observed, it may have been below detectable limits of the pyrosequencing technique rather than absence from the site.

A comparison of our results to other 16S rRNA pyrosequencing studies indicates that although similar microbiomes were identified, some differences were evident. Callaway et al. (2010) compared the rumen and fecal microbiome of six cattle (n=3 Jersey cows and n=3 Angus steers) adapted to a high grain feedlot diet. In the rumen, they found that the three most abundant genera within the rumen were *Prevotella* (18.21%), *Succinivibrio* (11.81%), and organisms from the Bacteroidales order (11.69%) whereas the hindgut was characterized by *Clostridium* (19.74%), *Bacteroides* (10.45%), and organisms from the Bacteroidales order (4.83%). In another microbiome study, Dowd et al. (2008) studied the fecal microbiome of 20 Holstein dairy cattle fed a total mixed ration (mixture of chopped alfalfa hay, cracked corn, soybean meal, cottonseed meal and trace mineral salts). Their results indicate that the top three most abundant

genera within fecal samples were *Clostridium* (19.0%), *Bacteroides* (9.26%), and *Porphyromonas* (7.34%). In our study, the most predominant genera in the rumen were *Prevotella* (21.08%), unclassified organisms from the RF16 family (9.64%), and uncultured organisms from the Prevotellaceae family (9.41%) while the hindgut was characterized by uncultured organisms from the Ruminococcaceae family (24.33%), uncultured organisms from the Lachnospiraceae family (8.04%), and uncultured organisms from the Peptostreptococcaceae family (8.02%). Additionally, Callaway et al. (2010) reported a Firmicutes:Bacteroidetes ratio of approximately 0.35 and 1.20 in rumen and fecal samples. In comparison, our study found a 0.44 and 5.5 Firmicutes:Bacteroidetes ratio within rumen and hindgut microbiomes, respectively. Although the only gross similarity seen between our study and other studies were the abundance of *Prevotella* within the rumen, it is likely a ramification of dietary differences between studies and differences in methodologies used for downstream pyrosequencing data analysis (i.e. classification database and taxonomy used).

One of the objectives of the current study was to examine the similarity of microbial genera in the rumen and hindgut. It was anticipated that they would be fairly similar given that these physiological locations are anatomically linked and the passage of digesta is exclusively in the direction from rumen to hindgut. It is important to note that the colonizing success of these immigrating species from the rumen (mainland) to the new surrounding habitats in the hindgut (island) is dependent on the diversity of its competitors. However, composition can also be modulated by rates of immigration, as described in the theory. Therefore, an increase in distance between the mainland (rumen) and the island (hindgut) would result in a lower immigration rate and consequently a

lower microbiome similarity.

To examine the distance-decay relationship, research conducted by Bell (2010) was compared to the current study. Bell (2010) created a synthetic microbial ecosystem by placing sterile leaf litter microcosms at variable distances in an English woodland. He examined the relationship between microbial community similarity and distance between microcosms by quantifying similarity as a function of distance. There was an inversely proportional relationship between distances and microbial community similarity, only until a threshold was reached, beyond which the relationship approached a plateau.

It was predicted here that the rumen-hindgut system would follow the distance-decay law given that it was expected that the rate of immigration of rumen bacteria (through ruminal digesta flow) throughout the digestive tract would decrease as distance from the rumen increases. Thus, the similarity of ecological niches throughout the gut would nonetheless also decline as a function of relative distance from the rumen. This prediction would be correlated with the low microbial community structure similarities seen in the rumen and hindgut samples tested (Table 7.2 and 7.3), however microbial composition at varying distances from the rumen was not measured. Given that situation, it is suggested that sampling the digestive tract at different locations would provide a more conclusive test of the distance-decay hypothesis in the ruminant gut. Lending support to the present hypothesis, Khafipour et al. (2011) showed that the rumen and cecum microbiomes were vastly different while a high similarity existed between cecum and fecal microbiomes.

The island biogeography theory also makes mention of competitive exclusion and dispersal barriers as a function of immigration and colonization success. Environmental

factors such as fluctuations in pH, enzyme production, rates of digesta passage, and host immunity throughout the digestive tract can also act as dispersal barriers and thus influence establishment of microbial species (Van Soest 1994). Bell (2010) also conducted a transplant experiment where microcosms located at one extremity of the study area were transplanted to the opposite extremity. Their study showed that within a few days, the microbial community of the transplanted microcosms resembled that of the controls in the new location. Bell (2010) concluded that local environmental factors play a major role in determining microbial community structure beyond the distance-decay limitation as seen in the present study of the rumen-hindgut system.

The species-area power law relationship in macro-ecosystems has been well established. This law can be stated algebraically as $S = cA^z$, were S is the number of species, A is the area sampled, c is a constant that varies with each system tested, and z is the species-area exponent (slope) (MacArthur and Wilson 1967). The logarithmic transformation is often used to linearize the relationship and thus the equation becomes: $\log S = \log c + z \log A$. In macro ecosystems, the z value is in the range of 0.2 to 0.3. A steeper slope is indicative of a strong positive relationship between area and species richness whereas a low z value represents little spatial specialization.

The species-area power law cannot be used to make comparisons between the rumen and hindgut because, as already discussed, the local environment is a powerful driver of microbiome composition (vastly different in rumen and hindgut). However, the rumen and hindgut of separate animals can be compared over time (animal growth will yield bigger islands) and useful comparisons made. To estimate the area of the rumen and hindgut, well known correlations between animal weight and gut size were used (Van

Soest 1994). Using this relationship it became evident that as the “container” (rumen or hindgut within an animal) size increased, as a result of animal growth, species richness also increased. Average *z* value for rumen and hindgut were 0.89 (Fig. 7.4A) and 0.64 (Fig. 7.4B), respectively. Much research has proposed a disproportionate positive correlation between body mass and mean retention time (Demment 1983; Demment and Van Soest 1985; Gordon and Illius 1994). More specifically, it has been shown that as the animal grows, the gastrointestinal tract volume increases by a factor of $BM^{1.0}$ while the energy requirements of the animal only increases by a factor of $BM^{0.75}$ (Parra 1978) thus mean retention time would theoretically increase by a factor of approximately $BM^{0.25}$. This phenomenon has been termed as the Jarman-Bell principle (Geist 1974). Some research suggests that these correlations will effectively allow larger animals with an increased gut capacity to have longer digesta retention time and thus adapt better to lower quality feed. Applying this body mass and mean retention time correlation to this study would imply that larger animals may have higher gut microbial growth and thus increased microbial richness due to lower energy requirements scaling in comparison to smaller animals (McNabb 2002; Owen-Smith 1988). Moreover, the steeper slopes seen in both sample locations can be ascribed to the heterogeneity of both environments and implies that an increase in island size will also result in a rapid increase in diversity (Bell et al. 2005).

There has been considerable debate in the literature regarding the potential for microorganisms to follow the species-area power law (Bell 2010; Bell et al. 2005; Green et al. 2004; Horner-Devine et al. 2004). Many have suggested that *z* values for microorganisms are close to zero due to their ubiquitous nature (Azovsky 2002; Finlay et

al. 1998; Green et al. 2004). This ubiquity may be attributed to their minute size which enables them to penetrate dispersal barriers, their greater adaptability compared to larger organisms, and their ability to adapt to numerous habitats due to their genomic plasticity. The data presented herein suggest that this is not true as the z values obtained are comparable to those reported by Bell et al. (2005) (z=0.26). Perhaps the ubiquity of bacteria is more accurately described by Baas Becking's (1934) classical tenet "*everything is everywhere, but, the environment selects'* ". Baas Becking's theory suggests that bacteria are widespread yet, only latently present in any given environment and must be selected for by the environment in order to flourish (De Wit and Bouvier 2006).

Further, comparisons of species richness and diversity (transformed to number of effective species) of the rumen and hindgut (Fig 7.2A & B) indicate that both indices were higher in the hindgut despite the rumen being 10-times as large as the hindgut (Van Soest 1994). The higher diversity seen in the hindgut is logical as it is likely a ramification of ruminal digesta flow disseminating microorganisms from the rumen to the hindgut. This action would allow the hindgut to have a higher functional redundancy in comparison to the rumen, which is a more highly specialized niche (Hobson and Stewart 1997). Additionally, the absence of protozoa, a key predator in the reticulo-rumen, may also help explain the higher bacterial richness seen in the hindgut.

In conclusion, when comparing the rumen and hindgut microbial communities, the presence/absence of species are comparable; however, the relative abundance of each species was markedly different between both gut locations. Ecological concepts from the island biogeography theory were useful in explaining microbial community structures

within the ruminant digestive tract and may be transferable to studies of the human gut.

CHAPTER EIGHT

GENERAL DISCUSSION

Phytomedicine is not new, but its application to animal agriculture has only recently been explored. The trigger for this renewed interest in phytomedicine stems from the rising concern over antibiotic resistance and regulatory pressure towards abolishing the use of in-feed antibiotics in livestock production systems. A variety of secondary plant metabolites are well known for their antimicrobial properties, thus their use in animal production systems warrants further investigation (Cowan 1999; Rochfort et al. 2008). However, adding phytochemicals to a ruminant feed is usually not economical or practical in western Canada. Nonetheless, one of our objectives was to identify a phytochemical rich plant and evaluate its potential to reduce the survival of *E. coli* spp, a fecal indicator for pathogenic bacteria.

In manuscript I, the condensed tannin (CT) concentrations of various forage legume species grown in western Canada was examined. Of the legume species tested, *Dalea purpurea* (purple prairie clover) was found to have the highest mean CT concentration of 68.6 g kg^{-1} followed by *Onobrychis viciifolia* (sainfoin) with 46.0 g kg^{-1} and *L. corniculatus* (birdsfoot trefoil) with 15.1 g kg^{-1}. Research has shown that ruminant production benefits such as bloat prevention (Li et al. 1996), reductions in fecal parasite egg count (Min and Hart 2003), and protection of plant protein from microbial degradation (Aerts et al. 1999; Waghorn et al. 1987), can be seen when CTs are included at moderate concentrations between 5 and 55 g kg^{-1}. Thus the CT concentrations

reported in *D. purpurea* and *O. viciifolia* has the potential to achieve the aforementioned benefits in ruminant production systems.

In addition to containing moderate levels of CTs, sainfoin is advocated by the forage industry as an alternative high-yield perennial forage with excellent grazing and nutritional qualities (Marais et al. 2000; Thompson et al. 1971). Therefore, the objective of the next study (manuscript II) was to determine if phytochemicals present in sainfoin could in fact inhibit and reduce the survival rate of non-pathogenic and pathogenic bacteria such as *E. coli* O157:H7.

In this study, the MIC of purified phenolic compounds (coumarate, ferulate, and cinnamate) and sainfoin extracts (silage or hay) against various strains of *E. coli* (pathogenic and non-pathogenic) was determined and compared against the MIC of ciprofloxacin. The results indicated that the purified compounds were more inhibitory than sainfoin extracts, a cocktail of less inhibitory phytochemical compounds. The method of extraction was not selective for the more active compounds in the forage, therefore, less inhibitory compounds were presumably extracted thereby diminishing the inhibitory activity of the extract.

In the *in vivo* portion of this study, a feeding trial was performed whereby steers were fed a sainfoin or alfalfa diet (hay or silage). The inhibitory effects of sainfoin were measured by quantifying generic fecal *E. coli* during the experimental period. Results showed that cattle fed sainfoin (hay and silage) tended to have a lower count of fecal generic *E. coli* during the experimental period without the development of adaptation. It is suspected that the reason that there was no adaptation to the sainfoin was because of the presence of multiple compounds in sainfoin which include CTs, flavonol glycosides,

nicotiflorin, narcissi, and various cinnamic acid derivatives (Barrau et al. 2005; Lu et al. 2000). By virtue of their different chemical structures, the modes of antimicrobial action are probably different. Consequently, resistance is not as likely to develop to a suite of compounds in a relatively short period of time.

As previously shown (manuscript II), secondary plant compounds alone are not as inhibitory as synthetic antibiotics. In the following study, a combined agent therapy approach was used to reduce and delay the onset of resistance. Combination therapy is a common practice in clinical medicine to overcome antimicrobial resistance mechanisms developed by bacteria. A widely used combination in human medicine includes the use of a ß-lactam with a ß-lactamase inhibitor to suppress the action of ß-lactam degrading enzymes (ß-lactamases) produced by bacteria (Lee et al. 2003). However, frequent use of these ß-lactamase inhibitors has allowed bacteria to adapt and overcome their effects (Chaibi et al. 1999).

A total of 19 plant compounds were screened for their inhibitory activity and synergistic interaction with ciprofloxacin against *E. coli* O157:H7. Of the compounds screened, geraniol (an essential oil), had one of the highest inhibitory activities, exhibited synergistic interactions with ciprofloxacin, and is generally regarded as safe by the World Health Organization (World Health Organization 2003). Thus, its activity with ciprofloxacin was explored in a batch culture mode. Results indicated a larger reduction in growth yield and cell yield when geraniol and ciprofloxacin were included as compared to ciprofloxacin alone. However, cell viability was lower in the geraniol treatment suggesting that the combination of ciprofloxacin and geraniol at sub-MIC concentrations may act antagonistically by forfeiting growth rates and cell yield as a

fitness cost to maintain cell viability. This type of compensatory mechanism in resistant phenotypes has been widely documented for several bacterial organisms and antimicrobials (Björkholm et al. 2001; Nagaev et al. 2001; Nilsson et al. 2003).

To test whether the combined use of geraniol and ciprofloxacin could reduce levels of ciprofloxacin resistance, *E. coli* O157:H7 cells were passaged 12 times in three different treatments where the concentration of ciprofloxacin, geraniol, or a combination of both was increased progressively. Interestingly, rather than decreasing the levels of resistance, a combination treatment of geraniol and ciprofloxacin promoted the development of ciprofloxacin resistance in *E. coli* O157:H7 by increasing the MIC 64-fold vs. 32-fold in the ciprofloxacin only treatment. It is suspected that this high-level resistance emerged as a result of a bet-hedging strategy employed by bacteria.

As a mechanism to cope with fluctuating environmental conditions, studies have shown that isogenic bacterial populations can hedge their bets by random expression of various fitness traits to increase the odds of survival for at least a subset of individuals within the clonal population (Beaumont et al. 2009). One such trait which has been shown to be involved in antimicrobial resistance is dormancy/persistence (Jones and Lennon 2010).

Unlike most antimicrobial resistance mechanisms, dormancy does not involve a genetic mutation but rather a reversible epigenetic change likely modulated by toxin-antitoxin modules (Adam et al. 2008; Lioy et al. 2010; Rotem et al. 2010). Upon removal of the stressor (i.e. antimicrobial) organisms will switch to an active metabolic state (Adam et al. 2008). Several indications in the present study support the bet-hedging strategy and dormancy/persister model: i) resistant *E. coli* O157:H7 cells lost resistance

following long-term storage in glycerol stocks at -20˚C in the absence of antibiotics, ii) secondary inhibitory zones were seen in E-test strip experiments suggesting a sub-population had a varying tolerance to ciprofloxacin, and iii) despite little to no growth being visible in cultures grown in the presence of antimicrobials (ciprofloxacin and geraniol) at their MIC upper limit breakpoint, recovery of highly tolerant cells was still possible. This study thus suggests that combination therapy using synthetic antibiotics and natural compounds has the potential to produce insidious effects leading to greater resistance in microorganisms.

In keeping with the previous theme, the subsequent study observed whether similar concepts of dormancy and bet-hedging could be observed at an ecosystem level. The applicability of dormancy in natural ecosystems was formally introduced in the 1970s by Stevenson (1977) where he hypothesized that the stability of aquatic microbial ecosystems exposed to unfavorable conditions was due to microorganisms entering and exiting out of a dormancy stage. Furthermore, Lennon and Stuart (2011) used rRNA-rDNA ratios of terminal restriction fragment polymorphism (TRFLP) to show that approximately 15% of total rumen bacteria are inactive whereas more than 50% of soil bacteria are inactive. The higher dormancy in soil bacteria can be ascribed to the inherently harsher conditions which exist in soil. As such, it was hypothesized during the present work that within the ruminant gut (rumen and hindgut), some microbial taxa would be inhibited by antimicrobial stressors present in sainfoin, while others would thrive due to better fitness traits and ensure the maintenance of microbial diversity of the ecosystem.

Overall, feeding a tanniniferous diet was associated with an increase in less

abundant cellulolytic organisms and a concurrent marked decrease in the initially highly abundant non-cellulolytic and proteolytic *Prevotella* spp in the rumen. Thus, it was suspected that the relative abundance of *Prevotella* spp. may have decreased due to a limiting amount of ammonia available as a nitrogen source (Purushe et al. 2010) as a consequence of the protective protein effect of CTs.

Since the proportion of rRNA to rDNA was not measured in the present work, changes in metabolic activity of organisms could not be detected. However, it was found that both the niche and unified neutral theory of biodiversity and biogeography were useful in explaining the microbial community dynamics of the ecosystems studied. The niche theory, a model branching from the island biogeography theory, implies that species within an ecosystem are modulated by biotic and abiotic environmental parameters required for growth and symbiotic interactions (Chase and Leibold 2003). In this study, it was evident that secondary plant compounds in sainfoin had a profound effect on cellulytic and non-cellulytic organisms within the rumen, suggesting that abiotic factors such as CTs and other secondary plant metabolites may play a critical role in structuring rumen microbial ecosystems. Additionally, the stressor mediated a shift in the microbial community structure characterized by an increase in rare species and decrease in abundant species. Nonetheless, richness remained comparable between treatment groups indicating support for the neutral model which suggests that species abundance changes must equal to zero (Hubbell 2001). An emerging concept in microbial ecology since the advent of high-throughput sequencing is the rare-biosphere concept (Sogin et al. 2006). By definition, the rare biosphere concept dictates that all microbial ecosystems are comprised of only a handful of abundant species and a vast number of rare species

(Lennon and Jones 2011; Sogin et al. 2006). Sogin et al. (2006) described the rare biosphere as a dynamic population sensitive to environmental fluctuations. Thus, a rare taxa may become abundant in response to an environmental change. It is proposed here that a fluctuation in environmental conditions created by feeding a tanniniferous diet resulted in a dominant population emerging from the rare biosphere of the rumen as a means to stabilize and maintain microbial community diversity and richness.

The importance of the gut microbial ecosystem on host health and the role it plays in the etiology of many diseases is a well-documented link (Prakash et al. 2011). However, much remains unknown regarding how biogeographical processes drive the microbial ecosystem structure of the entire gut system. Specifically in ruminants, improved manipulation of rumen metabolism can lead to increased feed conversion efficiency, reduced pathogen shedding, and mitigation of greenhouse gas emissions. As previously mentioned, the rumen microbial ecosystem is one of the most-well studied ecosystems, however, the ruminant hindgut has received little attention in comparison. Therefore, the island biogeography theory was applied to the ruminant gut microbial community in an attempt to garner a greater understanding of how biogeographical processes shape microbial communities of the gut. The island biogeography theory proposes that species richness of an island is dictated by the rate new species colonize the island and the rate at which species become extinct. This theory also implies that taxonomic richness of the island is controlled by island size, balance between immigration and extinction, number of predators, and dispersal barriers (MacArthur and Wilson 1967). Furthermore, the community structure of an island is defined by the distance between the island and mainland and island size. The theory implies that as

distance between the island and mainland increase, the community structure similarity will decrease as a result of lower immigration rate. And as island size increases, the rate of species extinction will decrease, thus defining the species-area relationship which assumes that species richness and diversity will increase as island size increases (MacArthur and Wilson 1967).

In this study (manuscript V), the microbial communities of the rumen and hindgut of cattle fed an alfalfa hay diet were characterized using high-throughput sequencing. The island biogeography theory was applied to the ruminant gut by considering the rumen as the mainland and hindgut as an island. Results showed that microbial community structures from the rumen and hindgut do not differ in members but rather in relative abundance of these members, and that few taxa were exclusive to either location. From all phyla identified in both rumen and hindgut, Fibrobacteres was the only phylum not identified in the hindgut.

Given that the rumen and hindgut are anatomically linked and the passage of digesta flows exclusively in the direction from rumen to hindgut, a high similarity in members of both communities would be expected. However, according to the island biogeography theory, the colonization success of members from the rumen to an island such as the hindgut would be limited by factors such as competitors, environmental conditions (pH, digestive enzymes, host immunity), and island size (MacArthur and Wilson 1967). The species-area relationship was utilized to define whether island size could be used as a predictor of species richness in a ruminant gut system. The species-area relationship is defined by $S = cA^z$, were S is the number of species, A is the area sampled, c is a constant that varies with each system tested, and z is the species-area

exponent (slope) (MacArthur&Wilson, 1967). Using well defined animal weight and gut size correlations (Van Soest 1994), a positive correlation was made between gut size and species richness.

Additional comparisons of the rumen and hindgut revealed that despite the rumen being markedly larger than the hindgut, higher species diversity was found in the hindgut. This finding can be explained by the perpetual dissemination of ruminal organisms to the hindgut and by the habitable conditions of the hindgut which allow for high colonization success. The higher diversity seen in the hindgut thus allows for the hindgut to harbor greater functional redundancy in comparison to the rumen, a highly specialized niche (Hobson and Stewart 1997). The inference of higher functional redundancy in the hingut is in line with the previous study (manuscript IV) which showed a less defined effect of tanniniferous feeding on the hindgut consortium. It is reasonable to suggest that a higher functional redundancy within an ecosystem yields a higher tolerance against environmental perturbations due to superior ecosystem stability.

CHAPTER NINE

CONCLUSIONS AND FUTURE STUDIES

9.1. FINAL CONCLUSIONS

Experimental manipulation of the ruminant gut can help identify physiological roles of gut bacteria and their influence on host health, and identify new strategies to improve livestock production as a whole. Studies within this thesis conclude that a shift in ruminant gut microbiome was elicited and maintained as a result of feeding a tanniniferous diet. Gut microbiota adaptation to a tanniniferous diet did not occur thus suggesting that provided a “threshold” is not surpassed, long-term adaptation of the indigenous gut consortium will not easily occur. In addition, these studies emphasize that local environmental conditions can have a profound impact on shaping gut microbial communities. The implications of this research may lead to the use of phytomedicinal compounds as a tool in best-management practices for ruminant manure.

9.2. FUTURE DIRECTIONS

The body of research presented herein focused on the use of secondary plant compounds to prevent and/or delay the onset of antimicrobial resistance in bacteria *in vitro*. Findings suggest that not only is it difficult to avoid the development of resistance to commercial antibiotics, but resistance to natural antimicrobials also occurs in bacteria following prolonged exposure above a tolerance threshold. Alternatively, the *in vivo* study completed suggested that feeding a tannin containing diet to steers did not elicit an adaption to CTs in the gut microbiota over an 11-week period, and this was likely due to the presence of secondary metabolites at concentrations below the tolerance threshold. From the results obtained during the present work, several areas requiring investigation were identified.

Future studies are required to:

1. Identify epigenetic elements involved in bet-hedging mechanisms of the persistent phenotype present in environmental bacterial isolates such as the ruminant gut. In addition, the prevalence of bet-hedging phenotypic mutants in the ruminant gut ecosystem should be determined.
2. Understand how population-based resistance develops in an isogenic and initially susceptible bacterial population.
3. Characterize the antimicrobial susceptibility of ruminant gut organisms following tanniniferous feeding.
4. Enable development of a tannin-producing alfalfa variety

CHAPTER TEN

REFERENCES

Aarestrup, F. M., Wegener, H. C. and Collignon, P. 2008. Resistance in bacteria of the food chain: epidemiology and control strategies. Expert Rev Anti Infect Ther **6**: 733-750.

Abraham, E. P. and Chain, E. 1988. An enzyme from bacteria able to destroy penicillin. 1940. Rev Infect Dis **10**: 677-678.

Adam, M., Murali, B., Glenn, N. O. and Potter, S. S. 2008. Epigenetic inheritance based evolution of antibiotic resistance in bacteria. BMC Evol Biol **8**: 52-63.

Aerts, R. J., Barry, T. N. and McNabb, W. C. 1999. Polyphenols and agriculture: beneficial effects of proanthocyanidins in forages. Agric Ecosyst Environ **75**: 1-12.

Aikin, D. E. and Rigsby, L. L. 1985. Influence of phenolic acids on rumen fungi. Agron **77**: 180-182.

Alexander, T., Yanke, J., Reuter, T., Topp, E., Read, R., Selinger, B. and McAllister, T. 2011. Longitudinal characterization of antimicrobial resistance genes in feces shed from cattle fed different subtherapeutic antibiotics. BMC Microbiol. **11**: 19-30.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. 1990. Basic local alignment search tool. J Mol Biol **215**: 403-410.

Amann, R. I., Ludwig, W. and Schleifer, K. H. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol Rev **59**: 143-69.

Ansorge, W. J. 2009. Next-generation DNA sequencing techniques. New Biotechnology **25**: 195-203.

Anuraga, M., Duarsa, P., Hill, M. J. and Lovett, J. V. 1993. Soil moisture and temperature affect condensed tannin concentrations and growth in *Lotus corniculatus* and *Lotus peduncul atus*. Aust J Agr Res **44**: 1667-1681.

Austin, P. J., Suchar, L. A., Robbins, C. T. and Hagerman, A. E. 1989. Tannin-binding proteins in saliva of deer and their absence in saliva of sheep and cattle. J of Chem Ecol **15**: 1335-1347.

Azovsky, A. I. 2002. Size-dependent species-area relationships in benthos: is the world more diverse for microbes? Ecography **25**: 273-282.

Baas Becking, L. G. M. 1934. Geobiologie of inleiding tot de milieukunde. W.P. van Stockum & Zoon, The Hague, the Netherlands. 263 pp.

Bae, H. D., McAllister, T. A., Yanke, J., Cheng, K. J. and Muir, A. D. 1993. Effects of condensed tannins on endoglucanase activity and filter paper digestion by *Fibrobacter succinogenes* S85. Appl Environ Microbiol **59**: 2132-2138.

Balaban, N. Q., Merrin, J., Chait, R., Kowalik, L. and Leibler, S. 2004. Bacterial

persistence as a phenotypic switch. Science **305**: 1622-1625.
Barahona, R., Lascano, C. E., Cochran, R., Morrill, J. and Titgemeyer, E. C. 1997. Intake, digestion, and nitrogen utilization by sheep fed tropical legumes with contrasting tannin concentration and astringency. J Anim Sci **75**: 1633-1640.
Barboza, P. S., Peltier, T. C. and Forster, R. J. 2006. Ruminal fermentation and fill change with season in an arctic grazer: responses to hyperphagia and hypophagia in muskoxen (*Ovibos moschatus*). Physiol Biochem Zool **79**: 497-513.
Bard, M., Albrecht, M. R., Navyash, G., Guynn, C. J. and Wtillwell, W. 1988. Geraniol interferes with membrane functions in strains of *Candida* and *Saccharomyces*. Lipids **23**: 534-538.
Barrau, E., Fabre, N., Fouraste, I. and Hoste, H. 2005. Effect of bioactive compounds from Sainfoin (*Onobrychis viciifolia* Scop.) on the *in vitro* larval migration of *Haemonchus contortus*: role of tannins and flavonol glycosides. Parasitology **131**: 531-538.
Barry, T. N. 1989. Condensed tannins: Their role in ruminant protein and carbohydrate digestion and possible effects upon the rumen ecosystem. Pages 153-169 *in* J. V. Nolan, R. A. Leng, D. I. Demeyer, eds. The roles of protozoa and fungi in ruminant digestion. Penambul Books, Armidale, Australia.
Barry, T. N. and Duncan, S. J. 1984. The role of condensed tannins in the nutritional-value of *Lotus pedunculatus* for sheep. 1. Voluntary intake. Br J Nutr **51**: 485-491.
Barry, T. N. and Forss, D. A. 1983. The condensed tannin content of vegetative *Lotus pedunculatus*, its regulation by fertiliser application, and effect upon protein solubility. J Sci Food Agr **34**: 1047-1056.
Barry, T. N. and Manley, T. R. 1986. Interrelationships between the concentrations of total condensed tannin, free condensed tannin and lignin in *Lotus* sp. and their possible consequences in ruminant nutrition. J Sci Food Agr **37**: 248-254.
Barry, T. N. and McNabb, W. C. 1999. The implications of condensed tannins on the nutritive value of the temperate forages fed to ruminants. Brit J Nutr **81**: 263-272.
Barry, T. N., McNabb, W. C., Kemp, P. D., Waghorn, G. C., Min, B. R. and Luque, A. 1999. The effect of condensed tannins in *Lotus corniculatus* upon reproductive efficiency and wool production in sheep during late summer and autumn. Proc New Zealand Soc Anim Prod **61**: 51-55.
Bate-Smith, E. C. 1975. Phytochemistry of proanthocyanidins. Phytochem **14**: 1107-13.
Bax, R. P. 1997. Antibiotic resistance: a view from the pharmaceutical industry. Clin Infect Dis **24 Suppl 1**: S151-S153.
Beauchemin, K. A., McGinn, S. M., Martinez, T. F. and McAllister, T. A. 2007. Use of condensed tannin extract from quebracho trees to reduce methane emissions from cattle. J Anim Sci **85**: 1990-1996.
Beaumont, H. J., Gallie, J., Kost, C., Ferguson, G. C. and Rainey, P. B. 2009. Experimental evolution of bet hedging. Nature **462**: 90-93.
Bell, T. 2010. Experimental tests of the bacterial distance-decay relationship. Int Soc Microbial Ecol J **4**: 1357-1365.
Bell, T., Ager, D., Song, J. I., Newman, J. A., Thompson, I. P., Lilley, A. K. and van der Gast, C. J. 2005. Larger islands house more bacterial taxa. Science **308**:

1884-1884.
Bell, T. A., Etchells, J. L., Singleto.Ja and Smart, W. W. G. 1965. Inhibition of pectinolytic and cellulolytic enzymes in cucumber fermentation by *Sericea*. J Food Sci **30**: 233-239.
Bellanger, X., Roberts, A. P., Morel, C., Choulet, F., Pavlovic, G., Mullany, P., Decaris, B. and Guedon, G. 2009. Conjugative transfer of the integrative conjugative elements ICESt1 and ICESt3 from *Streptococcus thermophilus*. J Bacteriol **191**: 2764-2775.
Benchaar, C., Petit, H. V., Berthiaume, R., Ouellet, D. R., Chiquette, J. and Chouinard, P. Y. 2007. Effects of essential oils on digestion, ruminal fermentation, rumen microbial populations, milk production, and milk composition in dairy cows fed alfalfa silage or corn silage. J Dairy Sci **90**: 886-897.
Bennett, R. and Wallsgrove, R. M. 1994. Secondary metabolites in plant defence mechanisms. New Phytol **127**: 617-633.
Berard, N. C., Holley, R. A., McAllister, T. A., Ominski, K. H., Wittenberg, K. M., Bouchard, K. S., Bouchard, J. J. and Krause, D. O. 2009. Potential to reduce *Escherichia coli* shedding in cattle feces by using sainfoin (*Onobrychis viciifolia*) forage, tested in vitro and in vivo. Appl Environ Microbiol **75**: 1074-1079.
Berard, N. C., Wang, Y., Wittenberg, K. M., Krause, D. O., Coulman, B. E., McAllister, T. A. and Ominski, K. H. 2011. Condensed tannin concentrations found in vegetative and mature forage legumes grown in western Canada. Can J Plant Sci **91**: 669-675.
Berge, A. C. B., Moore, D. A. and Sischo, W. M. 2006. Field trial evaluating the influence of prophylactic and therapeutic antimicrobial administration on antimicrobial resistance of fecal *Escherichia coli* in dairy calves. Appl Environ Microbiol **72**: 3872-3878.
Bhat, T. K., Singh, B. and Sharma, O. P. 1998. Microbial degradation of tannins - A current perspective. Biodegradation **9**: 343-357.
Bigger, J. 1944. Treatment of staphylococcal infections with penicillin by intermittent sterilisation. The Lancet **244**: 497-500.
Björkholm, B., Sjölund, M., Falk, P. G., Berg, O. G., Engstrand, L. and Andersson, D. I. 2001. Mutation frequency and biological cost of antibiotic resistance in *Helicobacter pylori*. Proc Natl Acad Sci **98**: 14607-14612.
Blondeau, J. M. and Vaughan, D. 2000. A review of antimicrobial resistance in Canada. Can J Microbiol **46**: 867-877.
Bouchard, K. 2011. Methane emissions and rumen microbial changes in steers fed condensed tannin containing diets under western Canadian conditions. Thesis, MSc, Dept Animal Sci. University of Manitoba, Winnipeg, MB, Canada. 123 pp.
Bown, M. D., Poppi, D. P. and Sykes, A. R. 1991. The effect of post-ruminal infusion of protein or energy on the pathophysiology of *Trichostrongylus colubriformis* infection and body composition in lambs. Aust J Agr Res **42**: 253-267.
Box, G. E. P. and Cox, D. R. 1964. An analysis of transformations. J R Stat Soc Series B Stat Methodol **26**: 211-252.
Braga, L. C., Leite, A. A., Xavier, K. G., Takahashi, J. A., Bemquerer, M. P.,

Chartone-Souza, E. and Nascimento, A. M. 2005. Synergic interaction between pomegranate extract and antibiotics against *Staphylococcus aureus*. Can J Microbiol **51**: 541-547.

Braoudaki, M. and Hilton, A. C. 2004. Adaptive resistance to biocides in *Salmonella enterica* and *Escherichia coli* O157 and cross-resistance to antimicrobial agents. J Clin Microbiol **42**: 73-78.

Broadhurst, R. B. and Jones, W. T. 1978. Analysis of condensed tannins using acidified vanillin. J Sci Food Agr **29**: 788-794.

Broderick, G. A. 1994. Desirable protein characteristics of forage legumes for improving protein utilization in ruminants. J Anim Sci: 2760-2773.

Brooker, J. D., O'Donovan, L., Skene, I. and Sellick, G. 2000. Mechanisms of tannin resistance and detoxification in the rumen. Pages 117-122 *in* J. D. Brooker, ed. Tannins in Livestock and Human Nutrition: Proceedings of an International Workshop. Australian Centre for International Agricultural Research, Adelaide, Australia.

Brooker, J. D., Odonovan, L. A., Skene, I., Clarke, K., Blackall, L. and Muslera, P. 1994. *Streptococcus caprinus* sp. Nov, a tannin-resistant ruminal bacterium from feral goats. Lett Appl Microbiol **18**: 313-318.

Brosh, A. 2007. Heart rate measurements as an index of energy expenditure and energy balance in ruminants: a review. J Anim Sci **85**: 1213-1227.

Bryant, M. P. 1974. Nutritional features and ecology of predominant anaerobic bacteria of the intestinal tract. Am J Clin Nutr **27**: 1313-1319.

Callaway, T. R., Dowd, S. E., Edrington, T. S., Anderson, R. C., Krueger, N., Bauer, N., Kononoff, P. J. and Nisbet, D. J. 2010. Evaluation of bacterial diversity in the rumen and feces of cattle fed different levels of dried distillers grains plus solubles using bacterial tag-encoded FLX amplicon pyrosequencing. J Anim Sci **88**: 3977-3983.

Callaway, T. R., Elder, R. O., Keen, J. E., Anderson, R. C. and Nisbet, D. J. 2003. Forage feeding to reduce preharvest *Escherichia coli* populations in cattle, a review. J Dairy Sci **86**: 852-860.

Canadian Council on Animal Care. 1993. Guide to the Care and Use of Experimental Animals. Canadian Council on Animal Care, Ottawa, ON, Canada. 298 pp.

Cape, J. L., Bowman, M. K. and Kramer, D. M. 2006. Computation of the redox and protonation properties of quinones: towards the prediction of redox cycling natural products. Phytochemistry **67**: 1781-1788.

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J., Fierer, N. and Knight, R. 2011. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proc Natl Acad Sci **108 Suppl 1**: 4516-4522.

Carulla, J. E., Kreuzer, M., Machmuller, A. and Hess, H. D. 2005. Supplementation of *Acacia mearnsii* tannins decreases methanogenesis and urinary nitrogen in forage-fed sheep. Aust J Agr Res **56**: 961-970.

Cavallarin, L., Tabacco, E. and GBorreani, G. 2007. Forage and grain legume silages as a valuable source of proteins for dairy cows. Ital J Anim Sci **6**: 282-284.

CFIA. 2008. Compendium of Medicating Ingredient Brochures (CMIB) 8th Edition,

Ottawa, ON, Canada. Available: http://www.inspection.gc.ca/animals/feeds/medicating-ingredients/eng/1300212600464/1320602461227 [June 5, 2011].

Chabot, S., Bel-Rhlid, R., Chenevert, R. and Piche, Y. 1992. Hyphal growth promotion in vitro of the VA mycorrhizal fungus, *Gigaspora margarita* Becker and Hall, by the activity of structurally specific flavonoid compounds under CO_2-enriched conditions. New Phytol **122**: 461-467.

Chaibi, E. B., Sirot, D., Paul, G. and Labia, R. 1999. Inhibitor-resistant TEM beta-lactamases: phenotypic, genetic and biochemical characteristics. J Antimicrob Chemother **43**: 447-458.

Chander, Y., Goyal, S. M. and Gupta, S. C. 2006. Antimicrobial resistance of *Providencia* spp. isolated from animal manure. Vet J **172**: 188-91.

Chase, J. M. and Leibold, M. A. 2003. Ecological Niches: Linking Classical and Contemporary Approaches. University of Chicago Press, Chicago, IL, USA. 212 pp.

Chesson, A., Stewart, C. S. and Wallace, R. J. 1982. Influence of plant phenolic-acids on growth and cellulolytic activity of rumen bacteria. Appl Environ Microbiol **44**: 597-603.

Chiquette, J., Cheng, K. J., Costerton, J. W. and Milligan, L. P. 1988. Effect of tannins on the digestibility of two isosynthetic strains of birdsfoot trefoil (*Lotus corniculatus* L.) using in vitro and in sacco techniques. Can J Anim Sci **68**: 751-760.

Chow, J., Lee, S. M., Shen, Y., Khosravi, A. and Mazmanian, S. K. 2010. Host-bacterial symbiosis in health and disease. Pages 243-274 *in* S. Fagarasan, A. Cerutti, eds. Mucosal Immunity. Elsevier Academic Press Inc, San Diego, CA, USA.

Chung, K. T., Wei, C. I. and Johnson, M. G. 1998. Are tannins a double-edged sword in biology and health? Trends Foods Sci Tech **9**: 168-175.

Clifford, M. N. and Scalbert, A. 2000. Ellagitannins - nature, occurrence and dietary burden. J Sci Food Agr **80**: 1118-1125.

Coen, J. A. and Dehority, B. A. 1970. Degradation and utilization of hemicellulose from intact forages by pure cultures of rumen bacteria. Appl. Microbiol. **20**: 362-368.

Conly, J. 2002. Antimicrobial resistance in Canada. Can Med Assoc J **167**: 885-891.

Costello, E. K., Stagaman, K., Dethlefsen, L., Bohannan, B. J. M. and Relman, D. A. 2012. The application of ecological theory toward an understanding of the human microbiome. Science **336**: 1255-1262.

Cowan, M. M. 1999. Plant products as antimicrobial agents. Clin Microbiol Rev **12**: 564-582.

Cressy, H. K., Jerrett, A. R., Osborne, C. M. and Bremer, P. J. 2003. A novel method for the reduction of numbers of *Listeria monocytogenes* cells by freezing in combination with an essential oil in bacteriological media. J Food Prot **66**: 390-395.

Cuenca-Estrella, M. 2004. Combinations of antifungal agents in therapy--what value are they? J Antimicrob Chemother **54**: 854-869.

De Wit, R. and Bouvier, T. 2006. ‘Everything is everywhere, but, the environment

selects'; what did Baas Becking and Beijerinck really say? Environ Microbiol **8**: 755-758.

Dehority, B. A. 2003. Rumen Microbiology. Nottingham University Press, Nottingham, England. 372 pp.

Demment, M. W. 1983. Feeding ecology and the evolution of body size of baboons. African Journal of Ecology **21**: 219-233.

Demment, M. W. and Van Soest, P. J. 1985. A nutritional explanation for body-size patterns of ruminant and nonruminant hervibores. Am Nat **125**: 641-972.

DeSantis, T. Z., Hugenholtz, P., Keller, K., Brodie, E. L., Larsen, N., Piceno, Y. M., Phan, R. and Andersen, G. L. 2006a. NAST: a multiple sequence alignment server for comparative analysis of 16S rRNA genes. Nucleic Acids Res **34**: W394-W399.

DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., Huber, T., Dalevi, D., Hu, P. and Andersen, G. L. 2006b. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl Environ Microbiol **72**: 5069-5072.

Di Pasqua, R., Hoskins, N., Betts, G. and Mauriello, G. 2006. Changes in membrane fatty acids composition of microbial cells induced by addiction of thymol, carvacrol, limonene, cinnamaldehyde, and eugenol in the growing media. J Agr Food Chem **54**: 2745-2749.

Dowd, S. E., Callaway, T. R., Wolcott, R. D., Sun, Y., McKeehan, T., Hagevoort, R. G. and Edrington, T. S. 2008. Evaluation of the bacterial diversity in the feces of cattle using 16S rDNA bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP). Biomed Central Microbiol **8**: 125-132.

Dubnau, D. and Losick, R. 2006. Bistability in bacteria. Mol Microbiol **61**: 564-72.

Duncan, S. H., Flint, H. J. and Stewart, C. S. 1998. Inhibitory activity of gut bacteria against *Escherichia coli* O157 mediated by dietary plant metabolites. FEMS Microbiol Lett, **164**: 283-288.

Durso, L. M., Harhay, G. P., Smith, T. P., Bono, J. L., Desantis, T. Z., Harhay, D. M., Andersen, G. L., Keen, J. E., Laegreid, W. W. and Clawson, M. L. 2010. Animal-to-animal variation in fecal microbial diversity among beef cattle. Appl Environ Microbiol **76**: 4858-4862.

Eloff, J. N. 1998a. A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. Planta Med **64**: 711-713.

Eloff, J. N. 1998b. Which extractant should be used for the screening and isolation of antimicrobial components from plants? J Ethnopharmacol **60**: 1-8.

Fang, T. J. and Lin, L. W. 1995. Comparative growth of *Listeria monocytogenes* and *Pseudomonas fragi* in a modified atmosphere/nisin model system. J Chin Agric Soc **33**: 391-403.

Filippich, L. J., Zhu, J., Oelrichs, P., Alsalami, M. T., Doig, A. J., Cao, G. R. and English, P. B. 1991. Hepatotoxic and nephrotoxic principles in *Terminalia-oblongata*. Res Vet Sci **50**: 170-177.

Finlay, B. J., Esteban, G. V. and Fenchel, T. 1998. Protozoan diversity: converging estimates of the global number of free-living ciliate species. Protist **149**: 29-37.

Fleming, A. 1945. Nobel Lecture. [Online] Available:

http://nobelprize.org/nobel_prizes/medicine/laureates/1945/fleming-lecture.html.
Flint, H. J. 1997. The rumen microbial ecosystem - some recent developments. Trends Microbiol **5**: 483-488.
Flint, H. J., Bayer, E. A., Rincon, M. T., Lamed, R. and White, B. A. 2008. Polysaccharide utilization by gut bacteria: potential for new insights from genomic analysis. Nat Rev Micro **6**: 121-131.
Frauenfeld, O. W., Zhang, T. J. and McCreight, J. L. 2007. Northern hemisphere freezing/thawing index variations over the twentieth century. Int J Climatol **27**: 47-63.
Friedman, M., Henika, P. R. and Mandrell, R. E. 2003. Antibacterial activities of phenolic benzaldehydes and benzoic acids against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica*. J Food Prot **66**: 1811-1821.
Frost, L. S., Leplae, R., Summers, A. O. and Toussaint, A. 2005. Mobile genetic elements: the agents of open source evolution. Nat Rev Microbiol **3**: 722-732.
Gao, W., Smith, D. W. and Li, Y. 2006. Natural freezing as a wastewater treatment method: E. coli inactivation capacity. Water Res **40**: 2321-2326.
Garrity, G. M., Bell, J. A. and Lilburn, T. G. 2004. Taxonomic outline of the prokaryotes. Bergey's Manual of Systemic Bacteriology. Springer Inc., New York, US. 399 pp.
Geishauser, T. 1993. Geishauser, An instrument for the collection and transfer of ruminal fluid and for the administration of water soluble drugs in adult cattle. Bovine Prac **27**: 38-42.
Geist, V. 1974. On the relationship of social evolution and ecology in ungulates. Integrative and Comparative Biology **14**: 205-220.
Gessel, P. D., Hansen, N. C., Moncrief, J. F. and Schmitt, M. A. 2004. Rate of fall-applied liquid swine manure: effects on runoff transport of sediment and phosphorus. J Environ Qual **33**: 1839-1844.
Getachew, G., Makkar, H. P. S. and Becker, K. 2000. Effect of polyethylene glycol on in vitro degradability of nitrogen and microbial protein synthesis from tannin-rich browse and herbaceous legumes. Brit J Nutr **84**: 73-83.
Goplen, B. P., Howarth, R. E., Sarkar, S. K. and Lesins, K. 1980. A search for condensed tannins in annual and perennial species of *Medicago*, *Trigonella*, and *Onobrichis*. Crop Sci **20**: 801-804.
Gordon, I. J. and Illius, A. W. 1994. The functional significance of the browser-grazer dichotomy in African ruminants. Oecologia **98**: 167-175.
Government of Canada. 1983. Feeds regulations (SOR/83-593). Ottawa. Available: http://laws.justice.gc.ca/eng/regulations/SOR-83-593/page-1.html.
Government of Canada. 1985. Feeds act (R.S.C., 1985, c. F-9). Ottawa. Available: http://laws-lois.justice.gc.ca/eng/acts/F%2D9/page-1.html.
Government of Canada. 1997. Controlled drugs and substances act (SOR/97-234). Ottawa. Available: http://laws-lois.justice.gc.ca/eng/regulations/SOR-97-234/page-1.html.
Government of Manitoba. 1999. The veterinary medical act (C.C.S.M. c. V30). Winnipeg, MB, Canada. Available:

http://web2.gov.mb.ca/laws/statutes/ccsm/v030e.php.

Government of Manitoba. 2010. The pharmaceutical act (C.C.S.M. c. P60). Winnipeg, MB, Canada. Available: http://web2.gov.mb.ca/laws/statutes/archive/2010-06-16/p060ei.php.

Grbic-Galic, D. 1986. O-demethylation, dehydroxylation, ring-reduction and cleavage of aromatic substrates by Enterobacteriaceae under anaerobic conditions. J Appl Bacteriol **61**: 491-497.

Green, J. L., Holmes, A. J., Westoby, M., Oliver, I., Briscoe, D., Dangerfield, M., Gillings, M. and Beattie, A. J. 2004. Spatial scaling of microbial eukaryote diversity. Nature **432**: 747-750.

Gutierrez, J., Williams, P. P., Davis, R. E. and Warwick, E. J. 1962. Lipid metabolism of rumen ciliates and bacteria. I. Uptake of fatty acids by *Isotricha prostoma* and *Entodinium simplex*. Appl Microbiol **17**: 548-551.

Hagerman, A. E., Robbins, C. T., Weerasuriya, Y., Wilson, T. C. and McArthur, C. 1992. Tannin chemistry in relation to digestion. J Range Manage **45**: 57-62.

Hamady, M., Lozupone, C. and Knight, R. 2010. Fast UniFrac: facilitating high-throughput phylogenetic analyses of microbial communities including analysis of pyrosequencing and PhyloChip data. ISME J **4**: 17-27.

Hammer, K. A., Carson, C. F. and Riley, T. V. 2004. Antifungal effects of *Melaleuca alternifolia* (tea tree) oil and its components on *Candida albicans*, *Candida glabrata* and *Saccharomyces cerevisiae*. J Antimicrob Chemother **53**: 1081-1085.

Haring, D. A., Huber, M. J., Suter, D., Edwards, P. J. and Luscher, A. 2008. Plant enemy-derived elicitors increase the foliar tannin concentration of *Onobrychis viciifolia* without a trade-off to growth. Ann Bot **102**: 979-987.

Haslam, E. 1966. Chemistry of vegetable tannins. Academic Press, New York. 179 pp.

Haslam, E. 1989. Plant polyphenols - vegetable tannins revisited. Cambridge University Press, Cambridge, U.K. 240 pp.

Hayes, D. J., Jensen, H. H. and Fabiosa, J. 2002. Technology choice and the economic effects of a ban on the use of antimicrobial feed additives in swine rations. Food Control **13**: 97-101.

Health Canada. 2002. Uses of antimicrobials in food animals in Canada: impact on resistance and human health. Report of the advisory committee on animal uses of antimicrobials and impact on resistance and human health. Available: http://www.hc-sc.gc.ca/dhp-mps/pubs/vet/amr-ram_final_report-rapport_06-27_cp-pc-eng.php [June 1, 2011].

Health Canada. 2009. Extra-Label Drug Use (ELDU). Veterinary Drugs. Available: http://www.hc-sc.gc.ca/dhp-mps/vet/label-etiquet/pol_eldu-umdde-eng.php [June 10, 2011].

Hemaiswarya, S., Kruthiventi, A. K. and Doble, M. 2008. Synergism between natural products and antibiotics against infectious diseases. Phytomedicine **15**: 639-652.

Hermann, M. L., Russell, J. R. and Barnhart, S. K. 2002. Evaluation of hay-type and grazing-tolerant alfalfa cultivars in season-long or complementary rotational stocking systems for beef cows. J Anim Sci **80**: 768-779.

Hespell, R. B., Akin, D. E. and Dehority, B. A. 1997. Bacteria, fungi, and protozoa of the rumen. Pages 59-141 *in* R. I. Mackie, B. A. White, R. E. Isaacson, eds.

Gastrointestinal Microbiology. Chapman & Hall, New York.
Hobson, P. N. and Stewart, C. S., (eds.) 1997. The Rumen Microbial Ecosystem. Chapman & Hall, London, UK. 744 pp.
Horigome, T., Kumar, R. and Okamoto, K. 1988. Effects of condensed tannins prepared from leaves of fodder plants on digestive enzymes in-vitro and in the intestine of rats. Br J Nutr **60**: 275-285.
Horner-Devine, M. C., Lage, M., Hughes, J. B. and Bohannan, B. J. 2004. A taxa-area relationship for bacteria. Nature **432**: 750-753.
Horvath, P. J. 1980. The nutritional and ecological significance of *Acer*-tannins and related polyphenols. Cornell University, Ithaca, NY, USA. 276 pp.
Hove, L., Topps, J. H., Sibanda, S. and Ndlovu, L. R. 2001. Nutrient intake and utilisation by goats fed dried leaves of the shrub legumes *Acacia angustissima*, *Calliandra calothyrsus* and *Leucaena leucocephala* as supplements to native pasture hay. Anim Feed Sci Tech **91**: 95-106.
Hu, Z. Q., Zhao, W. H., Hara, Y. and Shimamura, T. 2001. Epigallocatechin gallate synergy with ampicillin/sulbactam against 28 clinical isolates of methicillin-resistant *Staphylococcus aureus*. J Antimicrob Chemother **48**: 361-364.
Hubbell, S. P. 2001. The unified neutral theory of biodiversity and biogeography. Princeton University Press, Princeton, NJ, USA. 448 pp.
Hungate, R. E. 1942. The culture of *Eudiplodinium neglectum*, with experiments on the digestion of cellulose. Biol Bull **83**: 303-319.
Hungate, R. E. 1966. The Rumen and its Microbes. Academic Press. 533 pp.
Hungate, R. E. 1975. Rumen microbial ecosystem. Annu Rev Ecol Syst **6**: 39-66.
Hungate, R. E., Bryant, M. P. and Mah, R. A. 1964. The rumen bacteria and protozoa. Annu Rev Microbiol **18**: 131-166.
Huse, S. M., Welch, D. M., Morrison, H. G. and Sogin, M. L. 2010. Ironing out the wrinkles in the rare biosphere through improved OTU clustering. Environ Microbiol **12**: 1889-1898.
Hussain, I. and Cheeke, P. R. 1995. Effect of dietary *Yucca schidigera* extract on rumen and blood profiles of steers fed concentrate- or roughage-based diets. Anim Feed Sci Tech **51**: 231-242.
Hutchison, M. L., Walters, L. D., Moore, T., Thomas, D. J. and Avery, S. M. 2005. Fate of pathogens present in livestock wastes spread onto fescue plots. Appl Environ Microbiol **71**: 691-696.
Iason, G., Hodgson, J. and Barry, T. 1995. Variation in condensed tannin concentration of a temperate grass (*Holcus lanatus*) in relation to season and reproductive development. J Chem Ecol **21**: 1103-1112.
Ingham, S. C., Schoeller, E. L. and Engel, R. A. 2006. Pathogen reduction in unpasteurized apple cider: adding cranberry juice to enhance the lethality of warm hold and freeze-thaw steps. J Food Prot **69**: 293-298.
Jackson, F. S., McNabb, W. C., Barry, T. N., Foo, Y. L. and Peters, J. S. 1996. The condensed tannin content of a range of subtropical and temperate forages and the reactivity of condensed tannin with ribulose-1,5-bis-phosphate carboxylase (Rubisco) protein. J Sci Food Agr **72**: 483-492.
Jacob, M. E., Fox, J. T., Reinstein, S. L. and Nagaraja, T. G. 2008. Antimicrobial

susceptibility of foodborne pathogens in organic or natural production systems: an overview. Foodborne Pathog Dis **5**: 721-730.

Jones, G. A., McAllister, T. A., Muir, A. D. and Cheng, K.-J. 1994. Effects of sainfoin (*Onobrychis viciifolia* Scop.) condensed tannins on growth and proteolysis by four strains of ruminal bacteria. Appl Environ Microbiol **60**: 1374-1378.

Jones, S. E. and Lennon, J. T. 2010. Dormancy contributes to the maintenance of microbial diversity. Proc Natl Acad Sci **107**: 5881-5886.

Jones, W. T., Broadhurst, R. B. and Lyttleton, J. W. 1976. Condensed tannins of pasture legume species. Phytochemistry **15**: 1407-1409.

Jones, W. T. and Mangan, J. L. 1977. Complexes of the condensed tannins of sainfoin (*Onobrychis viciifolia* Scop.) with fraction 1 leaf protein and with submaxillary mucoprotein, and their reversal by polyethylene glycol and pH. J Sci Food Agr **28**: 126-136.

Joseph, R., Tanner, G. and Larkin, P. 1998. Proanthocyanidin synthesis in the forage legume *Onobrychis viciifolia*. A study of chalcone synthase, dihydroflavonol 4-reductase and leucoanthocyanidin 4-reductase in developing leaves. Aust J Plant Phyisol **25**: 271-278.

Jost, L. 2006. Entropy and diversity. Oikos **113**: 363-375.

Kalmokoff, M., Waddington, L. M., Thomas, M., Liang, K. L., Ma, C., Topp, E., Dandurand, U. D., Letellier, A., Matias, F. and Brooks, S. P. J. 2011. Continuous feeding of antimicrobial growth promoters to commercial swine during the growing/finishing phase does not modify faecal community erythromycin resistance or community structure. J Appl Microbiol **110**: 1414-1425.

Karczmarczyk, M., Walsh, C., Slowey, R., Leonard, N. and Fanning, S. 2011. Molecular characterisation of multidrug resistant (MDR) *Escherichia coli* isolates from Irish cattle farms. Appl Environ Microbiol **77**: 7121-7127.

Keren, I., Kaldalu, N., Spoering, A., Wang, Y. and Lewis, K. 2004. Persister cells and tolerance to antimicrobials. FEMS Microbiology Letters, **230**: 13-8.

Khachatourians, G. G. 1998. Agricultural use of antibiotics and the evolution and transfer of antibiotic-resistant bacteria. Can Med Assoc J **159**: 1129-1136.

Khafipour, E., Li, S., Plaizier, J. C., Dowd, S. E. and Krause, D. O. 2011. Microbiome analysis of the rumen, cecum, and feces of dairy cows with subacute ruminal acidosis. J Anim Sci **89, E-Suppl. 1**: 689-689.

Khanbabaee, K. and van Ree, T. 2001. Tannins: classification and definition. Nat Prod Rep **18**: 641-649.

Kim, M., Morrison, M. and Yu, Z. T. 2011. Status of the phylogenetic diversity census of ruminal microbiomes. FEMS Microbiol. Ecol. **76**: 49-63.

Kirby, W. M. 1944. Extraction of a highly potent penicillin inactivator from penicillin resistant staphylococci. Science **99**: 452-453.

Klieve, A. V., Swain, R. A. and Nolan, J. V. 1996. Bacteriophages in the rumen; types present, population size and implications for the efficiency of feed utilisation. Proc Aust Soc Anim Prod **21**: 92-94.

Kolar, M., Urbanek, K. and Latal, T. 2001. Antibiotic selective pressure and development of bacterial resistance. Int J Antimicrob Agents **17**: 357-363.

Koupai-Abyazani, M. R., McCallum, J., Muir, A. D., Lees, G. L., Bohm, B. A., Towers, G. H. N. and Gruber, M. Y. 1993. Purification and characterization of a proanthocyanidin polymer from seed of alfalfa (*Medicago-sativa* cv. beaver). J Agr Food Chem **41**: 565-569.

Krause, D. O., Denman, S. E., Mackie, R. I., Morrison, M., Rae, A. L., Attwood, G. T. and McSweeney, C. S. 2003. Opportunities to improve fiber degradation in the rumen: microbiology, ecology, and genomics. FEMS Microbiol Rev **27**: 663-693.

Krause, D. O. and Russell, J. B. 1996. How many ruminal bacteria are there? J Dairy Sci **79**: 1467-1475.

Kudva, I. T., Blanch, K. and Hovde, C. J. 1998. Analysis of *Escherichia coli* O157:H7 survival in ovine or bovine manure and manure slurry. Appl Environ Microbiol **64**: 3166-3174.

Kumar, R. 1983. Chemical and biochemical nature of fodder tree leaf tannins. J Agr Food Chem **31**: 1364-1366.

Kumar, R. A. and Singh, M. 1984. Tannins: their adverse role in ruminant nutrition J Agric Food Chem **32**: 447-453.

Kümmerer, K. 2004. Resistance in the environment. J Antimicrob Chemother **54**: 311-320.

Land, E. J., Ramsden, C. A. and Riley, P. A. 2004. Quinone chemistry and melanogenesis. Methods Enzymol **378**: 88-109.

Lee, H. H., Molla, M. N., Cantor, C. R. and Collins, J. J. 2010. Bacterial charity work leads to population-wide resistance. Nature **467**: 82-85.

Lee, N., Yuen, K. Y. and Kumana, C. R. 2003. Clinical role of beta-lactam/beta-lactamase inhibitor combinations. Drugs **63**: 1511-1524.

Lees, G. L. 1992. Condensed tannins in some forage legumes: Their role in the prevention of ruminant pasture bloat.

Lees, G. L. 1993. *Onobrychis viciifolia* Scop. (sainfoin): *in vitro* culture and the production of condensed tannins Pages 269-286 *in* Y. P. S. Bajaj, ed. Biotechnology in Agriculture and Forestry. Springer Verlag, Berlin, Germany.

Lees, G. L., Gruber, M. Y. and Suttill, N. H. 1995. Condensed tannins in sainfoin. II. Occurrence and changes during leaf development. Can J Bot **73**: 1540-1547.

Lees, G. L., Hinks, C. F. and Suttill, N. H. 1994. Effect of high temperature on condensed tannin accumulation in leaf tissues of big trefoil (*Lotus uliginosus* Schkuhr). J Sci Food Agr **65**: 415-421.

Lennon, J. T. and Jones, S. E. 2011. Microbial seed banks: the ecological and evolutionary implications of dormancy. Nat Rev Microbiol **9**: 119-129.

Lewis, S. M. and Dehority, B. A. 1985. Microbiology and ration digestibility in the hindgut of the ovine. Appl Environ Microbiol **50**: 356-363.

Li, M., Kai, Y., Qiang, H. and Dongying, J. 2006. Biodegradation of gallotannins and ellagitannins. J Basic Microbiol **46**: 68-84.

Li, Y. G., Tanner, G. and Larkin, P. 1996. The DMACA-HCl protocol and the threshold proanthocyanidin content for bloat safety in forage legumes. J Sci Food Agr **70**: 89-101.

Liggenstoffer, A. S., Youssef, N. H., Couger, M. B. and Elshahed, M. S. 2010.

Phylogenetic diversity and community structure of anaerobic gut fungi (phylum Neocallimastigomycota) in ruminant and non-ruminant herbivores. ISME J **4**: 1225-1235.

Lioy, V. S., Rey, O., Balsa, D., Pellicer, T. and Alonso, J. C. 2010. A toxin-antitoxin module as a target for antimicrobial development. Plasmid **63**: 31-39.

Looft, T., Johnson, T. A., Allen, H. K., Bayles, D. O., Alt, D. P., Stedtfeld, R. D., Sul, W. J., Stedtfeld, T. M., Chai, B., Cole, J. R. and others. 2012. In-feed antibiotic effects on the swine intestinal microbiome. Proc Natl Acad Sci U S A **109**: 1691-1696.

Lorenzi, V., Muselli, A., Bernardini, A. F., Berti, L., Pages, J.-M., Amaral, L. and Bolla, J.-M. 2009. Geraniol restores antibiotic activities against multidrug-resistant isolates from Gram-negative species. Antimicrob Agents Chemother **53**: 2209-2211.

Lowe, S. E., Theodorou, M. K. and Trinci, A. P. 1987. Cellulases and xylanase of an anaerobic rumen fungus grown on wheat straw, wheat straw holocellulose, cellulose, and xylan. Appl Environ Microb **53**: 1216-1223.

Lowry, J. B., McSweeney, C. S. and Palmer, B. 1996. Changing perceptions of the effect of plant phenolics on nutrient supply in the ruminant. Aust J Agric Res **47**: 829-842.

Lu, Y., Sun, Y., Foo, L. Y., McNabb, W. C. and Molan, A. L. 2000. Phenolic glycosides of forage legume *Onobrychis viciifolia*. Phytochemistry **55**: 67-75.

Luciano, F. B. and Holley, R. A. 2009. Enzymatic inhibition of allyl isothiocyanate and factors affecting its antimicrobial action against *Escherichia coli* O157:H7. Int J Food Microbiol **131**: 240-245.

Luscher, A., Haring, D. A., Heckendorn, F., Scharenberg, A., Dohme, F., Maurer, V. and Hertzberg, H. 2005. Use of tanniferous plants against gastro-intestinal nematodes in ruminants. International Society of Organic Agriculture Research, Adelaide, Australia. 272-276 pp.

MacArthur, R. H. and Wilson, E. O. 1967. The theory of island biogeography. Princeton University Press, Princeton, NJ, USA. 203 pp.

Makkar, H. P. S. 1993. Antinutritional factors in foods for livestock. British Society of Animal Production. 69-85 pp.

Makkar, H. P. S. 2003. Effects and fate of tannins in ruminant animals, adaptation to tannins, and strategies to overcome detrimental effects of feeding tannin-rich feeds. Small Ruminant Res **49**: 241-256.

Makkar, H. P. S., Blummel, M. and Becker, K. 1995. Formation of complexes between polyvinyl pyrrolidones or polyethylene glycols and tannins, and their implication in gas production and true digestibility in in vitro techniques. Br. J. Nutr. **73**: 897-913.

Makkar, H. P. S., Singh, B. and Dawra, R. K. 1988. Effect of tannin-rich leaves of oak *Quercus incana* on various microbial enzyme activities of the bovine rumen. Br J Nutr **60**: 287-296.

Mangan, J. L. 1959. Bloat in cattle XI. The foaming properties of proteins, saponins, and rumen liquor. New Zealand J Agric Res **2**: 47-61.

Mangan, J. L. 1988. Nutritional effects of tannins in animal feeds. Nutr Res Rev **1**: 209-

231.

Marais, J. P. J., Mueller-Harvey, I., Brandt, V. E. and Ferreira, D. 2000. Polyphenols, condensed tannins and other natural products in *Onobrychis viciifolia* (sainfoin). J Agric Sci **48**: 3440-3447.

Marten, G. C. and Ehle, F. R. 1984. Influence of quality variation in four legume species on weight gains of grazing heifers. Agron Abstr: 159.

McDermott, P. F., Walker, R. D. and White, D. G. 2003. Antimicrobials: modes of action and mechanisms of resistance. Int J Toxicol **22**: 135-143.

McDonald, M., Mila, I. and Scalbert, A. 1996. Precipitation of metal ions by plant polyphenols: optimal conditions and origin of precipitation. J Agr Food Chem **44**: 599-606.

McMahon, L. R., Majak, W., McAllister, T. A., Hall, J. W., Jones, G. A., Popp, J. D. and Cheng, K. J. 1999. Effect of sainfoin on in vitro digestion of fresh alfalfa and bloat in steers. Can J Anim Sci **79**: 203-212.

McMahon, L. R., McAllister, T. A., Berg, B. P., Majak, W., Acharya, S. N., Popp, J. D., Coulman, B. E., Wang, Y. and Cheng, K. J. 2000. A review of the effects of forage condensed tannins on ruminal fermentation and bloat in grazing cattle. Can J Plant Sci **80**: 469-485.

McNabb, B. K. 2002. The physiological ecology of vertebrates: a view from energetics. Cornell University Press, Ithaca, New York.

McNabb, W. C., Waghorn, G. C., Peters, J. S. and Barry, T. N. 1996. The effect of condensed tannin in *Lotus pedunculatus* upon the solubilization and degradation of ribulose 1,5-bisphosphate carboxylase protein in the rumen and on sites of digestion. Br J Nutr **76**: 535-549.

McSweeney, C. S., Palmer, B., Bunch, R. and Krause, D. O. 2001a. Effect of the tropical forage *Calliandra* on microbial protein synthesis and ecology in the rumen. J Appl Microbiol **90**: 78-88.

McSweeney, C. S., Palmer, B., McNeill, D. M. and Krause, D. O. 2001b. Microbial interactions with tannins: nutritional consequences for ruminants. Anim Feed Sci Technol **91**: 83-93.

Michelland, R. J., Monteils, V., Zened, A., Combes, S., Cauquil, L., Gidenne, T., Hamelin, J. and Fortun-Lamothe, L. 2009. Spatial and temporal variations of the bacterial community in the bovine digestive tract. J Appl Microbiol **107**: 1642-1650.

Millet, S. and Maertens, L. 2011. The European ban on antibiotic growth promoters in animal feed: From challenges to opportunities. Vet J **187**: 143-144.

Min, B. R. and Hart, S. P. 2003. Tannins for suppression of internal parasites. J Anim Sci **81 (E. Suppl. 2)**: E102-E109.

Min, B. R., Hart, S. P., Miller, D., Tomita, G. M., Loetz, E. and Sahlu, T. 2005. The effect of grazing forage containing condensed tannins on gastro-intestinal parasite infection and milk composition in Angora does. Vet Parasitol **130**: 105-113.

Min, B. R., McNabb, W. C., Barry, T. N., Kemp, P. D., Waghorn, G. C. and McDonald, M. F. 2000. The effect of condensed tannins in *Lotus corniculatus* upon reproductive efficiency and wool production in sheep during late summer. J Agric Sci **132**: 323-334.

Min, B. R., Pomroy, W. E., Hart, S. P. and Sahlu, T. 2004. The effect of short-term consumption of a forage containing condensed tannins on gastro-intestinal nematode parasite infections in grazing wether goats. Small Ruminant Res **51**: 279-283.

Mitaru, B. N., Reichert, R. D. and Blair, R. 1984. The binding of dietary-protein by sorghum tannins in the digestive-tract of pigs. J Nutr **114**: 1787-1796.

Mitchell-Olds, T., Gershenzon, J., Baldwin, I. and Boland, W. 1998. Research focus - chemical ecology in the molecular era. Trends in Plant Sci **3**: 362-365.

Moody, M. L., Zanton, G. I., Daubert, J. M. and Heinrichs, A. J. 2007. Nutrient utilization of differing forage-to-concentrate ratios by growing Holstein heifers. J Dairy Sci **90**: 5580-5586.

Morrill, W. L., Ditterline, R. L. and Cash, S. D. 1998. Insects, pests and associated root pathogens of sainfoin in western USA. Field Crops Res **59**: 129-134.

Morris, P., Carron, T. R., Robbins, M. P. and Webb, K. J. 1993. Distribution of condensed tannins in flowering plants of *Lotus corniculatus* var. japonicus and tannin accumulation by transformed root cultures. Lotus Newsl. **24**: 60-63.

Mowrey, D. P. and Volesky, J. D. 1993. Feasibility of grazing sainfoin on the southern great plains. J Range Manag **46**: 539-542.

Mueller-Harvey, I. 2006. Unravelling the conundrum of tannins in animal nutrition and health. J Sci Food Agr **86**: 2010-2037.

Murdiati, T. B., McSweeney, C. S. and Lowry, J. B. 1992. Metabolism in sheep of gallic acid, tannic-acid and hydrolyzable tannin from *Terminalia-oblongata*. Aust J Agr Res **43**: 1307-1319.

Nagaev, I., Björkman, J., Andersson, D. I. and Hughes, D. 2001. Biological cost and compensatory evolution in fusidic acid-resistant *Staphylococcus aureus*. Mol Microbiol **40**: 433-439.

Namba, T., Morita, O., Huang, S. L., Goshima, K., Hattori, M. and Kakiuchi, N. 1988. Studies on cardio-active crude drugs; I. Effect of coumarins on cultured myocardial cells. Planta Med **54**: 277-282.

National Committee for Clinical Laboratory Standards. 2002. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals. Approved standard. National Committee for Clinical Laboratory Standards, Wayne, PA, USA. 99 pp.

Nelson, K. E., Thonney, M. L., Woolston, T. K., Zinder, S. H. and Pell, A. N. 1998. Phenotypic and phylogenetic characterization of ruminal tannin-tolerant bacteria. Appl Environ Microb **64**: 3824-3830.

Niezen, J. H., Robertson, H. A., Waghorn, G. C. and Charleston, W. A. G. 1998. Production, faecal egg counts and worm burdens of ewe lambs which grazed six contrasting forages. Vet Parasitol **80**: 15-27.

Nilsson, A. I., Berg, O. G., Aspevall, O., Kahlmeter, G. and Andersson, D. I. 2003. Biological costs and mechanisms of fosfomycin resistance in *Escherichia coli*. Antimicrob Agents Chemother **47**: 2850-2858.

Novak, M., Wootton, J. T., Doak, D. F., Emmerson, M., Estes, J. A. and Tinker, M. T. 2010. Predicting community responses to perturbations in the face of imperfect knowledge and network complexity. Ecology **92**: 836-846.

O'Donovan, L. and Brooker, J. D. 2001. Effect of hydrolysable and condensed tannins on growth, morphology and metabolism of *Streptococcus gallolyticus* (*S. caprinus*) and *Streptococcus bovis*. Microbiology **147**: 1025-1033.

Oenema, O., Wrage, N., Velthof, G., Groenigen, J. W., Dolfing, J. and Kuikman, P. 2005. Trends in Global Nitrous Oxide Emissions from Animal Production Systems. Nutrient Cycling in Agroecosystems **72**: 51-65.

Oh, H. I., Hoff, J. E., Armstron, G. S. and Haff, L. A. 1980. Hydrophobic interaction in tannin protein complexes. J Agr Food Chem **28**: 394-398.

Okubo, T., Ishihara, N., Oura, A., Serit, M., Kim, M., Yamamoto, T. and Mitsuoka, T. 1992. In vivo effects of tea polyphenol intake on human intestinal microflora and metabolism. Biosci. Biotechnol. Biochem. **56**: 588-591.

Olasupo, N. A., Fitzgerald, D. J., Gasson, M. J. and Narbad, A. 2003. Activity of natural antimicrobial compounds against *Escherichia coli* and *Salmonella enterica* serovar Typhimurium. Lett Appl Microbiol **37**: 448-451.

Orpin, C. G. 1975. Studies on the rumen flagellate Neocallimastix frontalis. J Gen Microbiol **91**: 249-262.

Orpin, C. G. (ed.) and Mountfort, D. O. (ed.) 1994. Anaerobic fungi: taxonomy, biology, and distribution in nature. Marcel Dekker Inc., New York, NY, US. 290 pp.

Overbye, K. M. and Barrett, J. F. 2005. Antibiotics: where did we go wrong? Drug Discov Today **10**: 45-52.

Owen-Smith, R. N. 1988. Megaherbivores: the influence of very large body size on ecology. Cambridge University Press, Cambridge, UK. 369 pp.

Ozkose, E., Thomas, B. J., Davies, D. R., Griffith, G. W. and Theodorou, M. K. 2001. *Cyllamyces aberensis* gen.nov. sp.nov., a new anaerobic gut fungus with branched sporangiophores isolated from cattle. Can J Bot **79**: 666-673.

Ozutsumi, Y., Tajima, K., Takenaka, A. and Itabashi, H. 2005. The effect of protozoa on the composition of rumen bacteria in cattle using 16S rRNA gene clone libraries. Biosci, Biotechnol, Biochem **69**: 499-506.

Ozutsumi, Y., Tajima, K., Takenaka, A. and Itabashi, H. 2012. The mcrA gene and 16S rRNA gene in the phylogenetic analysis of methanogens in the rumen of faunated and unfaunated cattle. Anim Sci J **83**: 727-734.

Palmer, B., Jones, R. J., Wina, E. and Tangendjaja, B. 2000. The effect of sample drying conditions on estimates of condensed tannin and fibre content, dry matter digestibility, nitrogen digestibility and PEG binding of *Calliandra calothyrsus*. Anim Feed Sci Tech **87**: 29-40.

Parra, R., (ed.) 1978. Comparison of foregut and hindgut fermentation in herbivores. Smithsonian Institute Press, Washington, D.C., US. 205-229 pp.

Patra, A. K. 2012. Enteric methane mitigation technologies for ruminant livestock: a synthesis of current research and future directions. Environ Monit Assess **184**: 1929-1952.

Pedrós-Alió, C. 2007. Dipping into the rare biosphere. Science **315**: 192-193.

Pell, A. N. 1997. Manure and microbes: public and animal health problem? J Dairy Sci **80**: 2673-2681.

Pell, A. N., Woolston, T. K., Nelson, K. E. and Schofield, P. 2000. Tannins: biological

activity and bacterial tolerance. Pages 111-116 *in* J. D. Brooker, ed. Proc. Int. Workshop on Tannins in Livestock and Human Nutrition, Aust. Cent. Int. Agric. Res., Adelaid, Australia.

Pereira, R. V. V., Santos, T. M. A., Bicalho, M. L., Caixeta, L. S., Machado, V. S. and Bicalho, R. C. 2011. Antimicrobial resistance and prevalence of virulence factor genes in fecal *Escherichia coli* of Holstein calves fed milk with and without antimicrobials. J Dairy Sci **94**: 4556-4565.

Perez-Conesa, D., McLandsborough, L. and Weiss, J. 2006. Inhibition and inactivation of *Listeria monocytogenes* and *Escherichia coli* O157:H7 colony biofilms by micellar-encapsulated eugenol and carvacrol. J Food Prot **69**: 2947-2954.

Pichersky, E. and Gang, D. R. 2000. Genetics and biochemistry of secondary metabolites in plants: an evolutionary perspective. Trends Plant Sci **5**: 439-445.

Pillai, S. K., Moellering, R. C. and Eliopoulos, G. M. 2001. Antimicrobial combinations. Pages 365-440 *in* V. Lorian, ed. Antibiotics in Laboratory Medicine: Making a Difference. The Williams & Wilkins Co., Baltimore, MD, USA.

Pillai, S. K., Moellering, R. C. and Eliopoulos, G. M., (eds.) 2005. Antimicrobial combinations. 5th ed. The Lippincott Williams & Wilkins Co., Philadelphia, PA, USA. 889 pp.

Piller, N. B. 1975. A comparison of the effectiveness of some anti-inflammatory drugs on thermal oedema. Br J Exp Pathol **56**: 554-560.

Plaizier, J. C. 2004. Replacing chopped alfalfa hay with alfalfa silage in barley grain and alfalfa-based total mixed rations for lactating dairy cows. J Dairy Sci **87**: 2495-2505.

Poli, C., Hodgson, J., Cosgrove, G. P. and Arnold, G. C. 2006. Selective behaviour in cattle grazing pastures of strips of birdsfoot trefoil and red clover. 1. The effects of relative sward area. J Agr Sci **144**: 165-171.

Porter, L. J., Hrstich, L. N. and Chan, B. G. 1986. The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. Phytochemistry **25**: 223-230.

Prakash, S., Rodes, L., Coussa-Charley, M. and Tomaro-Duchesneau, C. 2011. Gut microbiota: next frontier in understanding human health and development of biotherapeutics. Biologics **5**: 71-86.

Pruesse, E., Quast, C., Knittel, K., Fuchs, B. M., Ludwig, W., Peplies, J. and Glöckner, F. O. 2007. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Res **35**: 7188-7196.

Purushe, J., Fouts, D. E., Morrison, M., White, B. A., Mackie, R. I., Coutinho, P. M., Henrissat, B. and Nelson, K. E. 2010. Comparative genome analysis of *Prevotella ruminicola* and *Prevotella bryantii*: insights into their environmental niche. Microb Ecol **60**: 721-729.

Reed, J. D. 1995. Nutritional toxicology of tannins and related polyphenols in forage legumes. J Anim Sci **73**: 1516-1528.

Reed, J. D., Horvath, P. J., Allen, M. S. and Vansoest, P. J. 1985. Gravimetric-determination of soluble phenolics including tannins from leaves by precipitation

with trivalent ytterbium. J Sci Food Agr **36**: 255-261.

Rochfort, S., Parker, A. J. and Dunshea, F. R. 2008. Plant bioactives for ruminant health and productivity. Phytochemistry **69**: 299-322.

Romero-Perez, G. A., Ominski, K. H., McAllister, T. A. and Krause, D. O. 2011. Effect of environmental factors and influence of rumen and hindgut biogeography on bacterial communities in steers. Appl Environ Microbiol **77**: 258-268.

Rotem, E., Loinger, A., Ronin, I., Levin-Reisman, I., Gabay, C., Shoresh, N., Biham, O. and Balaban, N. Q. 2010. Regulation of phenotypic variability by a threshold-based mechanism underlies bacterial persistence. Proc Natl Acad Sci **107**: 12541-12546.

Russell, J. B. and Rychlik, J. L. 2001. Factors that alter rumen microbial ecology. Science **292**: 1119-1122.

Sage, J. R. and Ingham, S. C. 1998. Survival of *Escherichia coli* O157:H7 after freezing and thawing in ground beef patties. J Food Prot **61**: 1181-1183.

Saiman, L. 2007. Clinical utility of synergy testing for multidrug-resistant *Pseudomonas aeruginosa* isolated from patients with cystic fibrosis: 'the motion for'. Paediatr Respir Rev **8**: 249-255.

Sato, M., Fujiwara, S., Tsuchiya, H., Fujii, T., Iinuma, M., Tosa, H. and Ohkawa, Y. 1996. Flavones with antibacterial activity against cariogenic bacteria. J Ethnopharmacol **54**: 171-176.

Scalbert, A. 1991. Antimicrobial properties of tannins. Phytochemistry **30**: 3875-3883.

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., Lesniewski, R. A., Oakley, B. B., Parks, D. H., Robinson, C. J. and others. 2009. Introducing Mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol **75**: 7537-7541.

Schofield, P., Mbugua, D. M. and Pell, A. N. 2001. Analysis of condensed tannins: a review. Anim Feed Sci Tech **91**: 21-40.

Schreurs, N. M., Tavendale, M. H., Lane, G. A., Barry, T. N., Lopez-Villalobos, N. and McNabb, W. C. 2007. Effect of different condensed tannin-containing forages, forage maturity and nitrogen fertiliser application on the formation of indole and skatole in in vitro rumen fermentations. J Sci Food Agr **87**: 1076-1087.

Shanks, O. C., Kelty, C. A., Archibeque, S., Jenkins, M., Newton, R. J., McLellan, S. L., Huse, S. M. and Sogin, M. L. 2011. Community structures of fecal bacteria in cattle from different animal feeding operations. Appl Environ Microbiol **77**: 2992-3001.

Sharp, R., Ziemer, C. J., Stern, M. D. and Stahl, D. A. 1998. Taxon-specific associations between protozoal and methanogen populations in the rumen and a model rumen system. FEMS Microbiol Ecol **26**: 71-78.

Shi, Z. C. 1988. Identification of the phenolic substances in bovine urine associated with oak leaf poisoning. Res Vet Sci **45**: 152-155.

Sikkema, J., de Bont, J. A. and Poolman, B. 1995. Mechanisms of membrane toxicity of hydrocarbons. Microbiol Rev **59**: 201-222.

Singh, B. and Bhat, T. K. 2003. Potential therapeutic applications of some antinutritional plant secondary metabolites. J Agr Food Chem **51**: 5579-5597.

Singh, S., McCallum, J., Gruber, M. Y., Towers, G. H. N., Muir, A. D., Bohm, B. A., KoupaiAbyazani, M. R. and Glass, A. D. M. 1997. Biosynthesis of flavan-3-ols by leaf extracts of *Onobrychis viciifolia*. Phytochem **44**: 425-432.

Smart, W. W., Stanley, N. W., Cope, W. A. and Bell, T. A. 1961. Inhibition of rumen cellulase by an extract from *Sericea* forage. J Dairy Sci **44**: 1945-1946.

Smith, A. H., Zoetendal, E. and Mackie, R. I. 2005. Bacterial mechanisms to overcome inhibitory effects of dietary tannins. Microb Ecol **50**: 197-205.

Sogin, M. L., Morrison, H. G., Huber, J. A., Welch, D. M., Huse, S. M., Neal, P. R., Arrieta, J. M. and Herndl, G. J. 2006. Microbial diversity in the deep sea and the underexplored "rare biosphere". Proc Natl Acad Sci **103**: 12115-12120.

Soulsby, L. 2007. Antimicrobials and animal health: a fascinating nexus. J Antimicrob Chemother **60**: i77-i78.

Stafford, H. A. and Lester, H. H. 1981. Proanthocyanidins and potential precursors in needles of douglas fir and in cell suspension cultures derived from seedling shoot tissues. Plant Physiol **68**: 1035-1040.

Stanton, T. B. and Canale-Parola, E. 1980. *Treponema bryantii* sp. nov., a rumen spirochete that interacts with cellulolytic bacteria. Arch Microbiol **127**: 145-156.

Stevenson , L. H. 1977. A case for bacterial dormancy in aquatic systems. Microb Ecol **4**: 127-133.

Stewart, C. S., Flint, H.J., Bryant, M.P. 1997. The rumen bacteria. Pages 10-72 *in* P. N. Hobson, Stewart, C.S., ed. The Rumen Microbiology Ecosystem. Blackie Academic & Professional, London, UK.

Tajima, K., Aminov, R. I., Nagamine, T., Matsui, H., Nakamura, M. and Benno, Y. 2001. Diet-dependent shifts in the bacterial population of the rumen revealed with real-time PCR. Appl Environ Microbiol **67**: 2766-2774.

Taylor, N. L. and Quesenberry, K. H. 1996. Red Clover Science. Kluwer Academic Publishers, Dordrecht, the Netherlands. 226 pp.

Terrill, T. H., Rowan, A. M., Douglas, G. B. and Barry, T. N. 1992. Determination of extractable and bound condensed tannin concentrations in forage plants, protein concentrate meals and cereal grains. J Sci Food Agr **58**: 321-329.

Terrill, T. H., Waghorn, G. C., Woolley, D. J., McNabb, W. C. and Barry, T. N. 1994. Assay and digestion of 14C-labelled condensed tannins in the gastrointestinal tract of sheep. Br J Nutr **72**: 467-477.

Thastrup, O., Knudsen, J. B., Lemmich, J. and Winther, K. 1985. Inhibition of human platelet aggregation by dihydropyrano- and dihydrofuranocoumarins, a new class of cAMP-phosphodiesterase inhibitors. Biochem Pharmacol **34**: 2137-2140.

Thompson, D. J., Beever, D. E., Harrison, D. G., Hill, I. W. and Osbourn, D. F. 1971. The digestion of dried lucerne (*Medicago sativa*, L) and dried sainfoin (*Onobrychis viciifolia*, Scop) by sheep. Proc Nutr Soc **30**: 14A-15A.

Tilman, D., Cassman, K. G., Matson, P. A., Naylor, R. and Polasky, S. 2002. Agricultural sustainability and intensive production practices. Nature **418**: 671-677.

Vamos-Vigyazo, L. 1981. Polyphenol oxidase and peroxidase in fruits and vegetables. Crit Rev Food Sci Nutr **15**: 49-127.

Van Soest, P. J. 1994. Nutritional Ecology of the Ruminant. 2nd ed. Cornell University Press, Ithaca, NY, USA. 476 pp.

Veening, J.-W., Smits, W. K. and Kuipers, O. P. 2008. Bistability, epigenetics, and bet-hedging in bacteria.(Report). Annu Rev Microbiol **62**: 193-210.

Waghorn, G. C. and McNabb, W. C. 2003. Consequences of plant phenolic compounds for productivity and health of ruminants. P Nutr Soc **62**: 383-392.

Waghorn, G. C. and Shelton, I. D. 1997. Effect of condensed tannins in *Lotus corniculatus* on the nutritive value of pasture for sheep. J Agr Sci **128**: 365-372.

Waghorn, G. C., Tavendale, M. H. and Woodfield, D. R. 2002. Methanogenesis from forages fed to New Zealand ruminants. Proc N Z Grassland Assoc **64**: 167-171.

Waghorn, G. C., Ulyatt, M. J., John, A. and Fisher, M. T. 1987. The effect of condensed tannins on the site of digestion of amino acids and other nutrients in sheep fed on *Lotus corniculatus* L. Brit J Nutr **57**: 115-129.

Wallace, R. J. 2004. Antimicrobial properties of plant secondary metabolites. Proc Nutr Soc **63**: 621-629.

Waller, P. J. 1997. Anthelmintic resistance. Vet Parasitol **72**: 391-405.

Wan, J., Wilcock, A. and Coventry, M. J. 1998. The effect of essential oils of basil on the growth of *Aeromonas hydrophila* and *Pseudomonas fluorescens*. J Appl Microbiol **84**: 152-158.

Wang, Y., Barbieri, L. R., Berg, B. P. and McAllister, T. A. 2007. Effects of mixing sainfoin with alfalfa on ensiling, ruminal fermentation and total tract digestion of silage. Anim Feed Sci Technol **135**: 296-314.

Wang, Y., Douglas, G. B., Waghorn, G. C., Barry, T. N. and Foote, A. G. 1996. Effect of condensed tannins in *Lotus corniculatus* upon lactation performance in ewes. J Agr Sci **126**: 353-362.

Wells, J. E., Berry, E. D. and Varel, V. H. 2005. Effects of common forage phenolic acids on *Escherichia coli* O157:H7 viability in bovine feces. Appl Environ Microbiol **71**: 7974-7979.

White, R. L., Burgess, D. S., Manduru, M. and Bosso, J. A. 1996. Comparison of three different in vitro methods of detecting synergy: time-kill, checkerboard, and E test. Antimicrob Agents Chemother **40**: 1914-1918.

Whitford, M. F., Forster, R. J., Beard, C. E., Gong, J. and Teather, R. M. 1998. Phylogenetic analysis of rumen bacteria by comparative sequence analysis of cloned 16S rRNA genes. Anaerobe **4**: 153-163.

Whittaker, R. J. and Fernandez-Palacios, J. M. 2007. Species numbers games: the macroecology of island biotas. Pages 77-106 Island Biogeography: Ecology, Evolution, and Conservation. Oxford University Press, New York, N.Y., USA.

Williams, A. G. 1986. Rumen holotrich ciliate protozoa. Microb Rev **50**: 25-49.

Williams, A. G. and Coleman, G. S. 1992. The rumen protozoa. Springer-Verlag, New York, NY, USA. 441 pp.

Williams, A. G. and Orpin, C. G. 1987. Polysaccharide-degrading enzymes formed by three species of anaerobic rumen fungi grown on a range of carbohydrate substrates. Can J Microbiol **33**: 418-426.

Witte, W. 2000. Ecological impact of antibiotic use in animals on different complex microflora: environment. Int J Antimicrob Agents **14**: 321-325.

Woodward, C. P., Waghorn, G. C., Lassey, K. R. and Laboyrie, P. G. 2002. Does feeding sulla (*Hedysarum coronarium*) reduce methane emission from dairy cows? Proc New Zealand Soc Anim Prod **62**: 227-230.

Woodward, S. L., Auldist, M. J., Laboyrie, P. J. and Jansen, E. B. L. 1999. Effect of *Lotus corniculatus* and condensed tannins on milk yield and milk composition of dairy cows. Proc New Zealand SocAnim Prod **59**: 152-155.

Woodward, S. L., Waghorn, G. C., Ulyatt, M. J. and Lassey, K. R. 2001. Early indications that feeding *Lotus* will reduce methane emissions from ruminants. Proc New Zealand Soc Anim Prod **61**: 23-26.

World Health Organization. 2003. The International Programme on Chemical Safety (IPCS). [Online] Available: http://apps.who.int/ipsc/database/evaluations/chemical.aspx?chemID=3549 [June 28, 2011].

World Health Organization. 2007. Report of the second WHO Expert Meeting, Copenhagen, May 29-31 2007. *in* WHO, ed. Critically important antibacterial agents for human medicine: categorization for the development of risk management strategies to contain antimicrobial resistance due to non-human use. WHO, Geneva. 34 pp.

World Health Organization. 2012. Evaluation of certain veterinary drug residues in food: seventy-fifth report on the Joint FAO/WHO Expert Committee on Food Additives. WHO Technical Report Series. World Health Organization, Geneva, Switzerland. 101 pp.

Wright, A.-D. G., Tajima, K. and Aminov, R. I. 2005. 16S/18S ribosomal DNA clone library analysis of rumen microbial diversity. Pages 163-174 *in* H. P. S. Makkar, C. S. McSweeney, eds. Methods in Gut Microbial Ecology for Ruminants. Springer, Dordrecht, The Netherlands.

Yamamoto, S. A. and Harris, L. J. 2001. The effects of freezing and thawing on the survival of *Escherichia coli* O157:H7 in apple juice. Int J Food Microbiol **67**: 89-96.

Yoshida, H., Kojima, T., Yamagishi, J. and Nakamura, S. 1988. Quinolone-resistant mutations of the gyrA gene of *Escherichia coli*. Mol Gen Genet **211**: 1-7.

Zhou, X., Ren, L., Meng, Q., Li, Y., Yu, Y. and Yu, J. 2010. The next-generation sequencing technology and application. Protein & Cell **1**: 520-536.

Ziemer, C. J., Sharp, R., Stern, M. D., Cotta, M. A., Whitehead, T. R. and Stahl, D. A. 2000. Comparison of microbial populations in model and natural rumens using 16S ribosomal RNA-targeted probes. Environ Microbiol **2**: 632-643.

Zoetendal, E. G., Smith, A. H., Sundset, M. A. and Mackie, R. I. 2008. The BaeSR two-component regulatory system mediates resistance to condensed tannins in *Escherichia coli*. Appl Environ Microbiol **74**: 535-539.

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