# Probiotic and dietary manipulation of the gastrointestinal tract microbiota in beef cattle: towards improved performance under different dietary management in Canadian Prairies

A Thesis Submitted to the Faculty of Graduate Studies of The University of Manitoba

In partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

by

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# Thesis Abstract

Microbial symbionts inhabiting the digestive tract of ruminants contribute to a wide range of metabolic functions essential for the survival of host. However, abrupt changes in the composition of diet can adversely affect the composition of these microbial communities, leading to metabolic disorders that could compromise the performance of the animals. Development of frothy bloat following exposure of over-wintered steers to legume pastures and impairment of ruminal fermentation as a result of feeding high-grain diets are common metabolic disorders affecting beef cattle in Canadian Prairies. Mitigation strategies to control these metabolic conditions are mainly based on dietary interventions, providing moderate protection to cattle. Understanding the contribution of microbiota to development of these metabolic conditions would pave the way towards implementing more effective strategies. In the first experiment of this thesis, the effects of supplementing the probiotic strain *Propionibacterium acidipropionici* P169 on the microbiota and metabolic profile of feedlot cattle receiving a high-grain diet were evaluated. Overall, the data suggest that P169 confers its beneficial effect via favoring the growth of cellulolytic bacteria, thus inhibiting the overgrowth of amylolytic bacteria and subsequent drop in the ruminal pH. In the second experiment, dynamics of rumen microbiota during development of frothy bloat and following administration of mitigating strategies was characterized (i.e., use of mixed alfalfa and sainfoin pasture and supplementing drinking water with Alfasure™). The data suggest that rapid proliferation of amylolytic bacteria following consumption of alfalfa contribute to development of frothy bloat. Dietary interventions used in this study prevented the development of clinal bloat, increased the proportions of cellulolytic bacteria, and alleviated bloat-associated dysbiosis in the composition of rumen microbiota. Finally, the third study evaluated the interrelationships of anaerobic rumen fungi (ARF) and ruminal bacteria in the context of frothy bloat. In general, ARF showed a large number of negative interrelationships with ruminal bacteria in normal rumen conditions. However, development of frothy bloat decreased the total number of relationships between ARF and bacteria, implying a disruption of microbe-microbe interrelationships (i.e., dysbiosis). Collectively, this thesis provides novel insights into the contribution of rumen microbiota to common metabolic disorders of beef cattle.

# Acknowledgements

I would like to thank my co-advisors, Dr. Tim McAlister and Dr. Emma McGeough, for providing guidance, feedback and kind advice throughout my PhD studies. My thanks also go to my advisory committee; Dr. Robert Forster, Dr. Surya Acharya and Dr. Peter Eck who kept me on track and provided insightful discussions about the research in committee meetings. I would like to express my special appreciation to my former advisor, Dr. Ehsan Khafipour, for his scientific, technical and financial support, and more importantly patience and encouragement throughout my graduate studies. I gratefully acknowledge the funding received towards my PhD from Agriculture Agri-Food Canada (AAFC). I also appreciate the financial support that I received through scholarships/awards provided by University of Manitoba, including IGSS, UMGF, Faculty of Graduate Studies conference travel awards, and Graduate Students Association travel awards. I would like to thank all fellow graduate students, technical, and research staff at the Department of Animal Science for their friendship, support and willingness to help. Many thanks to administrative staff at the Department of Animal Science, Mei Ding, Kathy Graham, Margaret Ann Baker, and Sandra Anderson for providing support during all these years.

# Dedication

I dedicate this dissertation to my beloved husband, best friend and role model of grit, determination and diligence, Hooman Derakhshani, whose constant support and encouragement throughout the challenging times of my graduate studies and life have given me strength to reach for starts and fulfill my dreams! I'm truly thankful to have you in my life. I also dedicate this thesis to my splendid parents, Nasrin Alivand and Reza Azad, and my kind brother, Amir Azad, who have always believed in me and whose love, thoughts, well-wishes and inspiration have sustained me throughout.

# Foreword

This thesis is written following a manuscript format and is composed of three manuscripts. All manuscripts have been formatted based on the BMC Microbiology Journal. The titles of these manuscripts and list of contributing authors are as follows:

**1) Manuscript 1:** Effect of *Propionibacterium acidipropionici*, P169 on the Rumen and Fecal Microbiomes of Beef Cattle Fed a Corn-Based Finishing Diet

**Contributing authors:** Elnaz Azad, Nelmy Narvaez, Hooman Derakhshani, Awfa Y. Allazeh, Yuxi Wang, Tim A. McAllister, and Ehsan Khafipour

**Published in Beneficial Microbes:** doi: 10.3920/BM2016.0145

**Contribution of authors:** T.A.M. and E.K. contributed to the conception and design of the study. E.A. conducted the laboratory work. E.A., N.N., A.Y.A., and Y.W. were responsible for sample collection and DNA extraction. E.A. and H.D. sequenced samples. E.A., H.D. and E.K. developed the bioinformatics and statistical models and analyzed the microbiome data. E.A. drafted the manuscript and prepared all tables and figures. All authors reviewed the manuscript critically for important intellectual content, and approved the submitted versions and agree to be accountable for all aspects of the work ensuring that questions related to the accuracy or integrity of any part of the work were appropriately investigated and resolved

**2) Manuscript 2:** Characterization of the rumen and fecal microbiome in bloated and non-bloated cattle grazing alfalfa pastures and subjected to bloat prevention strategies

Contributing authors: Elnaz Azad, Hooman Derakhshani, Robert Forster, Robert J Gruninger, Surya Acharya, Tim A. McAllister, and Ehsan Khafipour

**Published in Scientific Reports:** doi: 10.1038/s41598-019-41017-3

**Contribution of authors:** T.A.M., S.A. and R.J.F. contributed to the conception and design of the study. E.A. conducted the laboratory work. R.J.G., R.J.F. and E.A. were responsible for sample collection and DNA extraction. E.A. and H.D. sequenced samples. E.A, H.D and E.K. developed the bioinformatics and statistical models and analyzed the microbiome data. E.A. drafted the manuscript and prepared all tables and figures. All authors reviewed the manuscript critically for important intellectual content, and approved the submitted versions and agree to be

accountable for all aspects of the work ensuring that questions related to the accuracy or integrity of any part of the work were appropriately investigated and resolved

**3) Manuscript 3:** Interrelationships of Fiber-Associated Anaerobic Fungi and Bacterial Communities in the Rumen of Bloated Cattle Grazing Alfalfa

Contributing Authors: Elnaz Azad, Kelsey B Fehr, Hooman Derakhshani, Robert Forster, Surya Acharya, Ehsan Khafipour, Emma McGeough, and Tim A. McAllister

**Published in Microorganisms:** doi: 10.3390/microorganisms8101543

**Contribution of authors:** T.A.M., S.A., R.J.F., and E.M. contributed to the conception and design of the study. E.A. conducted the laboratory work. R.J.F. and E.A. were responsible for sample collection and DNA extraction. E.A. and H.D. sequenced samples. E.A, H.D and E.K. developed the bioinformatics and statistical models and analyzed the microbiome data. E.A. and E.M. drafted the manuscript and prepared all tables and figures. All authors reviewed the manuscript critically for important intellectual content, and approved the submitted versions and agree to be accountable for all aspects of the work ensuring that questions related to the accuracy or integrity of any part of the work were appropriately investigated and resolved

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# List of Abbreviations

| Abbreviation | Definition |
|---|---|
| ARF | Anaerobic Rumen Fungi |
| BCVFAs | Branched-Chain Fatty Acids |
| CAZymes | Carbohydrate Active Enzymes |
| CFUs | Colony Forming Units |
| CTs | Condensed Tannins |
| CP | Crude Protein |
| DFM | Direct Fed Microbial |
| DDGS | Dried Distillers Grains Plus Solubles |
| DM | Dry Matter |
| FDR | False Discovery Rate |
| F1 | Fraction 1 |
| GIT | Gastrointestinal Tract |
| ITS | Internal Transcribed Spacer |
| MaAsLin | Multivariate Analysis with Linear Modeling |
| NGS | Next-Generation Sequencing |
| OTUs | Operational Taxonomic Units |
| PLS-DA | Partial Least Square Discriminant Analysis |
| PERMANOVA | Permutational Multivariate Analysis of Variance |
| PCoA | Principal Coordinate Analyses |
| RDA | Redundancy Analyses |
| SCFAs | Short-Chain Fatty Acids |
| TMR | Total Mixed Ration |
| VFAs | Volatile Fatty Acids |

# 1 General Introduction

Ruminants themselves lack the required enzymatic repertoire to effectively harvest energy from lignocellulose-rich plant material. This deficiency has been compensated by anatomical compartmentalization of their gastrointestinal tract (GIT), which promotes colonization by complex microbial communities consisting of prokaryotes (bacteria and archaea), eukaryotes (protists and fungi) and viruses [1]. These microbial symbionts, also referred to as commensal microbiota, have the ability to perform a number of essential protective, structural, and metabolic functions for their ruminants' hosts. These include sequential degradation and fermentation of complex indigestible polysaccharides, metabolism of the main dietary nutrients (i.e., carbohydrates, proteins, and lipids), biosynthesis of a wide range of vitamins and metabolites, competition with pathogens and maintaining GIT homeostasis [2-4]. In large herbivores such as cattle, the anatomical characteristics of rumen and hindgut represent voluminous anaerobic chambers in which up to 70% of GIT volume is devoted to microbial fermentation [5]. The major products of this microbial fermentations are short-chain fatty acids (SCFAs), which are utilized by ruminants contributing up to 70% of their total energy requirement [4].

Recent advancements in the field of molecular microbiology and next-generation sequencing (NGS) have revolutionized our understanding of the compositional and functional diversity of the GIT microbiota in ruminants [6]. It is now known that the GIT microbiota of adult ruminants, particularly those inhabiting the rumen and large intestine, harbors an immense species- and strain-level diversity with overlapping metabolic capabilities [7]. Therefore, despite high inter-animal differences that exist in the composition of rumen microbiota [8], the presence of a "core rumen microbiome" ensures that rumen ecosystem is functionally redundant and capable to carryout main metabolic activities that are essential for the survival of the ruminant.

Another important characteristic of a mature GIT ecosystem is the resilience of its microbial symbionts against perturbations by abiotic and biotic factors. From an ecological perspective, resilience is the amount of stress or perturbation that a system can

tolerate before it changes from one stable equilibrium state to another [9]. Owing to their complex composition and functional redundancy, the core microbiomes of the rumen and hindgut in mature cattle are highly robust against external perturbations and can continue to maintain their symbiotic relationship with the host animal under fluctuating conditions. However, under certain circumstances, the intensity and nature of the external perturbants can be beyond the ability of the core microbiome to resist these disruptions, resulting in the development of unhealthy profiles of microbiota that are compositionally and functionally impaired (i.e., microbiota dysbiosis). Amongst all biotic and abiotic factors, diet appears to play the most influential role in changing the composition and functionality of the GIT microbiota [10, 11]. Abrupt changes in the biochemical characteristics and nutrient composition of the diet can rapidly change the composition and metabolic profile within the GI tract, which in turn, may result in altered biochemical parameters in the rumen and hindgut, influencing the overall well-being of the host animal [12-16].

In the Canadian Prairies, the most common beef production strategy is the sequential transfer of weaned calves from the cow-calf system to the stocker (backgrounder) and from the stocker to feedlot (finishing) operations. As such, the composition and nutritional value of the diets that are offered to cattle vary tremendously across different sectors of the beef industry, ranging from low-quality forage offered to over-wintered cows to nutritive fresh legume forage offered to cows and stocker cattle during spring and summer seasons, to high-grain based diets that are offered to finishing feedlot cattle, where the diet can consist of more than 90% grain [17-19]. These dietary changes can alter the composition and functionality of the GIT microbiota, which, in turn, may result in increased susceptibility of beef cattle to metabolic disorders and impaired performance. Two of the most common metabolic disorders that result from complex interactions between diet and GIT microbiota of beef cattle include a) development of pasture (frothy) bloat following sudden exposure of beef cattle to high quality legume forages (such as alfalfa and clovers) that contain high concentrations of soluble proteins [20], and b) development of subacute and acute ruminal acidosis in feedlot cattle following exposure to high-grain diets [13, 21, 22].

For each of the abovementioned metabolic disorders several mitigating strategies have been developed. Control of pasture bloat can be achieved by practices that lower the amount of soluble protein and reduce the rate of feed digestion. The use of pluronic detergents, such as Alfasure™ [23], and inclusion of non-bloating legumes such as sainfoin in mixed legume pastures [20, 24] have proven useful in reducing the severity and incidence of pasture bloat. On the other hand, current management strategies for preventing acidosis in feedlot cattle include the addition of chemical buffers [25], ionophores [26], yeast fermentation products [27], and direct fed microbials (DFM) to the diet [28, 29]. As an example, direct fed microbial (DFM) supplementation with lactic acid utilizing bacteria has been reported to be effective in modulating ruminal pH [30, 31] and reducing the risk of subacute ruminal acidosis (SARA) in cattle consuming high-grain diets. Although the effectiveness of the referred mitigating strategies on the performance of cattle has been extensively investigated, the microbial mechanisms that underlie development of each metabolic condition and/or recovery of ruminal microbial populations following these mitigating strategies is poorly understood. Given the immense contributions of GIT microbiota to the physiology of cattle and susceptibility to metabolic disorders, the main objective of this thesis was to evaluate the dynamics of rumen and hindgut microbial communities (i.e., bacterial and fungi communities) during exposure to a) high-grain diet (Chapter 5), and b) development of alfalfa-induced frothy bloat (chapter 6 and 7). Further, the effect of mitigation strategies to prevent rumen and hindgut microbial dysbiosis resulting from high-grain diet and alfalfa-induced frothy bloat is evaluated.

# 2 Literature Review

## 2.1 Microbial Ecology of the Gastrointestinal Tract (Git) in Ruminants

### 2.1.1 Symbiotic Relationship Between the Gut Microbiota and Ruminants

The reticulorumen - the largest compartment of the ruminant digestive tract - hosts a diverse and complex microbial community consisting of bacteria, archaea, ciliated protozoa, and fungi [32]. The proportion of each of these domains of microorganisms varies in the rumen ecosystem. Using metagenomics, Brulc et al. [33]. reported approximately 95% of the rumen microbial community belonging to bacteria, 0.6–4% to archaea, and 1.5% to eukarya. Using metatranscriptomics, more recent studies [34, 35] have reported relatively higher contribution of eukaryotes, specifically protozoa and anaerobic rumen fungi, to the overall rumen microbial ecosystem. Each of these domains encompass a variety of species that together have formed a unique symbiotic relationship with their host animal. In this relationship, the host animal provides the required nutrients and a suitable environment for the microorganisms to thrive, with the microorganisms in turn fermenting feedstuffs that are indigestible to the host, making nutrients accessible to the animal [36]. Most notable is the ability of rumen microbial community to produce a wide range of enzymes required for the breakdown of plant proteins, structural and non-structural carbohydrates into volatile fatty acids (VFAs), which serve as the main source of energy for ruminants [37]. Degradation and fermentation of plant polysaccharides by rumen microorganisms is an extremely coordinated and complex bioconversion process in which the substrate for one microorganism is frequently the product of another [36, 38]. In addition, microbial proteins synthesized by these microorganisms can make up 50–80% of the total protein that is absorbed in the small intestine of ruminants [39].

The rumen microbial ecosystem is both stable and dynamic. This ecosystem is stable as it is capable of maintaining its main function i.e., conversion of plant material to VFAs under different dietary and management strategies. It is also dynamic, as the microbial community changes rapidly in response to dietary modifications in order to adapt to the consumption and introduction of new feed ingredients into the rumen [40]. Prior to the emergence of next-generation sequencing techniques, our historical

understanding of rumen microbiology was mainly based on anaerobic culture-based techniques pioneered by Robert Hungate [41]. However, recent advances in high-throughput NGS have enabled comprehensive culture-independent profiling of the rumen microbiota, revealing greater species and functional diversity than previously realized [33, 42].

#### 2.1.1.1 Bacterial Community

Bacteria predominate the rumen microbial ecosystem, with an estimated population of $10^9$-$10^{11}$ colony forming units (CFUs) per mL of ruminal content [1]. The majority of bacteria present in the rumen ecosystem are obligate anaerobes, although some groups of facultative anaerobes also dwell within this microbial ecosystem [1]. Several studies have demonstrated immense species diversity within the rumen bacterial community, suggesting the existence of complex inter-species metabolic interactions that result in the efficient utilization of plant carbohydrates [33, 42, 43]. The dominant bacterial phyla present in the rumen ecosystem are similar to those detected in the gastrointestinal tract of other mammals, consisting mainly of members of Bacteroidetes and Firmicutes, and to a lesser extent members of the Proteobacteria, Actinobacteria, Tenericutes and a few other minor phyla [42, 43]. An important characteristic of rumen bacterial community is the existence of overlapping metabolic capabilities across phylogenetically diverse species i.e., functional redundancy which ensures the ability of this microbial ecosystem to perform its main metabolic functions under different dietary conditions and despite temporal fluctuation in species composition [7].

Traditionally, rumen bacteria have been categorized as those floating freely in the rumen fluid and those adherent to solid feed particles, with the latter group accounting for more than 70% of the total bacterial population [1]. It is believed that only a small group of bacteria are responsible for the initial degradation of complex plant cell wall material within the rumen ecosystem. This principal cultured bacteria, also known as cellulolytic rumen bacteria, are mainly *Ruminococcus flavefaciens, Ruminococcus albus,* and *Fibrobacter succinogenes*, and to a lesser extent, *Butyrivibrio fibrisolvens* [4, 44]. The initial breakdown of plant fibers by cellulolytic bacteria provides nutrients for other bacterial populations to thrive, including starch utilizers such as *Ruminobacter*

*amylophilus, Streptococcus bovis, Succinomonas amylolytica,* several *Prevotella* spp., *Butyrivibrio fibrisolvens* and *Selenomonas ruminantium*, and pectin utilizers such as *Succinivibrio dextrinosolvens* and *Lachnospira multiparus* [45, 46]. In addition to cellulolytic and starch utilizers, another group of bacteria within the rumen ecosystem are protein utilizers, including *Ruminobacter amylophilus, S. ruminantium, Prevotella ruminicola,* and *B. fibrisolvens*. However, as mentioned earlier, our growing understanding of the functional redundancy among rumen bacterial species challenges these traditional functional categories. For instance, *P. ruminicola*, a species considered an active protein utilizer and ammonia producer in the rumen, is also an active hemicellulolytic bacterium [47]. Indeed, Bacteroidetes species isolated from the gastrointestinal tract of bovine and other mammalian species, in particular members of the genus *Prevotella,* possess diverse and extensive repertoires of proteolytic and carbohydrate active enzymes that enable them to contribute to multiple metabolic pathways [48, 49].

#### 2.1.1.2 Fungal Community

From a functionality perspective, rumen fungi can be classified into two broad categories. The first group includes both aerobic and facultative anaerobic fungi, which are transient within the rumen ecosystem and are thought to not play a critical role in ruminal fermentation. On the other hand, the second group consists of strict anaerobic fungi, which are considered to be indigenous members of the rumen microbial community that make a major contribution to ruminal fermentation [1]. The anaerobic rumen fungi (ARF) are primary colonizers of plant fibrous materials and play an important role in degradation of lignocellulosic components [50]. The life cycle of ARF in the rumen consists of two main stages, the zoospore stage which is represented by motile zoospores in the ruminal fluid, and the non-motile stage which are mycelia associated with plant fibrous materials [1]. Upon attachment to plant fragments, the zoospores of ARF germinate and grow germinal tubes, which then branch into rhizoidal structures capable of penetrating the lignocellulosic tissues of the plant cell wall [1]. Therefore, by physical destruction of plant cell wall, ARF act to promote biofilm formation and facilitate access of rumen bacterial populations to fermentable

carbohydrates within the cell interior [51, 52]. Moreover, ARF also possess a wide variety of hydrolytic enzymes required for the break-down of the major components of plant biomass, including cellulases, hemicellulases, xylanases, amylases, and proteases [50, 53]. Although the overall contribution of ARF to ruminal fermentation is lower than bacterial community, their cooperation with other ruminal microorganisms plays a deterministic role in utilization of plant fiber material. Several studies have evaluated potential interspecies interactions between ARF and rumen bacteria, indicating both synergistic and competitive interactions with respect to nutrient utilization [38, 54, 55].

Th ARF account for up to 10 % of the total microbial biomass in the rumen, with counts of ARF zoospores approximating to $10^3$–$10^4$ per mL of rumen fluid. Current classification of anaerobic fungi encompasses 18 distinct genera under the phylum *Neocallimastigomycota* [56]. Among these, *Neocallimastix*, *Piromyces*, *Caecomyces*, *Cyllamyces*, *Orpinomyces*, *Anaeromyces, Pecoramyces, and Buwchfawromyces* have been detected in the rumen and fecal contents of cattle [57-61].

### 2.1.1.3 Protozoal Community

Rumen protozoa encompass a diverse group of single-cell mobile eukaryotic organisms in rumen fluid with an estimated size of 20-200 um [62]. The vast majority of protozoa in the rumen are ciliates belonging to the phylum Ciliophora, although a few flagellate species belonging to the phylum Sarcomastigophora have also been identified [63]. Although protozoa can make up a large proportion of the rumen biomass, their contribution to rumen fermentation is not as vital as rumen bacteria. This has been evidenced by defaunation studies where this group of microorganism have been eliminated from the rumen ecosystem without causing a severe effect on the well-being of the host [64]. Nonetheless, protozoa can contribute to fiber digestion as defaunation has been associated with a decline in fiber digestion. Also, protozoa play an active role in feed digestion and contribute to buffering capacity within the rumen. Ciliate protozoa can engulf bacteria and other feed particles in the rumen, digest carbohydrates, proteins, and fats, and therefore produce fermentation end products such as acetate, butyrate, and $H_2$. In particular, ciliate protozoa can engulf and store starch granules, preventing their rapid

fermentation by rumen bacteria and slowing down the production of acids [65]. Therefore, in ruminants fed high-grain diets, protozoa can modulate ruminal pH by reducing total bacterial numbers through predation and reducing the rate of starch fermentation. On the other hand, as protozoa are capable of degrading dietary proteins and predating bacteria, their presence can reduce the flow and availability of protein in the small intestine [1]. Some protozoa can also contribute to rumen methanogenesis by participating in interspecies transfer of $H_2$ as donors and generating $H_2$ in organelles known as hydrgenosomes, which is passed to epi- and endo-symbiotic methanogens [63].

### 2.1.1.4 Methanogen Community

Rumen methanogens belong to the archaeal domain, classified under the phylum Euryarchaeota. These are strictly anaerobic microorganisms that lack the typical peptidoglycan component of the bacterial cell walls, and can constitute about 0.6-3.3% of the total rumen microbial mass [66, 67]. Large scale culture-independent studies have revealed that most rumen methanogens belong to three genera of *Methanobrevibacter*, *Methanomicrobium*, and members of the order Methanomassiliicoccales [66, 68, 69]. More recently, Henderson et al. [43] comprehensively characterized the microbial communities of rumen samples collected from 35 countries and reported 5 dominant groups of methanogens, including *Methanobrevibacter gottschalkii*, *Methanobrevibacter ruminantium, Methanosphaera* species, and members of the family Methanomassiliicoccaceae to be globally omnipresent, accounting for more than 90% of the archaeal populations in rumen samples. With respect to functionality, although methanogenic archaea make up a small proportion of the rumen microbial biomass, their contribution to overall rumen fermentation is critical. Methanogens are the main recipients of $H_2$ within the rumen ecosystem, using the electrons derived from $H_2$ (or Formate) to reduce $CO_2$ to $CH_4$ [70]. Efficient removal of $H_2$ from rumen fluid decreases the inhibitory effect of accumulation of reducing equivalents on microbial fermentation and results in more favorable pattern of VFA production by rumen bacteria [66, 71]. Methanogens have established a symbiotic relationship with protozoa and fungi whereby utilizing $H_2$ as electron recipient, they thermodynamically promote energy utilization by

these eukaryotes [45, 72]. In addition to protozoa, bacteria also participate in inter-species transfer of $H_2$ with methanogens. For example, degradation of plant cell wall by cellulolytic bacteria such as *Ruminococcus albus* and *R. flavefaciens* generates $H_2$ which can be utilized by methanogens [65, 73].

### 2.1.2 Inter-Species Interactions: Driving Forces of the Microbial Ecosystem

From an ecological perspective, microorganisms do not live in isolation but instead coexist in complex ecologies with various symbiotic relationships with their hosts and their communities. Within an ecological niche, microbes can exchange or compete for nutrients, signaling molecules, or immune evasion mechanisms, resulting in the development of positive i.e., co-occurrence: for example between mutualistic microbes, or those that benefit each other or negative relationships i.e., mutual-exclusion: for example between antagonistic microbes, or those that compete and co-exclude each other [74]. Profiles of these microbial interactions are both complex and dynamic, influenced by several factors including host genotype, diet, and other external perturbations such as antimicrobial usage [74]. In the context of the rumen ecosystem, the host animal provides an ideal environment for bacteria and other microbes to thrive i.e., continuous supply of nutrients, optimum temperature, high buffering capacity of rumen contents, and in turn the microbial community as a whole harvests energy and nutrients from the feedstuff [36]. Utilization of a common feed resource by various groups of microorganisms is the main driving force of inter-species interactions within the rumen ecosystem [45]. As mentioned earlier, sequential breakdown of plant cell walls by bacteria, protozoa and fungi, and $H_2$ exchange and electron transfer among bacteria, methanogens, fungi and protozoa are examples of cooperative strategies that rumen microorganisms have adapted to increase feed utilization efficiency and generate favorable rumen fermentation profiles. Another type of inter-species interaction among rumen bacteria is cross-feeding, i.e., mutual utilization of fermentation end products that enhances energy utilization by both species. A good example of this type of interaction is the metabolic exchange between *Prevotella ruminicola,* a predominantly amylolytic species that also degrades protein and

liberates $NH_3$, and *Ruminococcus albus,* a predominantly cellulolytic species that degrades cellulose and provides hydrolyzed cellulolytic products [75]. The $NH_3$, liberated by *P. ruminicola* can in turn be utilized as a N source by *R. albus*. On the other hand, rumen microbes have also evolved mechanisms to compete with each other for available niches and nutrient resources, resulting in negative or inhibitory interactions. Secretion of secondary metabolites such as bacteriocins is an example of one of the most widespread mechanisms by which bacteria inhibit the growth of competing species [76]. For example, *R. albus* and *R.* flavefaciens are two cellulolytic species that produce bacteriocins as they compete for cellulose [77].

Characterizing microbial interactions under healthy and diseased conditions could serve as a mechanistic approach to investigating the contribution of microbiota to host physiological status [78]. However, microbial interactions within diverse ecosystems are far more complicated than simple experimental models usually investigated under laboratory conditions. Alternatively, correlation network analyses have emerged as promising tools for predicting microbial interactions based on relative proportion of microbes inferred from sequence data [74]. In this type of analysis, relative proportions of different microbial groups at different taxonomic levels (most often at the species or genus level) are used to identify co-occurrence (i.e., positive correlation) or mutual exclusion (i.e., negative correlation) patterns which may imply biologically meaningful relationships [79]. Albeit, similar to other *in silico* methods, correlation network analyses also suffer from high levels of false positive and false negative signals, which requires careful biological interpretation of the results [74].

## 2.2 Common Dietary Challenges Associated with Impaired Diversity and Functionality of the GIT Microbiota of Beef Cattle

Rumen microbial communities respond dynamically to a variety of internal and external forces. Like any other microbial ecosystem, the nature of the response of rumen microbial community to physical, chemical, and biological factors relies on the intensity and frequency of the perturbations on one hand, and diversity and resilience of the endogenous microbial community on the other [7]. Several studies have explored the

effect of such perturbations on the composition of the rumen microbiome, including the effect of ambient temperature [80], changes in diet [13, 27], and introduction of exogenous microbial populations such as DFM [81]. Amongst all these factors, diet appears to play a central role in shaping the composition and functionality of ruminant's GIT microbiota at different stages of life [6, 43].

Throughout their life cycle, beef cattle consume diets with varying nutrient composition at different intake levels. This could range from low to high quality forages and grain-based diets, affecting both the total concentration and composition of nutrients that are available to rumen microbiota. This in turn can influence the composition, function, and fermentation profile of the rumen microbial community, and subsequently the performance and well-being of the host [15, 82].

### 2.2.1 The Impact of High-Grain Diets on the Diversity and Functionality of Rumen and Hindgut Microbiota of Finishing Steers

The increasing global demand for meat and milk production has forced beef and dairy producers to adopt new feeding management strategies, including feeding readily fermentable high-grain diets to increase production in feedlot sector and intensive dairy farming. However, high-grain diets can disrupt the normal fermentation profile of the rumen and frequently cause metabolic disorders such as acidosis [83]. Ruminal acidosis is characterized by pH depression and accumulation of organic acids in the rumen which reflects a disruption of balance between microbial production, microbial utilization and the rate of absorption of organic acids. It is one of the most common digestive disorders in ruminants and can have a negative impact on the performance of cattle and subsequent profitability [83]. A drop of ruminal pH can result in dysbiosis of rumen microbial composition and fermentation profile, as well as the physiological features of the rumen in terms of permeability and motility [84]. As such, dietary-induced accumulation of organic acids in the rumen can significantly affect the functionality of the rumen microbiota and ruminal physiology, therefore compromising animal productivity and health [13, 15, 83, 85].

Following abrupt increases in the supply of starch (i.e., transition from predominantly forage-based to grain-based diets), bacteria capable of growing rapidly on soluble polysaccharides (e.g., *Streptococcus bovis, Ruminobacter amylophilus,* and anaerobic lactobacilli) can dominate the rumen microbial community, leading to a rise in the production of VFAs and lactate [86-88]. This accumulation of acids and consequent reduction of pH to levels below 5.6 has a remarkable impact on microbial activity, physiological function of the rumen and animal performance [84]. According to traditional culture techniques, the abundance of many amylolytic bacterial species changes in response to accumulation of short chain fatty acids and the subsequent drop in rumen pH [84]. For example, the total count of *S. bovis* can rise from $10^4$-$10^7$ cfu per gram in ruminal contents of forage-fed animal to $10^{11}$ per gram of ruminal contents of the animals fed excess levels of fermentable carbohydrates. The predominance of *S. bovis* in grain-fed animals is attributable to its rapid growth rate (doubling time: 12 minutes) on soluble carbohydrates [89]. However, following adaptation of the rumen to high-grain diets, the counts of *S. bovis* dramatically decrease to levels similar to those in forage-fed cattle [90]. In response to elevated levels of lactic acid in the rumen, acid-tolerant lactic-acid utilizing bacteria, mainly *M. elsdenii* and *S. ruminantium* increase [91-93]. As long as absorption and utilization of lactic acid keeps up with production, ruminal pH stays within a normal range (5.6-6.2), and ruminal functions remain stable and normal [93]. However, when the metabolic activity of lactate producers exceeds that of utilizers, lactic acid, which has a lower rate of ruminal absorption compared to VFAs, can accumulate and the density and activity of fiber digesting bacteria declines [91, 94]. Fiber digesting bacteria are generally sensitive to rumen pH below 6.0, thus the fibrolytic activities of the rumen microbiome are also compromised during ruminal acidosis [95]. There are 100s of genes in the genome of fibrolytic bacteria, including a variety of carbohydrate active enzymes (CAZymes) [34, 96, 97]. These genes act together to break down cellulose, hemicellulose, starch and pectin in plant tissues [98]. As fiber digestibility decreases, so does the net energy content of the diet [99], which can result in impaired feed efficiency and growth of the animal.

In agreement with the findings of traditional culture based techniques, DNA-based evaluation of rumen microbial community of beef cattle during adaptation to a high-grain diet [12] revealed that lactic acid producing bacteria (e.g., *S. bovis*, *S. ruminantium* and *Prevotella bryantii*) and lactic acid utilizing bacteria (e.g., *M. elsdenii* and *S. ruminantium*) were enriched in response to high-grain diet, whereas the proportions of fibrolytic bacteria (e.g., *Butyrivibrio fibrisolvens and Fibrobacter succinogenes*) gradually decreased. Other studies have shown that excessive grain feeding and the subsequent drop in ruminal pH can also reduce the overall richness and diversity of the rumen microbiota, and alter the proportion of major bacterial phyla in rumen contents [12, 13, 100-102]. In general, high-grain diets result in a decrease in the proportion of Bacteroidetes while increasing the proportion of Firmicutes in rumen contents [13, 27, 100]. Members of the Firmicutes encode fewer glycan-cleaving enzymes than Bacteroidetes [49]. It has been suggested that grain-induced reduction in the proportion of Bacteroidetes may be indicative of a reduced functionality of rumen microbiota in terms of fiber degradation [103].

Undigested starch could also bypass the rumen and enter the hindgut. This could alter bacterial composition and fermentation profiles in the hindgut, resulting in development of hindgut acidosis [104, 105]. Exploring the hindgut bacterial communities in cattle fed a high-starch diet, revealed increased proportion of genera *Prevotella, Blautia,* and other unclassified Lachnospiraceae, whereas the proportion of several operational taxonomic units (OTUs) belonging to the family *Ruminococcaceae* and genus *Clostridium* declined [106]. Also, in grass-fed cattle, the total *E. coli* count has been reported to be about $2 \times 10^4$ cells per gram of colonic digesta, whereas this number increased to $6.3 \times 10^6$ cells per gram of hindgut digesta when the cattle received moderate amounts of grain (60% of dry matter (DM)) [107].

### 2.2.2 Impact of Grazing Legume Forages on the Rumen Fermentation Profile and Frothy Bloat

Frothy bloat is recognized as a serious digestive disorder due to the adverse, and often fatal, effects it has on animal production. One of the main reasons for production losses in the case of bloat is reduced feed intake and death [108-110]. Bloat is responsible

for 2-3% of the mortalities in beef cattle grazing alfalfa as well as in cattle housed within feedlots [20, 108, 111]. In feedlot cattle, introduction of large amount of cereal grain into the diet (80-90% of DM) changes the principal substrates of microbial fermentation from slowly digestible plant fibrous material to rapidly digestible starch [112]. Availability of readily digestible carbohydrates results in accelerated microbial fermentation, production of large amount of fermentation acids, and uncontrolled release of bacterial mucopolysaccharides into the rumen content. This, together with finely ground feed particles can serve as a nuclei for formation of stable froth within the rumen content, thus disrupting the normal function of rumen [112, 113]. On the other hand, in cattle grazing legume pastures, the composition of the grazed sward can affect the incidence of pasture bloat with increases observed when bloat-causing legumes such as alfalfa and clover are included. Alfalfa is considered a premier forage in North America due to its high yield, nutritional quality and stand persistence. The average weight gain of properly managed cattle on alfalfa pastures i.e., those not experiencing frothy bloat, is comparable to those of cattle housed in feedlots [114]. However, due to the bloat-prone characteristics of alfalfa and other bloat-causing legumes, producers have typically limited inclusion levels in pastures to less than 30% of forage DM [115]. According to the traditional theory of bloat, soluble proteins in highly-digestible high protein containing forages including alfalfa are responsible for the onset of bloat [116]. Early cut alfalfa (i.e., late bud, early bloom stage) has a crude protein (CP) concentration between 16 and 20%. Even at maturity, it contains 12 to 15% crude protein, which is notably higher than that of grass hay at the same stage of maturity [117]. Highly soluble proteins in alfalfa are rapidly released in the rumen and are thought to contribute to the stable froth that causes bloat [113]. Rumen bacteria readily produce extracellular carbohydrates as they form biofilms which are composed of cells embedded within a mucopolysaccharide matrix [118, 119]. During the formation of biofilms, bacteria frequently produce excessive quantities of mucopolysaccharides or “bacterial slime”. Fermentation gases can become trapped within these bacterial slime – soluble protein complexes and further contribute to formation of the stable foam that impairs the removal of fermentation gases via eructation [113]. As a result, intraruminal pressure increases, the rumen distends and can restrict the contraction of the diaphragm, ultimately resulting in the death of the animal through suffocation [20,

120]. Production of foam in ruminal contents is a normal occurrence, however the amount of the foam produced is usually limited and does not persist. The formation of foam was initially believed to be associated with a soluble protein known as 18S, or fraction 1 (F1) protein (enzyme ribulose 1,5-biphosphate carboxylase oxygenase), which are present in high amounts (up to 4.5%) in bloat-causing legumes such as alfalfa and red clover, especially during the vegetative stage of growth [121, 122]. Despite its important role in the onset of frothy bloat, later studies showed only a modest association between F1 protein and other protein fractions and the occurrence of bloat [123]. However, a positive correlation between soluble proteins and bloat reported in subsequent trials, supported the concept that alfalfa in early stages is more likely to cause bloat [124]. The fact that tannin containing forages (e.g., Sainfoin) and senescence decrease the frequency of bloat through chemical modification of proteins, also supports the intimate role of soluble proteins in the etiology of bloat [125-127]. The soluble protein and chlorophyll are higher in pre-feeding ruminal liquid contents of cattle that subsequently bloated as compared to those that did not. In contrast, despite the positive correlation between post-feeding chlorophyl content and bloat, no relationship was detected between post-feeding soluble protein concentration of ruminal contents and bloat. As chlorophyll content correlates with chloroplast particles, the high concentration of digestible protein fractions in ruminal fluid is probably associated with the accumulation of chloroplast particles [128, 129]. Upon entry of fresh forage into the rumen, the thin mesophyll cell walls of bloat-causing legumes break down and chloroplasts are rapidly released. The soluble proteins released from lamellar membranes of plant cell walls increase the viscosity of ruminal contents, which further contribute to the stabilization of the foam and consequently promote bloat [123, 130]. Despite the fact that soluble proteins are major contributing factors to bloat, they are not the only factor involved in the etiology of this disease.

Based on the theory proposed by Howarth et al. [116] highly digestible protein is one of the three factors that are generally accepted as prerequisites for the onset of bloat. The other hypothesized reasons for frothy bloat are the fine plant particles arising from rupture of chloroplasts [116] and also the ability of rumen bacteria to attach to these particles [131]. The importance of the contribution of these factors was previously

emphasized based on the fact that cell walls of bloat-causing legumes are more easily degraded compared to the thicker cell walls of bloat-safe legumes [132]. The vulnerable cell wall of bloat-causing legumes is more easily breached by ruminal microorganisms compared to the thick cell walls of bloat-safe legumes [133]. The bacterial communities attached to alfalfa chloroplast particles are believed to degrade soluble proteins to produce an extracellular matrix composed of muccopolysaccharides (slime) which contributes to the viscosity of ruminal contents and ultimately frothy bloat [131, 134]. The isoelectric point of the F1 protein is in the pH range of 5.4-5.6, which is close to the pH which promotes maximum foam stability in the rumen [135]. Based on a culture based study conducted by Hazlewood et al. [136], the principal proteolytic bacteria isolated from rumen contents of cattle fed fresh alfalfa were identified as *Streptococcus bovis* and *Butyrivibrio* spp. The use of a selective medium containing F1 protein supported the role of the aforementioned bacteria in utilization of F1 protein as the sole nitrogen source but it failed to identify other proteolytic bacteria which use the nitrogen directly from this fraction or degrade it without utilizing end products. However, the low incubation time used in this study likely did not support the isolation of slower growing bacteria which maybe functionally important, but present at low numbers [136]. Early culture-based studies by Hungate [137] suggested that changing diets alter both the types and numbers of microorganisms in the rumen. After studying the effect of diet on protein degrading activity in sheep, Siddons et al. [138] concluded that increased ruminal proteolytic activity in sheep fed alfalfa was associated with increased numbers of proteolytic bacteria in the rumen [139]. Moreover, the microbial fermentation of soluble CP – which are high in vegetative wheat – has been shown to promote the production of muccopolysacharide matrix associated with biofilms [140, 141]. The incidence of bloat is highly associated with the generation of this matrix, as it entraps the fermentation gases within froth culminating in impairment of the eructation mechanism, leading to bloat [131, 142, 143]. Steers grazing vegetative wheat containing high levels of soluble protein were found to produce larger amounts of ruminal gas and exhibited a propensity to bloat. *Steptococcus bovis* was the major biofilm producer among 6 rumen bacterial species that were incubated with soluble wheat protein, indicating its role in utilizing soluble protein substrate [144]. The potential contribution of rapid proliferation of bacterial biofilms and

excessive production of slime to the development of frothy bloat was also evaluated by Pita et al [120] who concluded that the formation of biofilms by certain groups of bacteria disrupts fermentation patterns, most likely by trapping the substrates necessary for the growth of some genera. Further, a reduction in both the abundance and diversity of carbohydrases in rumen samples from bloated cattle supported their hypothesis that carbohydrate metabolism is altered in cattle suffering from this disease. Phylogenetic analysis of rumen samples from cattle grazing vegetative wheat [145] showed distinct microbial profiles as compared to those associated with rumen samples from cattle grazing wheat at other stages of maturity. Soluble protein and carbohydrates released from the rapid fermentation of vegetative wheat forage was suggested to be associated with a higher abundance of Bacteroidetes in rumen fluid. The results of this study suggested a possible association between changes in the predominant ruminal bacteria and the incidence of bloat in steers grazing wheat forage. Taken together, it appears that a bloat-associated reduction in the diversity and functional potential of the rumen microbial community correlates with a disturbed metabolic fermentation profile of the rumen.

## 2.3 Modulation of Rumen Microbial Community to Alleviate the Adverse Effect of High-Grain Diets and Bloat-Causing Legumes

In general, the two main strategies for modulating the rumen microbial community and fermentation profile in cattle include modifying the composition of the diet (i.e., altering the composition and proportion of main ingredients, or addition of supplemental chemicals, enzymes, etc.) or directly altering the composition of certain microbial populations by addition of probiotics or selective antimicrobials [7, 146]. Nutritional management strategies for controlling adverse effects of high-grain diets in dairy and beef cattle have been the subject of extensive research over the past few decades (as reviewed by [147]), which are outside the scope of this thesis. In terms of direct manipulation of rumen microbial communities to improve performance of beef cattle fed high-grain diets, the use of feed additives such as ionophores and sodium bicarbonate have also gained increasing attention [148]. Sodium bicarbonate is a natural buffer secreted through saliva and traditionally added to the diet of high-grain fed beef cattle to improve buffering capacity of rumen and moderate ruminal pH [149, 150]. Among

ionophores, monensin has been the most widely used feed additive in the diets of ruminants worldwide. Through its antimicrobial activities, monensin can directly modulate the composition of rumen microbial community and change the amount and profile of VFAs in favor of propionate production, and improve feed efficiency [151, 152]. Direct fed microbials have been used to improve the rumen fermentation profile in cattle fed high-grain diets, an approach that significant component of this thesis.

### 2.3.1 Application of Direct Fed Microbial to Alleviate Adverse Effects of High-Grain Diets on Rumen Microbial Fermentation Profile

Direct fed microbials, including probiotics and fermentation products of beneficial microbes have long been examined for their ability to prevent metabolic disorders and improve the performance of dairy and beef cattle [153, 154]. Yeast and yeast culture products, especially those derived from *Saccharomyces cerevisiae*, and lactic acid producing and/or utilizing bacteria have been among the most heavily examined microbes with respect to improving the rumen fermentation profile in cattle fed high-grain diets [155]. A major limiting factor for the widespread use of probiotic strains in modulating rumen fermentation profile is the inability of these strains to establish active populations within the rumen ecosystem, thus requiring frequent, often daily, supplementation via feeding [7]. For a non-native (allochthonous) probiotic strain to survive and establish an active population in the rumen ecosystem, it needs to overcome numerous challenges including adaptation to the biochemical characteristics of ruminal contents, competing effectively with hundreds of native (autochthonous) ruminal strains that are well-adapted to the harsh environment of the rumen, and most importantly, being able to establish mutualistic relationships with existing members of the ruminal microbial community [7].

As mentioned earlier, an abrupt shift from a primarily forage diet to high-grain diets can disturb both the fermentation profile and composition of the rumen microbial community, often characterized by enrichment of lactate-producing bacterial species such as *Streptococcus bovis* and anaerobic lactobacilli. This can result in the accumulation of lactate in the rumen and a temporary increase in the proportion of lactate-utilizing species

such as *Megasphaera elsdenii* and *Selenomonas ruminantium.* However, if excessive production of lactate continues in the rumen, the development of acidosis would result in a subsequent decrease in the proportion of *M. elsdenii* and *S. ruminantium* which are pH-sensitive [84]. Hence, the use of DFM products to improve the performance of beef cattle on high-grain diets has mainly focused on probiotic strains that can modulate rumen pH. Among these, active dry yeast products containing viable cells of *S. cerevisiae* have shown great potential in stabilizing ruminal pH and improving the performance of dairy and beef cattle fed high-grain diets [154]. One potential mechanism by which *S. cerevisiae* may contribute to stabilization of ruminal pH is through competition with *S. bovis* for utilization of starch, hence limiting the production of lactate by this bacterial species [156]. Another mechanism by which *S. cerevisiae* could improve the buffering capacity of rumen is through promoting the growth of lactate-utilizing bacteria, such as *M. elsdenii* or *S. ruminantium,* by supplying growth factors such as amino acids, peptides, and vitamins that are essential for their growth [157-159].

Lactate-utilizing bacteria, in particular *M. elsdenii*, have also been examined for their ability to attenuate the adverse effects of low ruminal pH in beef and dairy cattle fed high-grain diets [160]. Although a moderate decrease in ruminal lactate concentrations has been reported in some studies [31, 161], the effects of *M. elsdenii* on ruminal pH and production performance have been inconsistent across studies, therefore limiting the adoption of this DFM in the beef and dairy industries. Bacteria belonging to the genus *Propionibacterium* are another group of probiotics that have been examined for their ability to improve the performance of cattle fed high-grain diets. This group of bacteria are known for their ability to produce propionate, a major precursor of hepatic gluconeogenesis and an electron acceptor that is associated with reduced $CH_4$ production [162] . Therefore, DFM supplementation of propionibacteria may have the potential to improve feed efficiency and metabolism of cattle by increasing hepatic gluconeogenesis [162] and lowering ruminal $CH_4$ production. Studies on dairy cows have shown that DFM supplementation of *Propionibacterium* strain P169 during transition period and early lactation can increase the ruminal concentration of propionate and improve milk production [163, 164]. In the rumen of feedlot cattle fed a high-concentrate diet,

supplementation of *Propionibacterium* decreased the abundance of amylolytic bacteria, particularly *S. bovis*, although compared to the control, no significant changes in the ruminal pH of supplemented steers were observed [165]. However, little is known about the effect of *Propionibacterium* probiotics on the composition and fermentation profile of either the rumen or hind-gut microbial communities and whether this group of probiotics have the potential to improve the growth performance of feedlot beef cattle.

### 2.3.2 Mitigating Strategies to Prevent Alfalfa-Induced Frothy Bloat in Cattle

As indicated earlier, despite being one of the few legumes with the potential of sustaining production performance and feed efficiency of beef cattle in a manner similar to that achievable in feedlots [114], the widespread use of pure alfalfa pastures has been limited due to high risk of frothy bloat. As such, bloat mitigation strategies to enhance the performance of beef cattle grazing alfalfa pastures have been investigated extensively. These mitigation strategies have mainly revolved around pasture management, such as replacing or seeding alfalfa pastures with low-bloat potential forages, modifying grazing management practices such as altering duration and frequency of grazing, or the use of additives administered to cattle via drinking water [113]. With respect to the use of low-bloat potential forages, development of alfalfa cultivars with a low ruminal digestion rate and the use of legumes containing high amounts of condensed tannins have been studied extensively in the Canadian Prairies [113, 166]. An alfalfa cultivar selected via a twenty-year breeding program at Agriculture Agri-Food Canada, AC Grazeland, showed a 15% reduction in initial rate of digestion and reduced the incidence rate of frothy bloat in cattle by 56% compared to an unselected wild-type alfalfa [109, 167]. Sainfoin, is another legume forage capable of reducing the incidence of frothy bloat, owing to its high content of condensed tannins (CTs) [166]. Condensed tannins can bind with plant proteins via hydrophobic and hydrogen bonds in a pH-reversible manner, thus reducing the degradation of forage proteins in the rumen and the rapid proliferation of rumen microbial populations, in particular proteolytic and amylolytic bacteria [168]. Both *in vitro* and *in vivo* experiments have shown that moderate concentrations of CTs can

reduce the growth rate of amylolytic and proteolytic rumen bacteria including *Clostridium proteoclasticum*, *Prevotella bryantii*, and *Streptococcus bovis* [169, 170].

The use of feed additives to mitigate frothy bloat has also been the subject of extensive studies. Ionophores, in particular monensin, have been successfully used to reduce the incidence of bloat in beef cattle grazing alfalfa pastures [171]. The antimicrobial mode of action of ionophores relies on their ability to alter bacterial cell membrane permeability, dissipating ion gradients and uncoupling energy expenditures from growth, leading to a disruption of vital cell functions [172]. The anti-bloat effects of monensin have been attributed to its ability to suppress slime-producing bacterial groups (e.g., *S. bovis*), as well as decreasing overall ruminal gas production [172].

Pluronic surfactants are another group of additives used to prevent frothy bloat via reducing the viscosity and stability of foam in rumen contents [20]. In Canada, a mixture of pluronic surfactants (i.e., Alfasure™) was tested by mixing it in drinking water or spraying it on alfalfa pasture, resulting in a nearly complete prevention of frothy bloat [173, 174]. However, to what extent pluronic surfactants may alter the overall composition of the ruminal microbial community remains largely unknown.

To date, most of our knowledge regarding the contribution of ruminal microorganisms to development of alfalfa-induced frothy bloat is limited to either culture-based studies or targeted amplification and quantification of the classical rumen bacteria. However, as most species of rumen microbiota are yet uncultured, it is most likely that important microbial signatures associated with development of frothy bloat have been overlooked. Similarly, there is little know about the global response of rumen microbiota of feedlot cattle to DFM supplementation by *Propionibacterium* probiotics. This thesis aims to use NGS techniques, i.e., amplicon sequencing of 16S rRNA genes of bacteria and internal transcribed spacer (ITS) sequences of fungi, to comprehensively characterize the composition of rumen and hindgut microbiota of beef cattle in relation to each of the above-mentioned metabolic disorders.

# 3 Overall Objectives

The main objectives of this thesis were:

1) Investigate the responses of the rumen and hindgut microbiota of high-grain fed feedlot cattle to DFM supplementation with lactic acid utilizing bacteria *Propionibacterium acidipropionici* P169

2) Investigate the role of rumen microbiota in the development of pasture bloat and the response of rumen and hindgut microbiota to bloat mitigating strategies:

- Determine changes in the composition of the rumen microbiota (i.e., bacterial and fungal communities) that underlie the development of alfalfa-induced frothy bloat
- Explore the global responses of rumen microbiota to dietary interventions to prevent bloat which include grazing mixed alfalfa – sainfoin pastures and the addition of the pluronic detergent Alfasure™ to the water of cattle grazing pure alfalfa pastures
- Determine the degree to which hindgut microbiota can be influenced by these bloat prevention strategies

# 4 Hypotheses

The overall hypotheses were that the above-mentioned metabolic disorders of stocker and feedlot beef cattle results from dietary-induced microbial dysbiosis, and that dietary interventions that are commonly used to mitigate these metabolic disorders improve the profile of ruminal fermentation via modulating the composition of the GIT microbiota.

Specifically, it was hypothesized that:

1. Direct fed microbial supplementation by the lactate utilizer strain *Propionibacterium acidipropionici* P169 will improve ruminal fermentation profile by decreasing lactate concentration and preventing dysbiosis of rumen and hindgut microbiota of beef cattle receiving a high-grain finishing diet.
2. Development of alfalfa-induced frothy bloat is associated with significant changes in the composition of bacterial and fungal communities of the gastrointestinal tract.
3. Development of alfalfa frothy bloat is associated with the rapid proliferation of bacterial biofilms and disruption of interrelationships of fiber-associated fungi and bacterial species within the rumen ecosystem.
4. Bloat mitigating strategies, including changes in pasture management (i.e., mixing alfalfa pasture with low-bloat potential legume saponin) and application of feed additives (i.e., addition of pluronic detergents Alfasure™ in drinking water) will prevent development of pasture bloat in beef cattle.
5. The abovementioned mitigation strategies will mitigate bloat-associated microbiota dysbiosis and restore interrelationships of bacterial and fungal communities in the rumen content of beef cattle grazing alfalfa pastures.

# 5 Effect of *Propionibacterium acidipropionici*, P169 on the Rumen and Fecal Microbiota of Beef Cattle Fed a Corn-Based Finishing Diet

## 5.1 Abstract

Direct fed microbial supplementation with lactic acid utilizing bacteria (i.e., *Propionibacterium acidipropionici* P169) has been shown to alleviate the severity of subacute ruminal acidosis in high-grain fed beef cattle. This study was carried out to explore the impact of P169 supplementation on modulating rumen and hindgut microbiota of high-grain fed steers. Seven ruminally-canulated high-grain fed steers were randomly assigned to two treatment groups: control diet (n=3) and the same diet supplemented with P169 added at a rate of $1 \times 10^{11}$ cfu/head/d (n=4). Samples were collected every 28 days for a 101 d period (5 time points) and subjected to qPCR quantification of P169 and high throughput sequencing of bacterial V4 16S rRNA genes. Ruminal abundance of P169 was maintained at elevated levels ($P = 0.03$) both in liquid and solid fractions post supplementation. Concomitant with decreased proportion of amylolytic (such as *Prevotella*) and key lactate-utilizers (such as Veillonellaceae and *Megasphaera*), the proportions of fibrolytic bacterial lineages (such as Ruminococcaceae, Lachnospiraceae, Clostridiaceae, and Christensenellaceae) were enriched in the rumen microbiota of P169-supplemented steers. These, coupled with elevated molar proportions of branched-chain fatty acids and increased concentration of ammonia in the rumen content of P169-supplemented steers, indicated an improved state of fibrolytic and proteolytic activity in response to P169 supplementation. Further, exploring the fecal microbiota of P169-supplemented steers revealed enrichment of major amylolytic bacterial lineages such as *Prevotella*, *Blautia*, and Succinivibrionaceae, which might be indicative of an increased availability of carbohydrates in the hindgut ecosystem following P169 supplementation. Collectively, the present study provides insights into the microbiota dynamics that underlie the P169-associated shifts in the rumen fermentation profile of high-grain fed steers.

## 5.2 Introduction

Feedlot cattle are fed high-grain diets in order to improve growth performance. However, this practice can lead to changes in diurnal feeding behavior (i.e., meal frequency, meal duration, and eating rate) and erratic feed intake both of which are often associated with subacute and acute ruminal acidosis [21, 22] and dysbiosis of the rumen microbiome [13, 175]. The sum effect of all these changes is to decrease nutrient intake and SCFA absorption [176] and thereby compromise animal performance. Current management strategies for preventing acidosis in feedlot cattle include adding chemical buffers [25], ionophores [26], yeast fermentation products [27], DFM [28], and live yeast and bacterial based probiotics to the diet [29]. Inoculation with lactic acid utilizing bacteria is reported to modulate ruminal pH [30, 31], suggesting that they may reduce the risk of SARA. These microbial products may be beneficial during the transition phase from a high-forage to a high-grain diet.

*Propionibacterium* is both a lactate utilizer and an important propionate producer, converting glucose and lactate to acetate and propionate in a process that may lower ruminal lactate levels [177]. Moreover, *Propionibacterium* contributes to increased energetic efficacy through production of propionate which is the major precursor used to form glucose during gluconeogenesis [163]. Previous studies have shown that inclusion of *Propionibacterium acidipropionici* P169 in dairy cattle diets increased milk yield and ruminal propionate concentration [163, 164]. However, microbial populations inhabiting different ecological niches of the gastrointestinal tract are not isolated from each other and any substantial change in the profile of microbial fermentation is, most often, as a result of complex inter-species interactions. Therefore, the objectives of the present study were to explore the global dynamics of the rumen and hindgut microbiota in response to DFM administration of *P. acidipropionici* P169, and to gain a better understanding of mechanisms that underlie changes in the rumen fermentation profile of P169-supplemented cattle.

## 5.3 Methods

The details of animal trial and animal care protocols were described previously [178]. In brief, the experiment was conducted on 7 British × Charolais crossbreed steers fitted with rumen cannulas; with four of these animals being randomly assigned to P169 supplementation and three to the control diet. The control steers received 10 g $hd^{-1}d^{-1}$ of a maltodextrin carrier and the treatment group received 10 g $hd^{-1}d^{-1}$ of a maltodextrin carrier containing *P. acidipropionici* strain P169 at $1 \times 10^{11}$ cfu. Steers were housed in individual pens (1.8 m × 6.1 m) at the Lethbridge Research Centre, Alberta, Canada. Upon arrival at the center, steers were fed a growing diet composed of 81% corn silage, 13% corn grain, 4.5% corn dried distillers grains plus solubles (DDGS), and 1.5% supplement (DM basis). Steers were adapted from the growing to the finishing diet by gradually replacing corn silage with equal amounts of corn grain and corn DDGS at 7-d intervals over a 5-week period. During the finishing stage, the diet contained 56.2% dry rolled corn, 27.6% corn DDGS, 12% corn silage and 4.2% of a mineral and vitamin supplement (DM basis). The diet was formulated to meet or exceed the NRC (1996) nutrient requirements of beef cattle. All feed ingredients were mixed as a total mixed ration (TMR) in a Calan data ranger (American Calan, Northwood, NH, USA) and fed once daily ad libitum. The carrier containing P169 was top-dressed daily onto the TMR of each steer immediately after feed delivery. Steers had unrestricted access to clean drinking water at all times and were fed for 101 days until they reached the targeted slaughter weight.

### 5.3.1 Sample Collection

At the beginning of the finishing stage and every 28 d thereafter, rumen liquid and solid contents and grab-fecal samples were collected from each steer. Samples were also collected just prior to termination of the study when the steers reached slaughter weight. This resulted in each steer being sampled 5 times over the duration of the study. Representative samples of total rumen contents (25 g) were collected from the steers via the ruminal cannula 3 h post-feeding. Samples were collected from the reticulum, ventral and caudal sacs, mixed and placed on crushed ice. Samples were separated into rumen

liquid and solid fractions by squeezing rumen contents through four layers of cheesecloth and the resulting fluid was designated the rumen liquid fraction. The solid rumen contents were also retained for further analyses. Both rumen and fecal samples were snap-frozen by initially immersing them in liquid nitrogen, followed by storage at -80°C until analyzed.

### 5.3.2 DNA Extraction and Quantitative PCR

Ruminal and fecal samples were thawed at 4ºC overnight. Then 10 g of each sample was carefully homogenized cryogenically using Geno/Grinder 2010 (SPEX Sample Prep, NJ, USA). DNA was extracted using ZR-96 Fecal DNA Kit (Zymo Research, CA, USA), which included a 2-minute bead-beating step (at 1750 strokes per minute using Geno/Grinder 2010) for mechanical disruption of bacterial cells. The DNA obtained was stored at -20ºC in aliquots of 100 ng/µl (stock) of AE buffer. The DNA samples were normalized to 20 ng/µl for sequencing and 2 ng/µl for real-time quantitative PCR (qPCR). All DNA samples were quality checked by gel electrophoresis and PCR amplification of the 16S rRNA gene using universal primers 27F (5'-GAAGAGTTTGATCATGGCTCAG-3') and 342R (5'-CTGCTGCCTCCCGTAG-3') as described [13]. Amplicons were verified by agarose gel electrophoresis.

The qPCR reactions were conducted for absolute quantification of *P. acidipropionici* P169 using strain specific primers F-GTCCTTTCTTAACCGCTCGGG and R- GACTCCGTCGTCCGTCATAAG [179]. Genomic DNA was extracted from a pure culture of *P. acidipropionici* P169 and used to generate standard curves. The initial $10^9$ copies were calculated based on the size of P169 genome deposited in Genebank of NCBI, the number of targeted gene copies in each genome and the DNA concentration. A serial dilution containing known gene copy numbers of P169 (ranging from $10^1$ to $10^9$) was then used to determine the absolute quantity of P169 in each sample by relating the CT values to the standard curve. Reactions were performed on a Bio-Rad CFX384 Real-Time PCR Thermal Cycler (Bio-Rad, Mississauga, ON, Canada) in triplicate. A total volume of 20 µl per reaction was used, with 3 µl of DNA template, 400 nM forward primer, 400 nM reverse primer, and 10 µl of SsoFast EvaGreen Supermix (Bio-Rad,

Mississauga, ON, Canada). Reactions consisted of an initial enzymatic activation step at 98°C for 2 min followed by 39 amplification cycles of denaturation at 98°C for 4 sec and annealing/extension at 60°C for 4 sec. The melt cycle involved a temperature ramp from 65 to 95°C, with a 5 sec hold at each 0.5°C step of the ramp.

### 5.3.3 Illumina Library Construction and Sequencing

The V4 region of 16S rRNA gene was targeted for PCR amplification and construction of sequencing library using modified F515/R806 primers [180] as described by [181]. In brief, duplicate PCR reactions consisted of an initial denaturing step at 94°C for 3 min followed by 33 amplification cycles at 94°C for 45 sec, 50°C for 60 sec, and 72°C for 90 sec; finalized by an extension step at 72°C for 10 min in an Eppendorf Mastercycler pro (Eppendorf, Hamburg, Germany). PCR products were then purified using ZR-96 DNA Clean-up Kit™ (ZYMO Research, Irvine, CA, USA). The 150 paired-end sequencing reaction was performed on a MiSeq platform (Illumina, San Diego, CA, USA) at the Gut Microbiome and Large Animal Biosecurity Laboratories (Department of Animal Science, University of Manitoba, Winnipeg, Canada). The sequencing data were deposited into the Sequence Read Archive (SRA) of NCBI (http://www.ncbi.nlm.nih.gov/sra) and can be accessed via accession number SRR3194028.

### 5.3.4 Bioinformatics Analysis

The FLASH assembler v 1.2.11 [182] was used to merge and fix the overlapping paired-end Illumina fastq files so that reads with a minimum number of 25nt overlapping region and below 25% mismatch were kept for subsequent analysis. The output fastq file was then analyzed by downstream computational pipelines of the open source software package QIIME v 1.7.0 [183]. Assembled reads were demultiplexed according to the barcode sequences and exposed to additional quality-filters so that reads with ambiguous calls and those with phred quality scores (Q-scores) below 20 were discarded. Chimeric reads were filtered using UCHIME and sequences were assigned to OTUs using the QIIME implementation of UCLUST at 97% pairwise identity threshold. Taxonomies were assigned to the representative sequence of each OTU using RDP classifier and

aligned with the Greengenes Core reference database [184] using PyNAST algorithms. The resulting OTU-table was normalized to an even depth of 18,000 sequences per sample for all downstream analyses including calculation of alpha- and beta-diversity indices. Permutational multivariate analysis of variance (PERMANOVA) [185] was used to calculate *p* -values and test for significant differences of UniFrac distances between microbiota of different treatment groups.

Partial least square discriminant analysis (PLS-DA; SIMCA P+ 13.0, Umetrics, Umea, Sweden) was used to explore the association of microbial genera with treatment groups. The goodness of fit of the model was evaluated with $R^2$ and the predictive value of the model was evaluated using the $Q^2$ estimate. The PLS-regression coefficients were used to identify genera that were most characteristic of each treatment group. The results of PLS-DA were visualized by PLS-DA loading scatter plots. The UNIVARIATE procedure of SAS [186] was used to test the normality of residuals for alpha-diversity data as well as phyla and genera relative abundances across treatment groups and sampling sites. Non-normally distributed data were log transformed and then used to evaluate the effect of treatment (control vs. P169) and sampling site (liquid vs. solid fractions for rumen samples) on microbial diversity using MIXED procedure of SAS (SAS, 2011) fitted in a repeated measure design where the effect of individual steers were included in the model as a random factor. All pairwise comparisons among the groups were tested using a Tukey studentized range adjustment. The differences between groups were considered significant at $p < 0.05$, with trends discussed at $p < 0.10$.

## 5.4 Results

### 5.4.1 Effects of Treatment on Microbial Richness and Diversity

Illumina paired-end sequencing of the V4 region of 16S rRNA gene generated on average 35,007 (n=35, SD= 6331), 37,501 (n=34, SD= 7490) and 37,823 (n=35, SD= 12,400 high quality sequences for rumen liquid, solid and fecal samples, respectively. A summary of richness and diversity indices of rumen and fecal microbial communities are given in Table 1 and Table 2, respectively.

**Table 1. Summary statistics for diversity indices observed in rumen microbial communities of steers receiving *Propionibacterium acidipropionici* P169.**

| Site of sampling | Treatment | Mean results for indicated variable | | | | |
|---|---|---|---|---|---|---|
| | | Observed OTUs | Goods-Coverage (%) | Richness[1] Chao1 | Diversity[2] Shannon | Diversity[2] Simpson |
| Liquid | Control | 1477.00 | 96.6 | 2125.87 | 6.359 | 0.948 |
| | P169 | 1442.15 | 96.8 | 2060.25 | 6.361 | 0.938 |
| Solid | Control | 1491.43 | 96.6 | 2233.91 | 6.538 | 0.953 |
| | P169 | 1614.35 | 96.5 | 2326.90 | 6.586 | 0.940 |
| SED[3] | | 176.65 | 0.003 | 268.5 | 0.394 | 0.021 |
| *p* -value | Treatment | 0.830 | 0.836 | 0.967 | 0.942 | 0.539 |
| | Site of sampling | 0.257 | 0.344 | 0.112 | 0.249 | 0.782 |

[1] Based on Chao1 estimator of species richness.
[2] Based on Shannon and Simpson diversity estimators
[3]SED = standard error of difference between treatments.

**Table 2. Summary statistics for diversity indices observed in fecal microbial communities of steers receiving Propionibacterium acidipropionici P169.**

| | Mean results for indicated variable | | | | |
|---|---|---|---|---|---|
| | Observed | Goods-Coverage (%) | Richness[1] | Diversity[2] | |
| Treatment | OTUs | | Chao1 | Shannon | Simpson |
| Control | 1975.75 | 96.9 | 2553.40 | 7.679 | 0.977 |
| P169 | 1947.86 | 96.7 | 2656.04 | 7.637 | 0.977 |
| SED[3] | 40.68 | 0.004 | 341.91 | 0.288 | 0.005 |
| *P*-value | 0.896 | 0.703 | 0.767 | 0.884 | 0.958 |

[1] Based on Chao1 estimator of species richness.
[2] Based on Shannon and Simpson diversity estimators
[3]SED = standard error of difference between treatments.

Estimation of Good's coverage for both rumen and fecal samples were calculated to be above 96%. The Chao1 estimates of species richness for all samples started to plateau at approximately 18,000 sequences per sample (Figure 1), indicating sufficient sampling depth and sequence coverage. All Chao1, Shannon and Simpson indices were similar between control and P169 treatments in rumen liquid, solids and fecal samples.

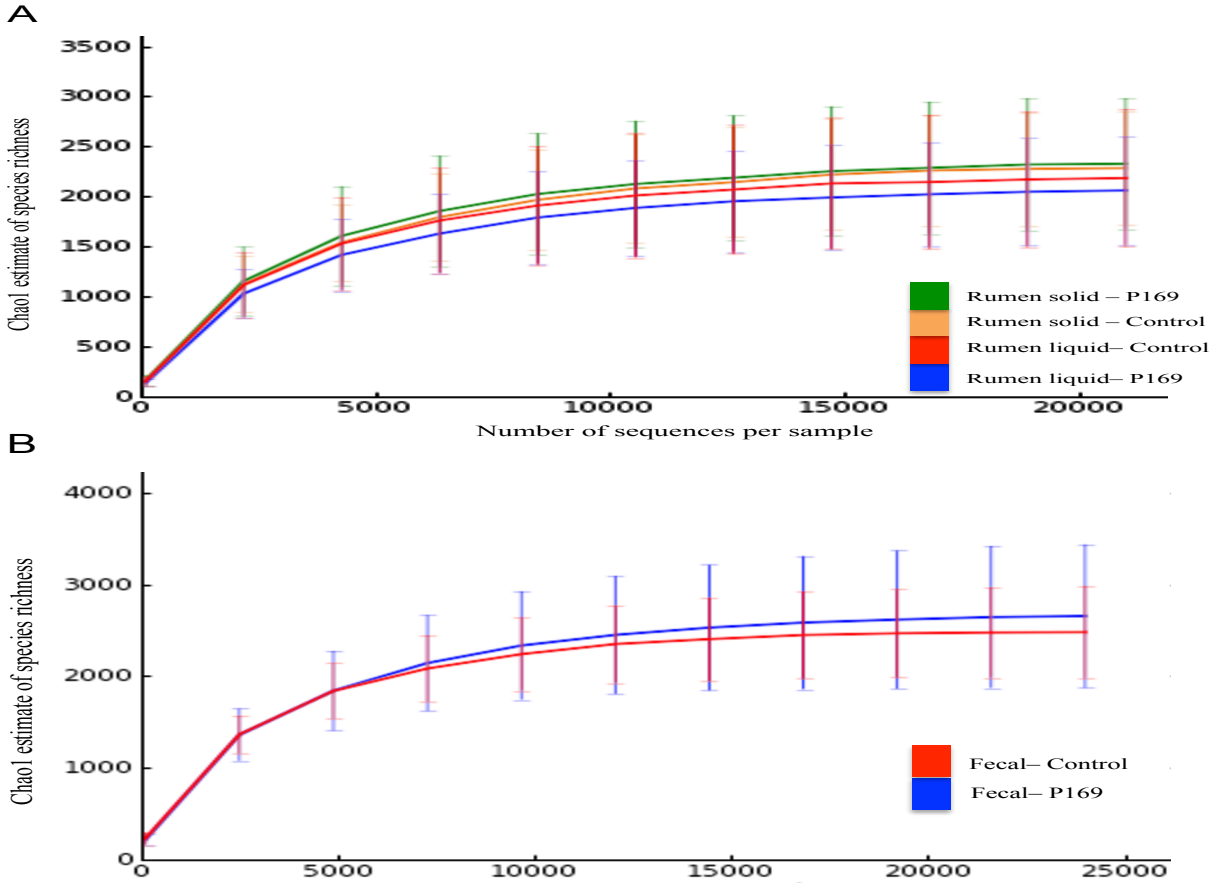


**Figure 1. Rarefaction analysis based on Chao1 estimator of species richness.**

A) Effect of probiotic supplementation (P169) on species richness of liquid and solid rumen fractions (rarefied at an even depth of 21,000 sequences per sample), B) Effect of probiotic supplementation (P169) on the species richness of fecal microbial communities rarefied at an even depth of 24,000 sequences per sample. Error bars indicate the 95% confidence intervals.

β-diversity, which was calculated using weighted UniFrac distances, was also similar between rumen microbiota of control and P169 groups ($p$ =0.18). However, a trend was observed when unweighted UniFrac distances were employed ($p$ =0.08; Figure 2, A and B). The microbial communities of the liquid and solid fractions of rumen contents clustered distinctly ($p$ =0.03) based on weighted UniFrac analyses, and a trend in clustering pattern was observed ($p$ =0.08) when unweighted UniFrac was employed. Inclusion of P169 in the diet did not affect the fecal microbial profiles (Figure 2, C and D).

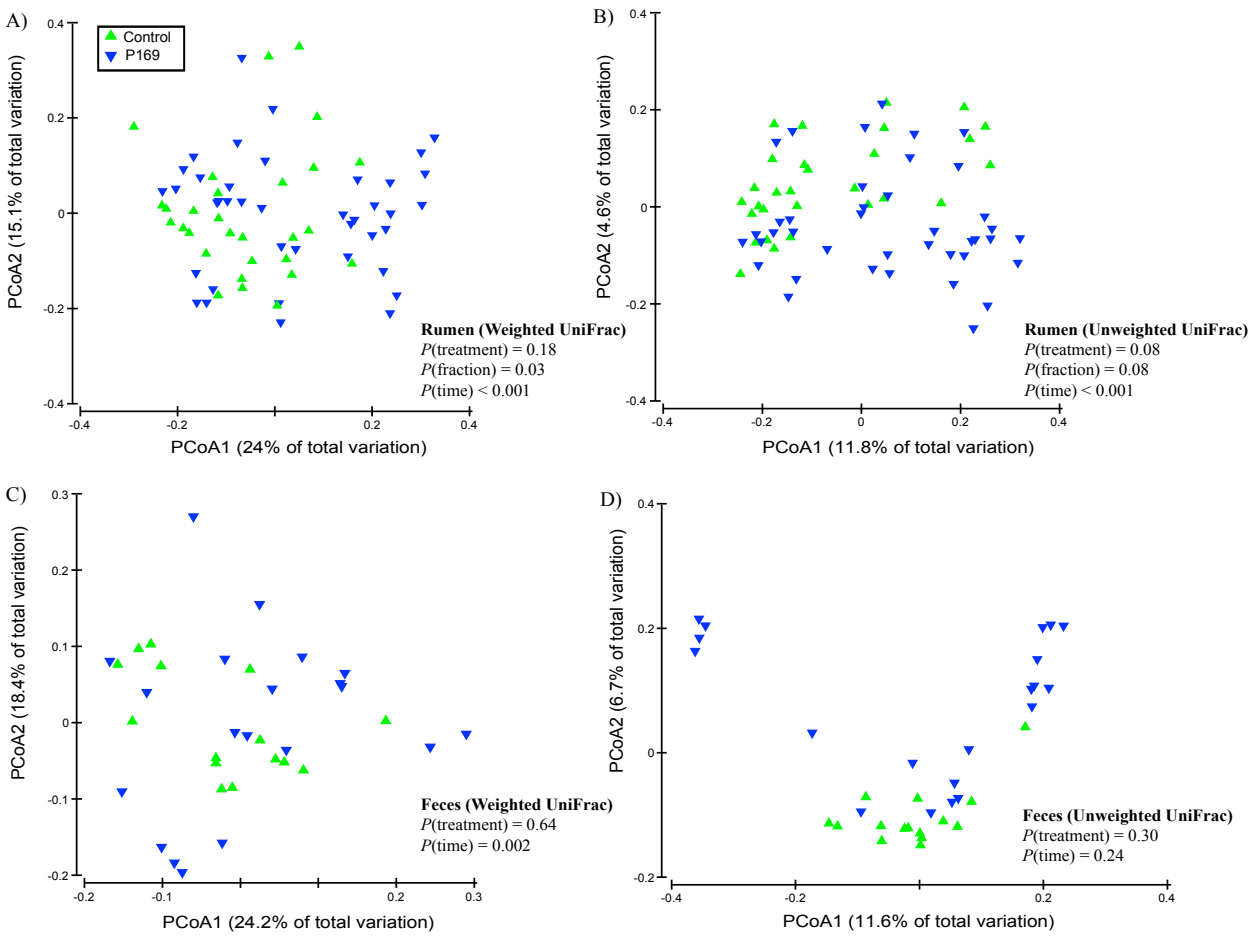



**Figure 2. Principal coordinates analysis (PCoA). Comparing the dissimilarities between microbial communities of P-169 supplementation versus control diet**

A) weighted UniFrac distances of rumen microbial communities B) uweighted UniFrac distances of rumen microbial communities C) weighted UniFrac distances of fecal microbial communities, and D) unweighted UniFrac distances of fecal microbial communities. *p* -value for each comparison was obtained from PERMANOVA and considered significant at $p < 0.05$.

For both rumen and fecal microbiota, PERMANOVA analysis of UniFrac distances revealed significant ($p < 0.05$) impact of time of sampling on the profile of microbial communities (Figure 3 a-c). However, the interactions between the impact of

treatment and time of sampling on the composition of microbiota were not significant in either sampling site.

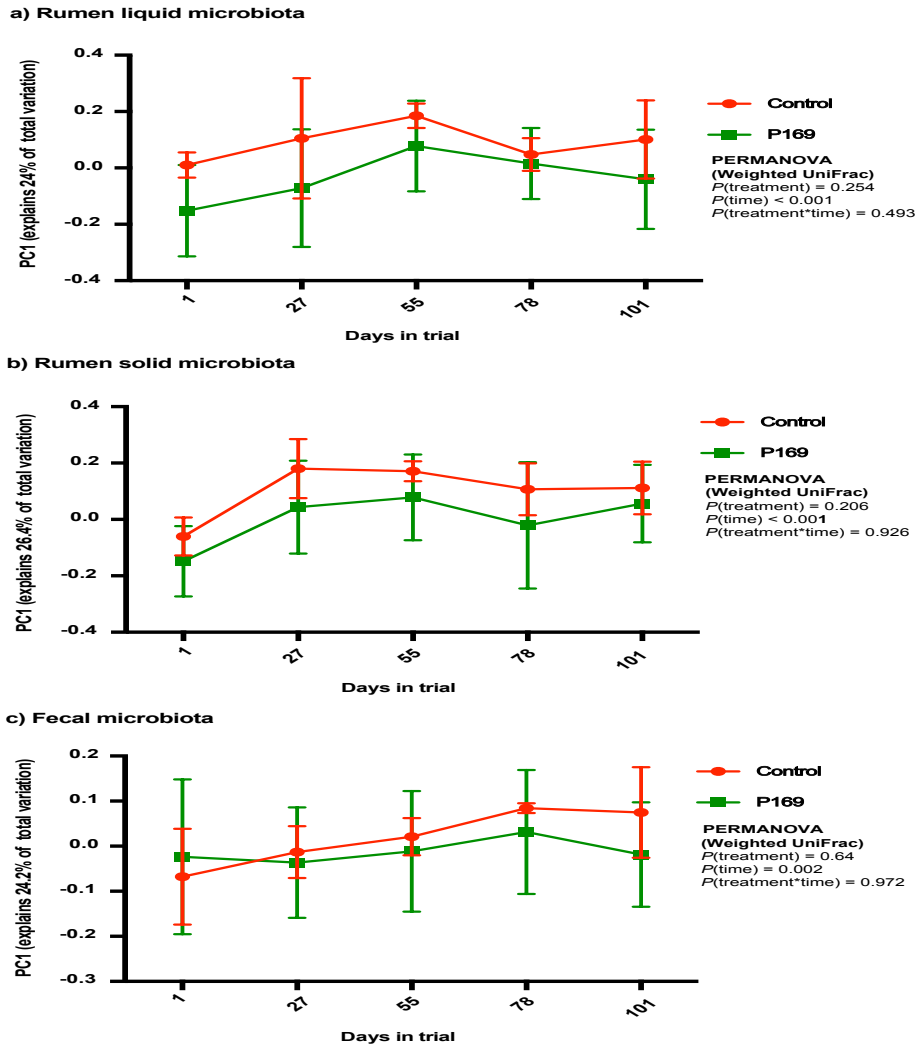



**Figure 3. Compositional dynamics of rumen and hindgut microbial communities overtime.**

Principal coordinate analyses (PCoA) of weighted UniFrac distances of a) rumen liquid, b) rumen solid, and c) fecal microbial communities have been projected over sampling time points. The Y-axis shows the differences between microbial communities as explained by first component (PC1) of the PCoA analysis. The X-axis indicates the sampling time points relative to the starting date of the experiment. Color codes have been used to differentiate between control (red) and P169 (green) groups. Error bars

indicate the 95% confidence interval. $p$ -value for each comparison was obtained from PERMANOVA and considered significant at $p < 0.05$.

### 5.4.2 Determining the Core Microbiota of Rumen and Hindgut

Operational taxonomic unit alignment at 97% similarity threshold resulted in identification of 21,316, 21,277, and 21,679 unique OTUs in rumen liquid, rumen solid, and fecal samples obtained from all steers across both treatment and control groups. The core microbiota was described as those representative OTUs that were present in 100% of the samples. As such, the core microbiota of rumen liquid and solid fractions consisted of 22 and 23 unique OTUs, accounting for 22% and 27% of the total non-singleton OTUs, respectively. Taxonomic classification of these OTUs identified Bacteroidetes, Firmicutes, Proteobacteria and Cyanobacteria as the core rumen bacterial phyla (Figure 4 a and b). On the other hand, the core microbiota of hindgut consisted of 22 unique OTUs, accounting for 25% of the total non-singleton OTUs, which all belonged to the phylum Firmicutes (Figure 4c). 21 out of 22 core OTUs identified in the liquid fraction of rumen content were shared with the core OTUs of the solid fraction (Figure 4d). Most abundant core OTUs of the rumen liquid were those belonging to genera *Butyrivibrio*, unclassified Ruminococcaceae, unclassified S24-7, *Desulfovibrio*, and *Bulleidia*. Predominant OTUs that were specifically core in rumen solid fraction included those belonging to genera *Sharpea*, *Prevotella*, and *Phascolarctobacterium*. In contrast, few commonalities were observed between the core microbiota of hindgut and rumen. Unclassified Coriobacteriaceae and Lachnospiraceae were the only OTUs that were common between the two ecosystems. The most abundant OTUs that were specifically core in fecal samples included unclassified members of Clostridiaceae and Peptostreptococcaceae, as well as genera *Turicibacter*, *Ruminococcus*, and *Clostridium*.

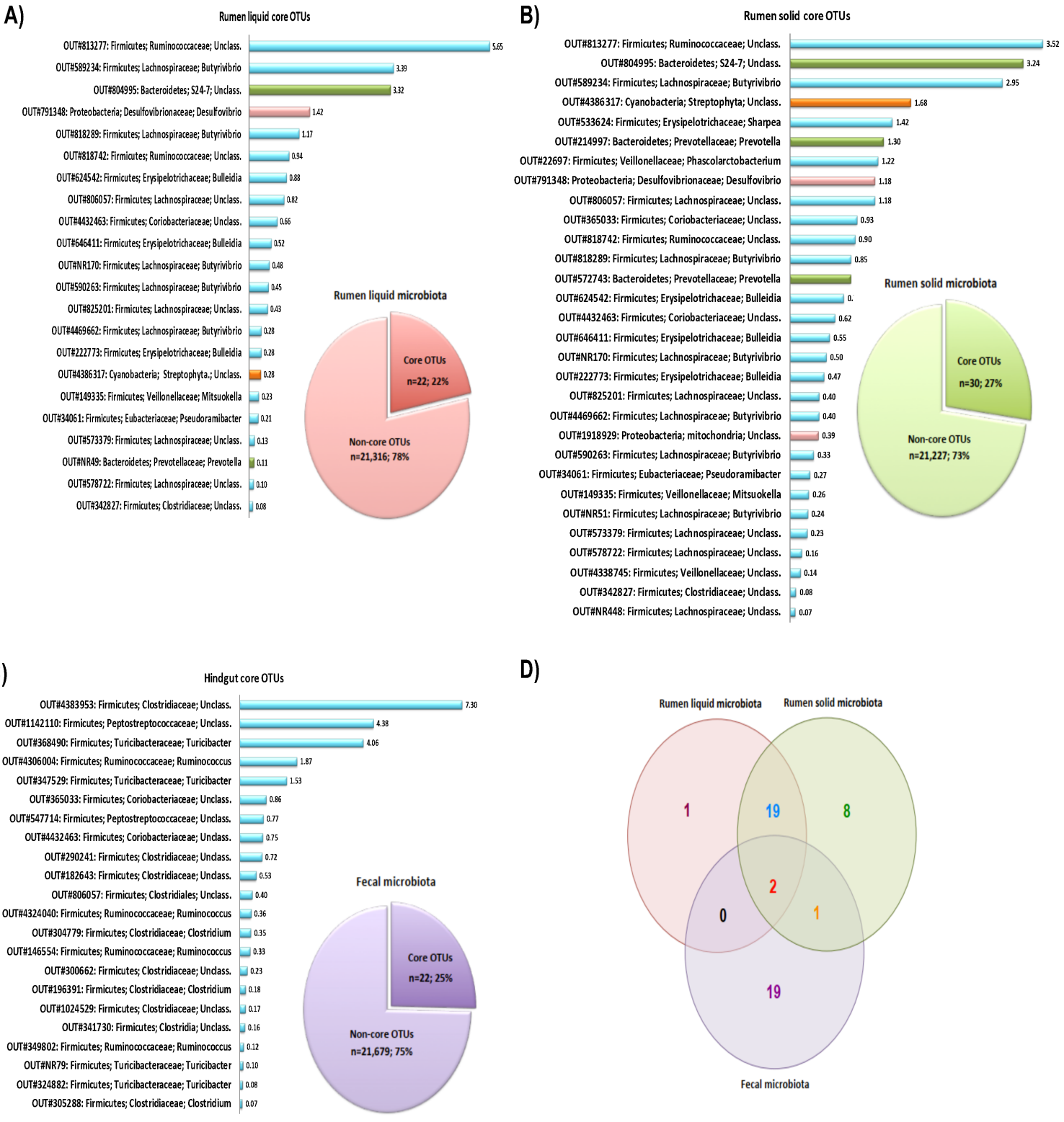

A)
Rumen liquid core OTUs
OUT#813277: Firmicutes; Ruminococcaceae; Unclass. 5.65
OUT#589234: Firmicutes; Lachnospiraceae; Butyrivibrio 3.39
OUT#804995: Bacteroidetes; S24-7; Unclass. 3.32
OUT#791348: Proteobacteria; Desulfovibrionaceae; Desulfovibrio 1.42
OUT#818289: Firmicutes; Lachnospiraceae; Butyrivibrio 1.17
OUT#818742: Firmicutes; Ruminococcaceae; Unclass. 0.94
OUT#624542: Firmicutes; Erysipelotrichaceae; Bulleidia 0.88
OUT#806057: Firmicutes; Lachnospiraceae; Unclass. 0.82
OUT#4432463: Firmicutes; Coriobacteriaceae; Unclass. 0.66
OUT#646411: Firmicutes; Erysipelotrichaceae; Bulleidia 0.52
OUT#NR170: Firmicutes; Lachnospiraceae; Butyrivibrio 0.48
OUT#590263: Firmicutes; Lachnospiraceae; Butyrivibrio 0.45
OUT#825201: Firmicutes; Lachnospiraceae; Unclass. 0.43
OUT#4469662: Firmicutes; Lachnospiraceae; Butyrivibrio 0.28
OUT#222773: Firmicutes; Erysipelotrichaceae; Bulleidia 0.28
OUT#4386317: Cyanobacteria; Streptophyta.; Unclass. 0.28
OUT#149335: Firmicutes; Veillonellaceae; Mitsuokella 0.23
OUT#34061: Firmicutes; Eubacteriaceae; Pseudoramibacter 0.21
OUT#573379: Firmicutes; Lachnospiraceae; Unclass. 0.13
OUT#NR49: Bacteroidetes; Prevotellaceae; Prevotella 0.11
OUT#578722: Firmicutes; Lachnospiraceae; Unclass. 0.10
OUT#342827: Firmicutes; Clostridiaceae; Unclass. 0.08
Rumen liquid microbiota
Core OTUs n=22; 22%
Non-core OTUs n=21,316; 78%
B)
Rumen solid core OTUs
OUT#813277: Firmicutes; Ruminococcaceae; Unclass. 3.52
OUT#804995: Bacteroidetes; S24-7; Unclass. 3.24
OUT#589234: Firmicutes; Lachnospiraceae; Butyrivibrio 2.95
OUT#4386317: Cyanobacteria; Streptophyta; Unclass. 1.68
OUT#533624: Firmicutes; Erysipelotrichaceae; Sharpea 1.42
OUT#214997: Bacteroidetes; Prevotellaceae; Prevotella 1.30
OUT#22697: Firmicutes; Veillonellaceae; Phascolarctobacterium 1.22
OUT#791348: Proteobacteria; Desulfovibrionaceae; Desulfovibrio 1.18
OUT#806057: Firmicutes; Lachnospiraceae; Unclass. 1.18
OUT#365033: Firmicutes; Coriobacteriaceae; Unclass. 0.93
OUT#818742: Firmicutes; Ruminococcaceae; Unclass. 0.90
OUT#818289: Firmicutes; Lachnospiraceae; Butyrivibrio 0.85
OUT#572743: Bacteroidetes; Prevotellaceae; Prevotella
OUT#624542: Firmicutes; Erysipelotrichaceae; Bulleidia 0.
OUT#4432463: Firmicutes; Coriobacteriaceae; Unclass. 0.62
OUT#646411: Firmicutes; Erysipelotrichaceae; Bulleidia 0.55
OUT#NR170: Firmicutes; Lachnospiraceae; Butyrivibrio 0.50
OUT#222773: Firmicutes; Erysipelotrichaceae; Bulleidia 0.47
OUT#825201: Firmicutes; Lachnospiraceae; Unclass. 0.40
OUT#4469662: Firmicutes; Lachnospiraceae; Butyrivibrio 0.40
OUT#1918929: Proteobacteria; mitochondria; Unclass. 0.39
OUT#590263: Firmicutes; Lachnospiraceae; Butyrivibrio 0.33
OUT#34061: Firmicutes; Eubacteriaceae; Pseudoramibacter 0.27
OUT#149335: Firmicutes; Veillonellaceae; Mitsuokella 0.26
OUT#NR51: Firmicutes; Lachnospiraceae; Butyrivibrio 0.24
OUT#573379: Firmicutes; Lachnospiraceae; Unclass. 0.23
OUT#578722: Firmicutes; Lachnospiraceae; Unclass. 0.16
OUT#4338745: Firmicutes; Veillonellaceae; Unclass. 0.14
OUT#342827: Firmicutes; Clostridiaceae; Unclass. 0.08
OUT#NR448: Firmicutes; Lachnospiraceae; Unclass. 0.07
Rumen solid microbiota
Core OTUs n=30; 27%
Non-core OTUs n=21,227; 73%
C)
Hindgut core OTUs
OUT#4383953: Firmicutes; Clostridiaceae; Unclass. 7.30
OUT#1142110: Firmicutes; Peptostreptococcaceae; Unclass. 4.38
OUT#368490: Firmicutes; Turicibacteraceae; Turicibacter 4.06
OUT#4306004: Firmicutes; Ruminococcaceae; Ruminococcus 1.87
OUT#347529: Firmicutes; Turicibacteraceae; Turicibacter 1.53
OUT#365033: Firmicutes; Coriobacteriaceae; Unclass. 0.86
OUT#547714: Firmicutes; Peptostreptococcaceae; Unclass. 0.77
OUT#4432463: Firmicutes; Coriobacteriaceae; Unclass. 0.75
OUT#290241: Firmicutes; Clostridiaceae; Unclass. 0.72
OUT#182643: Firmicutes; Clostridiaceae; Unclass. 0.53
OUT#806057: Firmicutes; Clostridiales; Unclass. 0.40
OUT#4324040: Firmicutes; Ruminococcaceae; Ruminococcus 0.36
OUT#304779: Firmicutes; Clostridiaceae; Clostridium 0.35
OUT#146554: Firmicutes; Ruminococcaceae; Ruminococcus 0.33
OUT#300662: Firmicutes; Clostridiaceae; Unclass. 0.23
OUT#196391: Firmicutes; Clostridiaceae; Clostridium 0.18
OUT#1024529: Firmicutes; Clostridiaceae; Unclass. 0.17
OUT#341730: Firmicutes; Clostridia; Unclass. 0.16
OUT#349802: Firmicutes; Ruminococcaceae; Ruminococcus 0.12
OUT#NR79: Firmicutes; Turicibacteraceae; Turicibacter 0.10
OUT#324882: Firmicutes; Turicibacteraceae; Turicibacter 0.08
OUT#305288: Firmicutes; Clostridiaceae; Clostridium 0.07
Fecal microbiota
Core OTUs n=22; 25%
Non-core OTUs n=21,679; 75%
D)
Rumen liquid microbiota
Rumen solid microbiota
1
19
8
2
0
1
19
Fecal microbiota

**Figure 4. Identification of the core microbiota of rumen and hindgut.**

The core microbiome of rumen liquid (a), rumen solid (b), and fecal (c) microbiota were defined as those representative OTUs that were present in 100% of the samples. Taxonomic assignment was performed at 97% similarity threshold against Greengenes database (OTU IDs are identical to OTU identifiers of the Greengenes database with the exception of those remarked by "NR" which are novel OTUs identified by de novo OTU picking strategy and do not have matching representatives in the Greengenes database). VENN analysis (d) was performed to identify the similarities between the core OTUs of different sampling sites.

### 5.4.3 P169 quantification and composition of bacterial phyla

The qPCR quantification of P169 in the rumen digesta revealed a significant increase in the gene copy numbers of this bacterium in supplemented as compared to control steers ($p$ = 0.03; Figure 5). Taxonomic classification of rumen microbial communities revealed the presence of 25 bacterial phyla across all samples.

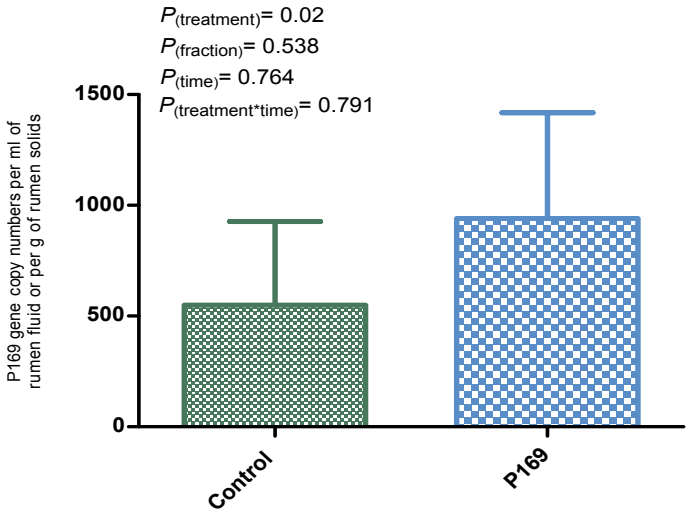



**Figure 5. q-PCR quantification of *Propionibacterium acidipropionici* P169.**

Absolute quantification (gene copy numbers) of P169 in rumen digesta of supplemented versus control group. The effect of treatment (control vs. P169) and fraction (liquid vs. solid fractions for rumen samples) on the abundance of P169 was evaluated using MIXED procedure of SAS (SAS, 2011) fitted in a repeated measure design. The effect of steer was treated as random factor. P-value for each comparison was considered significant at $p < 0.05$.

Table 3 demonstrates the relative abundances of the high (above 1% of community) and low (between 0.1 and 1% of community) abundant bacterial phyla. High abundant phyla included Firmicutes, Bacteroidetes, Tenericutes, Actinobacteria, Cyanobacteria and Proteobacteria whereas the low abundant phyla consisted of Fibrobacteres, Spirochaetes, Synergistetes and Verrucomicrobia.

**Table 3. Comparison of the proportion of the high (above 1% of community) and low (between 0.1 and 1% of community) abundant bacterial phyla between the rumen microbiota of the steers receiving *Propionibacterium acidipropionici* P169 and control group.**

| phylum | Percentage of sequences in: | | | | SED[1] | *p* -value | | |
|---|---|---|---|---|---|---|---|---|
| | Liquid | | Solid | | | Treatment | Fraction | Trt*Fraction |
| | Control | P169 | Control | P169 | | | | |
| Above 1% of population | | | | | | | | |
| Actinobacteria | 3.580 | 2.817 | 3.940 | 2.930 | 1.429 | 0.922* | 0.119* | 0.597* |
| Bacteroidetes | 27.728 | 25.373 | 22.425 | 21.283 | 4.071 | 0.668 | 0.033 | 0.778 |
| Cyanobacteria | 0.255 | 0.463 | 2.603 | 1.693 | 0.490 | 0.710* | <0.001* | 0.042* |
| Firmicutes | 54.589 | 56.831 | 59.192 | 59.283 | 8.633 | 0.888 | 0.141 | 0.651 |
| Proteobacteria | 10.070 | 10.319 | 8.457 | 10.515 | 5.137 | 0.670* | 0.876* | 0.691* |
| Tenericutes | 1.595 | 2.127 | 1.791 | 2.152 | 0.861 | 0.237* | 0.621* | 0.858* |
| Between 1% and 0.01% of population | | | | | | | | |
| Fibrobacteres | 0.025 | 0.044 | 0.056 | 0.194 | 0.125 | 0.683* | 0.083* | 0.624* |
| Spirochaetes | 0.664 | 0.279 | 0.229 | 0.344 | 0.374 | 0.190* | 0.544* | 0.979* |
| Synergistetes | 0.037 | 0.158 | 0.019 | 0.074 | 0.094 | 0.083* | 0.106* | 0.442* |
| Verrucomicrobia | 0.008 | 0.050 | 0.001 | 0.040 | 0.023 | 0.873** | 0.974** | 0.994** |

All pairwise comparisons among the groups were tested using a Tukey studentized range adjustment test.

*Statistical analyses were conducted on $\log_{10}$-transformed data following MIXED procedure of SAS.

** *p*-value obtained following GLIMMIX procedure of SAS.

[1]SED = standard error of difference between least squares means of treatments.

Similarly, taxonomic classification of fecal microbial communities revealed the presence of 17 bacterial phyla. In fecal samples, the most abundant phyla were Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria, Spirochaetes and Tenericutes, with Cyanobacteria, Elusimicrobia, Fibrobacteres, Synergistetes and Verrucomicrobia present in low abundance (Table 4).

**Table 4. Comparison of the proportion of high (above 1% of community) and low (between 0.1 and 1% of community) abundant bacterial phyla between the fecal microbiota of steers receiving *Propionibacterium acidipropionici* P169 and control group.**

| Phylum | Percentage of sequences in | | | |
|---|---|---|---|---|
| | Control | P169 | SED[1] | *p*-Value |
| | Above 1% of population | | | |
| Actinobacteria | 4.851 | 3.594 | 1.053 | 0.246 |
| Bacteroidetes | 15.335 | 15.412 | 1.468 | 0.958 |
| Firmicutes | 74.804 | 74.755 | 2.136 | 0.982 |
| Proteobacteria | 0.992 | 2.033 | 0.913 | 0.779* |
| Spirochaetes | 0.952 | 1.497 | 0.987 | 0.587 |
| Tenericutes | 2.300 | 1.811 | 0.855 | 0.574 |
| | Between 1% and 0.01% of population | | | |
| Cyanobacteria | 0.044 | 0.079 | 0.035 | 0.656* |
| Elusimicrobia | 0.000 | 0.001 | 0.002 | 0.408 |
| Fibrobacteres | 0.033 | 0.005 | 0.020 | 0.937** |
| Synergistetes | 0.0008 | 0.002 | 0.001 | 0.997** |
| Verrucomicrobia | 0.002 | 0.111 | 0.130 | 0.404 |

All pairwise comparisons among the groups were tested using a Tukey studentized range adjustment test

*Statistical analyses were conducted on $\log_{10}$-transformed data following MIXED procedure of SAS.

** *p*-value obtained following GLIMMIX procedure of SAS.

[1]SED = standard error of difference between least squares means of treatments.

### 5.4.4 Effects of P169 on Microbiota Composition

Table 3 provides a summary of statistic of relative abundances of bacterial phyla in the rumen liquid and solid fractions of control and P169 steers. P169 did not affect the proportion of abundant phyla in the community. Among the low-abundance phyla, P169 only tended to increase ($p$ =0.08) the proportion of Synergistetes. Among abundant phyla, the proportion of Bacteroidetes was higher ($p$ =0.03) in the liquid fraction of the rumen content whereas Cyanobacteria was enriched ($p$ <0.001) in the solid fraction. In low-abundance phyla, the proportion of Fibrobacteres tended to be higher ($p$ =0.08) in the solid fraction as compared to the liquid fraction of rumen contents.

Phylogenetic comparisons at the genus level revealed several alterations in response to P169; including a significant increase ($p < 0.05$) in the proportion of *Phascolarctobacterium*, unclassified members of Clostridiaceae and Lachnospiraceae and tendency for increases in the proportions of unclassified members of Ruminococcaceae ($p = 0.07$) and Christensenllaceae ($p = 0.09$). P169 decreased ($p < 0.05$) the proportion of *Prevotella*, *Succinivibrio*, *YRC22,* and unclassified members of Veillonellaceae in rumen contents.

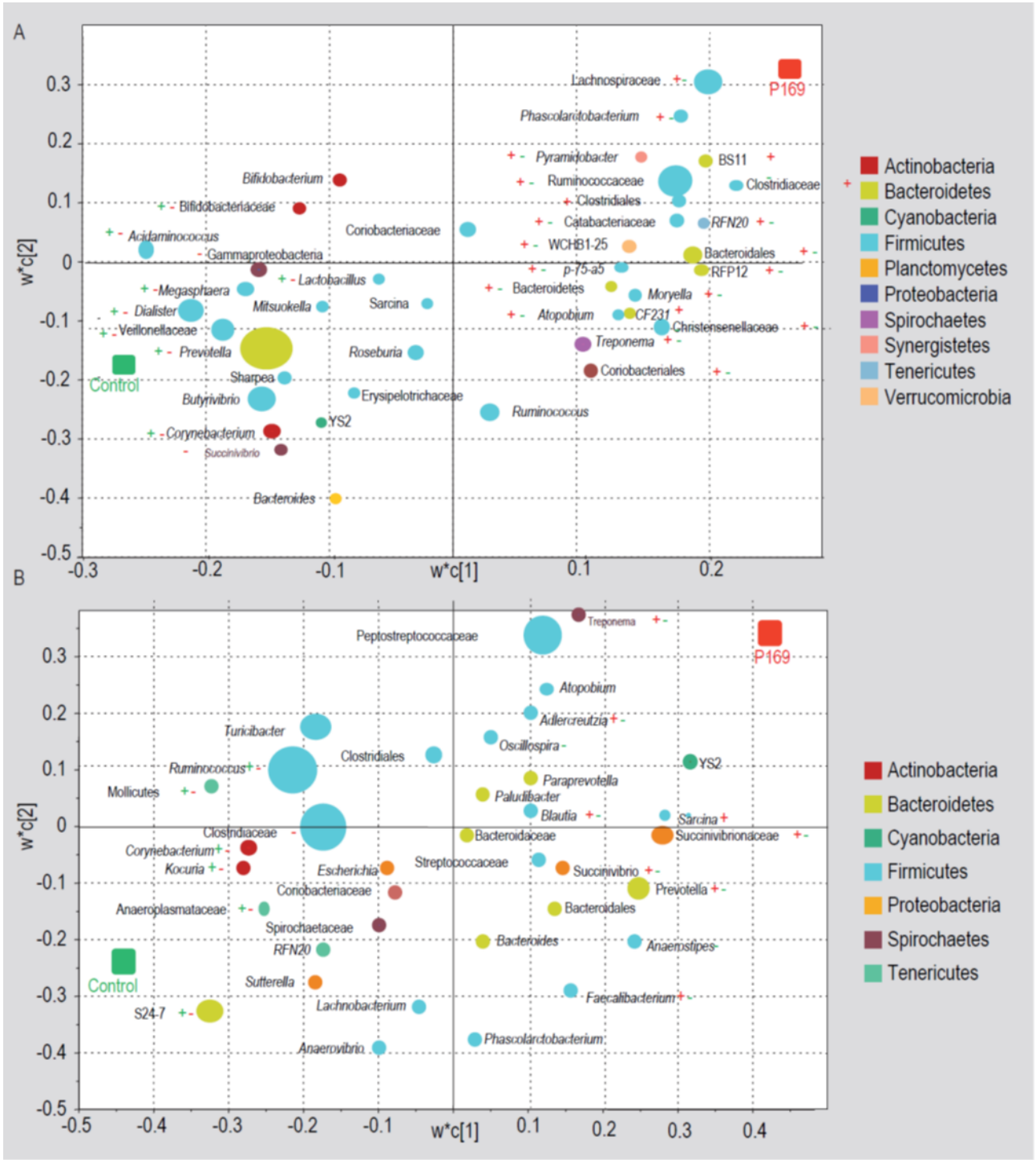
At the phyla level, relative abundances of the bacterial communities in the feces of steers were not affected by treatment (Table 4). However, some effects were noted at the genus level; for example within Firmicutes, genus *Sarcina* tended to increase ($p$ =0.08) in the feces of steers supplemented with P169. Additionally, the relative abundances of genus *Anaerovibrio* decreased ($p$ =0.03) and there was a tendency for a reduction in genus *Ruminococcus* ($p$ =0.07) in steers supplemented with P169. Within Bacteroidetes, relative abundance of members of the S24-7 family also tended to decline ($p$ =0.06) in the P169 group. Among the Spirochaetes, relative abundance of genus *Treponema* increased ($p$ =0.04).

Figure 6a presents the PLS-DA loading plots based on the relative abundances of bacterial genera in the rumen liquid and solid and their association with treatment groups. PLS-DA supported the above mentioned results obtained from ANOVA and additionally

revealed negative correlations between P169 and the ruminal proportions of genera *Megasphaera, Lactobacillus,* and *Dialister.*

Figure 6b represents the PLS-DA loading plots based on the relative abundance of bacterial genera in fecal microbiota. There was a positive association between some members of the Firmicutes (genera *Adlercreutzia*, *Faecalibacterium*, *Blautia* and *Sarcina)*, Proteobacteria (genus *Succinivibrio*), Bacteroidetes (genus *Prevotella)* and Spirochaetes (genus *Treponema*) and supplementation with P169, whereas several members of Firmicutes (genus *Ruminococcus),* Tenericutes (genus *Mollicutes* and family Anaeroplasmataceae), Actinobacteria (*Corynebacterium* and *Kocuria)* and Bacteroidetes **(**S24-7) were inversely associated with P169.

Figure 6c presents PLS-DA comparison between liquid and solid fractions of rumen contents and revealed a positive association of the members of families Erysipelotrichaecea and Coriobacteriaceae with the solid fraction while *WCHB1-25, BF311*, *Bacteroides*, *BS11*, Peptostreptococcaceae and *Turicibacter* were positively associated with the liquid fraction of rumen contents.


A
B
w*c[1]
w*c[2]
Control
P169
Actinobacteria
Bacteroidetes
Cyanobacteria
Firmicutes
Planctomycetes
Proteobacteria
Spirochaetes
Synergistetes
Tenericutes
Verrucomicrobia
Lachnospiraceae
Phascolarctobacterium
Pyramidobacter
BS11
Ruminococcaceae
Clostridiaceae
Clostridiales
Catabacteriaceae
RFN20
WCHB1-25
Bacteroidales
p-75-a5
RFP12
Bacteroidetes
Moryella
Atopobium
CF231
Christensenellaceae
Treponema
Coriobacteriales
Ruminococcus
Bifidobacterium
Bifidobacteriaceae
Acidaminococcus
Coriobacteriaceae
Gammaproteobacteria
Lactobacillus
Megasphaera
Mitsuokella
Sarcina
Dialister
Veillonellaceae
Prevotella
Roseburia
Sharpea
Butyrivibrio
Erysipelotrichaceae
YS2
Corynebacterium
Succinivibrio
Bacteroides
Peptostreptococcaceae
Treponema
Atopobium
Adlercreutzia
Turicibacter
Oscillospira
Clostridiales
Ruminococcus
YS2
Mollicutes
Paraprevotella
Paludibacter
Blautia
Sarcina
Clostridiaceae
Bacteroidaceae
Succinivibrionaceae
Corynebacterium
Kocuria
Escherichia
Streptococcaceae
Succinivibrio
Coriobacteniaceae
Prevotella
Anaeroplasmataceae
Bacteroidales
Spirochaetaceae
RFN20
Bacteroides
Anaerostipes
Sutterella
Faecalibacterium
S24-7
Lachnobacterium
Phascolarctobacterium
Anaerovibrio

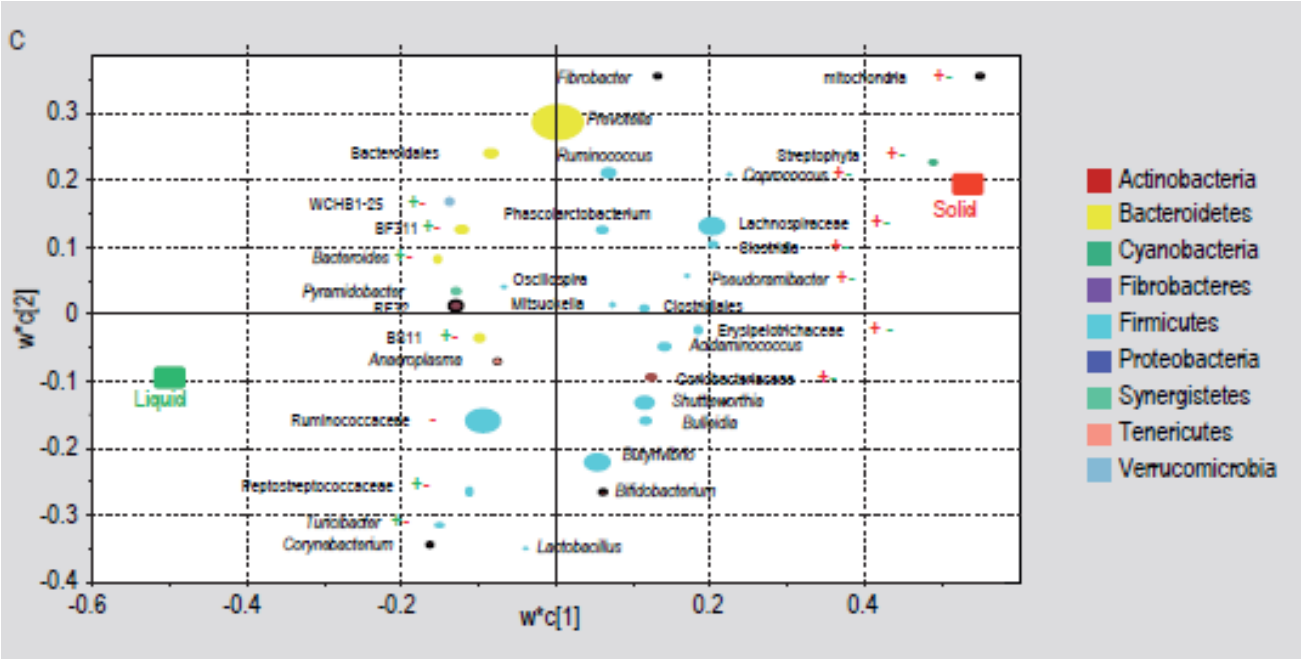



**Figure 6. Partial least square (PLS) discriminant analysis of rumen microbiota.**

The PLS-DA was performed on microbial communities based on the relative abundances of bacterial genera and their association with dietary treatments in (A) rumen and (B) faecal microbiota, or (C) with different fractions of rumen content. The genera are coloured according to their corresponding phyla. The size of the symbols (circles) is indicative of taxa abundance. Taxa closer to either the control or Propionibacterium acidipropionici P169 treatment indicate positive association with the relevant experimental treatments. The (+) or (-) indicate taxa that had positive or negative correlation to each treatment group. The goodness of fit of the model was evaluated with R2 and the predictive value of the model was evaluated using the Q2 estimate (A: R2 (0.83) and Q2 (0.54); B: R2 (0.86) and Q2 (0.57); and C: R2 (0.81) and Q2 (0.58)). Estimates were calculated based on inclusion of three PLS components in the model. Some sequences could only be affiliated to phylum (p), order (o), or family (f) levels.

## 5.5 Discussion

The current study evaluated the efficacy of the DFM supplementation of *Propionibacterium acidipropionici* strain P169 in modulating the rumen microbiota of beef cattle fed high concentrate corn-based diets. The ability of P169 to directly impact rumen fermentation profile has been attributed to its metabolic potential in converting lactate and glucose to propionic and acetic acid [187]. As compared to other *Propionibacterium* spp., P169 has been found to have higher affinity for utilizing lactate and synthesis of propionate [188, 189]. The mode of action of most DFM products on ruminal fermentation and animal performance is species-specific and dose-dependent [177], requiring successful integration and persistent of the target strain within the rumen microbial ecosystem [190].

Traditionally, propionibacteria have been described as natural (autochthonous) inhabitants of the rumen ecosystem (Oshio *et al.,* 1988). In the present study, in addition to the P169-supplemented steers, the rumen content of control animals also contained low abundances of P169, suggesting that this bacterium may be native to the rumen ecosystem. Generally, autochthonous microbes of rumen ecosystem are well adapted to the harsh environment of rumen, and therefore, compared to non-native (allochthonous) bacteria, are more competent at utilizing limited nutrient resources and establishing active populations within the rumen ecosystem. However, insights from previous ruminal dosing studies suggest that the ability of P169 to alter ruminal VFAs profile relies largely on its supplementation dose; indicating that, on its own, this bacterium is not competent enough to overgrow other autochthonous lactate utilizer of the rumen. While some production studies have demonstrated the ability of P169 supplementation ($6 \times 10^{11}$ cfu) to significantly increase the molar proportion of propionate in the rumen fluid of lactating dairy cows [163, 164] and steers [191], others have failed to show such impact when P169 was administered at a lower dose ($5 \times 10^{9}$ cfu; [192]).

### 5.5.1 Dynamics of rumen and hindgut microbiota in response to P169-supplementation

As reported by Narvaez et al (2014), P169 supplementation did not impact the ruminal propionate concentration of the steers in the present study (Supplementary Table 3). It has been suggested that P169 preferentially converts lactate to propionate as opposed to glucose to propionate [189]. It is possible that the lack of a direct impact of P169 on molar proportion of propionate in this study may be attributed to the gradual adaptation of steers to high-grain diet, resulting in low ruminal level of lactic acid in control (= 0.184mM) and P169-supplemented (= 0.376mM) groups, which were far below the typical levels measured during acidotic challenge studies (> 5mM; [84]. Notwithstanding, the ruminal content of P169-supplemented steers had elevated levels of NH3-N and increased molar proportions of butyrate and branched-chain fatty acids (BCVFAs) as opposed to the control group, which showed higher molar proportions of acetate (Supplementary Table 3). These observations are in general agreement with the findings of Ghorbani et al. [193] who also reported increased concentrations of NH3-N and butyrate in the rumen content of steers fed high-grain diet and supplemented with *Propionibacterium* strain (P15); suggesting that at low ruminal level of lactate, *Propionibacterium* strains may have higher tendency to convert acetate to butyrate.

The trophic network of rumen ecosystem is complex and the mechanisms by which DFM products alter the cross-feeding patterns of rumen microbiota are poorly understood. Following adaptation to high-concentrate diets, even if ruminal pH is within the normal range, amylolytic bacteria predominate the rumen ecosystem at the expense of a reduction in cellulolytic bacteria. It has been hypothesized that reduced fibre degradation can limit the access of proteolytic bacteria to proteins and indirectly diminishing protein degradation [194]. The products of protein degradation, BCVFAs and ammonia, are important growth factors for cellulolytic bacteria [194], optimum fermentation of plant cell walls and digestibility of organic matter [195]. In the present study, concomitant with elevated ruminal levels of BCVFAs and ammonia, the proportion of several cellulolytic bacterial lineages such as Ruminococcaceae, Lachnospiraceae, Clostridiaceae, and Christensenellaceae were enriched in the rumen

microbiota of P169-supplemented steers, suggesting an improved state of fibrolytic and proteolictic activity in response to P169 supplementation. Results from transition dairy studies [196-198] also suggest that the proportions of same bacterial lineages are suppressed during the postpartum period when dairy cattle are exposed to high-grain diets. On the other hand, as a trade-off between amylolytic and fibrolytic microbial populations, the proportions of major amylolytic and lactate utilizers such as *Prevotella*, Veillonellaceae, and *Megasphaera* were decreased in ruminal content of P169-supplemented steers. *M. elsdenii* is among the most potent lactate-utilizer in the rumen ecosystem [199]. Therefore, one explanation for the numerical increase of lactate in the rumen content of the P169-supplemented steers could be the decrease in the proportions of potent lactate utilizers such as *Megasphaera* and Veillonellaceae.

Following feeding high starch diet, the bypass of undigested starch from the rumen and small intestine may stimulate fermentation by hindgut bacteria, increase the acidity of hindgut contents and alter its microbial composition [14, 200]. In an acidotic challenge conducted by Mao et al. [201], exposure of animals to high-starch diet resulted in increased proportion of genera *Prevotella, Blautia*, and other unclassified Lachnospiraceae within the hindgut microbiota, whereas the proportion of several OTUs belonging to Ruminococcaceae and *Clostridium* decreased. Showing a similar trend, the hindgut microbiota of P169-supplemented steers in the present study was also enriched with major amylolytic bacterial lineages such as *Prevotella, Blautia*, and Succinivibrionaceae, whereas the proportion of other bacteria such as *Ruminococcus* and Clostridiaceae were decreased. This observation, coupled with the decreased proportion of amylolytic bacteria in the rumen microbiota of P169-supplemented animals, may be indicative of an increased bypass of undigested carbohydrates from the rumen to the hindgut, and consequently, increased microbial fermentation in the hindgut. However, due to the lack of direct measurements of pH and metabolites in the feces of steers, the present study remains inconclusive on whether or not the observed shifts in the composition of hindgut microbiota confers beneficial influence on the overall fermentation profile of the steers.

### 5.5.2 Comparative Analysis of the Core Microbiota of Rumen and Hindgut Ecosystems

The microbial populations of the rumen are compartmentalized into those suspended in the rumen fluid, those associated with solid feed particles, and those associated with the rumen-wall tissue [202]. In agreement with several other reports [203-205], the present study also revealed a distinct clustering pattern between the microbiota of solid and liquid fractions of the rumen. At the phylum level, the proportion of Bacteroidetes was significantly higher within the liquid fraction, whereas an expected enrichment of Fibrobacteres was observed in the microbiota of solid fraction. Due to fibre-degrading and cellulolytic activities of Fibrobacteres species (e.g*., F. succinogenes*), members of this bacterial lineage are usually associated with plant fibrous materials of the rumen ecosystem [206]. On the other hand, Bacteroidetes (e.g., *Bacteroides* spp. and *Prevotella* spp.) possess large repertoires of genes involved in carbohydrate metabolism [49], making them capable of harvesting energy from the readily fermentable carbohydrate pools that enter rumen liquid following ingestion of high-grain diets. In addition to Fibrobacteres, Cyanobacteria were also enriched in the microbiota of solid fraction of the rumen. The contribution of Cyanobacteria to rumen fermentation is as yet poorly understood. Due to horizontal gene transfers that had occurred between endosymbiont Cyanobacteria and plastid during the course of plant evolution [207], it is difficult to distinguish whether the sequences mapped to this phylum originate from plant chloroplast or Cyanobacterial rumen dweller.

In the present study, comparison of the core microbiota of liquid and solid fractions of the rumen revealed a high level of commonality between the two sites with the predominance of OTUs belonging to Firmicutes, Bacteroidetes and Proteobacteria. This finding is in general agreement with the observations of Jami and Mizrahi [8] and Petri et al. [27], who also reported the predominance of OTUs belonging to these bacterial phyla in the core rumen microbiota of cattle fed high-grain diets. A notable finding of the present work was the exclusive presence of phylogenetically related OTUs in the core microbiota of either fractions of the rumen content. Genera *Butyrivibrio*, *Prevotella*, and unclassified Lachnospiraceae had different representative OTUs that were

either shared between the core microbiota of the two fractions or exclusively found in the core microbiota of solid fraction. This observation implies niche-specific adaptation and/or metabolic preferences of closely related species/strains within these bacterial lineages, and underscores the need to deploy higher-resolution sequencing/bioinformatics approaches that enable recapturing microbial shifts at the strain-level.

In contrast with the results from rumen, the core microbiota of fecal samples was composed entirely of representative OTUs belonging to the phylum Firmicutes. Ecological characteristics of the gastrointestinal tract compartments vary along longitudinal axes, resulting in development of region-specific microbiota that are compositionally and functionally distinct [208, 209]. Our results are in agreement with previous studies [201, 209, 210] reporting the predominance of OTUs belonging to Firmicutes in the hindgut microbiota of cattle. Collectively, these findings imply that the hindgut ecosystem provide a selective environment which favor the growth of specific bacterial lineages within the Firmicutes over other main bacterial phyla such as Bacteroidetes and Proteobacteria. Increased Firmicutes:Bacteroidetes ratio has been linked to increased energy-harvesting capacity of the gut microbiota [211]. Hence, it may be speculated that compared to rumen microbiota, the microbial communities of the hindgut have evolved to become more efficient in harvesting energy from limited nutrient resources that escape ruminal fermentation.

## 5.6 Conclusions

Collectively, the present study provided a comprehensive description of the dynamics of rumen and hindgut microbiota of high-grain fed steers in response to DFM supplementation with *Propionibacterium acidipropionici* strain P169. Concomitant with decreased proportion of amylolytic and lactate-utilizer bacterial lineages, the proportion of major cellulolytic bacteria increased in the rumen content of P169-supplemented steers. These, together with elevated molar proportions of branched-chain fatty acids and increased concentration of ammonia in the rumen content of supplemented steers, indicated an improved state of fibrolytic and proteolytic activity in response to P169 supplementation. Future investigations under acute or subacute acidosis conditions can

provide further insights into whether or not P169-supplementation, and its associated shifts in rumen and hindgut microbial communities, can beneficially influence compromised gastrointestinal fermentation profile of the acidotic high-grain fed ruminants.

## Bridge to chapter 6

In the experiment reported in chapter 5, I evaluated the efficacy of daily supplementation of probiotic strain P169 in modulating the rumen and hindgut microbiota of beef cattle fed a high grain diet. I observed that daily supplementation of P169 was associated with increased proportions of cellulolytic bacteria and molar concentrations of branched-chain fatty acids within the rumen ecosystem, indicating potential beneficial effects of this probiotic strain through favoring the growth of cellulolytic bacteria and restoring a more balanced rumen microbial community. As mentioned in the general introduction of this thesis, feeding high concentrate diets is one of the main metabolic challenges that beef cattle experience during their production cycle in Canadian Prairies. Another major metabolic disorder common in Canadian Prairies is pasture bloat, caused by exposing overwintered beef cattle to high-quality legume forages such as alfalfa. Several intervention strategies have been developed to mitigate the adverse effect of alfalfa-induced frothy bloat on the well-being and production performance of beef cattle. These include supplementation of drinking water with pluronic detergents such as Alfasure and the inclusion of non-bloating legumes such as sainfoin in mixed alfalfa pastures. While beneficial effects of these bloat preventive strategies on the production performance of cattle have been well studied, their impact on different microbial populations inhabiting the rumen and hindgut of cattle has not been investigated.

**Rationale**: Abrupt changes in the composition of diet can directly impact the composition of rumen microbial community (i.e., rumen microbial dysbiosis) and impair ruminal fermentation profile. Transition of overwintered beef cattle to high-quality alfalfa pastures has been associated with rapid development of frothy bloat and impaired performance of cattle. The aim of the next chapter of my thesis was to evaluate the dynamics of rumen microbial community during development of frothy bloat and to further assess the impact of bloat preventative strategies such as supplementation of Alfasure and grazing mixed sainfoin-alfalfa pasture on the composition and functional properties of rumen and hindgut microbiota.

# 6 Characterization of the Rumen and Fecal Microbiome in Bloated and Non-Bloated Cattle Grazing Alfalfa Pastures and Subjected to Bloat Prevention Strategies

## 6.1 Abstract

Frothy bloat is an often fatal digestive disorder of cattle grazing on legume pastures. The aim of this study was to investigate ruminal and fecal microbiota dynamics associated with development of alfalfa-induced frothy bloat and to further explore how bloat preventive strategies influence the composition of these microbial communities. In a 3-period crossover experiment, twelve rumen-cannulated steers were sequentially subjected to: 1) pure alfalfa pasture, 2) pure alfalfa pasture supplemented with Alfasure™, and 3) alfalfa – sainfoin mixed pasture. Eleven out of 12 steers in pure alfalfa pasture developed clinical bloat, whereas Alfasure™ treatment prevented the development of bloat in all 12 steers and alfalfa – sainfoin prevented bloat in 5 out of 11 steers. Overall, development of frothy bloat was associated with considerable shifts in the microbiota profile of rumen contents. In particular, the microbiota of solid rumen contents from bloated steers contained higher species richness and diversity. Bacterial genera enriched in the rumen microbiota of bloated steers included *Streptococcus*, *Succinivibrio* and unclassified Myxococcales, whereas *Fibrobacter* and *Ruminococcus* were proportionally higher in the rumen contents of non-bloated steers. Overall, we observed that bacterial genera and predicted functional pathways associated with the metabolism of complex plant polysaccharides were enriched within bloated rumen contents.

## 6.2 Introduction

Frothy bloat is an often-fatal digestive disorder of cattle grazing on highly digestible legumes or wheat pastures [20]. Although ruminants themselves lack the required enzymatic repertoire to harvest energy from lignocellulose-rich plant material, microbial symbionts inhabiting the rumen ecosystem can sequentially break down and ferment plant cell wall carbohydrates and thus support the energy requirement of

ruminants [4]. Legume forages contain high concentrations of digestible proteins, which upon release into the rumen result in the rapid proliferation of ruminal microbes and an increase in fermentative activities and gas production [20]. This rapid proliferation of microbes, in particular bacterial biofilms, also results in production of an excessive amount of bacterial slime (i.e., exopolysaccharides), which in turn increase the viscosity of the rumen content and entrap fermentation gases in a stable foam that cannot be expelled by eructation [20, 212]. In addition, legumes also possess glycosides (i.e., saponins) with foaming properties that can play a secondary role in development of bloat [213]. Among all North American forage legumes, alfalfa (*Medicago sativa*) has been the most widely adopted due to its high nutritive quality and yield. However, the occurrence of bloat is a major limitation to its greater use in grazing systems [214]. Several preventive strategies have been deployed in an attempt to mitigate bloat in cattle grazing alfalfa pastures. These include breeding of the bloat-reduced alfalfa (AC Grazeland [167]), the use of alcohol ethoxylate/pluronic detergents, such as Alfasure™ [127], and the inclusion of non-bloating legumes such as sainfoin (*Onobrychis viciifolia*) in mixed legume pastures [215, 216].

Understanding the contribution of rumen microbiota to the development of frothy bloat is of high importance for implementing effective preventive strategies against this digestive disorder. Howarth et al. [217] proposed that highly digestible high-protein forages, such as alfalfa, clover, and wheat, result in proliferation of certain ruminal microbial populations [20] that promote the occurrence of bloat. To date, most of our knowledge regarding the contribution of rumen microbes to bloat is limited to either culture-based studies [218, 219], or targeted amplification and quantification (qPCR) of classical rumen bacteria [220, 221]. However, the majority of microbes inhabiting the rumen ecosystem are unculturable [222] or lack the genomic information for targeted amplification and therefore have likely been overlooked as important components within the rumen microbiome that contribute to the development of frothy bloat. More recently, Pitta et al. [223] used high-throughput sequencing to explore the association of the rumen microbiome with development of frothy bloat in steers grazing vegetative wheat pastures. They observed that wheat-induced frothy bloat was associated with an enrichment of

several bacterial genera within the phylum Firmicutes, including *Clostridium*, *Eubacterium*, and *Butyrivibrio,* and an underrepresentation of genes encoding for oligosaccharidases. While this study generated valuable insight into the role of the rumen microbiome in development of wheat-induced frothy bloat, the association of rumen and hindgut microbiota with development of alfalfa-induced frothy bloat remains unknown. Given the important contribution of rumen bacterial community to degradation of plant fibrous material, the main objectives of our study were to a) determine changes in the composition and functional properties of the rumen bacterial community underlying the development of alfalfa-induced frothy bloat, b) explore the response of rumen bacteria to dietary interventions to prevent bloat which include grazing mixed pastures and the addition of the pluronic detergent Alfasure™, and c) to investigate the degree that fecal microbiota can be influenced by these bloat prevention strategies.

## 6.3 Methods

### 6.3.1 Ethics Statement

Protocol (1214) for this experiment was reviewed and approved by the Animal Care Committee (ACC) of the Lethbridge Research Centre (LRC), Lethbridge, Alberta. Cattle were cared for according to the guidelines of the Canadian Council on Animal Care (CCAC, 1993).

### 6.3.2 Experimental Design and Assessment of Bloat Scores

Twelve mature (3-4 y old) ruminally-fistulated Angus steers were equally allocated in a repeated measurement design that subjected all animals to 3 different treatments. The treatments included: 1) pure alfalfa pasture; steers grazed on pure stands of alfalfa for 6 h where bloat occurred; 2) steers grazed on pure stands of alfalfa for 6 h but treated with the pluronic detergent Alfasure™ (0.25 mL $L^{-1}$; Rafter 8 Products Inc., Calgary, AB, Canada) to prevent development of bloat; and 3) steers grazed for 6 h on mixed sainfoin – alfalfa pastures in which a minimum of 15% of the standing pasture DM was composed of sainfoin. Within this group, we had two subgroups of a) steers that did not experience bloat, and b) steers that did experience clinical bloat. A primary

adaptation period (baseline phase) of 3 weeks preceded the experiment during which all steers were fed dry alfalfa hay.

The experiment was conducted in 3 periods each divided into an adaptation/washout phase (7 d: steers fed alfalfa hay) and sampling phase (4 d: steers grazed on pastures). At the start of the experiment, all groups were released at 0830 into either pure vegetative alfalfa (with and without Alfasure™) or mixed vegetative alfalfa – sainfoin pastures. The steers were allowed to freely graze for 6 h and at 1430 all steers were removed from the pasture and retained in a pen overnight without feed, but with free access to water. On pasture, all steers had access to water, but those steers on the Alfasure™ treatment had access only to water that contained Alfasure™ at a concentration that delivered the product at a dose of 9 mL per head per day. Steers were closely monitored every 30 min during grazing and for 2 h after grazing to record the incidences of clinical bloat according to the procedure described by Majak et al. [224]. In brief, bloat scores were assigned as follows: 0 = Normal (no visible sign of bloat), 1 = Slight (slight distention of the left side of the animal), 2 = Marked: (marked distention of the left side of the animal with asymmetrical (egg-shape) look when walking away from the observer), and 3 = Severe (severe distention above the top of back and inside from right side of the animal). A single steer bloating on one day was counted as one case of bloat. If the assessment indicated that the sum of bloat incidences reached 9 or more in any of the three groups within the sampling phase, then steers were crossed over between pastures and the experiment was repeated until an additional 9 cases of bloat were obtained. For the purpose of this study, rumen and fecal samples collected from steers with a bloat score of 0 were considered as non-bloated and those collected from steers with a bloat score between 1-3 were considered as bloated.

### 6.3.3 Rumen Sample Collection and Processing

Prior to the grazing experiment, rumen digesta (from both cranial and caudal ventral sacs of the rumen) and fecal samples were collected from each steer at the end of the primary adaptation period (baseline samples). In period 1, after the steers were introduced to the pasture, rumen digesta and fecal samples were collected from steers on

4 consecutive days, immediately after they were removed from their assigned paddock in the afternoon. The experimental procedure for period 2 and 3 were exactly the same as period 1, but steers were crossed among treatment paddocks.

Approximately 50 g of ruminal digesta was collected from each of the cranial and caudal ventral sacs of the rumen. Digesta was transferred into a heavy-walled 250-mL beaker and squeezed with a Bodum coffee maker plunger (Bodum Inc., Triengen, Switzerland). Aliquots of fluid digesta (5 mL) were placed in aluminum foil dishes and flash-frozen in liquid nitrogen and stored at −80°C. Solid residue was suspended in 30 mL cold (4 ºC) grinding buffer (100mM Tris-HCl, 500mM EDTA, 1.5MNaCl, 1 mg mL#1 proteinase K, pH 8.0). The suspension was placed in a shallow aluminum foil dish and flash frozen in liquid nitrogen. All samples were stored at -80 ºC until further processing.

### 6.3.4 DNA Extraction and Quality Check

To extract genomic DNA, each frozen sample was coarsely ground under liquid nitrogen in a precooled porcelain mortar. Samples were then transferred into a precooled Retsch RM 100 Mortar Grinder equipped with a stainless-steel mortar bowl and pestle (F. Kurt Retsch GmbH and Co. KG, Haan, Germany) and ground for a further 5 min under liquid nitrogen. Liquid nitrogen was added to the mortar bowl during grinding as needed to maintain the grinding mixture in a semi-fluid state. The ground samples were transferred to a 200-mL wide-mouth centrifuge bottle. The sample was then slowly poured into a 50 mL falcon tube. Genomic DNA was then extracted from 150-250mg of each sample using QIAamp DNA Stool Mini Kit (Qiagen Inc., Mississauga, ON, Canada). The extracted DNA was quality checked using agarose gel electrophoresis and quantified by Picogreen dsDNA (Invitrogen, Eurgene, OR, USA). DNA samples were then normalized to 20 ng/μL, and quality checked by PCR amplification of the 16S rRNA gene using universal primers 27F (5'-GAAGAGTTTGATCATGGCTCAG-3') and 342R (5'-CTGCTGCCTCCCGTAG-3') as described by Khafipour et al. [13]. Amplicons were verified by agarose gel electrophoresis.

### 6.3.5 Library Construction and Illumina Sequencing

The V3-V4 region of 16S rRNA gene was targeted for PCR amplification using modified primers as described by Derakhshani et. al [181]. PCR reaction for each sample was performed in duplicate and contained 1.0 µL of pre-normalized DNA, 1.0 µL of each forward and reverse primers (10 µM), 12 µL HPLC grade water (Fisher Scientific, Ottawa, ON, Canada) and 10 µL 5 Prime Hot MasterMix (5 Prime Inc., Gaithersburg, MD, USA). Reactions consisted of an initial denaturing step at 94°C for 3 min followed by 35 amplification cycles at 94°C for 45 sec, 50°C for 60 sec, and 72°C for 90 sec; finalized by an extension step at 72°C for 10 min in an Eppendorf Mastercycler pro (Eppendorf, Hamburg, Germany). PCR products were then purified using ZR-96 DNA Clean-up Kit (ZYMO Research, Irvine, CA, USA) to remove primers, dNTPs and reaction components. The V3-V4 library was then generated by pooling 200 ng of each sample as quantified by Picogreen dsDNA (Invitrogen, Burlington, ON, Canada). This was followed by multiple dilution steps using pre-chilled hybridization buffer (HT1) (Illumina, San Diego, CA, USA) to bring the pooled amplicons to a final concentration of 5 pM, as measured by a Qubit 2.0 Fluorometer (Life technologies, Burlington, ON, Canada). Finally, 15% of PhiX control library was spiked into the amplicon pool to improve the unbalanced and biased base composition, a known characteristic of low diversity 16S rRNA libraries. Customized sequencing primers were synthesized and purified by polyacrylamide gel electrophoresis (Integrated DNA Technologies, Coralville, IA, USA) and added to the MiSeq Reagent Kit V3 (600-cycle) (Illumina, San Diego, CA, USA). The 300 paired-end sequencing reaction was performed on a MiSeq platform (Illumina, San Diego, CA, USA) at the Gut Microbiome and Large Animal Biosecurity Laboratories, Department of Animal Science, University of Manitoba, Canada. The sequencing data are uploaded into the Sequence Read Archive (SRA) of NCBI (http://www.ncbi.nlm.nih.gov/sra) and can be accessed through accession numbers SRR7350213-SRR7350413.

### 6.3.6 Bioinformatics and Statistical Analysis

The PANDAseq assembler [225] was used to merge and fix the overlapping paired-end Illumina fastq files. All the sequences with low quality base calling scores as

well as those containing uncalled bases (N) in the overlapping region were discarded. The output fastq file was then analyzed by downstream computational pipelines of the open source software package QIIME [183]. Assembled reads were demultiplexed according to the barcode sequences, chimeric reads were filtered using UCHIME [226] and sequences were assigned to Operational Taxonomic Units (OTU) using the QIIME implementation of UCLUST [227] at a 97% pairwise identity threshold. Taxonomies were assigned to the representative sequence of each OTU using the RDP classifier [228] and aligned with the Greengenes Core reference database [184] using PyNAST algorithms [229]. A phylogenetic tree was built with FastTree 2.1.3. [230] for further comparisons between microbial communities. Within community richness (Chao 1 estimator of species richness) and diversity (Shannon's index) were calculated using QIIME at an even depth of 22,000 and 8,000 sequences per rumen and fecal samples, respectively. To compare microbial composition among samples, β-diversity was measured by calculating the weighted and unweighted UniFrac distances [231]. Principal coordinate analysis (PCoA) was applied on resulting distance matrices to generate two-dimensional plots using PRIMER v6 software [232]. Finally, open source software PICRUSt (phylogenetic investigation of communities by reconstruction of unobserved states) [233] was used to predict the functional genes of the classified members of the rumen microbiota, and assign them to corresponding KEGG [234] orthologs (KOs) and pathways (level 2 and 3).

### 6.3.7 Unsupervised Clustering Analysis

To test for discrete clustering pattern of samples, the relative abundance of the OTUs were binned into genus-level taxonomic groups and filtered to retain the most abundant genera across all samples (cutoff value of > 0.1% of community). The resulting relative abundance table was normalized (values divided by the Euclidean length of the row vector) to correct for compositionality and also aide in the heatmap-visualization of differentially abundant genera. The dissimilarity of samples were calculated based on Bray–Curtis measure using the R "vegan" package and the resulting matrix was subjected to unsupervised hierarchical clustering using the R "dendextend" package [235]. The resultant data were visualized over the heatmap of abundance matrix using the R

"complexheatmap" package [236]. Genera were also clustered based on their Spearman's correlation coefficient using R "complexheatmap" package.

### 6.3.8 Statistical Analysis

The UNIVARIATE procedure of SAS (SAS 9.3, 2012) was used to test the normality of residuals for alpha biodiversity data. Non-normally distributed data were log transformed and then used to assess the effect of infection using the MIXED procedure of SAS. All pairwise comparisons among the groups were tested using Tukey's studentized range distribution. The PERMANOVA [185] was used to calculate *p* -values and test for significant differences of β-diversity (Bray-Curtis dissimilarity, weighted and unwieghted UniFrac distance matrices) among treatment groups. Label permutations were used in PERMANOVA to estimate the distribution of test statistics under the null hypothesis that within-group distances are not significantly different from between-group distances [237]. In all statistical models, the effect of individual steers was included in the model as a random factor to account for individuality of microbiota in repeated measurement design.

Multivariate analysis with linear modeling (MaAsLin[238]) was used to determine significant associations of bacterial genera/functional pathways with treatment groups. MaAsLin included a general linear model with treatment groups as categorical predictor variables and arcsine-square root transformed relative abundances of bacterial genera/functional pathways as the response variable. In addition, MaAsLin also accounted for other potential confounders (covariates) that could affect the profile of microbiota, including rumen fraction (liquid and solid), period, and subject (steers). Multiple hypotheses were adjusted by Benjamini and Hockberg false discovery rate (FDR). Unless otherwise indicated, significant associations were considered below a *q*-value threshold of 0.05.

## 6.4 Results

Following introduction to pure alfalfa pasture, 11 out of 12 steers developed clinical bloat. In contrast, Alfasure™ completely prevented bloat and alfalfa – sainfoin was moderately effective as 5 out of 11 steers bloated (Table 5).

**Table 5. Summary of study design and incidences of bloat**

| Sequence[1] | Period 1 | | Period 2 | | Period 3 | |
|---|---|---|---|---|---|---|
| | Treatment[2] | Bloat status[3] | Treatment | Bloat status | Treatment | Bloat status |
| S1 | AA | NB=4 | AS | NB=3 | PA | NB=0 |
| | | B=0 | | B=1 | | B=4 |
| S2 | AS | NB=0 | PA | NB=1 | AA | NB=3 |
| | | B=4 | | B=3 | | B=0 |
| S3 | PA | NB=0 | AA | NB=3 | AS | NB=2 |
| | | B=4 | | B=0 | | B=1 |

[1]Sequence by which steers (4 within each sequence) entered 3 periods of the experiment.
[2]Within each period, steers were subjected to one of the three treatment groups including 1) pure alfalfa pasture (PA), 2) pure alfalfa pasture supplemented with Alfasure™ (AA), and 3) alfalfa – sainfoin mixed pasture (AS).
[3]Bloat status indicates the number of steers within each treatment groups that either developed clinical bloat (B; bloat scores 1-3) or were non-bloated (NB; bloat score 0).

Illumina paired-end sequencing of rumen microbial communities generated an average of 47,211 and 42,544 high quality sequences per rumen and fecal sample, respectively, with a median sequencing length of 426 nt covering the full length of the V3-V4 hypervariable region of the 16S rRNA gene. At 97% similarity threshold, alignment of non-singletone OTUs to the Greengenes database resulted in classification of OTUs into 17 and 15 different bacterial phyla for rumen and fecal samples, respectively. Rumen samples were predominated by Bacteroidetes (56.92%) and Firmicutes (30.44%), followed by Fibrobacteres 5.25%), Spirochaetes (1.85%) and

Proteobacteria (0.63%). Fecal samples were predominated by Firmicutes (63.36%) and Bacteroidetes (28.51%), followed by Spirochaetes (0.90%) Proteobacteria (0.83%) and Tenericutes (0.34%).

### 6.4.1 Impact of Dietary Interventions on Biodiversity of Rumen Microbiota

Comparisons of alpha-diversity indices were performed at an even depth of 22,000 sequences per sample. Figure.7 a and b compare richness and diversity of rumen microbiota among bloated steers grazed on pure alfalfa pasture and non-bloated steers that received Alfasure™ or were grazed alfalfa – sainfoin pastures. In general, the solid fraction of rumen content was found to be more affected by treatment. Rumen microbiota of bloated steers showed greater richness as compared to both those that did not bloat in Alfasure™ and alfalfa – sainfoin groups, while the Shannon's diversity index of bloated cattle was higher ($p < 005$) than that of steers that did not bloat while grazing alfalfa – sainfoin pasture (Figure 7. A and B). There was no significant difference between the richness and diversity of liquid fractions among treatments. Similarly, comparison of UniFrac distances among treatment groups also revealed that the microbiota of solid rumen content of bloated steers grazing pure alfalfa clustered distinctly ($p_{(PERMANOVA)} < 0.05$) from steers that did not bloat while receiving Alfasure™ or grazing alfalfa – sainfoin, whereas the overall composition of the microbiota of liquid rumen fraction was only different between steers that bloated while grazing alfalfa and those that did not bloat Alfasure™. Within both solid and liquid fractions of the rumen, no clustering pattern was observed between the microbiota of steers that did not bloat as a result of receiving Alfasure™ or grazing alfalfa – sainfoin (Figure 7. C and D).

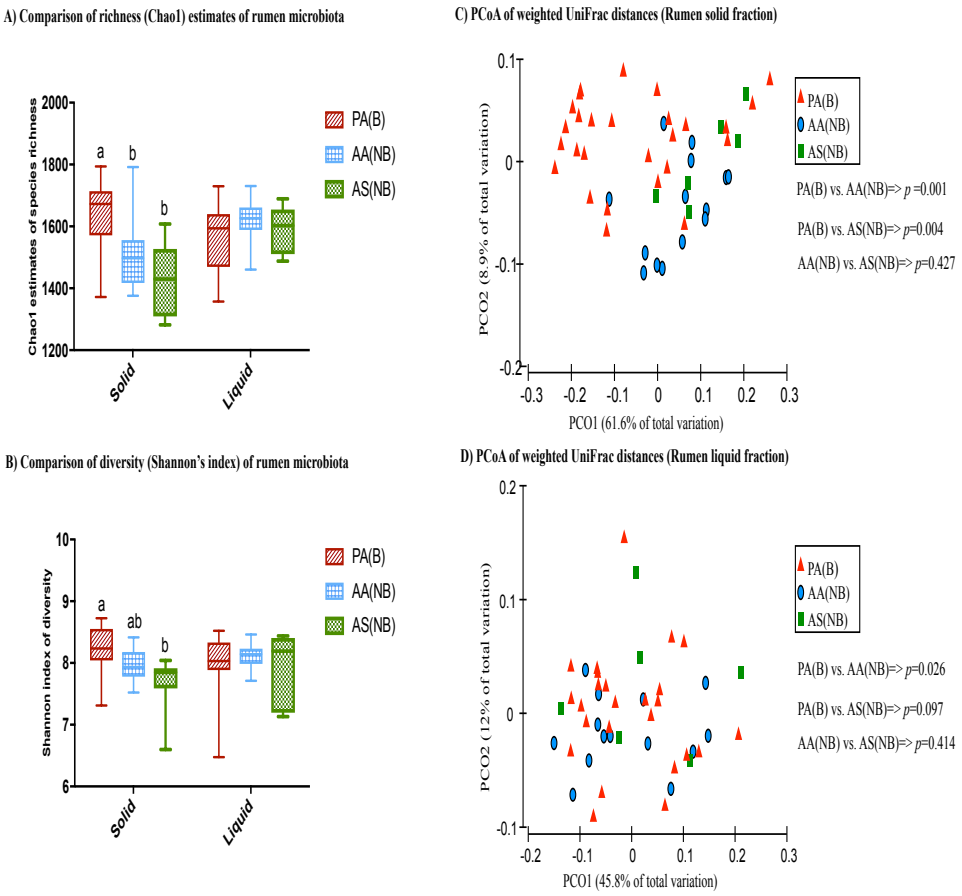



**Figure 7. Comparison of alfa- and beta-diversity metrics of microbial communities.**

A and B) Comparison of within community richness (Chao1 estimates), and alfa-diversity (Shannon's index) of the microbiota of liquid and solid fraction of rumen contents among treatment groups. Box-Whisker plots show average values of richness and diversity: boxes denote interquartile range with a line at the median, whiskers indicate minimal and maximal values, and error bars indicate the standard error for each treatment group. Different superscripts denote statistical significance among group means ($p$ <0.05) C and D) Principal coordinate analysis (PCoA): comparison of between community diversity based on weighted UniFrac distances of microbial communities in solid and liquid fractions of rumen content. The $p$ -value for each comparison was obtained from PERMANOVA and considered significant at $p$ <0.05. Color codes have been assigned to differentiate between treatment groups: red indicates samples obtained from steers grazed on pure alfalfa and developed clinical bloat (PA(B)), blue indicates

samples obtained from non-bloated steers grazed on pure alfalfa supplemented with Alfasure™ (AA(NB)), and green indicates samples obtained from non-bloated steers grazed on mixed pastures of pure alfalfa and sainfoin (AS(NB)). Bloat status was indicated with B (developed clinical bloat; bloat scores 1-3) or NB (non-bloated; bloat score 0).

In addition, unsupervised cluster analysis based on the proportion of main bacterial genera (> 0.1% of community) revealed that the microbiota of rumen samples from steers that bloated as result of grazing pure alfalfa clustered distinctly ($p_{(PERMANOVA)} = 0.001$) form the microbiota of rumen samples collected during the baseline adaptation phase (steers fed dry alfalfa hay). Same analysis also revealed distinct clustering patterns ($p_{(PERMANOVA)} = 0.01$) between the microbiota of solid and liquid fractions of rumen contents (Figure 8).

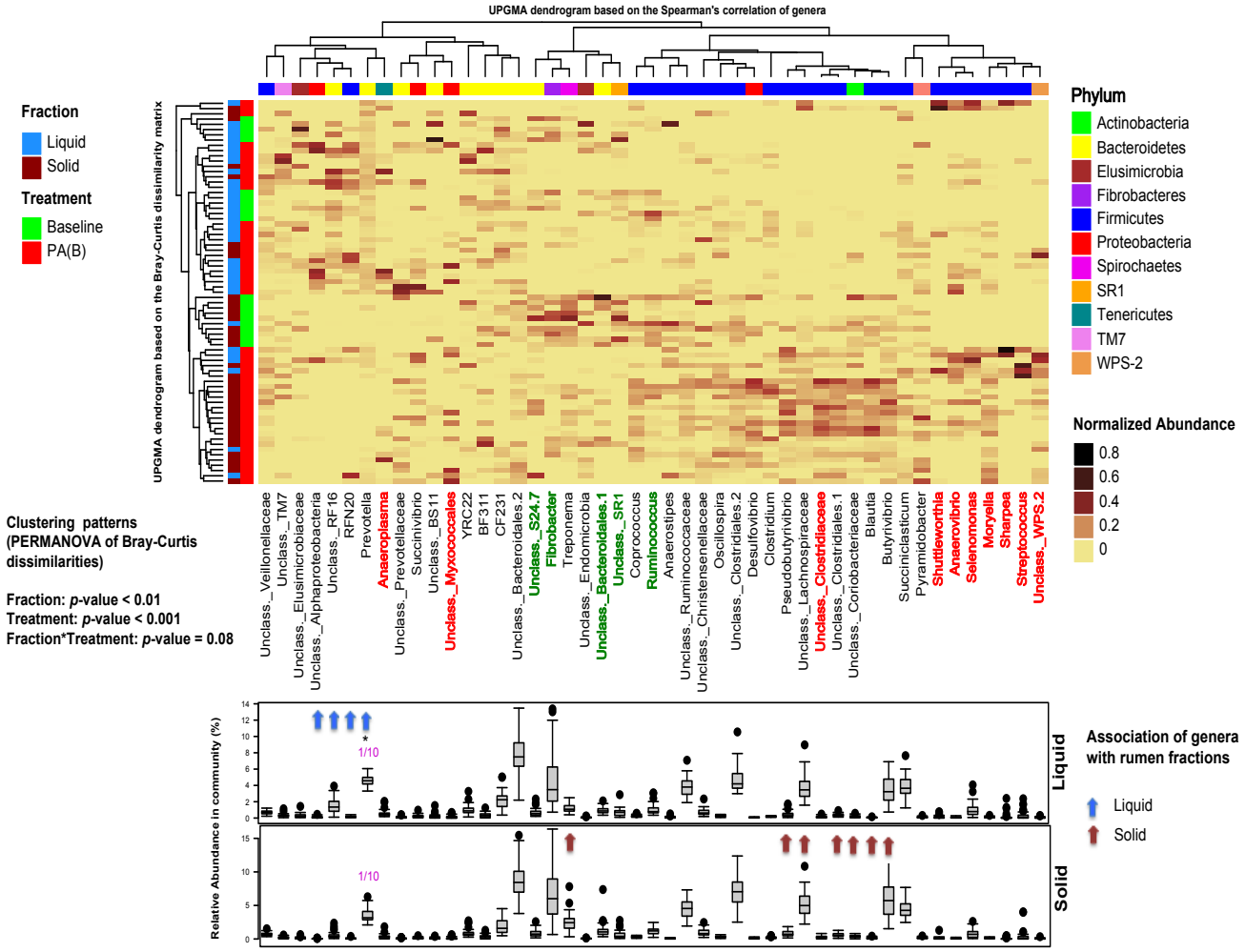



**Figure 8. Unsupervised cluster analysis of rumen microbial communities.**

Rows correspond to samples and columns correspond to abundant genera (> 0.1% of community). The "Normalized Abundance" key relates colors to the normalized proportions of genera (relative abundance of each genus divided by the Euclidean length of the column vector). The left dendogram shows clustering pattern of samples based on Bray–Curtis dissimilarities (using unweighted pair group method with arithmetic averaging (UPGMA)). The significance of clustering patterns was calculated based on 9999 permutations and p-values calculated based on PERMANOVA. The top dendogram shows correlation (cooccurrence) of genera based on Spearman's correlation coefficient. The "Phylum" key relates the top annotations to the corresponding phylum of each genus. The “Fraction” and “Treatment” keys relate samples to their originating rumen fraction (liquid or solid) and treatments groups (baseline: steers grazed on alfalfa hay prior to introduction to alfalfa pasture, PA(B): steers grazed on pure alfalfa pasture and developed clinical bloat). The bottom box-plots show relative abundances of genera in liquid (top) and solid (bottom) fractions. The association of genera with rumen fractions" key relates

color codes to the signature bacterial genera of each fraction (identified using MaAsLin, considering significance cut-off q-value of 0.05 and adjusted for all potential confounders including treatment groups, rumen fractions, subject (steers)). Color codes highlight association of bacterial genera with treatment groups (red: significant association with PA(B), and green: significant association with baseline). "*" The relative abundance of genus Prevotella has been scaled to 1/10 in order to assist visualization of other abundant genera.

### 6.4.2 Association of Rumen Microbiota with Dietary Treatment and Bloat Incidence

Comparison of the main bacterial genera between the microbiota of rumen samples from steers that bloated on pure alfalfa and those collected during the baseline adaptation phase revealed that several co-occurring genera within phyla Firmicutes (including *Streptococcus*, *Selenomonas*, *Sharpea*, *Shuttleworthia* and unclassified Clostridiaceae), Proteobacteria (including unclassified Myxococcales), and Tenericutes (*Anaeroplasma*) were proportionally more abundant, whereas other genera within phyla Bacteroidetes (including unclassified members of the order Bacteroidales and family S24.7), Fibrobacteres (genus *Fibrobacter*), and Firmicutes (genus *Ruminococcus*) were proportionally higher in the rumen of steers during the baseline adaptation phase (Figure 8). To further determine key bacterial genera and functional genes that were associated with the occurrence of bloat or lack thereof, the proportion of abundant rumen genera and functional pathways (KEGG level 2 and 3) were compared among treatment groups (Figures 9). The feature bacterial genera that were found to be consistently overrepresented within the rumen microbiota of bloated steers included unclassified Myxococcales and *Succinivibrio* (within phylum Proteobacteria), and *Streptococcus* (phylum Firmicutes), whereas genera *Fibrobacter* and *Ruminococcus* were overrepresented in the rumen of non-bloated steers.

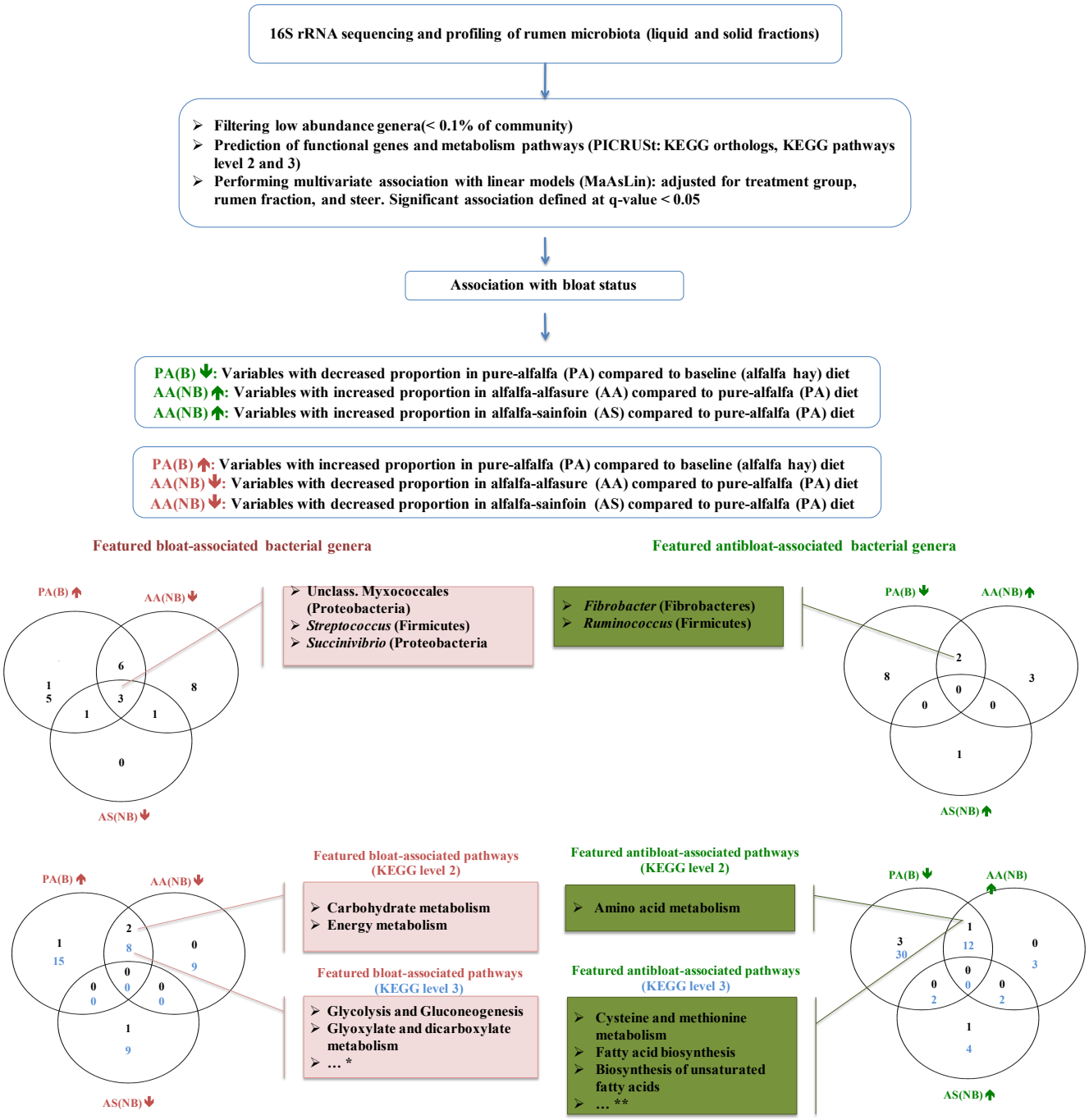



**Figure 9. Schematic diagram of data analysis and statistical approaches for identifying feature bacterial genera and functional pathways (KEGG level 2 and 3) associated with bloat status.**

Abbreviations are used to define treatment groups; PA(B): steers grazed on pure alfalfa pasture and developed clinical bloat; AA(NB): non-bloated steers grazed on pure alfalfa pasture supplemented with Alfasure™; AS(NB) non-bloated steers grazed on mixed

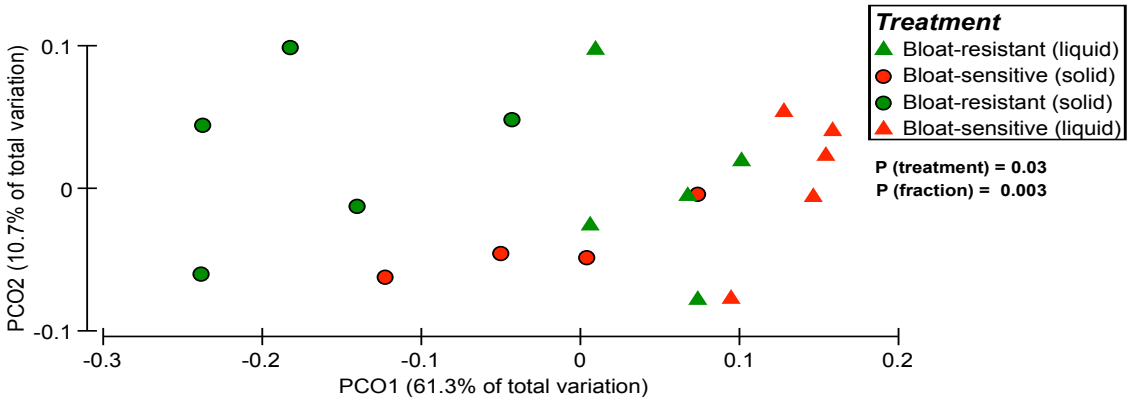
pastures of pure alfalfa and sainfoin (AS(NB)). Bloat status was indicated with B (developed clinical bloat; bloat scores 1-3) or NB (non-bloated; bloat score 0). * See supplementary file 3 for summary statistics of associative analyses and a complete list of shared/exclusive bacterial genera and KEGG pathways among treatment groups.

With regards to predicted functional pathways, in general, carbohydrate metabolism pathways (e.g., glycolysis and gluconeogenesis) were significantly enriched within the rumen microbiota of bloated steers, whereas amino acid metabolism pathways, including cysteine and methionine metabolism were enriched within the rumen microbiota of non-bloated steers (Figure 9).

MaAsLin was also used to explore the association of abundant bacterial genera with solid and liquid rumen fractions independent of treatments. Most of the genera that were significantly associated with solid fraction of rumen contents belonged to the phylum Firmicutes, including *Butyrivibrio*, *Blautia*, *Pseudobutyrivibrio*, and unclassified members of the families Lachnospiraceae and Clostridiaceae. In contrast, genera within phyla Bacteroidetes (including *Prevotella* and unclassified members of family RF16), and Proteobacteria (unclassified Alphaproteobacteria) were significantly overrepresented within the microbiota of the liquid fraction of rumen contents (Figure 8).

Finally, the baseline rumen microbiome of steers that did not bloat while grazing alfalfa – sainfoin pasture were compared to those that did bloat by comparison of beta-diversity based on UniFrac distances which revealed distinct clustering patterns between the two groups of steers ($p_{(\text{PERMANOVA})} = 0.03$; Figure 10.a). Comparison of abundant bacterial genera between the two groups also revealed increased in the proportions of several Firmicutes genera, including *Buleidia*, unclassified Ruminococcaceae, and unclassified Colostridiales, within the rumen microbiota of steers that did not bloat, whereas *Prevotella* was proportionally increased in the baseline rumen microbiota of bloated steers (Figure 10.b).

**A)**


Treatment
Bloat-resistant (liquid)
Bloat-sensitive (solid)
Bloat-resistant (solid)
Bloat-sensitive (liquid)
P (treatment) = 0.03
P (fraction) = 0.003
PCO2 (10.7% of total variation)
PCO1 (61.3% of total variation)
0.1
0
-0.1
-0.3
-0.2
-0.1
0
0.1
0.2

**B)**

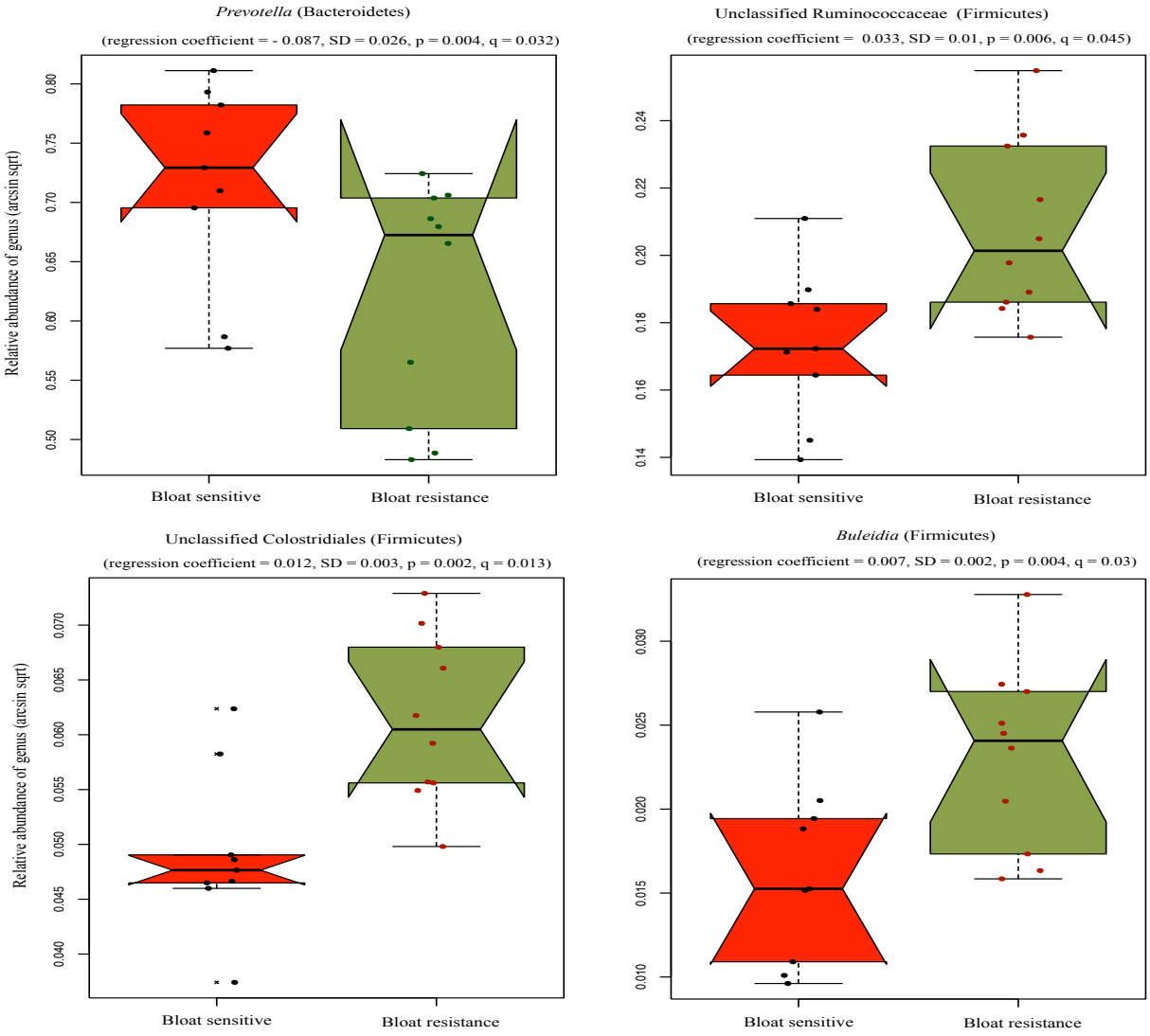

*Prevotella* (Bacteroidetes)
(regression coefficient = - 0.087, SD = 0.026, p = 0.004, q = 0.032)
Relative abundance of genus (arcsin sqrt)
Bloat sensitive
Bloat resistance
Unclassified Ruminococcaceae (Firmicutes)
(regression coefficient = 0.033, SD = 0.01, p = 0.006, q = 0.045)
Bloat sensitive
Bloat resistance
Unclassified Colostridiales (Firmicutes)
(regression coefficient = 0.012, SD = 0.003, p = 0.002, q = 0.013)
Relative abundance of genus (arcsin sqrt)
Bloat sensitive
Bloat resistance
*Buleidia* (Firmicutes)
(regression coefficient = 0.007, SD = 0.002, p = 0.004, q = 0.03)
Bloat sensitive
Bloat resistance

**Figure 10. Characterization of bloat-sensitive and bloat-resistant rumen microbiota.**

A) Principal coordinates analysis (PCoA) of weighted UniFrac distances: between community diversity was compared to test for different clustering patterns of baseline rumen microbiota (samples obtained during baseline adaptation when steers were fed dry alfalfa hay prior to grazing alfalfa pasture) from bloat-sensitive (steers that developed bloat on mixed pasture of alfalfa and sainfoin) and bloat resistant (steers that did not develop bloat on mixed pasture of alfalfa and sainfoin). Color codes have been assigned to differentiate between bloat-sensitive (red) and non-sensitive (green) steers. Triangles denotes samples from liquid fraction of rumen digesta and circles denote samples obtained from solid fraction. The p-value for each comparison was obtained from PERMANOVA and considered significant at $p < 0.05$ B) Association of bacterial genera with treatment groups: box plot of relative abundances of genera (arcsin square root transformed) indicate the median (horizontal solid line), interquartile range between the first and third quartiles (box), variability outside the upper and lower quartiles (whiskers), and outliers. Color codes indicate the treatment groups: red indicates samples obtained from bloat-sensitive animals and green indicates samples obtained from bloat-resistant animals.

### 6.4.3 The Impact of Dietary Treatments and Bloat Incidence on Fecal Microbiota

Unlike rumen microbiota, the richness and diversity of fecal microbiota was not affected by treatments (Figure 11.A and B). With respect to the beta-diversity of fecal microbiota, only unweighted UniFrac distances differed ($p_{(PERMANOVA)} = 0.005$) between the microbiota of steers that bloated while grazing alfalfa and those that received Alfasure™.

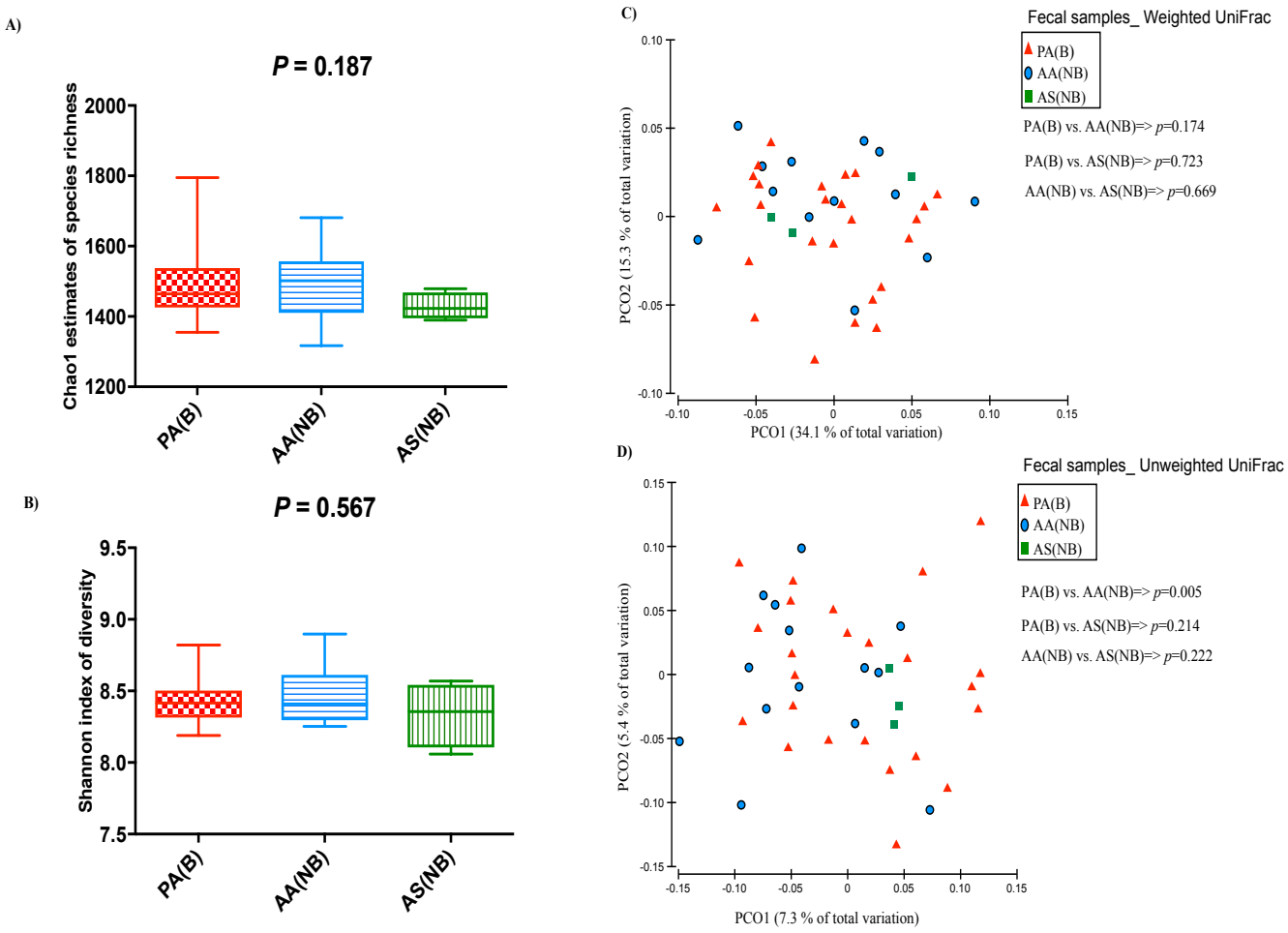



**Figure 11. Comparison of biodiversity metrics of fecal microbial communities.**

A and B) Comparison of within community richness (Chao1 estimates) and diversity (Shannon's index) among treatment groups. Box-Whisker plots show average values of richness and diversity: boxes denote interquartile range with a line at the median, whiskers indicate minimal and maximal values, and error bars indicate the standard error for each treatment group. *p* -value for each comparison was obtained from ANOVA (SAS 9.3) and considered significant at $p < 0.05$. C and D) Principal coordinate analysis (PCoA): comparison of between community diversity based on weighted and unweighted UniFrac distances of fecal microbial communities. *p* -value for each comparison was obtained from PERMANOVA and considered significant at $p < 0.05$. Color codes have been assigned to differentiate between treatment groups: red indicates samples obtained

from steers grazed on pure alfalfa and developed clinical bloat (PA(B)), blue indicates samples obtained from nonbloated steers grazed on pure alfalfa supplemented with Alfasure™ (AA(NB)), and green indicates samples obtained from non-bloated steers grazed on mixed pastures of pure alfalfa and sainfoin (AS(NB)). Bloat status was indicated with B (developed clinical bloat; bloat scores 1-3) or NB (non-bloated; bloat score 0).

In addition, unsupervised clustering analysis based on the proportions of main bacterial genera (> 0.1% of community) revealed distinct clustering pattern between the microbiota of fecal samples collected during the baseline adaptation phase and those that bloated while grazing alfalfa ($p_{(\text{PERMANOVA})} < 0.001$; figure 12). *Phascolarctobacterium* and unclassified Peptococcaceae within the phylum Firmicutes, unclassified Bacteroidetes belonging to families Rikenellaceae, S24-7, and RF16 were overrepresented within the fecal microbiota of bloated steers grazing alfalfa, whereas *Fibrobacter*, *Prevotella*, *Selenomonas* and unclassified Lachnospiraceae were overrepresented within the fecal microbiota of samples collected during the baseline adaptation phase.

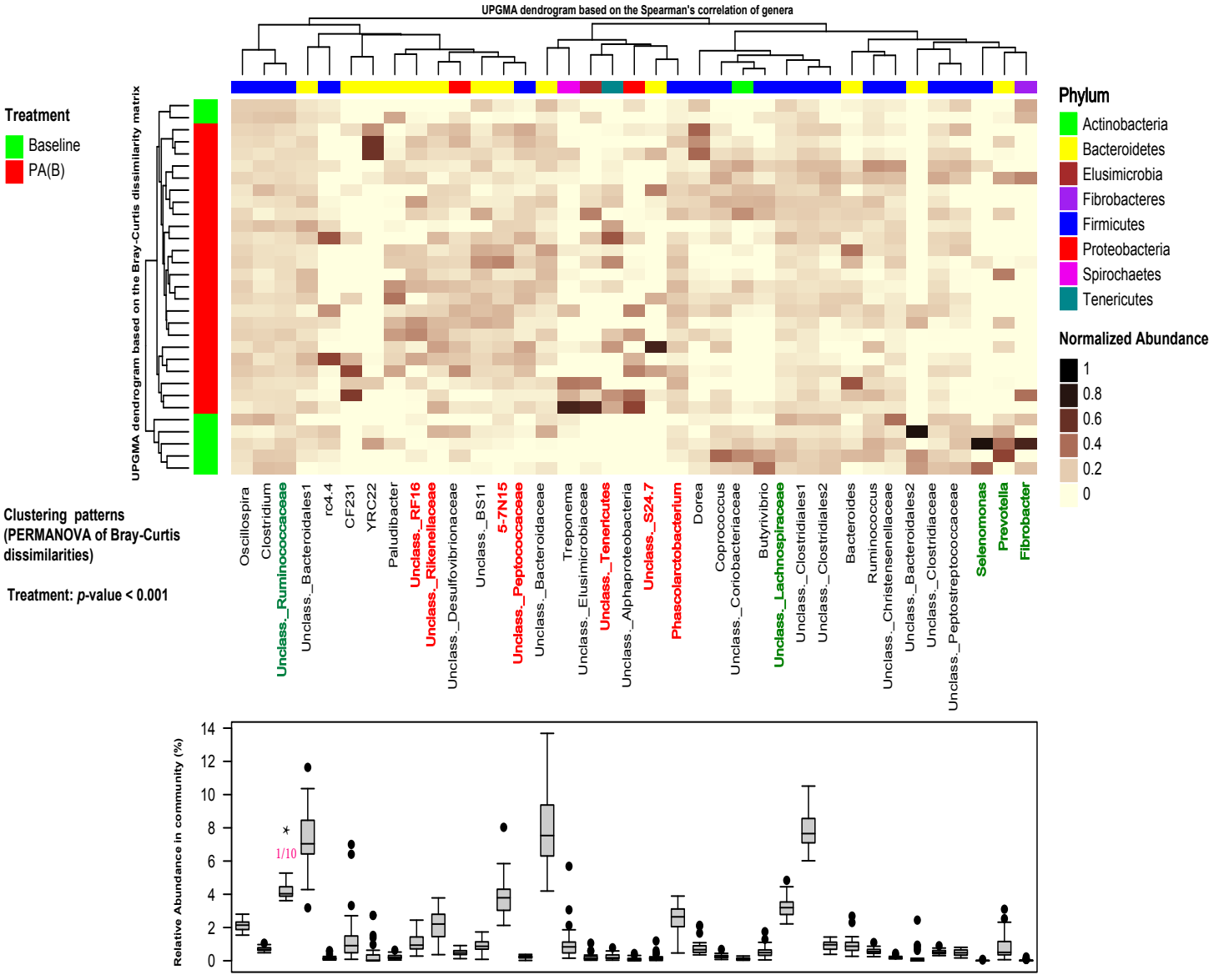

UPGMA dendrogram based on the Spearman's correlation of genera
Treatment
Baseline
PA(B)
UPGMA dendrogram based on the Bray-Curtis dissimilarity matrix
Phylum
Actinobacteria
Bacteroidetes
Elusimicrobia
Fibrobacteres
Firmicutes
Proteobacteria
Spirochaetes
Tenericutes
Normalized Abundance
1
0.8
0.6
0.4
0.2
0
Oscillospira
Clostridium
Unclass._Ruminococcaceae
Unclass._Bacteroidales1
rc4.4
CF231
YRC22
Paludibacter
Unclass._RF16
Unclass._Rikenellaceae
Unclass._Desulfovibrionaceae
Unclass._BS11
5-7N15
Unclass._Peptococcaceae
Unclass._Bacteroidaceae
Treponema
Unclass._Elusimicrobiaceae
Unclass._Tenericutes
Unclass._Alphaproteobacteria
Unclass._S24.7
Phascolarctobacterium
Dorea
Coprococcus
Unclass._Coriobacteriaceae
Butyrivibrio
Unclass._Lachnospiraceae
Unclass._Clostridiales1
Unclass._Clostridiales2
Bacteroides
Ruminococcus
Unclass._Christensenellaceae
Unclass._Bacteroidales2
Unclass._Clostridiaceae
Unclass._Peptostreptococcaceae
Selenomonas
Prevotella
Fibrobacter
Clustering patterns
(PERMANOVA of Bray-Curtis
dissimilarities)
Treatment: p-value < 0.001
Relative Abundance in community (%)
14
12
10
8
6
4
2
0
1/10

**Figure 12. Unsupervised cluster analysis of fecal microbial communities.**

Rows correspond to samples and columns correspond to abundant genera (> 0.1% of community). The "Normalized Abundance" key relates colors to the normalized proportions of genera (relative abundance of each genus divided by the Euclidean length of the column vector). The left dendogram shows clustering pattern of samples based on Bray–Curtis dissimilarities (using unweighted pair group method with arithmetic averaging (UPGMA)). The significance of clustering patterns was calculated based on 9999 permutations and p-values calculated based on PERMANOVA. The top dendogram shows correlation (co-occurrence) of genera based on Spearman's correlation coefficient. The "Phylum" key relates the top annotations to the corresponding phylum of each genus. The ”Treatment” key relates samples to treatments groups (baseline: steers grazed on alfalfa hay prior to introduction to alfalfa pasture, PA(B): steers grazed on pure alfalfa pasture and developed clinical bloat). The bottom box-plots show the relative abundances of genera. The associations of genera with treatment groups were identified using MaAsLin, considering significance cut-off q-value of 0.05 and adjusted for potential confounders including treatment groups and subject (steers)). Color codes have been used to highlight bacterial genera that were associated with treatment groups (red: significant association with PA(B); q-value < 0.05 and green: significant association with baseline; q-value < 0.05). “*” The relative abundance of Unclassified Ruminococcaceae has been scaled to 1/10 in order to assist visualization of other genera.

## 6.5 Discussion

Composition and functionality of rumen microbiota can be influenced by a wide array of host and environmental factors among which diet appears to play a central role in shaping the overall profile of rumen microbial ecosystem [6]. In beef cattle, grazing of wheat and alfalfa pastures has been associated with frothy bloat[20, 216], with recent evidence suggesting that this condition may be linked to a perturbed rumen microbiome in cattle grazing wheat pastures [239]. In the present study, we described bloat-associated shifts in the composition of rumen and fecal bacterial communities of steers grazing alfalfa pastures. Whereas only slight differences existed between the fecal microbiota of

bloated versus non-bloated steers, we observed that the development of frothy bloat was associated with dramatic shifts in the diversity of rumen microbiota, particularly those associated with the solid fraction of rumen contents. Increased species-richness and diversity of fiber-associated microbiota suggested that development of frothy bloat might be in part due to the rapid proliferation of bacterial biofilms. In particular, bacterial genera and predicted functional pathways that are associated with metabolism of complex plant polysaccharides were enriched within the rumen content of bloated steers.

In the current study, we demonstrated that bloat preventive strategies, including supplementation of drinking water with Alfasure™ and grazing of mixed alfalfa – sainfoin pasture, can prevent or reduce the development of alfalfa-induced frothy bloat and change the composition of rumen microbiota. Alfasure™ is a pluronic detergent known to prevent pasture bloat via reducing rumen fluid viscosity and disrupting the stability of the stable froth that causes bloat. This may be in part due to the ability of pluronic detergents to prevent the formation of micelle within the rumen ecosystem, which in turn can disrupt gas bubbles and prevent them from coalescing [240]. Sainfoin is a bloat-safe forage that contains condensed tannins [24]. Tannins are a family of polyphenolic compounds capable of binding to soluble proteins, polysaccharides and other macromolecules [241]. Thus, by binding to plant proteins and preventing them from being solubilized into the ruminal fluid, condensed tannins of sainfoin can prevent the formation of proteinaceous gas-trapping foam within rumen contents and therefore prevent frothy bloat. It has been demonstrated that inclusion of sainfoin with alfalfa at a concentration of 10-12% (DM basis) contributes to reduction in the incidence of bloat [242, 243].

Transition from alfalfa hay to alfalfa pasture resulted in increased richness and diversity of the rumen microbiota of steers that bloated while grazing alfalfa. Increased microbial diversity is an intrinsic characteristic of biofilm formation and proliferation; a principle referred to as "insurance hypothesis" and known to be the predominant growth mode for microbial communities in natural environments [244, 245]. The advent of diverse genetic pool allows associated microbial consortia to form structured communities - embedded in extracellular matrices - where end products produced by one

colony can be sequentially used by closely associated colonies [245, 246]. In the current study, increased diversity of rumen microbiota of bloated steers compared to non-bloated group was concomitant with general enrichment of predicted bacterial genes responsible for carbohydrate metabolism. This could indicate the robustness of ruminal biofilm for degrading complex plant polysaccharides and production of the exopolysaccharide slime that is associated with bloat[1]. Further, we observed that increased microbiota diversity in bloated animals was more pronounced in the solid fraction of rumen contents. One possible explanation could be that in bloated rumen ecosystem, increased viscosity results in entrapment of end products (metabolites) of the enzymatic degradation of plant polysaccharides surrounding solid particles, which in turns may result in uncontrolled proliferation of solid-associated biofilms.

Transition from hay to alfalfa-pasture also resulted in sharp compositional shifts in the rumen microbiota of grazing steers. In particular, our associative analysis revealed that a group of co-occurring bacterial genera within the phylum Firmicutes - including *Streptococcus*, *Selenomonas*, *Shuttleworthia* and unclassified Clostridiaceae - were enriched within the rumen contents of bloated steers; whereas other genera such as *Ruminococcus* and *Fibrobacter* were associated with baseline alfalfa hay diet. *Ruminococcus* spp. (e.g., *R. flavefaciens* and *R. albus*) and *Fibrobacter succinogenes* are commonly regarded as main cellulose-degrading species within the rumen ecosystem [44] and are predominantly present in high fiber diets [12]. In contrast, *Streptococcus* and *Selenomonas* are dominant bacterial genera within rumen microbiota of ruminants receiving high soluble carbohydrate diets [12, 247] or high quality fresh forage diets with high levels of soluble protein [248]. Some species of these genera, including *Streptococcus bovis* and *Selenomonas ruminantium,* are known to produce and store high amounts of reserve polysaccharides in their cytoplasm which can serve as precursors for extracellular polymeric substances of biofilms [249, 250]. Previously, Min et al. [251] reported the *in vitro* ability of *S. bovis* to actively produce biofilms. This same study also revealed increased *in vivo* biofilm production in rumen ecosystem of steers exposed to wheat forage. Unfortunately, the DGGE approached used by Min et al. [251] lacked the required resolution for accurate classification of bacterial genera and their association

with bloat. Most recently, Pitta et al. [239] investigated the association between wheat-induced frothy bloat and the dynamics of rumen microbiome where they observed *Ruminococcus*, *Lactobacillus*, and *Prevotella* to be associated with normal rumen condition whereas *Clostridium*, *Eubacterium*, and *Butyrivibrio* were associated with rumen contents from bloated cattle. The authors concluded that the latter group of bacteria can utilize the oligosaccharides trapped in the biofilm which they hypothesized could be related to the high proportion of genes within these Firmicutes that contribute to carbohydrate metabolism. In the present study, we observed that bloat-associated enrichment of *Streptococcus*, *Selenomonas*, *Shuttleworthia* and unclassified Clostridiaceae was associated with increased proportion of predicted microbial genes that were annotated to carbohydrate metabolism pathways. These findings led us to speculate that bloat-associated biofilms naturally select for genera that have higher potential for rapid metabolism of soluble carbohydrates trapped within exopolysaccharide slime. Ironically, Pitta et al. [239] reported that abundance and diversity of CAZymes were greatly reduced in the rumen content of bloated steers on wheat pasture. Discrepancies between our observations and those of Pitta et al. [239] might have been in part due to possible differences in the etiology and pathogenesis of alfalfa-induced versus wheat-induced frothy bloat, or perhaps because of methodological differences between the two studies. However, it should be noted that metagenomics approaches, either based on whole-genome sequencing or predicted from marker genes, face fundamental limitations to directly reflect the functional activities of microbial communities. Indeed, parallel metagenomics and metatransciptomics investigation of human gut microbiome has revealed that certain microbial clades possess metabolic activities that tend to be consistently overexpressed (mRNA is found to be more abundant than the equivalent DNA); whereas other genes can be consistently under expressed within microbial communities (DNA is found to be more abundant than the equivalent mRNA); demonstrating that translational activity of a given metagenome is highly dynamic and largely regulated by metabolome profile (i.e., availability/deficiency of certain metabolites in the ecosystem) [252]. Thus, further metaomics investigations of the rumen microbiome are required in order to infer microbial functions that underlie the development of frothy bloat.

Multiple comparisons among treatment groups resulted in identification of feature genera that were consistently overrepresented in the rumen ecosystem of bloated steers, including *Streptococcus*, *Succinivibrio*, and Unclass. Myxococcales, and those that were consistently overrepresented in the rumen contents of non-bloated steers, including *Fibrobacter* and *Ruminococcus*. As indicated earlier, Pitta et al. [239] also observed that the proportion of *Ruminococcus* was decreased in the bloated rumen content of steers grazing wheat pasture. This, together with our observation regarding simultaneous decreases in proportions of *Fibrobacter* and *Ruminococcus,* suggests that development of frothy bloat disfavors the growth of main fiber-degrading bacterial lineages. However, it is noteworthy to mention that some species of *Ruminococcus,* including *R. bromii,* could be main contributors to amylolytic activities rather than fibrolytic [253], warranting further in depth metagenomics analyses of bloat associated shifts in rumen microbiome. in the rumen On the other hand, we observed that members of the phylum Proteobacteria, including *Succinivibrio* and Myxococcales, were enriched in the rumen content of bloated steers. Proteobacteria have been reported to enrich during adaptation to high-grain diets [13, 254] and appear to be more tolerant of low rumen pH [27]. Pitta et al. [239] also observed increased proportion of the phylum Proteobacteria in the rumen content of bloated steers on wheat pastures. Simultaneous DNA and RNA analyses of rumen microbiome of steers fed grain-based diet [255] suggested that metabolic contributions of Proteobacteria are greater than their proportion in rumen community. The metabolic activity of family Myxococcales in rumen ecosystem is currently unknown, however, *Succinivibrio* spp. have been identified as part of the core-rumen microbiota of cattle which predominate during adaptation to high starch diets [27, 256]. *Succinivibrio dextrinosolvens*, is a predominant rumen dweller that contribute to fermentation of a variety of carbohydrates and its end products (succinate and formate) can serve as intermediates of rumen fermentation that are further used by other microorganisms [256, 257]. Our results suggest that Proteobacteria play central role in development of alfalfa-induced frothy bloat, likely via contribution to rapid metabolism of complex polysaccharides.

Another interesting finding of the present study was identification of steers that were more susceptible to development of frothy bloat when grazing mixed alfalfa – sainfoin pastures. Comparison of the baseline rumen microbiota of this group of steers with those that were resistant to development of bloat when grazing on mixed alfalfa – sainfoin pastures revealed distinct clustering patterns. In particular, we observed that the proportion of *Prevotella* was significantly higher in the baseline rumen microbiota of bloat-sensitive steers. *Prevotella* spp. have long been identified as predominant rumen dwellers [47, 258] which possess extensive amylolytic and proteolytic activities. *In vitro* co-culture of *Prevotella* species (i.e., *P. ruminicola*) with cellulolytic strains such as *Fibrobacter succinogenes* and *Ruminococcus flavefaciens* resulted in improved digestion of forage cellulose [259]. This synergistic effect is most likely due to the diverse pool of polysaccharide-degrading enzymes that are found in ruminal *Prevotella* spp. [260] and their ability to provide metabolic end/by products that can be further utilized by other cellulolytic bacteria and contribute to sequential breakdown of plant cell wall by rumen biofilms. Increased proportion of *Prevotella* in the rumen microbiota of bloat-sensitive steers led us to speculate that the metabolic activity and end/by products of this bacterial lineage may accelerate the formation and proliferation of bloat-associated biofilms.

The bypass of undigested rumen contents to the intestines can modify fermentation profile and microbiota composition of the hindgut ecosystem [14, 200, 261]. In the present study, transition from baseline diet to alfalfa pasture was associated with moderate shifts in the composition of fecal microbiota of steers. More specifically, we observed an underrepresentation of fiber degrading bacterial lineages such as *Fibrobacter* and unclassified Ruminococcaceae in the fecal microbiota of bloated steers, which might be indicative of disrupted fermentation profile of the hindgut ecosystem as a result of frothy bloat. However, we failed to observe any notable influence of dietary interventions on the fecal microbiota of bloated versus non-bloated steers grazed on alfalfa pastures. Comparison of baseline fecal microbiota between bloat-sensitive steers with those that did not develop bloat on mixed alfalfa – sainfoin pastures also did not reveal any significant difference. Overall, our data suggest that rapid screening of fecal microbiota

does not offer a viable strategy to identify steers that may be susceptible to development of frothy bloat on alfalfa pastures.

## 6.6 Conclusions

In conclusion, our experiment showed that pasture management strategies, including supplementation of drinking water with Alfasure™ and grazing of mixed alfalfa – sainfoin pasture, can prevent or reduce the development of alfalfa-induced frothy bloat in cattle. Our results indicate that development of frothy bloat is associated with considerable shifts in the microbiota profile of rumen contents, in particular within the solid fraction. Increased species-richness and diversity of plant particle-associated bacterial communities might imply that development of frothy bloat is in part due to the rapid proliferation of fiber-adherent biofilms. We further observed that proportions of bacterial genera and predicted functional pathways that are associated with metabolism of complex plant polysaccharides were enriched within bloated rumen contents. On the other hand, only slight differences existed between the fecal microbiota of bloated versus non-bloated steers, suggesting that hindgut microbial ecosystem remain more stable across different dietary treatments.

## Bridge to chapter 7

In the experiment reported in chapter 6, I evaluated the dynamics of rumen bacterial community during development of frothy bloat and further assessed the impact of bloat preventative strategies including supplementation of Alfasure and grazing mixed sainfoin-alfalfa pasture on the composition and functional properties of rumen bacteria. I observed that bloat-associated shifts were more pronounced in the composition of bacterial communities in solid fraction of rumen contents. Rumen anaerobic fungi are primary colonizers of fibrous plant materials which contribute to physical deconstruction of plant cell walls, and therefore, act as an extension of bacterial biofilms. Despite important contributions of ARF to digestion of plant materials, the potential contribution of these group of microorganisms to metabolic disorders of cattle, in particular frothy bloat remain largely unknown.

**Rationale**: Rumen anaerobic fungi play central role in biodegradation of plant fibrous material. This group of fungi can facilitate access of bacteria to plant cell wall through physical destruction of fibers, but some members of ARF may also compete with ruminal bacteria over available resources due to the availability if overlapping enzymatic repertoire and metabolic pathways. The aim of the next chapter of my thesis was to determine the composition of ARF associated with solid fraction of the rumen content, and to comprehensively evaluate the dynamics of this group of fungi during development of frothy bloat and in response to bloat preventative strategies. Further, I sought to characterize bloat-associated shifts in the interaction profile of ARF and rumen bacterial community.

# 7 Interrelationships of Fiber-Associated Anaerobic Fungi and Bacterial Communities in the Rumen of Bloated Cattle Grazing Alfalfa

## 7.1 Abstract

Frothy bloat is major digestive disorder of cattle grazing alfalfa pastures. Among the many factors identified to contribute to the development of frothy bloat, the disruption of rumen microbiota appears to be of central importance. Anaerobic rumen fungi play an important role in sequential breakdown and fermentation of plant polysaccharides, thus serving as extensions of bacterial biofilms. In the present study, we investigated the dynamics of ARF during development of alfalfa-induced frothy bloat and in response to bloat preventive treatments. By sequencing the ITS1 region of metagenomic DNA from solid fraction of the rumen content, we were able to identify 8 distinct genera of the ARF, including *Neocallimastix*, *Caecomyces*, *Orpinomyces*, *Piromyces*, *Cyllamyces*, *Anaeromyces*, *Buwchfawromyces, and* unclassified Neocallimastigaceae. Overall, transition of steers from baseline hay diet to alfalfa pastures was associated with drastic changes in the composition of fungal community, while the overall composition of ARF did not differ significantly among bloated and non-bloated steers on different treatments. By performing correlation network analysis among the proportion of ARF and ruminal bacterial communities, we were able to identify hub fungi species that showed considerable number of negative correlations with several bacterial species, suggesting the presence of inter-kingdom competition between these two major groups of rumen microorganisms. Interestingly, the number of negative correlations among ARF and bacteria decreased during development of frothy bloat, indicating the disruption of normal microbial profile within bloated rumen ecosystem. Better understanding of fungal-bacterial interactions that differ among bloated and non-bloated rumen ecosystem could advance our understanding of the etiology of frothy bloat.

## 7.2 Introduction

Alfalfa (*Medicago sativa* L.) is a perennial legume with the potential to improve productivity and sustainability of pasture-based beef production [114]. However,

susceptibility of cattle to frothy bloat, a common digestive disorder associated with grazing of vegetative alfalfa can cause mortalities [113] and has hampered the widespread inclusion of this forage in grazing systems. Among the many factors identified to contribute to the development of frothy bloat, the disruption of rumen microbial fermentation appears to be of central importance [113]. In a functioning rumen, interactions among different species of bacteria, fungi, archaea, and protozoa results in the sequential breakdown and fermentation of non-structural and structural plant polysaccharides [1]. However, the high concentration of fermentable proteins and carbohydrates in vegetative alfalfa, can promote the unrestrained proliferation of bacteria within the rumen. These bacteria in turn produce excessive amounts of exopolysaccharides (i.e. slime) and fermentation end products, increasing the viscosity of rumen fluid and generating a stable foam that traps gas and prevents it from being expelled by eructation [113, 262]. Several mitigation strategies have been developed to control the incidence of frothy bloat in alfalfa grazing systems [113]. These include selection for bloat-resistant alfalfa cultivars (e.g. AC Grazeland [167]), the use of water-soluble pluronic detergents that reduce the viscosity of rumen contents [263], and incorporation of non-bloating forages containing condensed tannins into alfalfa pastures [264]. Although plant factors and physiological mechanisms underlying the development of frothy bloat are reasonably well-described, contribution of the different groups of ruminal microorganisms to bloat and how they may be manipulated to prevent it are poorly understood.

Changes in the rumen bacterial communities during alfalfa-induced frothy bloat and in response to bloat preventatives have been previously described [265]. Anaerobic rumen fungi (ARF) also play an important role in sequential breakdown and fermentation of plant polysaccharides. These strictly anaerobic microorganisms are known as primary colonizers of fibrous plant materials [50]. Upon attachment to plant cell walls, the zoospores of ARF produce rhizoidal structures capable of penetrating the lignocellulosic tissues of the plant cell wall, thereby providing access to fermentable carbohydrates within the cell interior that are not readily accessible by rumen bacteria [51, 52]. In addition to physical deconstruction of the plant cell wall, ARF also possess a wide variety of polysaccharide degrading enzymes, including cellulases, hemicellulases, amylases and

xylanases that contribute to the enzymatic hydrolysis of plant polysaccharides [50, 53]. Due to this diverse enzymatic repertoire, interspecies interactions and nutrient competition between ARF and rumen bacteria has been the subject of several *in vitro* studies [38, 54, 55]. However, given the immense diversity and complexity of rumen microbiota, *in vitro* assays fail to provide a comprehensive picture of microbe-microbe interactions within the rumen ecosystem. Alternatively, correlation network analysis has emerged as a promising tool for exploring co-occurrence patterns within complex microbial communities, providing insights into potential interspecies interactions and microbial community dynamics within the rumen ecosystem [74, 266].

Currently, ARF are classified into 18 distinct genera belonging to the phylum Neocallimastigomycota [56]. Among these, *Neocallimastix*, *Piromyces*, *Caecomyces*, *Cyllamyces*, *Orpinomyces*, *Anaeromyces, Pecoramyces, and Buwchfawromyces* have been previously identified in the rumen and fecal contents of cattle using a combination of culture-dependent and marker-gene sequencing methodologies [57-61]. These genera can be distinguished by morphological features, or through genetic variations in the ITS region of the ribosomal RNA (rRNA) locus [267, 268]. In the present study, we hypothesized that rapid proliferation of bacterial biofilm during development of frothy bloat can disrupt interrelationships of anaerobic fungi with fiber-associated bacterial communities. We further investigated if the administration of the pluronic detergent, Alfasure™ in water and inclusion of condensed tannin containing sainfoin would preclude the development of this dysbiotic state in cattle grazing alfalfa pastures.

## 7.3 Methods

### 7.3.1 Ethics Statement

Protocol (1214) for this experiment was reviewed and approved by the Animal Care Committee of the Lethbridge Research and Development Centre (LRDC), Agriculture and Agri-Food Canada, Lethbridge, Alberta. Cattle were cared for according to the guidelines of the Canadian Council on Animal Care (CCAC, 1993) [269].

### 7.3.2 Experimental Design, Animal Management and Assessment of Bloat Scores

The animal experiment for this study was the same as previous chapter, only selecting a subset of samples (i.e., the solid fraction of rumen contents) for the study of ARF. In brief, 12 ruminally-fistulated Aberdeen Angus steers were allocated to a 3 × 3 repeated measurement design, subjecting all animals to three different treatments evenly distributed across three 11-day time periods. An adaptation period (baseline phase) of 3 weeks preceded the experiment during which all steers were offered ad libitum alfalfa hay. Steers were then subjected to three different treatments: (1) pure alfalfa pasture (PA), (2) pure alfalfa pasture with Alfasure™ (AA), and (3) mixed alfalfa - sainfoin pasture (AS). During both PA and AA treatments, steers grazed pure stands of alfalfa, but steers in AA treatment additionally received the pluronic detergent Alfasure™ (0.25 mL/L) in their drinking water to prevent bloat. This dosage delivered ≈ 9.5 g/d of poloxalene to each animal. During the AS treatment, steers grazed alfalfa pasture containing sainfoin which accounted for ≈ 15% of pasture DM. Each experimental period consisted of 11 days, an adaptation/washout phase (7 days, during which confined steers were offered alfalfa hay ad libitum) and a sampling phase (4 days, during which steers were grazed on pastures). On each of the sampling days, all groups were released at 08:30 into either pure vegetative alfalfa (PA and AA treatments) or mixed vegetative alfalfa–sainfoin pastures (AS treatment). Stocking densities were adjusted by using electric fencing to ensure that pastures were always vegetative and steers were allowed to graze freely for 6 h until 14:30. Steers were then removed from the pasture and housed in a pen with freely available water, but no feed.

Steers were monitored every 30 min during grazing and for 2 h after grazing to record incidences of bloat according to the protocol described by Majak et al. [224]. In summary, bloat scores were assigned as follows: 0 = Normal (no visible sign of bloat), 1 = Slight (slight distention of the left side of the animal), 2 = Marked: (marked distention of the left side of the animal with an asymmetrical egg-shape as observed from the rear), and 3 = Severe (severe distention on the left side so that it is observable along the top of back from the right side of the animal). A single steer bloating on one day was counted as

single bloat incidence. In the PA treatment, once the sum of bloat incidences reached 9 or more over the sampling period, steers were crossed over between treatments and the period was repeated until an additional 9 cases of bloat occurred on the PA treatment.

### 7.3.3 Rumen Sample Collection and Processing

For the purpose of this study, rumen samples collected from steers that did not bloat (score of 0) were compared to those from steers that achieved a bloat score between 1 to 3. Baseline samples of rumen digesta were collected from each steer at the end of the primary adaptation period. All rumen samples consisted of composites collected from the cranial, caudal and ventral sacs. In all treatment periods, after the steers were introduced to the pasture, rumen digesta were collected between days 8 and 11, immediately after they were removed from their assigned paddock in the afternoon.

Approximately 50 g of total ruminal digesta was collected and transferred into a heavy-walled 250-mL beaker and squeezed with a Bodum coffee maker plunger (Bodum Inc.,Triengen, Switzerland). Solid residue was suspended in 30 mL of cold (4 ºC) grinding buffer (100mM Tris-HCl, 500mM EDTA, 1.5M NaCl, 1 mL proteinase K, pH 8.0). The extruded liquid was placed in a shallow aluminum foil dish and flash frozen in liquid nitrogen. All samples were stored at -80 ºC.

### 7.3.4 DNA Extraction and Quality Check

For the purpose of this study, frozen solid residues were ground under liquid nitrogen in a precooled porcelain mortar. Samples were then transferred into a precooled Retsch RM 100 Mortar Grinder equipped with a stainless-steel mortar bowl and pestle (F. Kurt Retsch GmbH and Co. KG, Haan, Germany) and ground for a further 5 min under liquid nitrogen. As needed, liquid nitrogen was added to the mortar bowl during grinding to maintain the mixture in a semi-fluid state. Genomic DNA was then extracted from 150-250 mg of each sample using QIAamp DNA Stool Mini Kit following the manufacturer's protocol (Qiagen Inc., Mississauga, ON, Canada).

### 7.3.5 Library Construction and Illumina Sequencing

For bacterial communities, sequencing libraries targeting the V3-V4 region of the 16S rRNA gene were constructed as described previously [265]. For preparation of fungal sequencing libraries, the fungal-specific primer set of the Earth Microbiome Project, ITS1f (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS2 (5′-GCTGCGTTCTTCATCGATGC-3′) was used to target the internal transcribed spacer region of the fungal genomes [270]. PCR reaction for each sample was performed in duplicate and contained 1.0 µL of pre-normalized DNA (20 ng/µL), 1.0 µL of each forward and reverse primer (10 µM), 12 µL of molecular grade water (Fisher Scientific, Ottawa, ON, Canada) and 10 µL of 5 Prime Hot MasterMix (5 Prime Inc., Gaithersburg, MD, USA). Reactions consisted of an initial denaturing step at 94°C for 2 min followed by 35 amplification cycles at 94°C for 45 sec, 52°C for 45 sec, and 70°C for 60 sec; finalized by an extension step at 70°C for 10 min in an Eppendorf Mastercycler pro (Eppendorf, Hamburg, Germany). PCR products were then purified using ZR-96 DNA Clean-up Kit (ZYMO Research, Irvine, CA, USA) to remove primers, dNTPs and reaction components. Fungal ITS2 libraries were then generated by pooling 200 ng of each sample as quantified by Picogreen dsDNA (Invitrogen, Burlington, ON, Canada). This was followed by multiple dilution steps using pre-chilled hybridization buffer (Illumina, San Diego, CA, USA) to bring the pooled amplicons to a final concentration of 5 pM, as measured by a Qubit 2.0 Fluorometer (Life technologies, Burlington, ON, Canada). Finally, 15% of PhiX control library was spiked into the amplicon pool to improve the unbalanced and biased base composition of the fungal ITS libraries. Customized primers for sequencing reads R-1 (5′-TTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCC-3′), R-2 (5′-CGTTCTTCATCGATGCVAGARCCAAGAGATC-3′) and R-Index (5′-TCTC GCATCGATGAAGAACGCAGCCG-3′) were synthesized and purified by polyacrylamide gel electrophoresis (Integrated DNA Technologies, Coralville, IA, USA) and added to the MiSeq Reagent Kit V3 (600-cycle) (Illumina, San Diego, CA, USA). The 300 paired-end sequencing reaction was performed on a MiSeq platform (Illumina, San Diego, CA, USA) at the Department of Animal Science, University of Manitoba, Canada. Bacterial and fungal sequencing data were uploaded into the Sequence Read

Archive (SRA) of NCBI (http://www.ncbi.nlm.nih.gov/sra) and can be accessed through accession number PRJNA663462.

### 7.3.6 Bioinformatics

The default settings of FLASH assembler (version 1.2.11 [182]) were used to merge the overlapping paired-end Illumina fastq files. UPARSE algorithm (version 9 [71]) was used for quality filtering of the reads based on maximum expected error value = 1.0 (using options "-fastq_filter" and "-fastq_maxee 1"). The UNOISE2 algorithm [271] was then used to further refine sequencing reads and generate amplicon sequence variants (ASVs). Compared to traditional OTU clustering methodologies, the use of ASVs provide several advantages for microbial marker gene data analyses [272]. For bacterial ASVs, taxonomies were assigned by UCLUST consensus taxonomy assigner (version = 1.2.22) [227] using the GreenGenes database (release May 2013). For fungal ASVs, taxonomies were assigned by SortMeRNA classifier (version 2.0, 29/11/2014) [273] using the UNITE dynamic database (version 8, 04/02/2020 [274]). Unassigned ASVs, and those assigned to the class of Chloroplast and family of Mitochondria were removed from the final bacterial ASV table. Unless specified otherwise, all samples were rarefied to 25,000 bacterial ASV/sample for downstream analyses. Due to the importance of anaerobic rumen fungi in fiber degradation and ruminal fermentation, the fungal ASV table was filtered so that only reads belonging to the Neocallimastigomycota (strict anaerobe rumen fungi) were retained. The subsequent fungal ASV table was rarefied to 7,000 ASV/sample for downstream analyses.

### 7.3.7 Statistical Analysis

Differences in Shannon index (diversity) and Chao1 index (richness) of rumen fungi between the baseline hay diet (n=12) and each treatment diet [PA, n=11; AA, n=10; and AS, n=11] were assessed using the Wilcoxon signed-rank test for matched-pairs. Diversity and richness were also compared between steers that developed bloat (n=6) and those that did not (n=5) while grazing alfalfa-sainfoin pastures. The Wilcoxon signed-rank test was selected for this comparison after determining that ASV count data for ARF were non-normally distributed.

Microbiota present in at least 50% of samples were used to assess rumen fungi (79 ASVs) and bacterial composition (1518 ASVs). Redundancy analyses (RDA; Vegan package) [275] were performed to assess the observed variation in the composition of rumen fungi explained by diet (overall). For each treatment, diet was compared to the baseline and bloat-status within the alfalfa-sainfoin diet. Identical analyses were performed to assess the variation in rumen bacterial composition explained by diet and bloat-status. The ASV counts were center-log transformed using the CoDaSeq package [276] after imputation of zeros using a Bayesian multiplicative replacement method implemented in the zCompositions package [277].

Correlation network analysis (CoNet [278]) was used to assess co-occurrence and mutual exclusion relationships between bacterial and fungal ASVs and identify fungal hub ASVs that showed the highest number of positive/negative correlations within bacterial communities. In CoNet's ensemble method, a combination of different correlation and dissimilarity measures (Spearman's rank correlation coefficient, Kendal correlation coefficient, Bray-Curtis dissimilarity distance, and Kullback-Leibler Divergence) were used to infer co-occurrence networks. In brief, for each measure, distributions of all pairwise scores between the nodes (a node representing the relative abundance of an ASV that was found in at least 50% of the samples) were computed. For each measure and edge (an edge representing a positive or negative correlation between two nodes), 1000 permutations were conducted, including a renormalization step for Spearman correlations in order to address the issue of compositionality introduced by different sequencing depths among samples. For all correlation and dissimilarity measures, *p* values were computed as the probability of the null value (represented by the mean of the null distribution) under a Gauss curve generated from the mean and standard deviation of the bootstrap distribution. Measure-specific *p*-values were then merged using Brown's method [279] and subjected to Benjamini-Hochberg's FDR correction. An edge was considered significant and kept in the final network if (a) it was supported by at least three measures, (b) it had a merged *p* value below 0.05, and (c) it was within the 95% confidence interval defined by the bootstrap distribution. Using the above methodology, an analysis was conducted including all steers with both bacterial and fungal data (the

overall network), as well as a stratified analysis to assess the potential for unique interactions within those steers that bloated and those that did not. The 4 ASVs that had the most connecting edges (most interactions) within the overall network, within the no-bloat network and/or within the bloat network (10 ASVs in total) were further assessed as potentially important ASVs (i.e. hub ASVs). A second redundancy analysis was performed to assess observed variation in rumen bacterial communities explained by the selected fungal "hub ASVs". The observed variation in overall bacterial composition explained by each fungal hub ASV was assessed, along with the total variation explained by all selected hub ASVs (using an $R^2$ adjusted for multivariate analysis). Total variation explained by hub ASVs was also assessed within bloated and non-bloated steers.

An analysis of variance permutation test (lmPerm package) [280], to associate diet and bloat status with center-log transformed ASV abundances (for 133 ASVs present in at least 10% of samples). In all statistical models, the effect of individual steers was included in the model as a random factor to account for individuality of microbiota in repeated measurement design. Correction of *p*-values across ASVs was done using the Benjamini-Hochberg procedure. Pairwise comparisons were performed for the 51 ASVs identified as being significantly associated with diet/bloat-status combination after *p*-value correction ($p_{FDR}<0.05$). Specifically, the baseline diet was compared to other diet/bloat-status combinations [AA (no bloat, n=10); PA (bloat, n=11); AS (bloat, n=6); and AS (no bloat, n=5] using the Wilcoxon signed-rank test for matched pairs and *p*-values were corrected across ASVs (within each test) using the Benjamini-Hochberg procedure. Further, to assess the effect of bloat within diet, bloat status was compared within the alfalfa-sainfoin diet using the same *p*-value correction method.

## 7.4 Results

### 7.4.1 Bloat Incidence

Grazing steers on PA pasture resulted in in 11 out of 12 steers developing frothy bloat over the course of the three periods, with one steer being removed from the trial after the first period due to lameness. Conversely, the addition of Alfasure™ to drinking water completely prevented bloat in all steers across all treatment periods. Grazing steers

on AS pasture was moderately effective at preventing bloat, with 5 out of 11 steers not bloating on this treatment (Table.6).

**Table 6. Summary of study design and incidences of bloat**

| Period | Treatment[1] | | | | | |
|---|---|---|---|---|---|---|
| | PA | | AA | | AS | |
| | Bloat incidence[2] | Steers | Bloat incidence | Steers | Bloat incidence | Steers |
| Period 1 | NB | 0 | NB | 4 | NB | 0 |
| | B | 4 | B | 0 | B | 4 |
| Period 2 | NB | 0 | NB | 3 | NB | 3 |
| | B | 3 | B | 0 | B | 1 |
| Period 3 | NB | 0 | NB | 3 | NB | 2 |
| | B | 4 | B | 0 | B | 1 |

[1]Within each period, steers were subjected to one of the three treatment groups including 1) pure alfalfa pasture (PA), 2) pure alfalfa pasture supplemented with Alfasure™ (AA), and 3) alfalfa – sainfoin mixed pasture (AS).

[2]Bloat incidence indicates the number of steers within each treatment groups that either developed clinical bloat (B; bloat scores 1-3) or did not bloat (NB; bloat score 0).

### 7.4.2 Sequencing Results and Phylogenetic Diversity of the Fiber-Associated Microbial Communities

Taxonomic assignment of ASVs resulting from fungi-specific ITS primer set revealed the presence of 3 dominant fungal phyla across all samples, including Neocallimastigomycota 78.67% (SD =18.30%), Ascomycota 13.18% (SD =14.57%), and Basidiomycota 1.85% (SD =0.28%). The final ASV table was filtered so that only the Neocallimastigomycota were retained for downstream analysis. After quality filtering, the final ASV table for fiber-associated ARF contained an average of 111,724 (SD = 67,191) ASVs per sample, comprised of 189 unique ASVs. Within the Neocallimastigomycota, 8 distinct genera were identified across all samples, including *Neocallimastix* 14.70% (SD =8.20%), *Caecomyces* 28.06% (SD =16.96%), *Orpinomyces* 18.00% (SD =13.08%), *Piromyces* 21.11% (SD =15.42%), unclassified

Neocallimastigaceae 13.06% (SD =12.73%), *Cyllamyces* 10.89% (SD =14.64%), *Anaeromyces* 0.60% (SD =0.59%) and *Buwchfawromyces* 0.45% (SD=0.71%). The final ASV table for bacterial communities contained an average of 55,575 (SD = 18,676) reads per sample, comprised of 5,554 unique ASVs. Taxonomic assignment of ASVs resulting from V3-V4 primer set revealed the presence of five dominant phyla including Bacteroidetes 54.27% (SD = 9.64%), Firmicutes 28.71% (SD = 6.94%), Fibrobacteres 6.89% (SD = 3.91%), Spirochaetes 4.09% (SD = 1.86%), and Proteobacteria 0.78% (SD = 0.34%). Detailed description of bacterial communities and their associations with bloat development and preventive strategies in this experiment have been previously described [265].

### 7.4.3 Diversity of Anaerobe Rumen Fungi is Associated with Diet and Bloat-Status

Fungal richness (Chao1 index) and diversity (Shannon index) of rumen contents were compared between the alfalfa hay and the grazing treatments (Figure 13A), as well as between steers which bloated and those that did not when grazing AS (Figure 13B). Overall, fungal richness was lowest in the baseline hay diet, and increased with the grazing of AA and AS pastures. The greatest increase (compared to baseline) was observed when cattle were grazing pure-alfalfa. Meanwhile, compared to the baseline diet, diversity increased ($p = 0.03$) only in steers that did not bloat (i.e., baseline vs. AA, $p=0.03$; and baseline vs. AS no-bloat, $p = 0.03$). Steers that did not bloat while grazing AS also had higher ($p=0.02$) fungal richness compared to those that bloated (Figure 13B). Overall, fungal diversity did not correlate with the diversity of rumen bacteria (*rho*=0.23, $p=0.15$), even after stratification by bloat-status (No bloat, *rho*=0.24, $p=0.26$; Bloat, *rho*=0.074, $p=0.78$).

### 7.4.4 Composition of Rumen Fungi

Overall, composition of rumen fungi was largely associated with diet (i.e. baseline hay diet vs. alfalfa pastures), which explained over 30% of the observed variation in the ARF composition (Figure 13C and D). Within the AS treatment, although development

of bloat did not influence the overall composition of ARF, it did influence the bacterial composition ($P < 0.05$); Figure 13C).

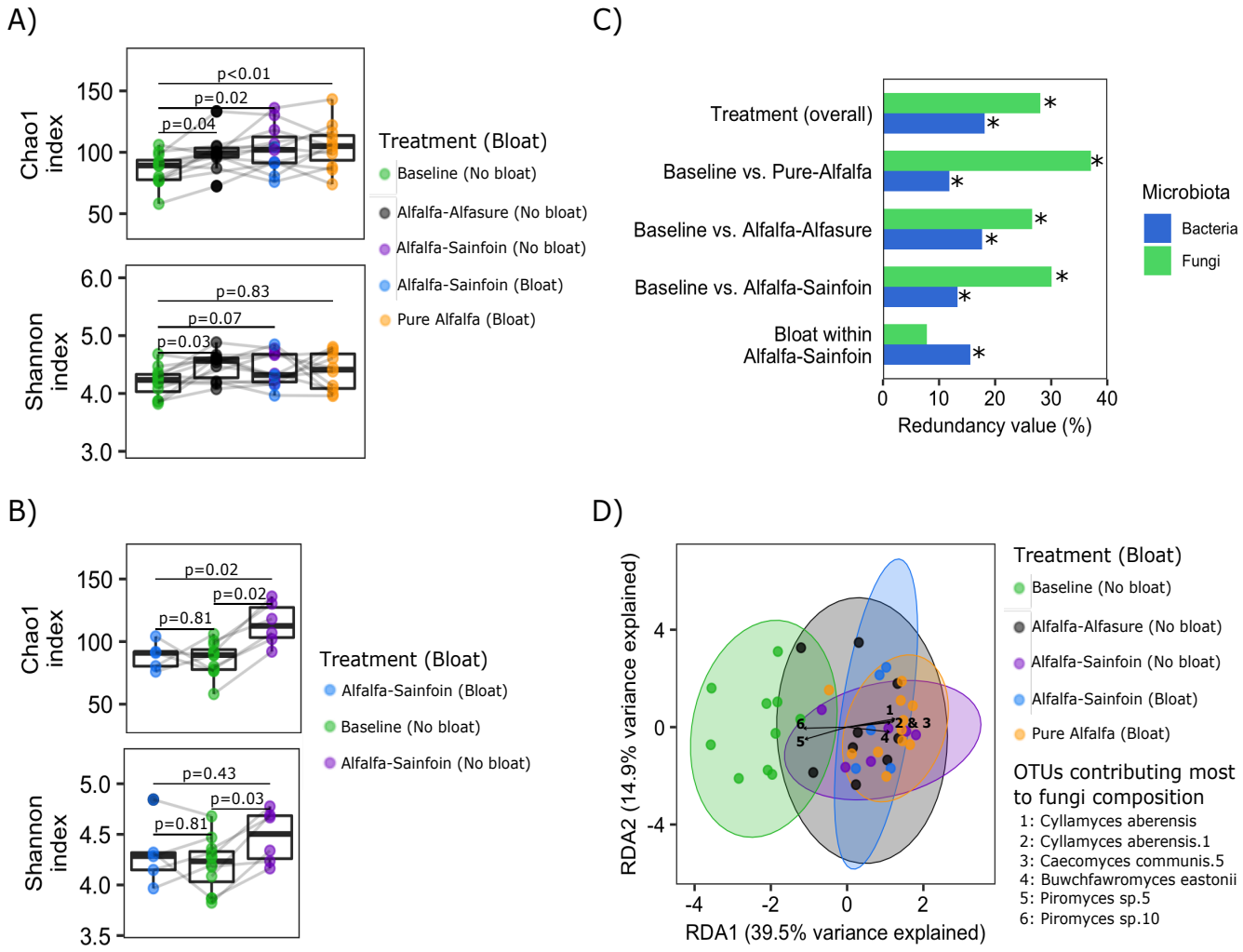



**Figure 13. Comparisons of richness, diversity and composition of rumen fungi communities in steers fed alfalfa hay or grazing alfalfa or alfalfa-sainfoin pastures.**

(A) Comparison of richness (Chao1 index) and diversity (Shannon index) among treatment groups and (B) comparisons of richness (Chao1 index) and diversity (Shannon index) among baseline hay diet and bloated and non-bloated steers on the alfalfa-sainfoin diet. Boxes show the interquartile range with the line at the median and whiskers showing minimum and maximum values. A line between two individual dots indicates the same

steer in different groups. Comparisons were tested using Wilcoxon signed-rank test. (C) Summary statistics of redundancy analyses assessing the association of the overall composition of fungi and bacterial communities with treatments in general (top), individual pair-wise compassions between baseline hay diet and each of the alfalfa pasture treatments (three middle), and bloated vs. non-bloated steers within the alfalfa-sainfoin treatment (bottom). Redundancy values indicate the percentage of observed variation in fungi composition explained by each factor [Treatment overall, n=44; Pairwise comparisons: Baseline vs. Prue-Alfalfa, n=22; Baseline vs. Alfalfa-Alfasure™, n=20; Baseline vs. Alfalfa-Sainfoin, n=22; Bloat vs. non-bloat in Alfalfa-Sainfoin, n=11]. * = $p$ <0.05. (D) Redundancy analysis biplot depicting differences in fungal composition between treatment groups/bloat status. Arrows indicate 7 ASVs that accounted for most of the differences in fungal composition (e.g., *Neocallimastigaceae* sp.7 and sp.3 (arrows ID 1 and 2) tend to be enriched in the rumen content of steers on baseline diet).

The ADONIS pairwise comparison also revealed considerable differences in the overall composition of ARF between the baseline hay diet and other treatments (baseline vs. PA, $R^2$=0.44 and $p$<0.01, baseline vs. AA, $R^2$=0.36 and $p$<0.01; baseline vs. AS (no-bloat) $R^2$=0.30 and $p$<0.01; baseline vs. AS (bloat) $R^2$=0.35 and $p$<0.01). Certain ASVs were found to make the largest contribution to differences in the composition of ARF between treatments; *Piromyces* sp.5 and *Piromyces* sp.10 were associated with the baseline diet whereas in general, *Cyllamyces aberensis*, *Cyllamyces aberensis*.1, *Caecomyces communis.5*, and *Buwchfawromyces eastonii* were associated with steers grazing alfalfa (Figure 13D).

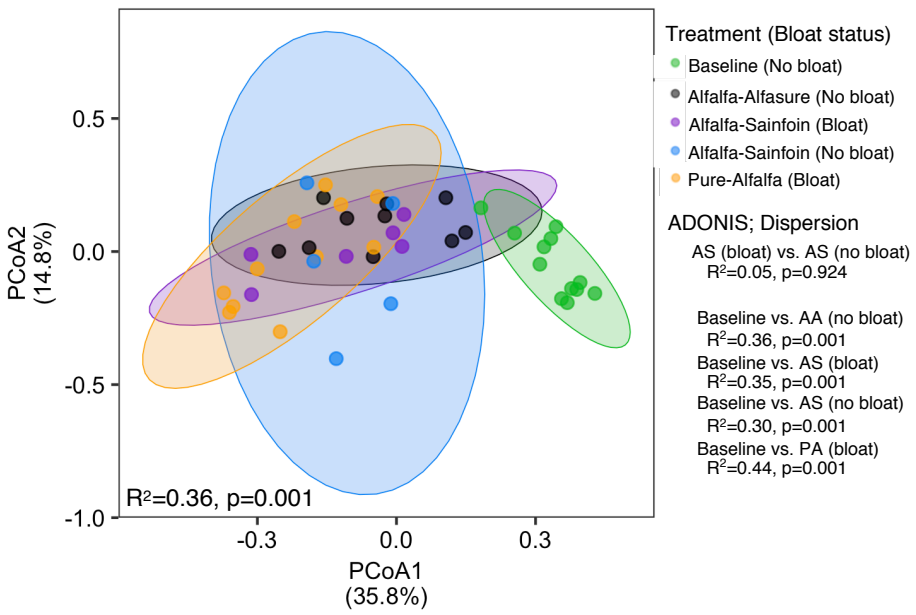



**Figure 14. Beta-diversity of ruminal fungal communities.**

Principal coordinate analysis (PCoA) was used for visualization of Bray-Curtis dissimilarities of the fungal communities in the rumen content of steers subjected to different dietary regimens. Color codes were used to differentiate treatment groups and bloat status. The ADONIS package of R was used for performing Permutational Multivariate Analysis of Variance (PERMANOVA) to assess the effect of dietary treatments/bloat status on the overall composition of fungal communities. ADONIS R2 for the overall model and pair-wise comparisons show the explanatory power of diets/bloat status to differentiate ruminal fungal communities. For all tests, p values $< 0.05$ were considered significant.

The relative proportions of fungal genera were altered following transition from baseline diet to alfalfa pastures (Figure 15A). We observed that regardless of bloat incidence or inclusion of sainfoin or Alfasure™, transition from the baseline diet to alfalfa pastures enriched *Cyllamyces, Caecomyces, Anaeromyces* and *Buwchfawromyces,* whereas; *Neocallimastix*, *Piromyces* and unclassified Neocallimastigaceae accounted for a larger proportion of the fungal population in the rumen of steers consuming the baseline diet. Compared to other genera, *Orpinomyces* appeared to be more universally distributed

among steers regardless of treatment. Among all possible pair-wise comparisons, the proportion of *Neocallimastix*, *Piromyces* and unclassified Neocallimastigaceae were significantly lower in bloated steers on AS compared to the baseline diet (Figure 15B).

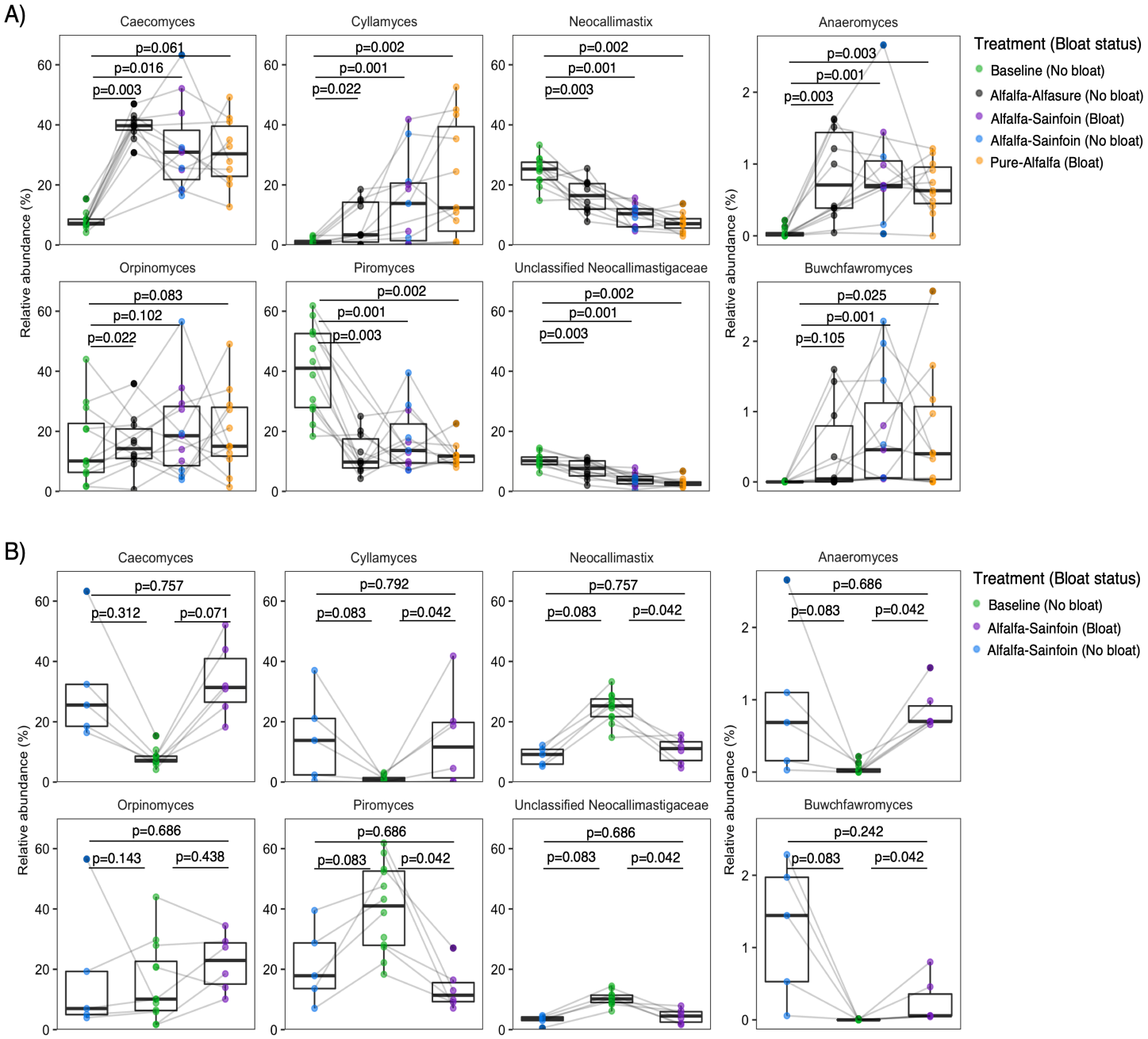



**Figure 15. Pair-wise comparisons of the proportion of anaerobic rumen fungal genera.**

Comparisons (A) between baseline and each of the treatments, and (B) among steers that did and did not bloat while grazing alfalfa-sainfoin and the baseline alfalfa hay diet. Boxes show the interquartile range with a line at the median and whiskers showing minimum and maximum values. A line between two individual dots indicates the same steer in different groups. Comparisons were tested using Wilcoxon signed-rank test on center log-ratio proportions. The FDR corrected p-values were obtained using Benjamini-Hochberg procedure.

### 7.4.5 Co-occurrence Patterns of ARF with Rumen Bacterial Community in Relation to Treatments

Co-occurrence patterns between fungal and bacterial ASVs were assessed across all steers (Figure 16A), as well as between steers that did and did not bloat. Overall, a large percentage of observed interrelationships among fungal and bacterial ASVs were mutually exclusive (i.e. negative relationship between the proportion of two ASVs; including 81% of relationships in the overall network, 88% in bloat network, and 77% in non-bloated network), suggestive of the presence of competitive interactions among fungi and bacteria within the rumen. Within each network, the top 4 ASVs having the highest number of connections (i.e. significant relationships) with other ASVs in the network were all fungi. Figure 2B depicts fungal hub ASVs with a considerable number of connections to various rumen bacteria (showing > 10 significant +/- relationships in one network). These fungal hub ASVs were next assessed in downstream analyses for their potential influence on the composition of the rumen bacterial population (Figure 16C). Interestingly, redundancy analysis revealed that *Buwchfawromyces eastonii,* a major hub-ASV identified in the network analysis of steers without bloat, had the highest explanatory power regarding variations in ruminal bacterial composition (Figure 16C). Similarly, *Piromyces* sp.5, *Caecomyces communis.*5 and *Piromyces* sp.7, identified as hub-ASVs by network analysis of non-bloated steers, could also explain more than 5% of the overall variation in ruminal bacterial composition. The only major fungi hub-ASV in the network of steers with bloat (*Neocallimastix frontalis.*5), generally did not explain as much variation in rumen bacterial composition (Figure 16C). Assessments of the combined influence of hub-fungi on ruminal bacterial composition revealed that changes

in the proportion of hub-fungi were associated with bacterial composition in rumen contents from both non-bloated ($R^2_{adj}$=9.7%, $p < 0.01$) and bloated steers ($R^2_{adj}$=4.9%, $p < 0.01$; Figure 16C).

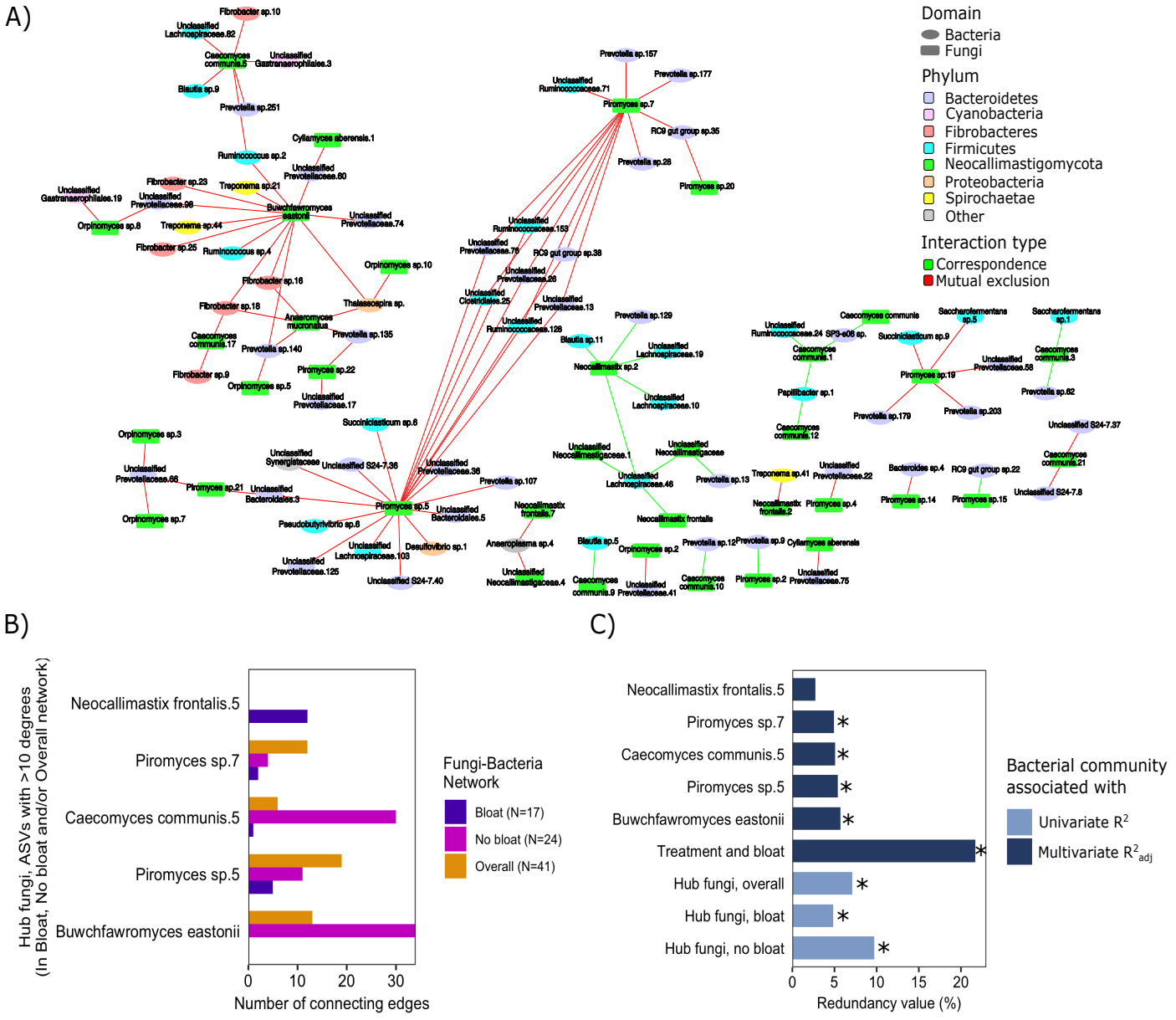



**Figure 16. Co-occurrence patterns between fungi and bacteria and association of hub fungal amplicon sequence variants "ASVs" with bacterial composition.**

(A) The overall network showing associations between fungi and bacteria among all steers and across all treatments (separate networks showing fungal-bacterial associations in bloated and non-bloated steers can be found in Supplementary Figures 2a and b, respectively). Nodes (representative of amplicon sequence variants "ASVs") are colored based on originating phyla, and edges (representing significant co-occurrence/competition relationships) are colored based on the type of relationships (red = negative relationship or mutual co-exclusion and green = positive relationship or co-occurrence). Only edges showing significant associations ($p$ (FDR) < 0.05) supported by at least 3 measures of correlation and/or dissimilarity are included in the network (Spearman rank correlation, Kendal correlation, Bray-Curtis and Kullback-Leibler dissimilarity). (B) Potential hub fungi ASVs identified across all networks (non-bloated steers, bloated steers and overall networks). The number of connecting edges indicates the number of associations each fungi ASV has with bacterial ASVs in each network. The y-axis shows ASVs with the most connecting edges (potential hub fungi) for each of the three networks. (C) Redundancy analyses showing the contribution of hub fungi ASVs to observed variation in overall bacterial composition (Redundancy value using center log-ratio proportions of fungal ASVs). Individual hub fungi were assessed in a single redundancy model adjusted for dietary treatment and bloat status (top 6 bars colored in dark blue). Three additional models (bottom 3 bars colored in light blue) show the combined contribution of all 10 hub fungi to observed variations in overall bacterial composition of the rumen content of all steers (Hub fungi, overall), overall bacterial composition of the rumen content of non-bloated steers (Hub fungi, no bloat), and overall bacterial composition of the rumen content of bloated steers (Hub fungi, bloat). * indicates $p < 0.05$.

The proportions of ARF identified as hub ASVs also differed between the baseline hay diet and other treatment groups, as well as among steers that did and did not bloat while grazing AS (Figure 17A and B). Compared to the baseline diet, the proportions of hub ASVs belonging to *Caecomyces communis*, *Cyllamyces aberensis*, *Anaeromyces* and *Buwchfawromyces eastonii* increased in the rumen contents from steers grazing alfalfa pastures. Among hub ASVs, *Buwchfawromyces eastonii* was among the ones that

contributed most to the difference in fungal composition between samples (Figure 13D), as well as being a hub ASV with over 30 connections with bacterial ASVs. The proportion of this fungi ASV increased when steers switched from baseline diet to alfalfa pastures. However, the proportion of this ASV was lower in the rumen content of steers that bloated on the AS treatment compared to the ones that did not bloat (Figure 17B). Although after FDR correction no ASVs differed significantly between bloated and non-bloated steers on AS treatment, which is likely in-part due to the low sample size of this comparison (n=6 bloat vs. 5 non-bloat), before FDR correction *Buwchfawromyces eastonii* was found to be enriched in the rumen content of steers that did not bloat on the AS treatment ($\boldsymbol{p}$ = 0.03).

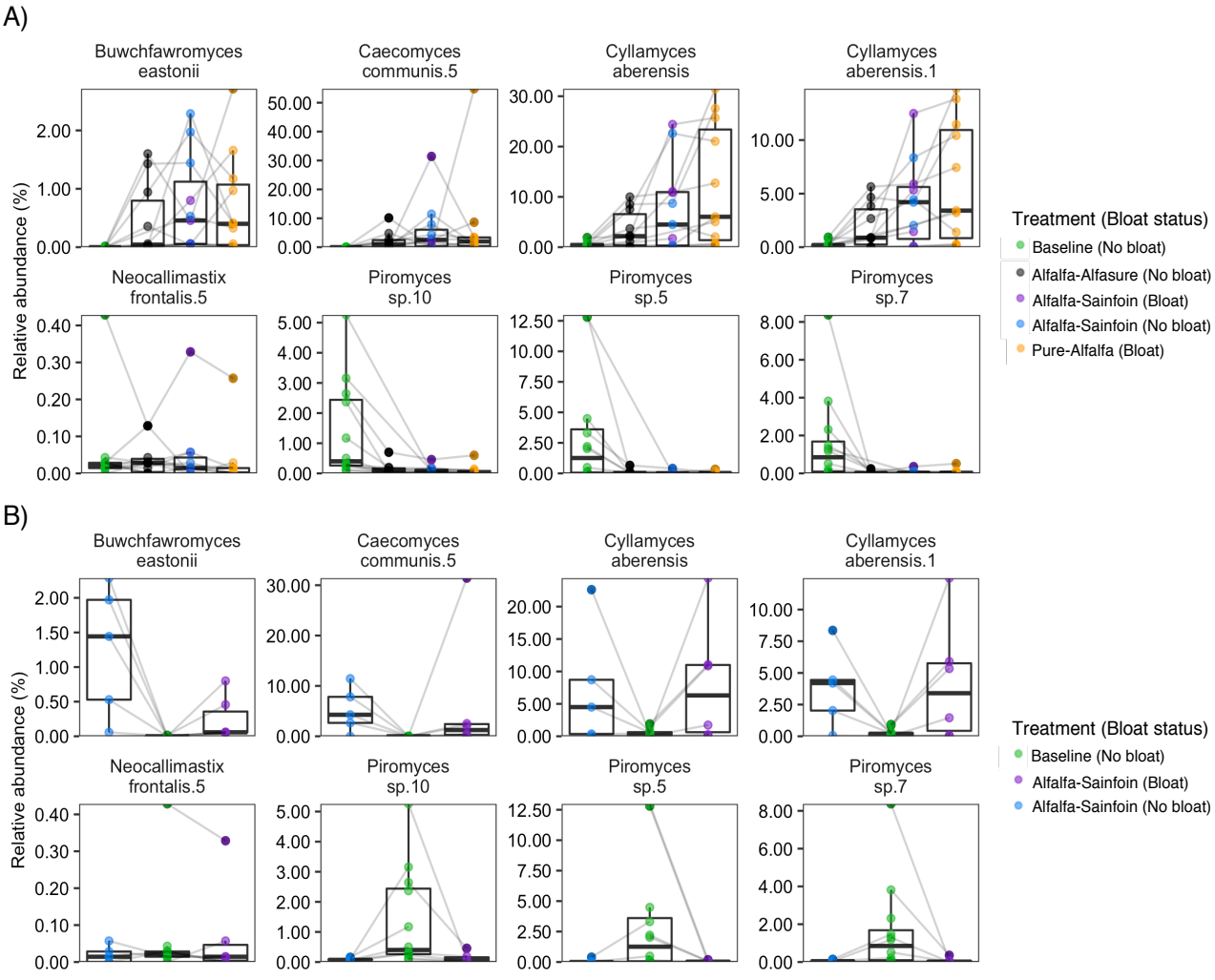



**Figure 17. Comparison of the proportions of anaerobic rumen fungi "ARF"**

Anaerobic rumen fungi identified as hub ASVs between the baseline hay diet and other treatment groups, as well as among steers that did and did not bloat on Alfalfa-Sainfoin "AS" treatment. Summary of statistics can be found in the supplementary data provided in the online version of the manuscript.

## 7.5 Discussion

The transition from alfalfa hay to alfalfa pastures and subsequent development of frothy bloat has previously been associated with the perturbation of rumen bacterial communities [281], but its impact on rumen fungal communities has not been investigated. In the current study, we characterized the fungal communities of the solid fraction of rumen digesta and their relationship to bacterial communities. In doing so, we provided a comprehensive description of the changes in the composition of anaerobic fungi during development of frothy bloat and characterized their co-occurrence patterns with rumen bacterial communities. In particular, we identified fungal hub species that were negatively correlated with a number of rumen bacterial species, suggestive of competitive interactions among these two major groups of ruminal microorganisms. We further observed that transition from alfalfa hay to alfalfa pasture, and subsequent development of frothy bloat was associated with drastic changes in the composition of ARF, but only exhibited moderate changes with the use of pluronic detergent or condensed tannin containing forages as preventatives.

Anaerobic rumen fungi are a phylogenetically unique group within the phylum Neocallimastigomycota. In the present study, the majority of sequencing reads obtained using the fungi-specific primer pair belonged to the phylum Neocallimastigomycota (~78%), enabling us to provide a comprehensive description of the ARF community within the rumen ecosystem. It is noteworthy to mention that our sequencing protocol was also able to detect aerobic fungal species belonging to the phyla Ascomycota and Basidiomycota. These fungi may represent epiphytic fungal populations inhabiting forages and other feed components, and likely do not contribute directly to ruminal fermentation. However, in a recent study utilizing metatranscriptomic data to extract 18S rRNA genes [282], authors also detected members of the phyla Ascomycota and

Basidiomycota in the rumen contents of cattle, and suggested that these fungi may play an active role in the scavenging of oxygen that enters the rumen during feed and water consumption. We identified eight distinct genera of the phylum Neocallimastigomycetes, including *Neocallimastix, Caecomyces, Piromyces, Anaeromyces, Orpinomyces, Cyllamyces*, and *Buwchfawromyces* in over 80% of the ruminal samples collected from steers on both baseline and alfalfa pasture. Studies using ITS1 primer sets different than the one used in the present study have reported recovery of less than 23% of the sequencing reads assigned to ARF [57] and identified fewer ARF genera, including *Piromyces, Anaeromyces, Cyllamyces, Neocallimastix*, *Caecomyces* and *Orpionmyces* [57, 283]. By using a similar primer set to our study, Kumar et al. [60] reported recovery of around 1% of the sequencing reads belonging to ARF and identified 3 genera within this phylum, including *Cyllamyces, Caecomyces* and *Orpinomyces*. Given the similarity of the primer set used to amplify fungal ITS regions, differences observed in the coverage of ARF between the latter study and ours could be mainly explained by differences in the fraction of the rumen digesta that had been used for isolation of metagenomic DNA. In our study, we isolated DNA from the solid (i.e. fibrous) fraction of the rumen digesta collected via the rumen cannula, whereas Kumar et al. [60] isolated DNA from rumen liquid collected via stomach tube. The main role of ARF within rumen ecosystem is to facilitate the decomposition of plant cell walls and enzymatic degradation of plant cell wall polysaccharides [51, 52]. As such, the primary colonization site of ARF in rumen ecosystem is believed to be fibrous plant materials [50], explaining why sequences belonging to ARF were enriched in our study. Moreover, differences in the taxonomic classifier used for fungal sequences could also contribute to differences in the coverage of ARF between the two studies - SortMeRNA classifier vs. RDP classifier [284]. In the present study, in addition to seven previously described genera of the phylum Neocallimastigomycota, we were also able to identify a group of unclassified ARF within the family Neocallimastigaceae. Previous studies have found evidence of uncharacterized isolates of rumen fungi, some of which may belong to the family Neocallimastigaceae [285, 286].

Rumen fungi produce all the enzymes necessary for the degradation of plant material, and this enzymatic degradation may also be aided by mechanical deconstruction of plant cell walls as a result of the penetration of fungal rhizoids [287, 288]. Previous in-vitro research has shown that rumen fungi more readily degrade recalcitrant plant cell walls as compared to rumen bacteria, and that there is synergistic activity between fungi and bacteria in co-culture [289]. While fiber degradation is likely aided by both rumen bacteria and fungi, our network analysis suggests that the relationship between bacteria and fungi is generally competitive. Rumen bacteria and fungi also can compete for the same substrates (e.g. fiber) in the rumen. In support of this, our correlation network analysis showed many negative associations between rumen fungi and bacterial groups associated with fiber degradation, including members of Fibrobacteraceae, Ruminococcaceae, Christensenellaceae and the genus *Treponema* [290, 291]. Negative interactions between rumen fungi and cellulolytic bacterial species of *Ruminococcus* have been identified in previous *in vitro* studies [288, 292]. This was hypothesized to involve the release of a polypeptides by ruminococci that inhibited cellulose hydrolysis by fungi. In our network analysis, rumen fungi also showed negative associations with *Prevotella,* a primarily amylolytic/proteolytic genus that is among the most abundant bacteria in the rumen [290]. The diverse enzymatic activity of rumen fungi also includes proteolytic and amylolytic activity [50, 53, 293], suggesting potential competition of this group of fungi with *Prevoltella* spp. for common substrates. Based on our results, two fungal species may be of particular importance, *Orpinomyces* (sp.8) and the other an unclassified Neocallimastigaceae (sp.3) as they exhibited the most negative interaction with rumen bacterial species in cattle. In addition, they explained the largest amount of variation in overall bacterial composition of all the ten hub-fungi tested.

The negative relationship between bacterial and fungal species observed in our network analysis was particularly notable in cattle that did not bloat; this relationship appeared to be disrupted in cattle that experienced bloat, with a large reduction in the number of negative associations between fungi and bacteria. In particular, hub fungi species showing the majority of negative relationships with bacteria in non-bloated rumen contents, were no longer associated with overall bacterial composition during

bloat. The reduction in negative relationships between bacterial and fungal species following transition to alfalfa pasture and with the onset of bloat may be the result of reduced competition for substrates. As cattle were switched from the baseline hay diet to vegetative alfalfa pasture, increased availability of nutrients could provide a more permissive environment for competing species to proliferate at the same rate. Another possible explanation for the reduced negative correlations between fungi and bacteria could be the difference in the nature of rumen contents. During frothy bloat, rumen contents are enriched in bacterial slime, resulting from uncontrolled proliferation of slime-producing bacterial populations, such as *S. bovis* and *S. ruminantium [249, 250].* Thus, the slime-enriched rumen contents may not harbor a similar ratio of fungi:bacteria as that of normal rumen contents. Within the rumen of non-bloated cattle, a few positive associations were also identified between specific fungi and bacteria. These positive associations were primarily observed between *Neocallimastix* species and bacterial species within the genus *Treponema* and family Ruminococcaceae. *Treponema* spp. in the rumen are enriched by diets high in pectin, such as alfalfa hay [294, 295]. Likewise, we found that the relative abundance of *Neocallimastix* was highest in alfalfa hay as the pre-grazing baseline diet. It is possible that *Treponema* and *Neocallimastix* thrive under similar dietary conditions, but in a non-competitive manner.

Condensed tannins within sainfoin are thought to be the primary components responsible for the bloat preventive properties of this forage, mainly as a result of a reduction in the rate of fermentation of soluble proteins and plant cell walls [127, 296]. Sainfoin cultivars in our study possessed about 30 g of extractable condensed tannins per kg of forage DM [57]. Previous research has identified a reduction in the number of cellulolytic bacteria, such as *Ruminococcus* spp., with the inclusion of forages containing condensed tannins in the diet [297]. However, rumen fungi capable of degrading cellulose, such as *Neocallimastix patriciarum,* are thought to be less affected by condensed tannins than cellulolytic bacteria [297-301]. Of the fungi genera examined, only *Neocallimastix* was in a higher relative abundance in steers grazing alfalfa-sainfoin as compared to those grazing only alfalfa. Interestingly, *in vitro*, increases in pectin concentrations can reduce the extent to which condensed tannins limit the rate of

cellulose digestion [296, 302]. Our current research indicates positive interactions between pectinolytic bacteria *Treponema* spp.[294, 295] and *Neocallimastix* spp. [303, 304], though whether this is related to the interaction between pectin and tannins merits further investigation.

The addition of sainfoin appeared to increase the diversity of fungal species compared to the baseline hay diet and grazed alfalfa, only in steers which did not bloat. This suggests a link between the bloat-prevention mechanism of sainfoin and rumen fungal diversity. High diversity has been suggested as a general indicator of a stable microbial community that is resistant to change during environmental perturbations [305], such as frothy bloat. However, the reason why inclusion of sainfoin increased the species diversity of rumen fungi in only a subset of animals in our study remains unknown. This may be in part explained by differences in selective grazing of sainfoin by individual animals. Whether reduced sainfoin intake in the subgroup of steers that developed bloat could be the reason for reduced fungal diversity, and potentially development of bloat, warrants further investigation.

In the present study, transition from baseline hay diet to alfalfa pastures was associated with drastic changes in the composition of the fungal community. Based on PCoA of Bray-Curtis dissimilarity and RDA analysis, the composition of the fungal community during the baseline alfalfa hay diet differed from that observed in all other grazing treatments. However, the overall composition of the fungal community did not differ significantly among bloated and non-bloated steers on different treatments (i.e. PA, AA, and AS). This observation suggests that fungal populations were impacted more by the nature of the forage – i.e. whether the forage was conserved as hay or grazed – rather than the development of bloat. In contrast, our RDA analysis showed that a higher percentage of the variation in the composition of the ruminal bacterial community could be explained by the development of bloat. The observed differences in the response of these two groups of microorganisms to bloat could be attributed to their physiological characteristics. Bacterial populations can rapidly proliferate upon gaining access to the readily fermentable carbohydrates and proteins within vegetative alfalfa, whereas the long life-cycle of anaerobic rumen fungi (8–32 h) [45] results in these microorganisms

proliferating at a slower rate than bacteria. Furthermore, bacteria can produce excessive amounts of mucopolysaccharide “slime” that can interact with proteins to produce the stable froth that traps gases and results in bloat [113, 262].

**Strengths and Limitations:** An important strength of the present study was our ability to comprehensively assess the overall composition of the ARF, including uncultivated/uncharacterized members of this group, and explore their response to bloat development and mitigation strategies. In particular, ITS gene sequencing of the solid fraction of rumen contents enabled us to recover a considerably higher percentage of the sequencing reads that belonged to ARF as compared to previous studies. Whereas previous research on interactions between rumen fungi and bacteria has been mostly limited to *in vitro* microbe-microbe experiments, having access to both bacterial and fungal sequencing data enabled us to explore the global interrelationships among these two groups of microorganisms within the rumen ecosystem. However, care should be taken when interpreting the results of network analysis of amplicon sequencing data, as this type of analysis is prone to a high rate of false-positives [74]. Furthermore, while our experiment was carried out in a cross-over design that reduced inter-animal variability in the response of rumen microbiota (i.e. same steer receiving each of the treatments), a disadvantage of this approach is the potential carry-over effect between dietary treatments even after the 7-day adaptation phase that preceded the sampling period of each treatment. Another limitation of our study design that might also contribute to the development of bloat was the overnight feed deprivation prior to each grazing period, as this could affect both the grazing behavior of steers and the ruminal microbial profile. However, in this case this management strategy was deliberately employed to increase the likelihood of bloat occurring. Nonetheless, being able to comprehensively describe the composition of anaerobic fungi in healthy and bloated rumen ecosystems, our results pave the way towards future research on the functional contribution of the rumen fungi to the development of frothy bloat.

## 7.6 Conclusions

Our experiment provides novel insights into the dynamics of the rumen fungal community during adaptation to alfalfa pasture. In general, we observed that transition from baseline alfalfa hay diet to alfalfa pasture was associated with drastic changes in the diversity and composition of rumen fungal community whereas the overall composition of this group of microorganisms was less affected by the development of bloat. In contrast, we observed that the ruminal bacterial community differed considerably between bloated and non-bloated steers, implying that rumen bacteria are the main drivers of frothy bloat. Rumen fungi play an important role in fiber degradation and our results are in agreement with previous studies regarding general competition between rumen fungi and fiber degrading bacteria. Identification of novel fungal-bacterial interactions that differed among bloated and non-bloated rumen ecosystem merits further investigations to advance our understanding of the etiology of frothy bloat.

# 8 General Discussion and Conclusions

## 8.1 General Discussion

Recent insights regarding substantial contributions of commensal microbiota to physiology of animals have led to the concept of a "holobiont", suggesting that phenotypic traits of an animal is determined by the interplay between its own genotype and those of its symbiotic microbes [306]. The most important contribution of the commensal microbiota to host physiology is the modulation of metabolic and immune homeostasis [307, 308]. In the context of the rumen ecosystem, microbial symbionts contribute to a wide range of structural and metabolic functions that are critical to the survival and performance of their host, including degradation of indigestible plant materials, metabolism of dietary nutrients, and biosynthesis of vitamins. Indeed, metabolic products of rumen microbiota  i.e., VFAs and microbial proteins, serve as the main energy sources for ruminants [288]. Several studies have associated the composition and functional properties of the rumen microbiome to feed efficiency [309-311], DM intake and milk production [198, 312, 313]. Contributions of rumen microbiome to

physiology and well-being of ruminants have also been exemplified by studies associating metabolic disorders of cattle such as ruminal acidosis [13, 27, 314], milk fat depression [315] and frothy bloat [223] to changes in the diversity and composition of the rumen microbiota. Indeed, diversity is the central property of microbial ecosystems that gives rise to other functional properties such as stability, robustness, redundancy and resilience [316]. Microbial communities that are species-rich and diverse, are genetically and functionally redundant, and therefore able to maintain their core functions even in the face of environmental perturbations [7, 317]. Although the rumen hosts an incredibly diverse and functionally robust microbiome, severe perturbations such as abrupt changes in the amount and composition of diet can disturb the diversity of this microbial community and impair its ability to perform core metabolic functions [7].

The segmented nature of the beef industry in Canada represents critical time points during which cattle are exposed to diets of varying composition and nutritional values. The most critical time points in this process include transitioning of over-wintered stocker cattle from low-quality forage to nutritive and fresh legume pastures during spring and summer seasons, and then the subsequent exposure of feedlot steers to high-grain diets consisting of more than 90% concentrates [17-19]. These abrupt changes in the composition of diet can predispose cattle to metabolic disorders such as frothy bloat following exposure to high quality legume pastures [20] and ruminal acidosis following introduction of high-grain diets [13, 21, 22]. Ensuring acclimatization of the rumen microbiome prior to and during each of these transitional phases could reduce the risk of metabolic disorders and greatly improve productivity and well-being of cattle. In terms of frothy bloat, mitigating strategies that focus on lowering the amount of soluble proteins and reducing the rate of feed digestion in the rumen ecosystem have proven useful in reducing the incidence and severity of bloat. Inclusion of non-bloating legumes such as sainfoin in mixed legume pastures [20, 24] and the use of pluronic detergents capable of decreasing the viscosity of rumen contents [23] are examples of bloat mitigating strategies commonly practiced in Canada. Several mitigating strategies have also been developed for controlling metabolic disorders associated with feeding high-grain diets. These include addition of chemical buffers [25], ionophores [26], yeast fermentation products [27], and DFM in the diet of finishing steers [28, 29] to prevent ruminal

acidosis. Although the effectiveness of the referred mitigating strategies on the performance of cattle has been extensively investigated, the microbial mechanisms that underlie development of each metabolic condition and/or improvement of ruminal fermentation following each mitigating strategy is as yet poorly understood. In this thesis, the dynamics of rumen and hindgut microbiota of beef cattle during adaptation to high-grain diets and introduction to alfalfa pasture were investigated. Particularly, this thesis investigates the response of the rumen and hindgut microbiota to mitigating strategies including a) supplementation of *Propionibacterium acidipropionici* P169 to control the adverse effects of a high-grain diet in finishing steers, and b) dietary supplementation of sainfoin and Alfasure™ in the drinking water of cattle grazing alfalfa pastures to prevent frothy bloat.

In the first manuscript, the efficacy of the DFM, P169 in modulating the rumen and hindgut microbiota of beef cattle fed high concentrate corn-based diets was evaluated. The mode of action of P169 in modulating rumen fermentation profile has been attributed to its ability to convert lactate to propionic acid, thus preventing lactate accumulation and consequent drop in the pH of rumen contents [187]. However, as with many other DFM products, the ability of P169 to confer its beneficial effects greatly relies on the dosage level, frequency of administration and its successful integration within the host microbial ecosystem [177, 190]. In my experiment, I observed that administration of $10^{11}$ cfu/head/day of P169 significantly increased the abundance of this bacterium in the rumen contents steers compared to a control group. However, no increase in the ruminal concentration of propionate following P169-supplementation was observed, likely because gradual adaptation of steers to high-grain diet might have prevented the sudden accumulation of lactic acid in rumen contents. Notwithstanding, the observed increase in the concentration of branched-chain fatty acids in the rumen contents of P169-supplemented steers could be indicative of a beneficial effect of this probiotic strain, as BCVFAs are important growth factors for cellulolytic bacteria [194]. During adaptation to high-concentrate diets, even if ruminal pH remains within the normal range, amylolytic bacteria predominate the rumen ecosystem at the expense of cellulolytic bacteria. Interestingly, compared to the steers in the control group, P169-supplemented steers

had higher proportions of fibrolytic bacteria in their rumen contents, suggesting that P169 might confer its beneficial effect in rumen ecosystem in part by favoring the growth of cellulolytic bacteria and restoring a more balanced rumen microbial community. In addition to changing the composition of rumen microbiota, feeding high-grain diets can also result in the bypass of undigested starch from the rumen into the hindgut and change the composition of its microbial ecosystem [201]. In my study, I observed that proportions of several amylolytic lineages, including *Prevotella* and Succinivibrionaceae, were enriched in the hindgut microbiota of P169-supplemented steers whereas cellulolytic lineages, including members of the *Ruminococcus* and Clostridiaceae, were under represented. This observation could be indicative of an increase in the bypass of undigested carbohydrates from the rumen to the hindgut following P169-supplementation, and consequently, increased microbial fermentation in the hindgut. However, by failing to directly measure the pH and VFAs in the faeces of steers, our study remains inconclusive on whether or not the observed shifts in the composition of hindgut microbiota would be indicative of a beneficial impact on the overall fermentation profile of the rumen and hindgut ecosystems. Aside from evaluating the effect of P169 on the composition of rumen and hindgut microbiota, another noteworthy result was the comprehensive comparison of the core microbiota among different niches of the gastrointestinal tract in cattle. In particular, OTU-level comparison of the core microbiota among rumen liquid, rumen solids, and feces enabled us to identify species-level adaptation of bacteria within each niche of the GI tract. For instance, I observed that members of the phylum Bacteroidetes were proportionally more abundant within the liquid fraction of the rumen, whereas *Fibrobacteres* species were overrepresented in the solid fraction. Due to fibre-degrading and cellulolytic activities of *Fibrobacteres* species, members of this bacterial lineage are usually associated with fibrous plant materials in the rumen, whereas Bacteroidetes species, which usually possess large repertoires of genes involved in carbohydrate metabolism, were overrepresented in the liquid fraction of rumen contents. In this compartment they would have access to readily fermentable carbohydrate pools that enter the rumen following ingestion of high-grain diets. These findings are in general agreement with previous studies [8,

27, 206] and provide additional insights into the compartmentalization of the GI tract microbiota of cattle.

In the second manuscript, I comprehensively evaluated changes in the composition and predicted functional properties of the rumen and hindgut microbiota following introduction to alfalfa pasture and development of frothy bloat. Alfalfa is a highly nutritious legume with the potential to improve productivity of beef cattle to levels comparable to that obtained in feedlot systems [212]. However, susceptibility of cattle to frothy bloat upon grazing alfalfa pasture has greatly limited the wide-spread adoption of this legume in Canadian Prairie grazing systems. Despite extensive research on the etiology of frothy bloat, the role of rumen microbiota in the development of this metabolic disorder remains poorly understood. In the first section of this manuscript, I described bloat-associated shifts in the composition and predicted functional properties of the rumen microbiota following transition from hay diet to alfalfa pasture. I observed that transition to alfalfa pasture and development of frothy bloat was associated with increased diversity of the rumen microbiota and enrichment of predicted metabolic pathways responsible for the metabolism of complex carbohydrates. I attributed this observation to a rapid proliferation of plant fiber-associated biofilms within the rumen ecosystem, as increased microbiota diversity in bloated animals was more pronounced in the solid fraction of rumen contents. One potential explanation is that increased viscosity of rumen contents following consumption of alfalfa results in accumulation of metabolites of the enzymatic degradation of plant polysaccharides surrounding solid particles, which in turn may result in uncontrolled proliferation of solid-associated biofilms. Although in this thesis viscosity of rumen contents were not measured, I observed that *Streptococcus* and *Selenomonas* were enriched in the solid rumen content of bloated steers. Some members of these genera, including *S. bovis* and *S. ruminantium* are capable of producing and storing large amounts of reserved polysaccharides in their cytoplasm, which can serve as precursors for extracellular polymeric synthesis of biofilms [249, 250]. Alfasure™ was able to completely prevent the development of frothy bloat, whereas grazing alfalfa-sainfoin mixed pastures, reduced but did not eliminate the occurrence of bloat. By comparing the rumen microbiota of bloated and non-bloated steers following dietary interventions, I identified key bacterial genera

that were either positively (including *Streptococcus* and *Succinivibrio*) or negatively (including *Fibrobacter* and *Ruminococcus*) associated with development of frothy bloat. These observations partially agreed with Pitta et al. [318] who also reported a decrease in the proportion of *Ruminococcus* in the rumen contents of bloated steers grazing wheat pastures. Given the well-described contributions of *Fibrobacter* and *Ruminococcus* species to the break-down of plant cell walls [4, 319], it seems that development of frothy bloat disfavors the growth of the main fiber-degrading bacteria while increasing the growth of amylolytic bacteria. My experiments also had several shortcomings, most importantly: a) the relatively short washout phase between the different treatment periods of our crossover experimental design which might have resulted in carryover effect from previous treatments, and b) lack of comprehensive evaluation of bloat-associated shifts in the functionality of rumen microbiome via conducting metatranscriptomics. Nonetheless, my results provided novel insights into compositional dynamics of rumen microbiota during development of frothy bloat and in response to dietary interventions.

Lastly, my final manuscript sought to characterize bloat-associated shifts in the composition of anaerobic rumen fungi, and evaluated interrelationships of this group of microorganisms with the ruminal bacterial community in the context of development of frothy bloat. As observed in our second manuscript, bloat-associated shifts in the composition of rumen bacterial communities were more pronounced in the solid fraction of rumen contents. In the rumen ecosystem, anaerobic fungi are primary colonizers of fibrous plant materials which contribute to physical deconstruction of plant cell walls, and therefore, act as an extension of bacterial biofilms [52]. Despite important contributions of ARF to digestion of plant materials, the potential contribution of these group of microorganisms to metabolic disorders of cattle, in particular frothy bloat remain unknown. The comprehensive ITS sequencing protocol and bioinformatics pipeline used in this study enabled us to identify all of the rumen-associated ARF genera described in the literature, including the newly described genus *Buwchfawromyces*. Overall, I observed that regardless of development of frothy bloat, transition from baseline hay diet to alfalfa pastures was associated with significant changes in the composition of ARF, in particular enrichment of *Cyllamyces, Caecomyces, Anaeromyces*, and *Buwchfawromyces*. In

addition to having a large enzymatic repertoire for degradation of plant fibrous material, different species of ARF can also contribute to proteolytic and amylolytic activities within the rumen ecosystem [50, 53, 62]. While some species of ARF (e.g., *Piromyces communis*) can synergistically interact with rumen bacteria to promote microbial fermentation and cellulose digestion [320], others (e.g., *Neocallimastix frontalis*) have an antagonistic relationship with cellulolytic rumen bacteria [292]. In the present study, our correlation network analysis revealed that the relationship between bacteria and fungi in the rumen content of steers grazing alfalfa pastures were in general competitive, with certain species of *Orpinomyces* and unclassified Neocallimastigaceae exhibiting the highest number of negative interactions with the rumen bacterial community. Interestingly, the total number of negative relationships observed between ARF and rumen bacteria was considerably higher in the rumen contents of non-bloated than bloated cattle. The observed reduction in negative relationships between rumen bacteria and fungi following development of bloat may be an indicator of reduced competition for substrates (i.e., availability of large amount of nutrients following initial degradation of alfalfa). Another feasible explanation could be the accumulation of slime and uncontrolled proliferation of certain bacterial populations in frothy rumen contents, thus changing the ratio of fungi:bacteria in the rumen content of bloated steers.

## 8.2 Conclusions and Future Directions

The following conclusions can be drawn based on my thesis's research projects:

- Manuscript 1:
  - DFM supplementation of *P. acidipropionici* strain P169 at $10^{11}$ cfu/head/day significantly increased the abundance of this bacterium in the rumen content of supplemented steers.
  - P169 supplementation was not associated with a significant change in the overall composition of rumen microbiota. However, P169-supplemented steers had elevated molar proportions of branched-chain fatty acids in their rumen content.

  - The proportions of major fibrolytic bacteria were also increased in the rumen content of P169-supplemented steers, suggesting that P169 might confer its beneficial effect in rumen ecosystem by favoring the growth of cellulolytic bacteria and inhibiting the dominance of amylolytic bacteria following consumption of high-grain diets.
- Manuscript 2:
  - Transition from hay diet to alfalfa pasture and development of frothy bloat increased both the species-richness and diversity of the microbiota of solid fraction of rumen content, and resulted in enrichment of predicted metabolic pathways that are responsible for metabolism of complex carbohydrates
  - Increased diversity of fiber-associated microbiota suggests that development of frothy bloat might be in part due to the rapid proliferation of fiber-adherent biofilms. In particular, proportions of major amylolytic genera including *Streptococcus* and *Selenomonas* were enriched in the rumen content of bloated steers.
  - Our dietary interventions to prevent frothy bloat, including grazing of alfalfa-sainfoin mixed pastures and addition of the Alfasure™ to the drinking water of steers, had either complete (Alfasure™) or moderate (sainfoin) inhibitory effects on development of alfalfa-induced frothy bloat.
  - Rumen content of non-bloated steers receiving Alfasure™ and sainfoin had higher proportions of major fibrolytic bacterial groups including genera *Fibrobacter* and *Ruminococcus.* Overall, our results suggest that development of frothy bloat is associated with rapid proliferation and dominance of amylolytic rumen bacteria and that the referred dietary intervention could alleviate the bloat-associated dysbiosis in rumen microbiota by favoring the growth of fiber-degrading bacteria.
- Manuscript 3:
  - We observed that the changes in the composition of diet (transition from hay to alfalfa pasture) had a profound effect on the overall composition of the anaerobic rumen fungi. Rregardless of the development of frothy bloat, the proportion of genera *Cyllamyces, Caecomyces, Anaeromyces*, and

*Buwchfawromyces* were increased in the rumen content of steers grazing of alfalfa pasture.

- Correlation network analysis revealed a general competitive pattern (negative correlation) between ARF and rumen bacteria.
- Certain species of ARF, including *Orpinomyces* sp. and unclassified species belonging to the family Neocallimastigaceae, showed the highest number of negative interactions with rumen bacterial community, and were identified as hub ARF species contributing most to the overall composition of rumen microbiota.
- The proportion of negative relationships between ARF and rumen bacteria was higher in the rumen content of non-bloated steers than the bloated ones, suggesting the disruption of normal microbe-microbe interrelationships in the rumen content of bloated steers.

While my thesis provided novel insight into the compositional dynamics of rumen microbiota in response to critical dietary challenges of beef cattle, it remains inconclusive regarding changes in the functionality of rumen microbiome at both the metagenome (DNA) and metatranscriptome (mRNA) levels. With the aid of advanced assembly and binning bioinformatics pipelines, shotgun metagenomics can now enable us to explore rumen microbiome at the resolution of individual genomes (i.e. metagenome-assembled genomes; MAGs [42, 321]), therefore revealing the genetic repertoire of different microbial lineages and how they contribute to different metabolic processes in the rumen ecosystem. This approach would be particularly useful in studying the role of rumen microbiome in development of frothy bloat, as little is known about the strain diversity and functional properties of different bacterial species and anaerobic fungi that contribute most to degradation of plant cell walls and production of slime. Moreover, parallel metatransciptomics studies would also be required to precisely assess the functional contributions of rumen microbiome to each of the metabolic challenges described in my thesis. Indeed, combined metagenomics and metatransciptomics studies of the human gut microbiome have revealed that microbial transcriptional activities are extremely complex and unpredictable [252]. While some gene families can be consistently under-expressed in response to certain metabolic conditions, others could be over-expressed (mRNA being

proportionally more abundant than the equivalent DNA), making it evident that relying solely on 16S rRNA gene sequencing or metagenomics data to understand the true contributions of microbiome to host physiology could be imprecise.

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