ENGINEERING RUMEN METABOLIC PATHWAYS: WHERE WE ARE, AND WHERE ARE WE HEADING

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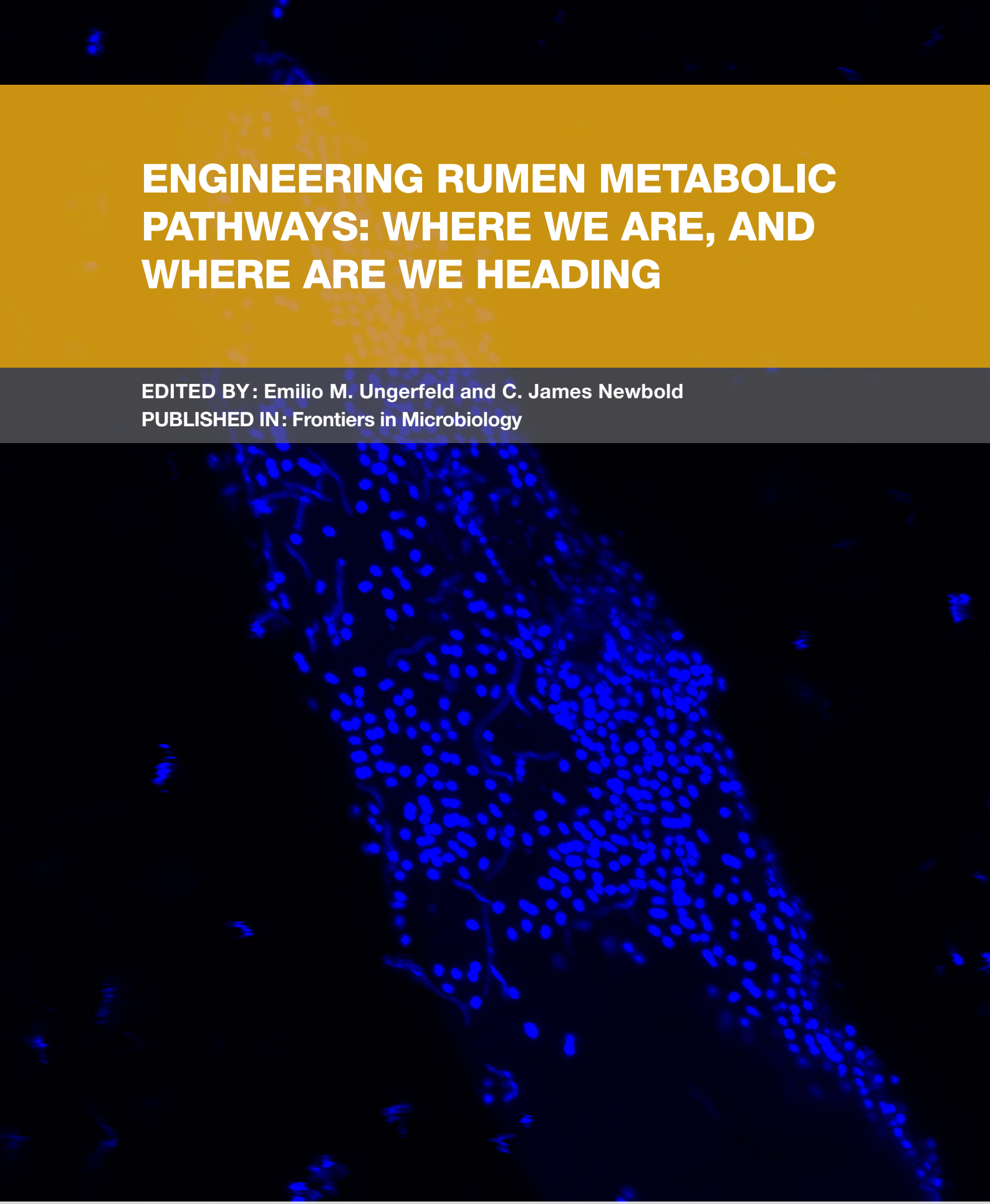

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ENGINEERING RUMEN METABOLIC PATHWAYS: WHERE WE ARE, AND WHERE ARE WE HEADING

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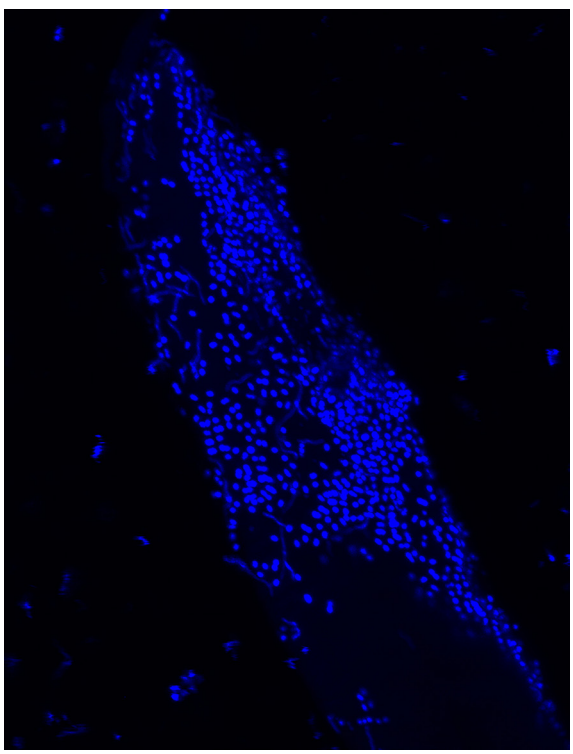

DAPI-stained bacteria obtained from the rumen of muskoxen. Photo taken at Lethbridge Research Centre, Agriculture & Agri-Feed Canada.

Ruminants were domesticated in the Middle East about 10,000 years ago and have since become an inseparable part of human diet, society, and culture. Ruminants can transform inedible plant fiber and non-protein nitrogen into meat, milk, wool and traction, thus allowing human utilization of non-tillable land and industrial by-products. The nutritional flexibility of ruminants is conferred by the rumen´s complex microbial community.

Driven by rising income and population growth in emergent economies, the global demand for livestock products, including milk and meat from ruminants, has been increasingly growing, and is predicted to continue growing in the next few decades. The increase in production necessary to satisfy this rising demand is putting much pressure on already dwindling natural resources. There are also concerns about the emissions of methane and nitrous oxide, potent greenhouse gases associated to ruminant production. The need to make ruminant production more efficient in the use of natural resources poses a big challenge to ruminant science, and within it, rumen microbiology.

Recent years have seen important advances in basic and applied rumen microbiology and biochemistry. The knowledge generated has significant implications for the efficiency and sustainability of ruminant production and the quality of ruminant products for human health. The present compilation is an update of recent advances in rumen microbiology and ruminant digestion and fermentation, including original research, reviews, and hypothesis and theory articles. We hope that the experimental results, discussion, models and ideas presented herein are useful to foster future research contributing to sustainable ruminant production.

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EDITORIAL
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Editorial: Engineering Rumen Metabolic Pathways: Where We Are, and Where Are We Heading

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Editorial on the Research Topic

Engineering Rumen Metabolic Pathways: Where We Are, and Where Are We Heading

The rumen plays a central role in the ability of ruminants to produce human edible food from resources that are otherwise not available for consumption by mankind. Fermentation in the rumen also has the potential to influence the health and well-being of both the host and humans through the nutritional quality of meat and milk, and the environment through potential deleterious environmental consequences of emissions of greenhouse gases and excessive nitrogen excretion in feces and urine. Given the importance of the rumen fermentation, it is perhaps not surprising that a great deal of effort has been devoted to investigating methods for manipulating this complex ecosystem, and the possibility of engineering rumen metabolic pathways is a highly attractive target.

By "Engineering" we do not refer specifically to genetic engineering; by "Engineering rumen metabolic pathways" we understand the manipulation of one or more rumen microbial processes toward the optimization of ruminant production and sustainability. As such, this e-book includes articles related to rumen fermentation, energy metabolism, and methane production. We have also included articles of microbial digestion and growth, on the understanding that these processes also impact ruminant production efficiency and sustainability. The e-book includes articles that report or review interventions to manipulate the rumen microbial community, as well as articles on basic experimental and theoretical research that help understanding the rumen microbial community toward its manipulation. The e-book compiled does not intend to be a comprehensive summarization of current knowledge or an update of all recent discoveries. It is proposed as a useful, rather than as a thorough, compilation of recent experimental and theoretical research on rumen microbiology and biochemistry.

An excellent overview of rumen microbial ecology is provided by Weimer. Weimer discusses how the rumen microbial community, while being complex and diverse, is remarkably stable due to its ecological redundancy in the role and physiology of microbial groups and the resilience to resist and recover from perturbations. Whereas these properties provide nutritional stability to the rumen and host, they also imply great challenges to engineer rumen function. Interestingly, recent research discussed by Yáñez-Ruiz et al. on how microbial colonization of the rumen soon after birth can be influenced by various factors, offers hope that early in the animals' life the rumen may be reprogramed in a somewhat stable way.

Protozoa are a very important group of microorganisms in the rumen microbial ecosystem. Newbold et al. review current knowledge about rumen protozoa and critically discuss their role in the microbial community and implications to the host's nutrition. They conclude that elimination of the ciliate protozoa increases microbial protein supply by up to 30% and reduces methane

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production by up to 11% whilst noting that as yet no method to control protozoa in the rumen that is safe and practically applicable has been developed.

Microbial protein synthesized in the rumen is the main and cheapest source of amino acids for ruminants. Hackmann and Firkins discuss in depth how energetically inefficient processes such as glycogen cycling and interconversion of volatile fatty acids can potentially impact microbial protein synthesis. Knowledge about these processes can allow designing strategies to minimize them and help optimizing microbial protein supply to the ruminant.

Soliva et al. investigated the efficiency of microbial synthesis from a nutritional viewpoint, revisiting the concept that there is a constant minimum requirements for rumen-degradable protein to support microbial digestion of organic matter across different feeds. Their study suggests that with tropical forages the required ratio of rumen degradable protein: apparently degraded organic matter might not be constant across high-fiber diets and that thresholds of either rumen degradable protein or ruminal ammonia concentration in existing models of rumen function may not be reflected appropriately by constants.

Microbial growth and protein synthesis requires utilization of ATP, which needs to be generated in fermentation. Efficiency and rate of ATP generation can thus impact microbial protein production. Hackmann and Firkins identified in most species of the genera *Butyrivibrio* and *Pseudobutyrivibrio* genes encoding for enzymes that acting in concert could allow for ATP generation through transmembrane electrochemical gradients. They propose the novel concepts that butyrivibrios could drive ATP generation by electron transport phosphorylation and that this energy conservation system might enhance the butyrivibrios' ability to overcome growth inhibition by unsaturated fatty acids.

Unsurprisingly given the current focus on greenhouse gas emissions from ruminant agriculture, methane production in the rumen, and its mitigation, provides a significant focus to our research topic. St-Pierre et al. review available information on the identification and occurrence of methanogenic archaea in the rumen and other gastrointestinal environments in herbivores, and speculate on how in the future such knowledge might lead to mitigation strategies targeting methanogens in the rumen. Latham et al. review the utilization of nitrate and nitrocompounds as methane-controlling strategies, as alternative electron acceptors that incorporate electrons at the expense of methane production and as direct inhibitors of methanogenesis. Despite demonstrated *in vitro* and *in vivo* efficacy of nitrate in mitigating rumen methane production, concerns remain over potential toxicity to the animal and increases in the emissions of nitrous oxide to the atmosphere, and further studies are required to quantify the risk versus the benefits as a practical approach in the field. Nitrocompounds, and more recently nitrooxycompounds, have been shown to effectively decrease methane production *in vitro* and *in vivo* in the long term, although as yet production benefits are still to be realized and the problem of accumulation of dihydrogen typically occurring along the inhibition of methanogenesis remains unresolved.

Whilst a decrease in methane emissions is expected to improve ruminant performance by decreasing energy losses as methane, decreases in methane production do not consistently result in greater performance. Benefits in energy efficiency caused by inhibiting methane production depend on the resulting alterations occurring in the flows of metabolic hydrogen. Changes in metabolic hydrogen flows as a consequence of inhibiting methane production have not been measured *in vivo* simultaneously with production of gases, but metabolic hydrogen balances can be calculated for published *in vitro* methanogenesis-inhibition experiments. Ungerfeld meta-analyzed shifts in metabolic hydrogen flows in 28 batch culture experiments and 16 continuous culture experiments in which methanogenesis was inhibited. He concluded that inhibiting methane production resulted in a moderate re-direction of reducing equivalents toward propionate in batch culture, but not in continuous culture. There was no benefit in heat of combustion in total volatile fatty acids by inhibiting methanogenesis, and a consistent decrease in metabolic hydrogen recovery. Guyader et al. further investigated the re-direction of metabolic hydrogen away from methane using three known methanogenesis inhibitors, nitrate, 3-nitrooxypropanol, and anthraquinone. Methane production was decreased by up to 75%, but despite increases in reduced volatile fatty acids production, recovery of metabolic hydrogen was still considerably lower than 100%, highlighting the need to identify and study unknown metabolic hydrogen sinks in the rumen.

Engineering the flows of metabolic hydrogen in the rumen requires understanding how these flows are physicochemically controlled. Through estimating the thermodynamic feasibility of the incorporation of accumulated dihydrogen into propionate and butyrate and reductive acetogenesis, Ungerfeld attempted to understand why dihydrogen generally accumulates when methanogenesis is inhibited. In that regard, insightful experiments by Denman et al., Martínez-Fernández et al., and Martínez-Fernández et al. examined the changes in the rumen microbiome occurring during methanogenesis inhibition. Denman et al. showed that inhibiting methanogenesis in goats stimulated the abundance of reads of enzymes from the propionate randomizing pathway, in agreement with increases in the abundance of microbial groups producing propionate. In contrast, inhibiting methanogenesis did not stimulate reductive acetogenesis. Martínez-Fernández et al. conducted metabolomic analyses that revealed an increase in amino acids, organic and nucleic acids in rumen fluid with inhibition of methanogenesis. Importantly, the main known fibrolytic bacteria, as well as protozoa and fungi, seemed mostly unaffected by inhibition of methanogenesis. Martínez-Fernández et al. successfully decreased accumulated dihydrogen in the methanogenesis-inhibited rumens of steers through the supplementation of phloroglucinol, a metabolic intermediate of flavonoids in the rumen.

Almost all measurement of dihydrogen concentration in rumen fermentation *in vitro* and *in vivo* have been conducted in the gas phase. Equilibrium between gaseous and dissolved dihydrogen has then been assumed to estimate the concentration of dissolved dihydrogen to calculate the thermodynamic feasibility of rumen pathways producing or incorporating dihydrogen. For the first time, Wang et al. simultaneously

determined the concentration of dihydrogen in the gas and fluid phases to discover that they were not at equilibrium, and that dissolved dihydrogen was supersaturated in the rumen of sheep. This has important implications for the understanding of rumen thermodynamics.

The rate of digestion of a substrate influences the profile of fermentation products. Two studies in this e-book have implications in the understanding of the kinetics of fiber digestion. Griffith et al. found responses in digestibility of barley straw in semi-continuous culture to fast-digesting inoculum when barley straw was ammonia fiber expansion treated, but no differences between fast and slow-digesting inocula in untreated barley straw. Oss et al. investigated the effect of inocula from cattle and bison and their mixtures on fermentation in semi-continuous cultures, finding that bison inoculum increased fiber and protein digestibility. Both studies confirm the role of the microbial community inoculated in semi-continuous cultures in determining the capacity to digest fiber. Although a challenging objective, the rate or the extent of fiber digestion or both might respond to the manipulation of the microbial community.

Complexities of the rumen acidosis phenomenon are unraveled in the research by Plaizier et al. These authors compared the impact on the rumen, cecal, and fecal bacterial communities during subacute acidosis induced by two contrasting diets in which part of the forage was replaced by either rapidly degradable starch pellets or pellets of ground alfalfa. Different changes in microbial communities induced by the different acidotic challenges confirm that our understanding of the acidotic rumen is still incomplete. A better understanding of the different ways the acidosis phenomenon occurs and the microbial populations involved may allow differentially manipulating key microbial groups to control different types of acidosis.

Bannink et al. present a thorough analysis of how mechanistic mathematical modeling can help understanding digestion, fermentation, absorption of volatile fatty acids, and the control of rumen pH. The authors also explain the contribution of dynamic modeling to predict rumen nitrogen balance and how prediction of the volatile fatty acids profile can be aided by recent developments in the characterization of the rumen microbiome.

Microbial digestion and fermentation in the rumen provides ruminants with the flexibility to use fiber and non-protein nitrogen unavailable to humans and other animals, but at the same time results in energy losses as methane, and often inefficient utilization of nitrogen, both of which have negative consequences for the environment. Making ruminant production more efficient and sustainable will ineludibly involve manipulating rumen microbial activity. Unlike the fragility of other ecosystems, the redundancy and resilience of the rumen microbial community makes it a challenging target to manipulate. Whilst currently metabolic engineering of the rumen to achieve theoretical potentials is not yet possible, we continue approaching that goal as we develop our understanding of rumen microbiology and biochemistry. Some main roadblocks to be tackled are digestion, metabolic hydrogen management, nitrogen and fatty acids metabolism, and acidosis. These areas should not be seen as separate compartments, and the existence of interphases can result in interventions on one aspect having consequences on others. We foresee future developments resulting from the integration of microbial ecology multiomics techniques, in particular regarding the expression of functional genes, with the application of physical-chemistry principles and the refinement of thermodynamic and kinetic measurements in the rumen environment.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Redundancy, resilience, and host specificity of the ruminal microbiota: implications for engineering improved ruminal fermentations

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The ruminal microbial community is remarkably diverse, containing 100s of different bacterial and archaeal species, plus many species of fungi and protozoa. Molecular studies have identified a "core microbiome" dominated by phyla Firmicutes and Bacteroidetes, but also containing many other taxa. The rumen provides an ideal laboratory for studies on microbial ecology and the demonstration of ecological principles. In particular, the microbial community demonstrates both redundancy (overlap of function among multiple species) and resilience (resistance to, and capacity to recover from, perturbation). These twin properties provide remarkable stability that maintains digestive function for the host across a range of feeding and management conditions, but they also provide a challenge to engineering the rumen for improved function (e.g., improved fiber utilization or decreased methane production). Direct ruminal dosing or feeding of probiotic strains often fails to establish the added strains, due to intensive competition and amensalism from the indigenous residents that are well-adapted to the historical conditions within each rumen. Known exceptions include introduced strains that can fill otherwise unoccupied niches, as in the case of specialist bacteria that degrade phytotoxins such as mimosine or fluoroacetate. An additional complicating factor in manipulating the ruminal fermentation is the individuality or host specificity of the microbiota, in which individual animals contain a particular community whose species composition is capable of reconstituting itself, even following a near-total exchange of ruminal contents from another herd mate maintained on the same diet. Elucidation of the interactions between the microbial community and the individual host that establish and maintain this specificity may provide insights into why individual hosts vary in production metrics (e.g., feed efficiency or milk fat synthesis), and how to improve herd performance.

Keywords: fermentation, host specificity, redundancy, resilience, rumen

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Introduction

The rumen is the characteristic defining feature and most voluminous digestive organ of ruminant animals, and the microbial fermentation that occurs within is largely responsible for providing the

energy and protein needs of the animal, in the form of volatile fatty acids (VFA) and microbial cell protein, respectively (Hungate, 1966). Because the ruminal fermentation is of central importance in ruminant nutrition, the prospects of manipulating the fermentation to improve the performance of the predominant ruminant livestock species (cattle, sheep and goats) has long attracted the attention of both microbiologists and animal scientists (Chalupa, 1977). In addition to its practical importance, the rumen is an ideal laboratory for elucidating fundamental principles of microbial ecology, for several reasons: i) The organ itself is of manageable size, and is contained within a unique animal unit; ii) access can readily provided by cannulation surgery; iii) the experimentalist can dictate the types and amounts of inputs (feed and water), and can accurately measure outputs; and iv) assembling a number of habitats for replicated studies is as simple as gathering the desired number of animal units. None of these useful properties are as easily embodied in other commonly studied microbial habitats (e.g., soils, lakes, etc.). And while it is clear that the ruminal microbial community is rich in terms of both its biomass density and its species diversity, this community operates under the same ecological principles as do the microbial communities in other habitats (Weimer, 1998).

Central to attaining the goal of improving the ruminal fermentation are fundamental questions regarding variability in the composition and activities of the ruminal microbiota, and the extent to which these factors impact overall animal performance. Historically attempts at ruminal manipulation have focused on using chemical agents (reviewed by Chalupa, 1977) or enzymes (reviewed by Beauchemin et al., 2003) as feed additives. However, recent advances in our understanding of the microbial community have allowed us to formulate strategies based on microbial agents (e.g., probiotics) that might contribute to a re-engineering of community dynamics and activities. These microbially based approaches, particularly those based on Domain Bacteria, are the subject of this analysis.

The Ruminal Microbiota

The ruminal microbial community consists of two groups of procaryotes (bacteria and archaea) and two groups of eucaryotes (protists and fungi). Bacteria and protists together account for well over 90% of the microbial biomass, and the bacteria in particular have been the focus of most quantitative studies on community composition.

Following the development of culture techniques suitable for strictly anaerobic bacteria, extensive efforts were made by microbiologists—particularly Hungate and others – toward isolation and characterization of ruminal bacteria. The vast majority of isolates were classifiable into about two dozen species, but even then it was noted (by comparison of colony counts in agar roll tubes versus direct enumeration of cells under the microscope) that only ∼8% of the bacterial community was cultivable using standard anaerobic techniques and media (Bryant and Burkey, 1953), although later refinements in media and technique improved this value somewhat (Bryant and Robinson, 1961; Leedle and Hespell, 1980). The development of molecular methods for characterizing microbial communities by sequencing genes for small-subunit rRNA genes, and more recently by metagenomic analysis employing next-generation sequencing (NGS), have provided a fuller appreciation for the very large number of bacterial species in a typical rumen. Over the past 5 years there have been over 30 publications that have quantified the microbial community composition at different taxonomic levels based on NGS technology alone; these studies have been well summarized in the recent review of McCann et al. (2014). It is clear from these studies that community composition data are influenced by a variety of factors which can be broadly grouped into those resulting from authentic differences in composition versus those in which the community composition results were influenced by the experimental methods employed (**Table 1**). The effect of methodology was highlighted by the landmark study of Henderson et al. (2013), who showed dramatic differences in the yield, quality, and taxonomic distribution of DNA that resulted from the use of different DNA isolation methods. Techniques that employed bead-beating of samples, particularly with phenol present during the beating step, gave superior yields of high-quality DNA compared to methods that employed commercial kits routinely used for DNA isolation from soils or feces. Of particular importance was their demonstration that, upon analysis of sequences by Titanium 454 pyrosequencing, isolation methods that gave low DNA yields also gave community compositions quite different from those obtained using high-yield isolation methods.

Further complications result from the use of different methods of analyzing the isolated DNA. Quantitative real-time PCR studies with genus-specific primers suggest that the genus *Prevotella* can constitute around half of the total bacterial 16S rRNA gene copy number (Stevenson and Weimer, 2007), a proxy for relative population size. By contrast, most studies that have employed pyrotag sequencing suggests that the phylum Bacteroidetes (which includes *Prevotella* and many other genera), while of major abundance, are substantially outnumbered by members of the phylum Firmicutes (de Menezes et al., 2011; Henderson et al., 2013; Mohammed et al., 2014). Similar disparities are observable by cross-comparison of different studies at almost any phylogenetic level. Overall, then, we must be cautious in interpreting quantitative abundance data across studies, and instead should focus primarily on differences among animals, treatments, and time-dependent dynamics within individual studies, or perhaps across studies that at least used similar methods of DNA isolation and analysis (Henderson et al., 2013).

One of the major problems in associating specific animal production responses with microbial community composition is that a substantial number of animals are typically required to demonstrate a statistically significant effect of treatment (e.g., diet), but the number of animals available for microbiological studies (e.g., ruminally cannulated cows for optimal sampling at a similar physiological state) is often limited. One approach to this dilemma is to conduct production experiments with a large number of cows, a small subset of which meet the desired sampling criteria (de Menezes et al., 2011), and then verify a lack of differences in production metrics between the subset of animals and

TABLE 1 | Factors that can affect the outcome of microbial community analysis in the rumen, with references for studies in which individual factors were examined systematically.

Factor	Reference
Factors resulting from authentic differences in the subject	
Animal type (species, breed, age, stage of ruminal development)	Lee et al. (2012), Li et al. (2012), Wu et al. (2012), Henderson et al. (2013), Rey et al. (2014)
Diet (ration ingredients and chemical composition)	Numerous studies[a]
Phase of ruminal contents (liquid, suspended solid, ruminal mat, epimural)	Numerous studies[b]
Plane of nutrition	Mohammed et al. (2012b)
Time of sample collection relative to time of feed presentation	Li et al. (2009), Welkie et al. (2010)
Ruminal pH	Khafipour et al. (2009), Palmonari et al. (2010), Mohammed et al. (2012b), Petri et al. (2013a,b)
Host specificity (animal individuality)	Weimer et al. (2010a,b), Jami and Mizrahi (2012), Mohammed et al. (2012b), Zhou et al. (2012)
Factors resulting from study methodology	
Method of sample collection (ruminal cannula, stomach tube)	Lodge-Ivey et al. (2009), Henderson et al. (2013)
Site of sampling (cranial/caudal, dorsal/ventral)	Li et al. (2009)
DNA isolation method	Henderson et al. (2013)
DNA analysis method (ARISA, qPCR, tRFLP, clone library construction, NGS)	de Menezes et al. (2011), Mohammed et al. (2014)

[a] *Tajima et al. (2001), Callaway et al. (2010), Fernando et al. (2010), Pitta et al. (2010), Weimer et al. (2010b), Welkie et al. (2010), de Menezes et al. (2011), Khafipour et al. (2011), Broadway et al. (2012), Mohammed et al. (2012a), Ramirez et al. (2012), Wang et al. (2012), Petri et al. (2013a,b), Zened et al. (2013), Zhang et al. (2013), Mohammed et al. (2014).*

[b] *Sadet et al. (2007), Pitta et al. (2010), Welkie et al. (2010), de Menezes et al. (2011), Fouts et al. (2012), Lee et al. (2012), Mohammed et al. (2012a, 2014), Henderson et al. (2013), Petri et al. (2013a).*

the larger set. Such experimental designs may not be appropriate for all studies, however. For example, ruminally cannulated cows that provide optimal sampling logistics may not be practical for methane emissions measurements due to potential losses of fermentation gases from the vicinity of the cannula (de Menezes et al., 2011; Beauchemin et al., 2012), though this would not be an issue for animals confined to chambers for collection of gaseous emissions.

Despite the caveats listed above with regard to methodologies, it is clear that molecular approaches, particularly NGS, have tremendously expanded our appreciation for the richness and complexity of the ruminal microbial community. As these quantitative phylogenetic assessments mount, we must tackle the challenge of relating this information to the physiologies and interrelationships of the different species. This will demand that we distinguish between microbial populations and their level of metabolic activity, a problem elegantly addressed using such novel methodologies as simultaneous DNA/cDNA quantification (Lettat and Benchaar, 2013) and metatranscriptomic profiling (Dai et al., 2015). Additionally, it will ultimately be necessary to isolate and characterize new microbial species, particularly phylotypes revealed to be abundant but which have heretofore escaped cultivation in the laboratory (Kobayashi, 2006).

Ecological Properties of the Ruminal Microbiome

Redundancy

With regard to the ruminal microbial community, we can define *redundancy* as the overlapping distribution of physiological capabilities across multiple microbial taxa. Conceptually, redundancy with respect to catabolism can be inferred from a comparison of the number of degradable substrates (or, for polymeric substrates, the number of different monomeric units and different linkages between monomeric units) in the feed on the one hand, and the number of species available to carry out the degradations. Despite the complexity of feeds ingested by the ruminant, there are a relatively modest number of potential "degradation points" (see **Tables 2** and **3** for a partial listing of biopolymers and soluble substrates, respectively). By contrast, the number of microbial species in the rumen is enormous. For example, in an oft-cited study Kim et al. (2011) used rarefaction analysis of 16S rRNA sequences archived in the Ribosomal Database Project to predict that 99.9% species coverage in the rumen would be obtained from 78,218 bacterial and 24,480 archaeal sequences, and most NGS studies have obtained 100s–1000s of operational taxonomic units (OTUs, a proxy for species) per individual ruminal sample. Dividing the large number of species by the relatively small number of degradation points provides a clear indication that, on average, there are many different species that can potentially contribute to the degradation of each substrate or linkage. Moreover, because many species can participate in the degradation of multiple substrates or attack multiple linkages within biopolymers, it is clear that the ruminal community is, from a metabolic standpoint, highly redundant in its composition. Although there are no specific surveys that have determined the number of species capable of degrading individual substrates in the rumen, it appears that catabolic redundancy is skewed heavily toward the most abundant and degradable substrates (or, for biopolymers, their monomeric units), and it would be surprising indeed if the number of species capable of degrading glucose did not greatly outnumber those capable of degrading, for example, oxalate. It thus appears that

TABLE 2 | Polysaccharides degraded by mixed ruminal microbes and pure culture of ruminal bacteria.

Structural polysaccharides	Structure	Mixed culture	Pure culture
Cellulose	β-1,4-glucan	Waldo et al. (1972), Van Soest (1973)	Hungate (1966)
Homoxylan	β-1,4-xylan	Weimer et al. (2000)	Suen et al. (2011)
Arabinoxylan	β-1,4-xylan with α-1 → 3 arabinose substituents		Hespell and Cotta (1995)
4-O-Methylglucuronoxylan	β-1,4-xylan with methylglucuronic acid substituents		Dehority (1965)
Pectins (forage)		Gradel and Dehority (1972)	Gradel and Dehority (1972)
Pectins (citrus)	Methoxylated α-1,4-galacturonic acid		Gradel and Dehority (1972)
Xyloglucan	β-1,4-glucan with β-1,6 xylose substituents		Suen et al. (2011)
Storage polysaccharides			
Starch	α-1.4-glucan	Mertens and Lofton (1980)	Cotta (1988)
	α-1,4 glucan with a-1,6 branching at ∼3–4% of residues		
Fructans	2,1-fructan with 1 → 2 glucosyl substituents		Ziolecki et al. (1992)
Glucomannan	β-(1 → 4)-linked mannose and glucose in a ratio of 1.6:1		Suen et al. (2011), Christopherson et al. (2014)
Laminarin	β(1 → 3):β(1 → 6) glucan (ratio of 3:1)		Teather and Wood (1982)
Lichenan	Repeating β-1,3 and β-1,4 glucan		Christopherson et al. (2014)

Where possible, references were selected to provide a spectrum of compounds within each class, or a spectrum of taxonomy of the degradative strains.

the ruminal community is composed of a mix of generalists that compete for a large number of abundant substrates, and specialists that face much less competition for a relatively smaller number of typically less abundant substrates. The redundancy of the ruminal microbial community is further suggested by the fact that, within studies, considerable changes in community composition often do not translate into changes in fundamental fermentation metrics such as pH, VFA concentrations or molar proportions of VFA (Welkie et al., 2010; Sandri et al., 2014). It must be borne in mind; however, that lack of significant differences in fermentation variables may reflect, at least in part, the use of a small number of animals per experimental treatment.

Aside from these generalizations, a more detailed understanding of redundancy is not easily won, for we are quickly mired in the difficulty of assigning *in situ* function to a very large number of species, only a few of which have been cultured in the lab. For example, two of the most abundant bacterial genera in the rumen are *Prevotella* and *Butyrivibrio*, and each rumen typically contains dozens to hundreds of OTUs from each of these genera, whose rRNA sequences vary sufficiently that current taxonomic fashion would classify into a large number of separate species (Pitta et al., 2010; Li et al., 2012; Mohammed et al., 2014). Cultured representatives of these genera display extremely broad degradative capabilities: hydrolysis of proteins and peptides; hydrolysis of starch and many hemicelluloses, and fermentation of many amino acids and most sugars (Kelly et al., 2010; Willems and Collins, 2011). Many of these capabilities can reside within a single bacterial strain. So while it is clear that representatives of these genera can participate in the conversion of a broad array of substrates, what is not clear is which particular degradative capability any particular species or strain might be carrying out *in situ*, in the presence of a large number of potential competitors and symbionts. The H_2-oxidizing, CO_2-reducing acetogens provide an object lesson in this regard: Such species have been isolated from the rumen (although they are not abundant there) but labeling studies have shown that essentially no acetate is produced from CO_2 in the rumen (LeVan et al., 1998). Moreover, theoretical calculations have shown that these acetogens would have difficulty competing with methanogens for available H_2, as suggested by their slower growth rates, poorer affinity for H_2, and smaller free energy yield per mol of H_2 consumed (Ungerfeld, 2013). Because CO_2-reducing acetogens are very versatile catabolically, it has been suggested that their presence in the rumen reflects their ability to subsist by degrading a wide range of organic substrates, rather than by reduction of CO_2 with H_2 to acetate (Rieu-Lesme et al., 1996). How this contention might be proven remains at this point rather elusive.

Resilience

Like any ecosystem, the organisms of the rumen respond to perturbation by internal or external forces. These forces may be physical (e.g., a change in temperature), chemical (e.g., ruminal pH, a change in diet composition, or an introduction of a plant toxin in the feed), or biological (e.g., the input of a non-native microbe). Perturbations may differ in intensity, frequency and duration, and these differences play a major role in determining the nature of the microbial response. Some perturbations are such common features of an animal's existence that they are taken for granted (e.g., variations in meal patterning or water consumption). Others are less obvious, such as gradual change in forage quality in a grazing plot over time.

The ruminal response to these internal and external perturbations can be examined using concepts developed from the field of macroecology. Over the years a complex and often contradictory terminology has evolved among ecologists to describe these responses. For our purposes, we will use the terminology of Westman (1978; see **Table 3**). Responses to perturbation can be described in terms of the system's *inertia* and its *resilience*. Inertia refers to system stability (i.e., how well it resists change), while resilience is a reflection of how the system responds once

TABLE 3 | Selected examples of reports of soluble substrate degradation by mixed ruminal microbes and pure culture of ruminal bacteria.

Nutritional strategy	Substrate class	Substrates[a]	Reference (mixed culture)	Reference (pure culture)
Generalist Specialist	Aldohexoses	Glucose, galactose, mannose	Hungate (1966)	Hungate (1966)
	Aldopentoses	Xylose, arabinose, ribose	Heald (1952)	Rasmussen (1993)
	Protein components	Amino acids, di-, and tri-peptides	Russell (2002)	Eschenlauer et al. (2002)
	Disaccharides	Cellobiose, lactose, maltose,	Heald (1952)	
	Carbohydrate oligomers	Cellodextrins,		Russell (1985), Shi and Weimer (1996)
		Maltodextrins	Kim et al. (1999)	
		Xylodextrins		Cotta (1993)
	Nucleic acids	DNA, RNA, nucleotide bases	Jurtshuk et al. (1958)	Cotta (1990)
	Ketohexoses	Fructose		
	Deoxyhexoses	Rhamnose, fucose, 2-deoxyglucose		Rasmussen (1993)
	Primary alcohols	Methanol, ethanol	Pol and Demeyer (1988), Pradhan and Hemken (1970)	Genthner et al. (1981)
	Sugar alcohols, polyols	Glycerol, mannitol, 1,2-propanediol	Lee et al. (2011)	Czerkawski et al. (1984)
	Cyclitols	*myo*-inositol, pinitol, quebrachitol	Lowry and Kennedy (1995)	
	Dicarboxyllic acids	Malonate, succinate, malate, fumarate	Russell and Van Soest (1984)	
	Hydroxyacids	Lactate, malate		Hino et al. (1994)
	Monolignols		Besle et al. (1995)	
	Phenolic acids	Ferulic acid, p-coumaric acid		Chesson et al. (1982), Long et al. (2003)
	Tricarboxylic acids	Citrate, aconitate, tricarballylate	Russell and Van Soest (1984)	
	Uronic acids	Galacturonic acid, glucuronic acid	Heald (1952)	
	Tannins			Nelson et al. (1998)
	Urea	Urea		Cook (1976)
	Amines	Cadaverine, histamine, putrescine, tyramine	Van Os et al. (1995)	
	Inorganic electron acceptors	Nitrate, sulfate, arsenate	Herbel et al. (2002), Van Zijderveld et al. (2010)	
	Hydroxyaromatic compounds	Phloroglucinol		Tsai and Jones (1975), Patel et al. (1981)
	Flavonoids	Rutin, quercitin, naringin, hesperidin	Simpson et al. (1969)	
	Plant toxins	Allyl cyanide	Duncan and Milne (1992)	
		Fluoroacetate	Camboim et al. (2012)	
		Mimosine	Jones and Megarrity (1986)	Allison et al. (1992)
		Nitro-1-propanol, nitropropionate		Majak (1992), Anderson et al. (1996)
		Oxalate	Belenguer et al. (2013)	Allison et al. (1985)
	Mycotoxins	Aflatoxin, ochratoxin, zearalenone	Kiessling et al. (1984)	
	Xenobiotic compounds	TNT, RDX	Fleischmann et al. (2004), Li et al. (2014)	

Substrates are arranged in rough order of their abundance in feeds and forages, which roughly parallels their degradability across a spectrum of nutritional strategies from generalists to specialists. Where possible, references were selected to provide a spectrum of compounds within each class, or a spectrum of taxonomy of the degradative strains. For pure cultures, not all individual compounds listed were degraded by all of the pure cultures examined.
[a]*Phloroglucinol, 1,3,5-trihydroxybenzene; RDX, Hexahydro-1,3,5-trinitro-1,3,5-triazine; TNT, 2, 4, 6-trinitrotoluene.*

it has been changed. As discussed by Westman (1978), resilience has four components that describe the extent to which, and the path by which, an original state may be restored (**Table 4**). In macroecology, the properties of inertia and resilience are typically (and most easily) examined at the level of an individual species, and are famously illustrated by particular examples of population levels of these species over time or across spatial domains (for example, the spruce budworm in Canadian boreal forests, or whitefish in the Great Lakes; see Holling, 1973). In habitats of high species diversity, interactions among organisms become more and more complex, and unraveling the factors that underlie inertia and resilience becomes progressively more difficult for individual species and more difficult yet for entire communities. Nevertheless, it is useful to extend these concepts from classical ecology to the microbial world, including the highly diverse microbial community of the rumen, as this provides a theoretical underpinning of discussions on how successfully, and under what conditions, the ruminal fermentation might be manipulated.

TABLE 4 | Characteristics that describe the stability and adaptability of the ruminal microbial community

Characteristic	Definition[a]	Likely status in the rumen
Inertia	Resistance to change	High, based on dosing studies
Resilience	Ability to restore its structure following acute or chronic disturbance	High, based on exchange studies
Components of resilience:		
Elasticity	Rapidity of restoration of a stable state following disturbance	Relatively high, based on exchange studies
Amplitude	Zone from which the system will return to a stable state	Very high, based on exchange studies
Hysteresis	Degree to which path of restoration is an exact reversal of path of degradation	Unknown
Malleability	Degree to which stable state established after disturbance differs from the original steady state	Low

[a]*Verbatim definitions of Westman (1978).*

Redundancy and resilience are two concepts from macroecology that appear to apply in microbial ecology as well. Other concepts may not be quite as readily transferred, at least for the ruminal habitat. For example, while the rumen may have a "core microbiome," it is not clear whether any of its members represent *keystone species,* i.e., species that have a disproportionately large influence on the ecosystem relative to their abundance, and whose disappearance would imperil ecosystem function (Mills et al., 1993). Clearly there are keystone functional groups of microbes (e.g., fibrolytics and methanogens), but within the rumen there are multiple species representing each of these groups, and this redundancy makes it unlikely that any one species would have a role in the habitat that would be sufficiently essential and irreplaceable to merit the designation of keystone species.

Host Individuality

Producers, especially those holding small herds who spend a lot of time interacting with their animals, are well aware of behavioral and production differences among individuals. An intriguing question is whether or not these inter-animal differences are a reflection of, or are even caused by, differences in the composition of the ruminal microbial community. The potential for a host-specific microbiological uniqueness of the ruminant was first noted in the protozoal community (Kofoid and MacLennan, 1933; Eadie, 1962), and much later in the fibrolytic bacterial community (Weimer et al., 1999), prior to the development of advanced molecular tools to characterize the gut community. The concept of host microbiome individuality has now achieved substantial attention, primarily as a result of recent studies of the human gut microbiome. Such studies have revealed that the human gut contains a "core microbiome" (i.e., a set of taxa present in all animals in the study), but also a large number of taxa whose presence or abundance varies among hosts. In numerous cases, the human gut microbiome has been shown to vary in a consistent manner with such clinical conditions as obesity or various intestinal maladies that may be grouped under the collective term *dysbiosis*. These sorts of studies have proliferated into a kind of microbiological cottage industry, and despite the welter of breathless press releases for public consumption, we are only occasionally reminded (e.g., by Hanage, 2014) that the "conclusion" of these studies have almost always been based on association, rather than on a rigorous demonstration of cause and effect.

As in the case of the human GI tract, it appears that the rumen of cattle (and probably other ruminant species) contains a core microbiome (Jami and Mizrahi, 2012; Petri et al., 2013b). Across studies there is general agreement that the core microbiome of cattle includes members of the phyla Firmicutes (especially genera *Ruminococcus* and *Butyrivibrio*) and Bacteroidetes (particularly genus *Prevotella*), along with some taxa present in lower abundance. Because each study used a relatively small number of animals, membership in the core microbiome would be expected to shrink upon inclusion of successively larger numbers of animals (i.e., upon generalization to a global population of hosts). Despite this, the core microbiome remains a useful concept because it focuses attention on the microbes that are likely to be either essential for, or at least major contributors to, overall ruminal fermentation. But we are again confronted with the familiar problems of assigning specific functions to the members of this community, whose cultured representatives often are nutritional generalists.

Once one gets beyond the core microbiome, it appears that there is extensive variability among individual animals with respect to the bacterial species composition of the rumen. Part of the variation is due to the presence of very low-abundance OTUs – often as singletons detected by NGS, but substantial inter-animal variation has been detected even when using relatively low-sensitivity, low-resolution methods such as automated riborsomal intergenic spacer analysis (ARISA, Welkie et al., 2010) or denaturing gradient gel electrophoresis (DGGE, Zhou et al., 2012). Host specificity in the rumen does not appear to be restricted to Domain Bacteria, as it has been observed for both the methanogenic archaeal and the protozoal communities (Zhou et al., 2012).

If the microbial community within each rumen is unique to its host, several questions arise: At what stage of life is this community assembled to the point where it can be regarded as compositionally and functionally unique? What environmental drivers determine the initial establishment and ultimate maintenance of each community? Can community composition and its resultant functionality be substantially altered by some combination of dietary manipulation and exogenous inoculation?

Responses to Experimental Manipulation of the Ruminal Microflora

Insights from Feeding Studies

A variety of microbial strains, some of them marketed commercially, have been tested as feed additives to improve animal performance, particularly in dairy and beef cattle. Much of this work has been summarized in excellent reviews (Yoon and Stern, 1995; Chaucheyras-Durand et al., 2008). Among the most heavily examined microbes have been *Saccharomyces* yeast, the filamentous fungus *Aspergillus oryzae* (AO), and lactic acid bacteria. In general, these strains have shown inconsistent production responses that are highly dependent on growth or lactation stage of the host, feeding level, and diet type. The few studies that have been conducted regarding persistence of the fed strains suggest that live cultures do not grow in the rumen, and in fact some of the products are as effective when added in non-viable forms (or in the case of AO, as a cell-free extract) as when added to the rations in live form (Yoon and Stern, 1995).

Even for strains that have shown occasional improvements in performance, it has proven difficult to convincingly demonstrate any of the proposed mechanisms of action. These proposed mechanisms range from consuming oxygen, to providing nutrients (e.g., vitamins or amino acids), to selectively enhancing growth of particular (usually unidentified) bacterial species. Based on our understanding of microbial ecological principles, the lack of persistence of the fed strains is not surprising, but the occasional demonstration of production improvement by direct fed microbials is sufficiently intriguing to warrant a more complete comparison of the microbial communities in animals fed these products versus control animals fed the same diet without the fed strains, using more modern techniques of molecular microbial ecology. There have been a few studies in this regard. For example, Mosoni et al. (2007) reported that sheep fed active dry yeast preparations along with a high-concentrate diet had enhanced levels of 16S rRNA corresponding to the cellulolytic bacteria *Ruminococcus albus* and *R. flavefaciens*. While the mechanism of action is not clear, it should be noted that these bacteria are among the most O_2-sensitive ruminal bacteria in culture, and thus might benefit from the known O_2-consuming capacity of the yeast. On the other hand, there is substantial evidence (discussed below) that cellulose digestion in the rumen is not limited by the number or activity of cellulolytic microbes, but by the accessibility of cellulose, so the effects on the cellulolytic community may be unrelated to the probiotic role of active dry yeast products, which may be complex and multifaceted.

Insights from Ruminal Dosing Studies

A clear indication of the inertia and resilience of the ruminal community is provided by dosing studies with ruminally cannulated animals. In one of the earliest examples, Varel et al. (1995) isolated a strain of *Clostridium longisporum* from the rumen of a bison, and strain of *C. herbivorans* from the pig intestinal tract. Both bacterial strains were more actively cellulolytic in pure culture than were the common isolates of "classical" ruminal cellulolytic species (*Fibrobacter succinogenes, R. albus*, and *R. flavefaciens*), and on this basis it was hypothesized that these *Clostridium* strains would be able to be established in the rumens of animals fed diets high in cellulosic feeds. Varel et al. (1995) then dosed fermenter-grown cultures (6 l of culture, plus 20 l of buffer) into three ruminally cannulated cows whose rumens had been nearly completely emptied; after dosing the cows were returned to hay feeding. The inoculated strains had a distinctive colony pigmentation and morphology that allowed their easy identification on selective agar medium. Despite the massive inoculation into nearly emptied rumens, the dosed strains were cleared to undetectable levels, usually within 24 h of inoculation. While the inoculated strains were of ruminal or hindgut origin, they represented species that are not considered abundant in the rumen, which suggested that these strains were not highly competitive in the ruminal habitat from the start, and under the experimental conditions used, the quantitatively modest residual host community was easily able to displace the dosed species in relatively short order.

As summarized in **Table 5**, other reported dosing experiments with fibrolytic strains of ruminal origin have proven no more successful, even when using more modern and sensitive detection methods. It thus appears that the native (*autochthonous*) fibrolytic microbes within each individual rumen are sufficiently well adapted in their native habitat to outcompete non-native (*allochthonous*) strains or species introduced from other habitats, including other rumens. Within the ruminal habitat, competition is likely intensified by fiber limitation (i.e., although fiber concentration is high on concentration basis, most fiber is inaccessible).

The situation with non-fibrolytic microbes is not always as discouraging. Establishment of dosed strains has in a few cases been dramatically successful. Undoubtedly the premier example of a successful ruminal introduction is provided by the case of *Synergistes jonesii*. The tropical leguminous shrub *Leucaena leucocephala* contains high levels of the non-protein amino acid mimosine, which is ruminally converted to the goitrogenic 3,4-dihydroxypyridine. Jones and Megarrity (1986) noted that Indonesian goats were sensitive to mimosine poisoning, while Hawaiian goats were not. They further showed that a single oral dosing of ruminal contents from these resistant Hawaiian goats into Indonesian goats conferred resistance to mimosine poisoning. Allison et al. (1992) isolated the microbial agent, a novel bacterial species they named *Synergistes jonesii*, and demonstrated that the bacterium was only capable of using mimosine and two other amino acids as growth substrates. Subsequent studies revealed that inoculation of this bacterium either as a pure culture, or as ruminal contents from mimosine-resistant animals, readily conferred mimosine resistance to the recipient animals (Hammond, 1995).

A second example of a successful inoculation concerns toxicity of fluoroacetate, a secondary plant metabolite that blocks the action of citrate synthase, an essential enzyme of the tricarboxylic acid cycle. Gregg et al. (1998) successfully

TABLE 5 | Ruminal dosing experiments with fibrolytic microbial strains.

Dosed strain	Source	Recipient animals	Result	Notes	Reference
Clostridium longisporum B6405 and *C. herbivorans* 54408	Bison (B6405), Pig (54408)	Three cannulated 6 years-old cows	Dosed strains not detected ($<10^3$ Cells/mL) within 24–48 h of dosing	Rumen nearly emptied prior to dosing, and feeding resumed immediately after dosing.	Varel et al. (1995)
Ruminococcus albus (Y1, LP9155 or AR72), or *R. flavefaciens* (SY3 or AR67)	Lab strains	Total of 16 cannulated adult Merino sheep	Strains dosed daily for 9 days at 5×10^{12} cells/dose reached abundances of up to 6.5% of the bacterial community but did not persist.	No improvement observed in dry matter digestibility of Rhodes grass incubated *in situ* during dosing period.	Krause et al. (2001)
R. flavefaciens NJ + probiotic	Wild moose	Six cannulated non-lactating dairy cows	Dosed strain (6.8×10^{11} cells) did not persist.	Dosed strain declined by $\sim 10^3$-fold within 24 h and was undetectable by 50 h after dosing.	Chiquette et al. (2007b)
		Calves (21–35 days old)	Dosed strain showed weak persistence.	Dosed strain detected at low levels ($\sim 10^2$ cells/mL) 7 days after cessation of dosing.	
R. flavefaciens 8/94-32	Norwegian reindeer	Three starved male reindeer	Dosed strain did not persist.	Population size of the abundant Ruminococcaceae family did not change. Some change in overall bacterial community composition observed.	Praesteng et al. (2013)
R. flavefaciens FD-1	Lab strain	Six lactating Murrah buffaloes	Equivocal results: Population of *R. flavefaciens* increased from 1.46×10^7/mL prior to dosing to 2.52×10^7/mL during the week after dosing concluded, but also increased in control buffaloes fed autoclaved cultures.	Very heavy oral supplementation of dosed strain [9×10^{14} cells (sic) on alternate days for 1 month].	Kumar and Sirohi (2013)
Ruminal fungi *Orpinomyces* sp. C-14 or *Piromyces* sp. WNG-12	Cattle	15 lactating Murrah buffaloes	Increased feed digestibility and up to 5.6% improvement in milk production.	Zoospore density higher in dosed animals, but level of dosage not reported.	Saxena et al. (2010)

TABLE 6 | Comparison of niche filling and niche replacement.

	Niche filling	Niche replacement
Initial status	Niche unoccupied	Niche occupied, sometimes by several competitors
Dosing and results	Single dosing often sufficient to establish dosed strain	Multiple doses sometimes (but often not) sufficient to establish dosed strain
Examples	*Synergistes jonesii* to impart resistance to mimosine toxicity	Most commercial probiotics; Experimental strains of fibrolytics and homoacetogens

introduced a dehalogenase gene from a *Pseudomonas* strain into a ruminally derived strain of *Butyrivibrio fibrisolvens*, and then successfully established the recombinant strain by dosing into sheep, which subsequently exhibited retention of the strain over several months along with a markedly enhanced tolerance to fluoroacetate. In the case of both mimosine and fluoroacetate protection, inoculation success is clearly the result of the dosed strain filling an open niche, aided by the strain's highly specialized metabolic capability and a substantial concentration of susceptible substrate. Interestingly, more recent work has revealed that fluoroacetate resistance appears to have developed naturally in some ruminal bacteria (Camboim et al., 2012).

Perhaps we should not be surprised by the general difficulty of establishing a single allochthonous microbial strain within a given rumen, unless that strain can fill an open niche (**Table 6**). At the risk of anthropomorphism, we may view the problem in more familiar sociological terms: In addition to abiotic stressors, an introduced strain is likely to encounter many enemies (direct competitors as well as their co-adapted symbionts, with whom they may have established productive mutualistic relationships worth defending) and few friends (unaffiliated microbes that might immediately benefit from cooperating with the introduced strain). This raises the question: can inoculation success be enhanced by introducing more complex assemblages, or even entire communities, of co-adapted species?

Insights from Exchanges of Ruminal Contents

The possibility that introduced strains can be established more effectively if accompanied by co-adapted community members can be examined via experiments in which ruminal contents are exchanged between pairs of ruminally cannulated animals. Early ruminal contents exchanges were conducted to test for specific physiological or nutritional outcomes rather than effects on the ruminal microbial community. Satter and Bringe (1969) exchanged ruminal contents between Holstein cows fed diets high in forages versus high in concentrates, and also switched the diets themselves coincident with the contents exchange. They observed that it took 5–6 days for the milk-fat depressing effects of the high-concentrate diet to be expressed in the recipient cow, and concluded that metabolic changes (including possible adaptation of the ruminal microbial community)

were more important in controlling milk fat synthesis than were the amount and proportions of different VFA (acetate and propionate), which differed between the exchanged contents and would be expected to act more immediately. Interestingly, these researchers conducted additional comparative experiments in which abrupt dietary switches were performed without ruminal contents exchange, but they did not conduct ruminal contents exchanges while maintaining the cows on their separate diets. Cockrem et al. (1987) exchanged ruminal contents in varying amounts between cattle divergently selected for bloat susceptibility, and noted that susceptibility was determined primarily by ruminal contents volume (i.e., the extent to which the rumens were re-filled) rather than by the source of the added ruminal fluid. Cole (1991) demonstrated that exchange of ∼50% of ruminal contents through the cannulas of fed and fasted sheep (which would be expected to have high and low rumen microbial activity, respectively) had no measurable effect on feed intake or on various digestion parameters upon return of the animals to feed. These authors concluded that improving "rumen function" (including the ruminal fermentation) is unlikely to improve feed intake, and thus would not improve production. Taken together, these early studies suggested that the ruminal microbial community might be highly resilient and host-specific.

The above exchange studies demonstrated the adaptability of animals in response to dramatic changes in ruminal contents, but did not directly address the microbiology of the rumen prior to or following the exchange. More recent development of methods for characterizing the microbial community has permitted such examinations. Obviously it is not possible to sterilize the rumen prior to or during the exchange to remove the resident microbiota from the recipient animal, but it is possible to remove almost the entire contents of the rumen, leaving behind a modest portion (a few per cent) of the original resident community that can then be challenged with a relatively massive amount of a co-adapted microbial community from a donor animal. Such studies have shown that retention of even a small fraction of the original resident community is sufficient to facilitate (within days to weeks) a reassortment of the bacterial community to resemble that of the cow prior to contents exchange, even when the donor inocula are obtained from herd mates fed the same diet and housed under apparently identical conditions (Weimer et al., 2010a). One interesting and unexpected result of these exchange experiments was that ruminal pH and VFA profiles returned to those of the recipient cows much more quickly (within 1 day) than did the bacterial community composition. This indicates that the animal exerts ultimate control over its ruminal chemistry through salivary buffering and through absorption and passage of VFA, and in fact this may be one of the means by which the animal provides its own selective pressure on the microbial community. Future experiments could include the use of pairs of animals that are inherently more similar in ruminal chemistry, and could explore the response of all the major microbial groups (not just bacteria), with more detailed characterization using NGS.

Microbial Ecology and the Prospects for Establishing Allochthonous Strains in the Rumen

The essential nutrient transformations within the rumen were identified in the 1950s and 1960s through the pioneering efforts of Hungate and others. From these studies it is apparent that, from an energy conservation and nutrient retention standpoint, the primary limitations to the ruminal fermentation are (1) the relatively slow rate and incomplete extent of fiber fermentation; (2) inefficient nitrogen utilization due to loss of feed protein by unproductive ruminal fermentation to ammonia; and (3) loss of feed energy to methane as a result of interspecies hydrogen transfer reactions. Ever since, rumen microbiologists have made these three limitations the foci of efforts to manipulate the ruminal fermentation.

Overall, there are two primary strategies for manipulating a given aspect of the ruminal fermentation: modifying the ration (including altering the proportions and compositions of the main ration ingredients as well as adding various chemical modifiers, enzymes, etc.), and directly altering the composition of the microbial community (primarily via feeding or addition of probiotics). This review shall consider only the latter strategy, with an emphasis on its likelihood for success based on our understanding of the principles of microbial ecology.

Dietary supplementation with probiotics has become fashionable in human nutrition, and is practiced to some extent in livestock agriculture in developed countries. The essential aim of probiotic feeding is to provide a more balanced and harmonious gut microbial community that improves a suite of gut functions including digestion and immune response. In many cases, probiotic strains appear to function transiently in the community and their effects are sustained only by frequent (daily or continuous) feeding – a marketing executive's dream comes true. Successfully establishing a probiotic strain would require that the strain surmount numerous challenges: surviving the stresses of delivery and inoculation (culture storage, air exposure, mixture into feeds); locating and accessing its food source within the rumen; competing effectively against community members already well-established to the unique characteristics of an individual ruminal habitat; avoiding predation and antagonistic agents (e.g., bacteriocins); establishing productive mutualistic interaction with particular community members; and growing at a rate sufficient to exceed the dilution rate of ruminal contents.

The general characteristics of the ruminal habitat and the associated challenges in establishing an allochthonous microbe therein parallel in many ways those of another familiar and economically important habitat, the anaerobic sludge digester used in biological waste treatment. In both habitats, a complex mixture of substrates of high organic matter content are degraded under semi-continuous conditions by a dense and diverse microbial community that operates at several trophic levels. Because of the large number of potential interactions among community members, establishing a new member in the community would likely require a number of accommodations by the existing community, and a failure of any one of these may be sufficient to

prevent retention and establishment of the new member. Thus it is instructive that there are few reports (Duran et al., 2006; Lu et al., 2009) describing the successful introduction of a probiotic microbial strain into the sludge digester community, and these have only been demonstrated at laboratory scale under which reactor conditions were controlled over a relatively narrow range.

Enhancing Fiber Fermentation

Enhancing plant cell wall digestibility in the rumen by microbial intervention has proven remarkably difficult. Plant fiber is a complex matrix of cellulose, hemicellulose, and lignin, along with smaller amounts of pectins and protein. Lignin itself is essentially indigestible under anaerobic conditions, but its effects do not end there. It also serves as a physical barrier that limits the accessibility of ruminal microbes and their fibrolytic enzymes to otherwise digestible structural polysaccharides. Digestibility can be enhanced by pretreatments (e.g., ammonia) that act primarily by removing lignin or disrupting its chemical bonding to hemicellulose, but such pretreatments generally are not cost effective for ruminant agriculture. Ruminants rely instead on an effective physical pretreatment (mastication and rumination) to improve the digestibility of relatively indigestible plant fiber, largely by increasing available surface area for enzymatic and microbial attack (Van Soest, 1994). There is ample experimental evidence that structural polysaccharide degradation follows first-order kinetics with respect to substrate concentration (Waldo et al., 1972; Van Soest, 1973), and its rate is limited by available surface area (Weimer et al., 1990), and not by the abundance and activity of the microbial population. In fact, several studies have revealed that the *in vitro* digestion rate of both neutral detergent fiber and cellulose does not decline until ruminal contents are diluted approximately five- or sixfold (Pell and Schofield, 1993; Mouriño et al., 2001).

If the rate and extent of fiber digestion are limited by substrate accessibility, one would expect that artificial augmentation of the ruminal community by exogenous addition of fibrolytic microbes would not discernibly improve fiber digestion, and dosing experiments by and large have supported this expectation. Most studies have been aimed specifically at quantifying persistence of dosed strains, without any reported measure of animal performance. Of these, the only report (Chiquette et al., 2007b; **Table 4**) in which dosing of a fibrolytic bacterial strain was associated with maintaining population sizes of $>10^7$ cells/mL for the represented species beyond the dosing period, used an aggressive (and frankly incredible) dosing schedule (reportedly 9×10^{14} cells per dose on alternate days over a 30 days period). In cases where fiber digestibility has been measured, dosing has not improved digestibility or animal performance even during periods of frequent dosing (Krause et al., 2001). These results are in accord with the work of Dehority and Tirabasso (1998), who showed that increasing the cell density of cellulolytic bacteria in the rumen 10-fold by feeding a diet high in purified wood cellulose did not improve *in situ* digestibility of alfalfa fiber.

Recent work with ruminal fungi suggest that this microbial group may show promise as a probiotic for improved fiber digestion (Puniya et al., 2014). Ruminal fungi typically account for only a few per cent of total microbial biomass in the rumen, but their unique ability to combine physical disruption of plant tissue by the growing appresoria, with enzymatic hydrolysis of cell wall polysaccharides may increase in available surface area of fiber that currently limits its rate of digestion in the rumen. Several early studies showed very modest improvement in nutrient digestibility upon dosing with ruminal fungi, but strain persistence was not measured. However, Saxena et al. (2010) reported modest improvement in milk production, as well as increased ruminal zoospore densities, in water buffalo dosed weekly for 180 days with two different fungal strains, (**Table 4**); unfortunately, the specific abundance of the individual species was not measured.

Decreasing Nitrogen Losses

Under modern agricultural production conditions, feed nitrogen (primarily in the form of protein) is inefficiently used by ruminants. It is primarily excreted as ammonia, which acts variously as an air pollutant and a substrate for nitrification to nitrate, which is both a water pollutant and a substrate for respiratory denitrification that yields nitrous oxide, a potent greenhouse gas, as an ancillary product. Use of biological approaches to decrease ruminal protein degradation have received relatively little attention, in part because non-biological interventions are available (e.g., roasting of soybeans to reduce protein reactivity), and in part because there appear to be few viable biological approaches. Decreasing ruminal proteolysis is challenging because the functionally redundant ruminal community contains many species that produce a variety of proteases, and that further ferment amino acids and di- and tripeptides. Some species can potentially be more troublesome than others. Pure cultures of some obligate amino acid fermenters (sometimes called hyper ammonia-producing bacteria, HAB) have specific rates of ammonia production up to 100-fold higher than do *Prevotella* species (Russell, 2002). However, the overall contribution of the HAB may be minor, as the classical HAB species (*Clostridium sticklandii, Clostridium aminophilum,* and *Peptostreptococcus anaerobius*) seem to comprise only a small fraction of the ruminal bacterial community [<0.7% of 16S rRNA gene copy number, as determined by qPCR (Wallace, 1996), while *Prevotella* is perhaps the most abundant genus in the rumen (Stevenson and Weimer, 2007)].

Methane Mitigation

Methane is the most fully reduced form of carbon, and thus its production is an ideal electron sink for balancing fermentations of organic matter in the rumen and other microbial habitats. However, enteric methanogenesis in ruminants represents a loss of 2–14% of feed energy, and is widely regarded as a substantial contributor to anthropogenic greenhouse gas emissions (McAllister et al., 1996). Much effort has been expended in investigating strategies for redirecting the flux of reducing equivalents in the rumen toward alternative electron acceptors, either through the use of chemical agents that inhibit methanogens or their symbionts, or through the direct addition or feeding of various exogenous electron acceptors. The chemical inhibitors often work well *in vitro* but show smaller and more transient effects

in vivo (McAllister et al., 1996). Alternative electron acceptors (e.g., nitrate, sulfate, or fumarate) can potentially be more effective on a long-term basis, but their use *in vivo* is not practical due their high cost or toxicity issues (particularly H_2S produced by sulfate reduction), and the potential requirement for additional inoculation of a direct-fed microbial (Jeyanathan et al., 2014). An alternative approach is to directly inhibit ruminal methanogens and allow the microbial community to redirect electron flow to other acceptors, especially C_3–C_6 VFA. However, careful *in vitro* product balance studies with methane inhibited cultures do not yield a full accounting of this electron flow (see Ungerfeld, 2015, for a detailed meta-analysis), suggesting that it may be necessary to employ additional interventions to realize significant methane mitigation.

One such strategy that has long interested microbiologists is to promote reductive acetogenesis ($4\ H_2 + 2\ CO_2 \rightarrow CH_3COOH + 2\ H_2O$) in the rumen, which would enhance the retention of feed energy in a form readily utilizable by the host. However, ruminal methanogens successfully fill the niche of H_2 utilization in the rumen, and their kinetic and thermodynamic advantages over known acetogens are well established from both pure culture comparisons, and mixed culture studies conducted *in vitro* (Fievez et al., 1999). On this basis, one could predict that acetogens would not establish themselves when exogenously added into the rumen. *In vitro* experiments have largely confirmed these expectations: Acetogens are known to colonize the rumen early in development (Morvan et al., 1994) and can be sustained in gnotobiotic lambs, but quickly disappear upon colonization by methanogens (Gagen et al., 2012). Perhaps the most interesting aspect of the methanogenesis/acetogenesis story is the fact that, as pointed out by Jeyanathan et al. (2014) acetogens can outcompete methanogens in the gut environments of some non-ruminant animals ranging from termites to wallabies to humans. Understanding the basis of competitive success of acetogens in these species may inform future efforts to mitigate methanogenesis in ruminants (Wright and Klieve, 2011).

Because of its intimate link to decreased feed efficiency, there have been some efforts to characterizing the methanogenic community of animals that differ in feed efficiency. Studies have revealed that beef steers grouped as low- or high-efficiency (high and low residual feed intake, respectively) differed in the relative proportions of individual methanogenic OTUs (viz., higher OTU diversity and higher densities of *Methanospaera stadtmanae* and *Methanobrevibacter* sp. strain Amb4 in less efficient animals), even though the total density of methanogenic cells did not differ (Zhou et al., 2009). How these proportional differences may be related to differing methane emissions among animals is not clear. Do particular methanogenic OTUs differ in methane output per unit of cell mass? Can efficiency be increased by ridding the community of specific OTUs?

Answering these questions will ultimately require isolation of individual methanogenic OTUs and performing quantitative comparisons of growth and product formation. In the meantime, culture-independent methods can provide useful perspective on how methane emissions might be de-coupled from the abundance of the total methanogenic population. Poulsen et al. (2013) have recently shown that dietary supplementation with rapeseed oil (RSO, 33 g/kg DM) decreased methane emissions from dairy cows by 6.2% and substantially decreased the relative population size of one specific group of methanogens, the relatively abundant Methanomassiliicoccales (formerly Rumen Cluster C – Thermoplasmata; Iino et al., 2013). Metatranscriptomic data suggest that this group (no representatives of which have been isolated) are methylotrophs that utilize either methylamines (MA) or methanol as methanogenic substrates, and that transcription of two Methanomassiliicoccales-specific methyl-CoM reductase genes decreased upon RSO supplementation. Ruminal concentrations of MA were significantly higher under RSO supplementation (0.22 mM vs. 0.04 mM without supplementation), suggesting that MA (possibly produced from choline and related compounds) are normal methanogenic precursors *in vivo*. Because the flux and passage of MA in the rumen is not known, it is not yet clear if these cellular-level decreases are sufficient to completely account for the observed decrease in methane emitted by the animal, nor is it clear whether the Methanomassiliicoccales may have additional capabilities to produce methane from other feed components. Regardless, this study reinforces the notion that the methanogens are not a physiologically monolithic group, and that methane mitigation strategies might be productively directed toward specific methanogenic subpopulations. Alternatively, Shi et al. (2014) have reported that, while sheep that produce high or low amounts of methane have similar levels of methanogens, expression of certain methanogen-specific genes is elevated in the higher-emitting animals. This suggests that strategies for mitigating methane emissions might be expanded to include regulation of gene expression rather than attempts to control methanogen population sizes *per se*.

Other Potential Microbial Interventions

Ruminal acidosis resulting from sudden increases in the amount or form of dietary starch is a serious health issue that also reduces productivity in both dairy and beef cattle. Acidosis can occur in both an acute and sub-acute form, and is typically ascribed to the activity of bacterial species such as *Streptococcus bovis* that produce lactic acid as a major fermentation end product (Russell, 2002). Lactic acid has a pK_a of ∼3.8 and is thus a much stronger acid than are the volatile fatty acids (pK_a∼4.8). Use of probiotic bacterial strains that consume lactic acid has been proposed as a means of attenuating acidosis, with particular efforts focused on *Megasphaera elsdenii* strain NCIMB 41125 (extensively reviewed by Meissner et al., 2010). Dosing experiments conducted on dairy cows at various stages of lactation and levels of production and fed different diets have yielded inconsistent effects on ruminal pH and on milk production and composition, although decreases in ruminal lactate concentrations were observed. Because relatively few ruminal microbial species are known to actively ferment lactate, it would seem that lactate utilization might provide *M. elsdenii* with an opportunity to fill an available niche in the rumen, but additional studies are necessary to quantify persistence of the dosed species. Interestingly, qPCR studies have revealed that *M. elsdenii* is barely detectable in most ruminal samples tested, although its abundance was strongly

elevated under conditions of both subacute acidosis (Khafipour et al., 2009) and milk fat depression (MFD; see Palmonari et al., 2010; Weimer et al., 2010b; Mohammed et al., 2014). Further research is needed to determine if this species has a causative role in MFD, or if it is merely associated with MFD through some other primary driver.

A second strategy to control acidosis is via probiotic addition of bacteria that rapidly ferment starch but do not produce appreciable amounts of lactic acid. Chiquette et al. (2007a) ruminally dosed *Prevotella bryantii* 25A daily for a 10 week period (3 week prepartum to 7 week postpartum) into cows fed a diet whose forage:concentrate ratio was 40:60. Relative to control cows, lactate concentrations in the dosed cows were halved (0.7 mM vs. 1.4 mM), VFA concentrations were higher, and there was a tendency toward increased milk fat percentage. Interestingly, ruminal ammonia was also elevated in the dosed strains, suggesting perhaps that the dosed strain contributed to undesirable degradation of protein and its hydrolytic products. Unfortunately the abundance or persistence of the dosed strain in the rumen was not quantified.

Practical Considerations for Probiotics

For any manipulation involving a probiotic, identification of the candidate microbial agent through laboratory and animal trials is just the beginning. Additional research is necessary to develop a formulation that retains activity over the course of shipping, storage and application under production conditions. A large body of information on formulation (often proprietary) has been gathered for specific microbial products in other applications (probiotics for human use, silage inoculants, etc.), and formulation technology has been developed for certain ruminal probiotics (particularly yeast and lactic acid bacteria). However, probiotic products based on microbes isolated from the rumen itself may face additional challenges due to their often strictly anaerobic nature, poor viability retention in stationary phase, and lack of a highly resistant resting state (spores, cysts, etc.) to resist environmental insult. Perhaps the most detailed formulation research on a true ruminal microbe has been obtained with *Megasphaera elsdenii*, noted above for its potential to control lactic acidosis (Meissner et al., 2010). This bacterium has been successfully packaged into a pouch containing separate compartments for dried cells and growth medium; squeezing of the pouch combines the ingredients at the point of use in a readily incubated form under anaerobic conditons. Once grown, the culture was dosed into the rumens of cannulated cows in research studies, but it is not clear how effectively it can be introduced into animals via feeding.

Concluding Remarks

From the above discussion, several salient points emerge. First, the microbial community is phylogenetically diverse, metabolically redundant, and both compositionally and functionally resilient. Second, the stability and host specificity of the community provide substantial barriers to manipulation of community composition and function. Third, successes in modifying the microbial community to improve animal performance have thus far been most dramatic for specialist microbes that fill an otherwise unoccupied niche. Fourth, recent advances in determining community composition and diversity have far outpaced our ability (or willingness) to dissect out the physiological and ecological roles of individual phylotypes, particularly those that impact animal performance. Elucidating these roles and exploiting them to manipulate the composition and function of the ruminal microbiome represents probably the most challenging and most important means by which microbiologists can advance the productivity, profitability, and sustainability of ruminant animal agriculture.

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REVIEW
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Manipulating rumen microbiome and fermentation through interventions during early life: a review

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The nutritional manipulations of the rumen microbiome to enhance productivity and health are rather limited by the resilience of the ecosystem once established in the mature rumen. Based on recent studies, it has been suggested that the microbial colonization that occurs soon after birth opens a possibility of manipulation with potential to produce lasting effects into adult life. This paper presents the state-of-the-art in relation to early life nutritional interventions by addressing three areas: the development of the rumen as an organ in regards to the nutrition of the newborn, the main factors that determine the microbial population that first colonizes and establishes in the rumen, and the key immunity players that contribute to shaping the commensal microbiota in the early stage of life to understand host-microbiome specificity. The development of the rumen epithelium and muscularization are differently affected by the nature of the diet and special care should be taken with regards to transition from liquid (milk) to solid feed. The rumen is quickly colonized by all type of microorganisms straight after birth and the colonization pattern may be influenced by several factors such as presence/absence of adult animals, the first solid diet provided, and the inclusion of compounds that prevent/facilitate the establishment of some microorganisms or the direct inoculation of specific strains. The results presented show how early life events may be related to the microbial community structure and/or the rumen activity in the animals post-weaning. This would create differences in adaptive capacity due to different early life experiences and leads to the idea of microbial programming. However, many elements need to be further studied such as: the most sensitive window of time for interventions, the best means to test long term effectiveness, the role of key microbial groups and host-immune regulations.

Keywords: early life, microbial colonization, rumen development, rumen microbiome, weaning

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INTRODUCTION

The forestomachs of ruminant animals contain a great diversity of prokaryotic (bacteria, archaea, virus) and eukaryotic (protozoa and fungi) micro-organisms that together breakdown and ferment the feed ingested by the host animal (Dehority, 2003). In the last decades there have been significant efforts to develop compounds that may shift the rumen fermentation toward more

efficient metabolic pathways by targeting key groups of microorganisms (i.e., archaea in case of methanogenesis, Hart et al., 2008). However, the utility of such compounds often appears limited as results are often inconsistent or short-lived. This is mainly due to the difficulty in modifying a well-established and fully matured microbial ecosystem in the rumen of adult animals. There is ample evidence of a strong host-microbiota specificity (Kittelmann et al., 2014), implying that after any alteration (i.e., rumen digesta swapping, exogenous bacteria application or antibiotic treatment), once ceased, the microbial community composition and fermentation profile will return to the original pre-treatment composition (Weimer et al., 2010). The developing rumen in the new-born provides a unique opportunity for potential manipulation of such a complex microbial ecosystem.

Early experience ingesting feeds increases preference for and later consumption of those feeds by animals (Provenza and Balph, 1990). Early dietary experiences have a greater and more lasting effect than those occurring later in life (Distel et al., 1994). Different processes (neurological, morphological, and physiological) may be involved during early in life and can be altered so that animals can better manage in the environment in which they are reared from birth.

Li et al. (2012), based on 454-pyrosequencing of 16S rDNA, reported that a total of 170 bacterial genera exists in the developing rumen of 14 days old calves, and that the microbiota was responsive to dietary modifications as well as physiological changes in the host. Earlier work reported that forage or concentrate diets fed around weaning had an impact on the bacterial population that established in the rumen (Eadie et al., 1959); however, the impact that this differentiation might have later in life on the rumen microbial ecosystem remained to be determined. Recent studies (Yáñez-Ruiz et al., 2010; Abecia et al., 2013, 2014a) suggested that it would indeed be possible to promote different microbial populations establishing in the rumen of the young animal by manipulating the feeding management early in life that persisted in later life. This would create differences in adaptive capacity due to different early life experiences, leading to the idea of microbial programming. However, despite significant research effort, there is still a lack of understanding of the mechanisms governing microbial/host cell interactions, the development of the rumen and its microbial community, and the implications for the host when microbial colonization patterns are altered, especially the long-term effects. This paper will critically review the information published on: (i) the development of the rumen as an organ in regards to the nutrition of the new-born, (ii) the factors (maternal, dietary, etc.) that determine the microbial population that first colonizes and establishes in the rumen and (iii) the key immunity players that contribute to shaping the commensal microbiota in the early stage of life to understand host-microbiome specificity. The aim of the review is to evaluate the importance of the multiple factors in shaping the rumen microbiome and the potential of early life rumen microbial programming based on current research and to identify gaps of knowledge for future research studies.

THE DEVELOPMENT OF THE RUMEN AS AN ORGAN AND THE INFLUENCE OF THE DIET

Young ruminants present at birth an undeveloped reticulo-rumen, therefore, until the system is fully matured they function as monogastrics fed on milk-based diets that are not digested in the rumen but in the abomasum (Church, 1988; Davis and Drackley, 1998). As stated by Heinrichs (2005) 'a smooth transition from a monogastric to ruminant animal, with minimal loss in growth, requires the development of the reticulo-rumen and its associated microbial population for efficient utilization of dry and forage-based diets'.

Development of the rumen is an important physiological challenge for young ruminants (Jiao et al., 2015). It entails growth and cellular differentiation of the rumen, and results in a major shift in the pattern of nutrients being delivered to the intestines and liver, and thus the peripheral tissues of the animal (Baldwin et al., 2004). The development of the rumen involves three distinct processes: (i) anatomical development (growth in rumen mass and growth of rumen papillae; Reynolds et al., 2004), (ii) functional achievement (fermentation capacity and enzyme activity; Rey et al., 2012; Faubladier et al., 2013), and (iii) microbial colonization (bacteria, fungi, methanogens, and protozoa; Fonty et al., 1987; Fouts et al., 2012). This section addresses the first process, while microbial colonization will be discussed in Section "Factors that Influence the Microbiota Establishing in the Rumen and Long Term Effects."

An inadequate development of the rumen will affect nutrient digestion and absorption (Baldwin et al., 2004). On the other hand, a complete development of the rumen facilitates digestion of feed components, which provides nutrients for the physiological requirements of the animal. The anatomical development of the rumen is a process that occurs following three phases: non-rumination (0–3 weeks); transitional phase (3–8 weeks), and rumination (from 8 weeks on; Wardrop and Coombe, 1960; Lane et al., 2002).

During the transition from a pre-ruminant to a ruminant animal, growth and development of the ruminal absorptive surface area (papillae) is essential to enable absorption and utilization of digestion end products, specifically rumen volatile fatty acids (Warner et al., 1956). The presence and absorption of volatile fatty acids stimulates rumen epithelial metabolism and may be key in initiating rumen epithelial development (Baldwin and McLeod, 2000). Different studies (Nocek et al., 1984; Greenwood et al., 1997) have shown that ingestion of dry feeds and the resultant microbial end products stimulate the development of the rumen epithelium. However, different volatile fatty acids stimulate such development differently, as butyrate is most stimulatory, followed by propionate. With decreasing rumen pH and increasing butyrate concentrations, butyrate metabolism by the epithelium increases concomitantly (Baldwin and McLeod, 2000). A continuous exposure to volatile fatty acids maintains rumen papillae growth, size, and function (Warner et al., 1956). Thus, it is expected that diets consisting of milk, concentrates, or forages affect the rumen epithelial growth to different extents (**Table 1**). Moreover,

TABLE 1 | Effect of different dietary interventions in early life on rumen development parameters.

Dietary treatment	Rumen weight, kg	Wall thickness, cm	Papillae, n/cm^2	pH	Study
Corn processing	ND	Affected (1.06–1.21)	ND	Affected (5.41–5.66)	Lesmeister and Heinrichs, 2004
Supplemental yeast	ND	No effect	ND	ND	Lesmeister et al., 2004
Supplemental molasses	ND	No effect	ND	ND	Lesmeister and Heinrichs, 2005
Carbohydrate composition	Increased (0.73–1.73)	Increased (0.86–1.32)		Affected (4.9–5.3)	Suárez et al., 2006
Milk allowance	Increased (1.37–1.89)	Increased (1.15–1.47)	Increased (71–86)	Decreased (6.22–5.66)	Khan et al., 2007
Milk allowance	Affected (0.58–1.35)	ND	ND	Affected (5.56–6.29)	Kristensen et al., 2007
Forage to concentrate ratio	Affected (0.95–1.45)	Affected (0.82–1.20)	ND	Affected (5.09–5.23)	Suárez et al., 2007
Starch sources	Affected (1.21–1.53)	Affected (1.55–1.95)	Affected (70–91)	Affected (5.46–5.79)	Khan et al., 2008
Provision of hay	Increased (1.59–1.89)	No effect	No effect	Increased (5.06–5.49)	Khan et al., 2011
Whole milk vs. milk replacer	No effect	ND	ND	No effect	Górka et al., 2011
Whole milk vs. milk replacer	Decreased (0.73–0.66)	ND	ND	Increased (6.12–6.57)	Abecia et al., 2014b
Milk replacer feeding strategy	No effect	ND	ND	Affected (6.2–6.9)	Silper et al., 2014

ND, not determined.

the establishment and activity of the rumen epithelial tissue-associated microbes (defined as epimural community) may be another factor that influences the extent of development of the rumen epithelium (Malmuthuge et al., 2012, see "Factors that Influence the Microbiota Establishing in the Rumen and Long Term Effects").

The chemical composition of the liquid (milk) feed and the effect of the oesophageal groove limit the process of physical and functional development of the rumen (Warner et al., 1956). In young ruminants fed only milk or milk replacer, the rumen development has been shown to be limited even up to 12 weeks of age (Tamate et al., 1962). Indeed, it has been reported a regression of rumen development when calves were changed from a solid diet and milk replacer to a solely milk/milk replacer diet (Harrison et al., 1960). Also, young ruminants receiving only milk/milk replacer had limited metabolic activity in the rumen epithelium and minimal absorption of volatile fatty acids (Heinrichs, 2005). Therefore, although milk based diet promote rapid and efficient growth of the young animal, it does not contribute to prepare the pre-ruminant to utilize solid diets.

Unlike liquid feeds, solid feeds are mainly directed to the reticulo-rumen for digestion (Church, 1988). Solid feed intake stimulates rumen microbial proliferation and production of volatile fatty acids, which have been shown to initiate rumen epithelial development, although, different solid feeds may differ in their ability to stimulate the development of the rumen. Both the chemical composition of feeds and the resultant microbial digestion end products have the greatest influence on the development of the rumen epithelium (Nocek et al., 1984).

Providing natural milk or milk replacer to newborn ruminants differs not only in their intrinsic differences in nutrient composition but also in the presence or absence of the dam. In ruminant farming two main systems for managing the young animals can be identified. In commercial dairy systems, calves are typically separated from the dam at a young age and fed either milk replacer or whole milk; on the contrary, in fattening systems, the newborns remain with the mother until weaning. It has been recently reported that kid goats reared with the dam had greater rumen development than their twins that were fed on milk replacer and isolated from adult animals, despite both groups having access to the same forage and concentrate offered *ad libitum* (Abecia et al., 2014b). This is accordance with De Paula Vieira et al. (2012), which showed that calves reared in the presence of older companions exhibited more frequent and longer visits to the feeder, which they hypothesized to be a consequence of social learning (Galef and Giraldeau, 2001). However, the advantage of the direct microbial inoculation through physical contact with the dam deserves further attention, as discussed in the following section.

In intensive farming, the supplementation with concentrates is the most common method of providing nutrients to the animal with emphasis on offering young ruminants concentrate solid starter at a relative early age (Jiao et al., 2015). Therefore, in the last years, research on rumen development has been mainly directed on this type of feeding system and the main factors that affect rumen development in ruminants fed a range of different diets (Owens et al., 1993), with the primary attention on diet composition (**Table 1**, Coverdale et al., 2004; Suárez et al., 2007; Khan et al., 2011). Feeding concentrate feeds in early life stimulates the development of the epithelium, while forages with large particle size or high fiber sources appear to be the primary stimulators of rumen muscularization and volume (Zitnan et al., 1998). Several recent studies have shown that another effective method to foster solid feed intake in calves, contrary to what it has been traditionally adviced, is to provide *ad libitum* access to poor quality (nutritionally) chopped straw or hay (Jiao et al., 2015). Castells et al. (2013) conducted a meta-analysis and concluded that there were no differences in gut fill between calves consuming no forage and calves consuming forage up to 5% of total solid feed consumption. Thus, it can be concluded that when forage consumption is less than 5% of the total solid feed intake, gut fill is negligible and thus advantages reported in performance and efficiency when feeding chopped forages to calves are not an artifact due to gut fill. Depriving calves from forage during the pre-weaning phase may offer yet another physiological and dietary adaptation challenge to young

calves during the transition when presented with forage for the first time. Phillips (2004) reported that calves fed fresh grass during the milk-feeding period spent more time eating on a pasture compared with those that received no forage before weaning. Recent data also shows that 22% of the variation in milk yield in first lactation is associated to the average daily gain during the first weeks of life (Soberon et al., 2012). However, the long-term effects of early life nutritional management in relation to rumen development are still largely unknown and there are factors that still need to be carefully considered such as composition of the starter, type of forage and timing of its introduction.

When addressing the development of the rumen, the following question arises: does the development of the organ determine which microbes colonize the rumen or do the microbes themselves shape the rumen development through their activity and specific signaling? In the adult animal, the diet is the main driver of the microbial community structure (McCann et al., 2014), but in the pre-ruminant both microbial colonization and rumen development may interact in a way that one influences the other. Also, it is still unknown to what extent the animal is genetically pre-determined to develop a certain type of rumen (i.e., epithelium, muscularization, contractions). Goopy et al. (2012) reported that low methane yield sheep were associated with a shorter mean retention time of particulate and liquid digesta, lesser amounts of rumen particulate content and a smaller rumen volume. Low methane yield sheep harbor a distinctive bacterial community structure (Kittelmann et al., 2014). Thus, it could be hypothesized that promoting a large rumen by feeding more forage in early life may determine the type of microbiota harbored in the rumen and consequently the digestion efficiency of the animal.

FACTORS THAT INFLUENCE THE MICROBIOTA ESTABLISHING IN THE RUMEN AND LONG TERM EFFECTS

Sequential Microbial Colonization of the Rumen

The gastrointestinal tract of most animals is supposed to be sterile and germ free right after birth; then, microbes from other adult animals and the surrounding environment subsequently colonize the rumen until a very complex and diverse microbial population develops (Ziolecki and Briggs, 1961). Several studies have shown that in young ruminants and during rumen development, ingested microbes colonize and establish in a defined and progressive sequence (Stewart et al., 1988). Ample evidence (Fonty et al., 1987; Morvan et al., 1994) now exists that a significant proportion of the strict anaerobes that become predominant in the mature rumen are already present in the rumen 1 or 2 days after birth. The use of molecular techniques has shown the complex microbial community that soon establishes in the non-mature rumen. All major types of rumen **bacteria**, including proteolytic and cellulolytic species, as well as some niche specialists, are present in the rumen microbial community of 14 days old calves (Li et al., 2012), whilst Jami et al. (2013) stated that "some rumen bacteria essential for mature rumen function could be detected as early as 1 day after birth". Rey et al. (2013) monitored the establishment of ruminal bacterial community in dairy calves from birth to weaning. They showed that the establishment is rapid after birth and sequential: *Proteobacteria* is gradually replaced by *Bacteroidetes* as the main Phyla. Between days 3 and 12, the bacterial community was composed of many bacteria present in the developed rumen, showing that the bacteria responsible for the degradation of feeds are present before the ingestion of solid substrate begins. Between days 9 and 15, diet influence seemed strongest and was associated with a change in the bacterial community structure. From 15 days on, the community no longer exhibited clear time related changes at phyla level although variations on the relative abundance of some genera did occur (**Table 2**).

Becker and Hsiung (1929) first demonstrated that the rumen **ciliate protozoa** are passed from animal to animal by direct transfer of saliva containing the active organisms as there is no resistant phase or cysts in their life cycle (Strelkov et al., 1933). Ciliate protozoa can normally be seen in the rumen of young ruminants within 2 weeks of birth with small entodinia established before large endomorphs and holotrich protozoa (Eadie, 1962). However, if animals are isolated from other ruminants shortly after birth no protozoa establish (Bryant and Small, 1960; Eadie, 1962), a property that has been widely used and continues to be used to study the role of protozoa in the rumen (Belanche et al., 2014).

Methanogenic archaea have been found in the undeveloped rumen of lambs well before the arrival of solid substrate to the rumen (2–4 days) and reach concentrations equivalent to those in adult animals around 10–14 days after birth (Fonty et al., 1987; Morvan et al., 1994). The development of molecular techniques allowed the detection of methanogenic archaea at earlier stages as probably they could not be detected by classical microbial counting (Gagen et al., 2012). Guzman et al. (2015) has

TABLE 2 | Age classification of bacterial groups colonizing the rumen from birth to weaning. Values expressed as range of mean percentages[a].

	Age (days)				
	3	7	14	28	42
Phyla					
Proteobacteria	46.6–70.4	16.9–18.7	6.45–16.9	1.8–27.6	12–27.6
Bacteroidetes	13.9–42.6	56.3–56.9	46–61.3	49.9–56.3	56.3–74
Firmicutes	5.05–13.9	13.9–17.5	13.9–34	13.9–42.1	10–13.9
Actinobacteria	0.05–4.9	0.55–4.9	0.95–4.9	0.25–4.9	4.9
Fusobacteria	4.7–5.55	4.7–5.30	0.2–0.55	0.2–0.3	0.2–0.4
Spirochaetes	0–0.4	0.1–0.4	0.4–2.60	0.4–0.85	0.4
Fibrobacteres	0–0.3	0–0.3	0.2–0.3	0.3–1.45	0.3–1.6
Tenericutes	0	0.80	0.20	0.90	0.95
Elusimicrobia	0	0	0.20	1.45	2.1
Lentisphaerae	0	0	0.15	0.20	0.31

[a]*Data collected from Li et al. (2012), Jami et al. (2013) and Rey et al. (2013).*

recently reported that at day 0 of life *M. mobile*, *M. votae,* and *Methanobrevibacter* sp. were detected in the rumen of neonatal dairy calves.

As reviewed by Stewart et al. (1988), anaerobic **fungi** established in the rumen of flock-reared lambs by 8–10 days after birth (Fonty et al., 1987). They were found in all lambs by 3 weeks of age and interestingly then were no longer detectable in 9 of the 11 lambs studied when a diet based on concentrate was provided. The fungal population was mainly composed of *Neocallimastix frontalis*; *Sphaeromonas communis* was found only sporadically. The early appearance of these fungi is another characteristic of the rumen. These microorganisms which had only previously been found in mature ruminants or when forage-rich diets are fed (Orpin and Joblin, 1988) are apparently able to develop in the rumen before solid substrate enters the rumen.

In addition to the colonization pattern of the different microbial groups in the rumen, special attention should be paid to the microbial community associated to the rumen wall. Stewart et al. (1988) stated that the **epimural bacterial community** is established shortly after birth and soon reaches concentration equivalent to those in the adult while the diversity of this community seem to change with age (Mueller et al., 1984a; Rieu et al., 1990). Mueller et al. (1984a) described 24 morphological types of bacteria associated to the rumen wall in 1- to 10-week old lambs by using scanning electron microscopy, although only seven types, found in both the lamb and the adult, could be considered indigenous members of the epimural community. This community follows a characteristic succession, with significant changes occurring in the generic composition through the first 10 weeks of life. According to Mueller et al. (1984b), the epimural community does not appear to be markedly different taxonomically from the bacterial community of rumen contents, since most isolated strains could be placed into common rumen genera. However, recent studies conducted using molecular tools disagree with that statement. Sadet et al. (2007) using PCR-DGGE found that the epithelial community differed from that of rumen contents. As expected, the nature of the feed influenced the bacterial communities from the solid and liquid rumen phases but no diet effect was observed in the rumen epithelial profiles, suggesting a strong host effect on this bacterial population. More recently, Malmuthuge et al. (2014) reported large differences between digesta and epimural bacterial communities in the rumen of pre-weaned calves, highlighting greater abundances of *Prevotella* and lower abundances of *Bacteroidetes* in digesta compared with epimural bacterial communities. Moreover, the apparent association between the development of the mucosal bacteria community with the expression of some key immune related genes in mucosal tissue (Malmuthuge et al., 2012), suggests that future work on rumen colonization should include the study of the epimural community.

Factors that Influence Early Life Colonization

Given that the different trophic niches in the rumen ecosystem are first occupied during early life and that a key turning point in microbial colonization is the introduction of solid feed in the diet (Rey et al., 2013), an important issue to address is whether management of the newborn alters the colonization pattern. As described in Section "Sequential Microbial Colonization of the Rumen," there is now ample evidence of the early colonization of the rumen by anaerobic microorganisms, however, very few studies have actually compared the colonization pattern of the undeveloped rumen in the context of the *factors* that facilitate (or prevent) the colonization of some microbial groups (i.e., maternal influence, offspring reared in isolation, liquid/solid feed, use of additives, etc).

Protozoa are not essential for the normal rumen functioning (Williams and Coleman, 1992); however, the presence/absence of protozoa has been associated with the structure of different bacterial and methanogens communities and different rumen fermentation pattern (Yáñez-Ruiz et al., 2007; Belanche et al., 2014). Adult ruminants harbor distinctive protozoal populations with key species such as *Polyplastron* and *Epidinium* indicative of types A and B populations, respectively (Williams and Coleman, 1992). The introduction of *Polyplastron* into the rumen of animals harboring a type B protozoal population leads to the elimination of type B protozoa, however, within most flocks sheep exist with approximately the same number of animals harboring a type A and type B populations, clearly some unknown host factor influences the colonization of the rumen of individual sheep by protozoa (Williams and Coleman, 1992). Skillman et al. (2004) used twin lambs to identify methanogens colonizing the rumen of young lambs. The similarities between the rumen methanogen populations found in twins suggested that the dam was the main source of methanogen inoculation. The **maternal** influence has been further supported in recent studies in terms of microbial development in pre-ruminants subjected to anti-methanogenic treatments (bromochloromethane, BCM). Abecia et al. (2013, 2014a) reported that the archaeal community establishing in the rumen of kids depended on whether the doe was treated or not with BCM. This suggests that any intervention applied in the early life of young animals raised by the dams should consider applying the same treatment to the doe.

Both Abecia et al. (2014b) and Belanche et al. (2015) showed a different colonization pattern for protozoa in artificially reared animals as compared to those raised by the dams. Abecia et al. (2014b) showed that natural milk feeding via the dam vs. artificial feeding with milk replacer resulted in consistently lower pH in the developing rumen of goat kids that stayed with the mothers. They hypothesized that naturally raised kids would have consumed more concentrate at an earlier stage as a result of social feeding learning. An environment with a different pH during the development would be more beneficial for some microbial groups (Palmonari et al., 2010) and may set a different microbial population in the adult animal.

Anderson et al. (1987) showed that introducing solid feed for early weaning (3 weeks) in calves promoted greater microbial abundance in the rumen as compared to calves weaned conventionally (6 weeks), but no assessment of the composition of the microbiota was performed. Early studies (Eadie et al., 1959; Ziolecki and Briggs, 1961) reported that giving forage or forage and concentrate around weaning determined the concentration

of some anaerobic bacteria (lactobacilli and lactate-utilizing cocci), although no information on the persistency of such effect was provided. Yáñez-Ruiz et al. (2010) reported that feeding forage vs. concentrate around weaning modified the bacterial population colonizing the rumen of lambs and that the effect persisted over 4 months, suggesting the possibility of further exploring the feasibility of manipulating the microbial populations present in the adult animal using diets or dietary additives fed early in life.

In addition to the introduction of solid diet around weaning, nutritional interventions in early life may include (i) the direct inoculation of specific microorganisms or (ii) the use of compounds (i.e., additives) that prevent or facilitate the colonization of some microbial groups. Feeding live microorganisms to ruminants is not a novel concept and extensive work has been published on the use of 'direct-fed microbials' (DFM; Martin and Nisbet, 1992; Jeyanathan et al., 2014). Theodorou et al. (1990) reported that the addition of the anaerobic rumen fungus *Neocallimastix* sp increased intake and liveweight gain in calves at weaning, whilst Ziolecka et al. (1984a,b) reported that a stabilized rumen extract enhanced live weight gain and stimulated rumen development in calves during weaning and Zhong et al. (2014) demonstrated that inoculation of fresh rumen fluid into the rumen of lambs for 7 days improved average daily gain and digestibility in early weaned lambs. Nakanishi et al. (1993) found that adding lactic acid bacteria to starter diets of Holstein calves stimulated rumination and ruminal development, however, no performance benefits were observed and possible microbial changes during rumen development were not determined. Lesmeister et al. (2004) evaluated the effect of supplementing yeast (*Saccharomyces cerevisiae*) culture on rumen development and growth performance in neonatal dairy calves. Although yeast cultures are widely used in ruminant nutrition, the concept of applying them in the diet of pre-ruminants deserves further assessment. They conclude that the addition of yeast in dairy calf starter at 2% enhanced dry matter intake and growth and slightly improved rumen development. Unfortunately they did not study either the effect on the rumen microbiota or the long-term effects in the animals. Other microbes targeting the rumen (i.e., *Megasphaera elsdenii*, propionibacteria) have been used as rumen probiotics but to our knowledge only in adult animals (Klieve et al., 2003).

A different experimental approach is to provide specific microbes in gnotobotically reared neonates. Gnotobitic lambs harboring either a simple or complex microbiota are an important method for investigating the role of specific microbes in the rumen. This approach has been used mainly to gain insight in the manipulation of microbes directly involved in H_2 transfer within the rumen. Hydrogenotrophic acetogens colonize first the rumen and then they are gradually replaced by methanogenic archaea as the rumen develops (Gagen et al., 2012). The early establishment of acetogenic and sulfate-reducing bacteria underlines the competition that exists between H_2-utilizing species. Naturally reductive acetogenesis is not a significant hydrogen sink in the rumen. However, in the absence of methanogenesis, acetogens contribute to H_2 capture and can sustain functional rumen. Fonty et al. (2007) demonstrated using gnotobiotic lambs, that in animals lacking ruminal methanogens, the introduction of acetogens made reductive acetogenesis the major hydrogenotrophic process and that the effects of such intervention applied after birth persisted 12 months later. They suggested that if reliable methods for eliminating methanogens from early life and maintained the inoculation with acetogens could be a feasible option to decrease methane emissions from adult animals. More recently, Gagen et al. (2012) used lambs that were born naturally, left with their dams for 17 h and then placed into a sterile isolator and reared aseptically. They were inoculated with cellulolytic bacteria and later with *Methanobrevibacter* sp.7 to investigate the effect of methanogen establishment on the rumen acetogen population since they lacked cultivable methanogens. Methanogens were present in lambs isolated 17 h after birth, though were undetectable using traditional cultivation techniques. Methanogen numbers were low in these lambs ($<10^4$ rrs copies per microgram of DNA) however, mcrA diversity was not dissimilar to that found in 2-year-old conventional sheep. This suggests that early colonizing methanogens may persist in the rumen and supports the potential of early life microbial programming.

With regards to the suppression of methanogens in early life, the use of compounds that inhibit the establishment of certain microbial groups or favor the development of others is now starting to attract attention. Abecia et al. (2013, 2014a) showed that application of BCM to young goat kids modified archaeal colonization of the rumen, which was linked to a reduction in methane emission of around 50%, with the effects persisting for 3 months after weaning and cessation of treatment in kids raised by does that received the same treatment as the kids.

Timing for Interventions in Early Life and Persistency of the Effects

Given that particular factors favor the establishment of certain microorganisms, we still need to know what the most sensitive window of time for interventions is in early life. Recent work (Rey et al., 2013; Abecia et al., 2014b; Guzman et al., 2015) showed that initial colonization occurs straight after birth and that it takes 3–4 weeks for the bacterial community structure to reach a certain degree of stabilization from birth, suggesting that this period is critical. However, this assumes that once the community is more or less constant, there is no room for 'programming' and this has not yet been fully confirmed. It is clear that nutrient supply and hormonal signals at specific times during development (both pre- and early post-natal) exert permanent changes in the metabolism of humans (Fall, 2011), as well as changes in performance, body composition, and metabolic function of the offspring of livestock (Wu et al., 2006). These changes occur through processes generically referred to as fetal programming and metabolic imprinting. The information available in ruminants suggests that microbial colonization occurs earlier than functional achievement (i.e., a functioning rumen), with anatomic development occurring last (Jiao et al., 2015). However, the actual window of time in which such changes

can be exerted in relation to microbial colonization and more importantly the persistency of the imprint needs to be further clarified. Therefore, there is urgent need to further address this question with more fundamental research. Rey et al. (2013) showed substantial colonization by the main bacterial groups in the first days of life. Likewise, Guzman et al. (2015) reported the presence of methanogenic archaea and fibrolytic rumen bacteria at day 0 in neonatal dairy calves, which suggests that the window for intervention starts straight after birth. Along these lines, there are some reported cases of human twins that harbor different gut microbiota (Clemente et al., 2012), which also offers promise for the potential of early life programming interventions. Studies in humans showed that early gut colonizers, such as those acquired from parents, can exert physiological, metabolic, and immunological effects for most of our lives (Faith et al., 2013).

HOST IMMUNE RESPONSE TO MICROBIOTA

The gastrointestinal tract has a diverse array of non-specific and specific protective mechanisms to allow it to coexist with resident microbiota (Hooper et al., 2012). The functions of nutrients absorption, symbiotic microbial tolerance and pathogenic microbial barrier, create a conflict in function requiring a complex system of physical, biochemical, and cellular mechanisms for protection of the intestinal mucosa against invading pathogens (Kuhn and Stappenbeck, 2012).

The training or education process that the immune system needs to go through to learn how to deal with microbial loads has been widely highlighted (Wu and Wu, 2012) and this is of particular importance during early life stages (Collado et al., 2012); however, the mechanisms involved in the 'tolerance' to the first colonizers of the rumen are largely unknown. The physiological elements involved in the case of the rumen may differ from other parts of the gastrointestinal tract due to the nature of the fermentation and the constant exposure of the rumen wall to microbial biomass. The forestomachs of the ruminant species are expanded esophageal portions lined by stratified squamous epithelium. As stated by Trevisi et al. (2014), scant information is available about the organization of the epithelial immune system in forestomachs as opposed to the impressive amount of data about the intestinal tract of both ruminant and non-ruminant species (Dommett et al., 2005). In general, the immune response in the mucosal areas of the gut is orchestrated by mucosal-associated lymphoid tissue (MALT) and gut-associated lymphoid tissue (GALT) in the gut. However, in the rumen no organized lymphoid tissue exists in the epithelium (Sharpe et al., 1977). The rumen epithelium includes up to a 15 cell layer, which can limit the permeability of large molecules. Therefore, the microbial equilibrium in the rumen is achieved by a combination of different mechanisms, illustrated in **Figure 1**: (i) constant supply of Immunoglobulins (IgA and IgG) via saliva (Williams et al., 2009), (ii) the activity of Toll-like receptors (TLRs, Malmuthuge et al., 2012), (iii) a group of genetically encoded pattern recognition receptors (Seabury et al., 2010); (iv) peptidoglycan recognition proteins (PGLYRP1, Malmuthuge et al., 2012), and (v) antimicrobial peptides defensins (Malmuthuge et al., 2012; Meade et al., 2014).

Blood serum in animals contains circulating antibodies to a wide range of Gram-negative bacteria, particularly enterobacteria (Landy and Weidanz, 1964). The antibodies are considered to be natural antibodies produced in the absence of overt infection (Boyden, 1966). Sharpe et al. (1969) found antibodies against specific strains of rumen bacteria in the blood of cows, sheep, goats, and horses, but not in pigs, rabbits, and humans. They showed the high specificity of the natural agglutinating antibodies in ruminants in absorption tests, which was further confirmed by the absence of agglutinins against a human *Escherichia coli* strain although they were detected against a rumen *E. coli* strain. A close relationship seems to exist between motility of the rumen microorganisms and their ability to stimulate natural antibodies, with antibodies being detected against motile *Butyrivibrio*, *Streptococci,* and *Lactobacilli* (Sharpe et al., 1969). Further work showed that antibodies against these organisms were present in bovine colostrum at the same level as serum and were transferred to the calf serum via colostrum (Sharpe et al., 1977). While not excluding the possibility that non-viable rumen bacteria leaving the abomasum could be a source of antigenic stimulus in the small intestine, an early study (Latham et al., 1971) investigated the caecum as an alternative site of antigenic stimulus. Many of the bacteria in the caecum are similar to those in the rumen, however, whereas the epithelium of the rumen is non-glandular and keratinized that of the caecum contains lymphatic tissue and plasma cells. The role of the caecum as active immune organ needs to be further studied.

Sharpe et al. (1977) used four gnotobiotic lambs, reared on milk, a starter ration and then grass cubes to understand the relationship between rumen microbial colonization in early life and antigen production. The lambs were inoculated with strains of *Veillonela*, *Prevotella ruminicola*, *Ruminicoccus*, *Selenomonas, Megasphaera, Lactobacillus, Butyrivibrio* and, in one case, *E. coli*. *Prevotella, Selenomonas,* and *Megasphaera* gave a strong immunological response, with antibodies to the former bacteria appearing at 20–40 days after inoculation and to the *Megasphaera* at 28–74 days. Agglutinins to *Veillonella* and *Ruminococcus* were weak and appeared only at 100–136 days after inoculation. As expected, no agglutinins were detected against non-inoculated bacteria. The gnotobiotic lambs did not receive colostrum and were born with only traces of immunoglobulins, but after 74–77 days had synthesized appreciable amounts of IgM and relatively little IgG. Since the primary antigenic response of an animal is to produce IgM, the preponderance of IgM is not surprising. At 140 days, IgG levels had risen considerably to similar levels as IgM. These results highlighted the strong link between rumen microbial colonization and specific antigen production. Unfortunately, no further work has been conducted in animals reared under different conditions in early life.

As noted earlier, saliva seems to be the main vehicle of introducing immunoglobulins in to the rumen. The levels of IgA and IgG in cattle serum, saliva, and rumen fluid have been studied recently in the context of exploring the possibility of vaccinating ruminants against specific rumen microorganisms (Subharat

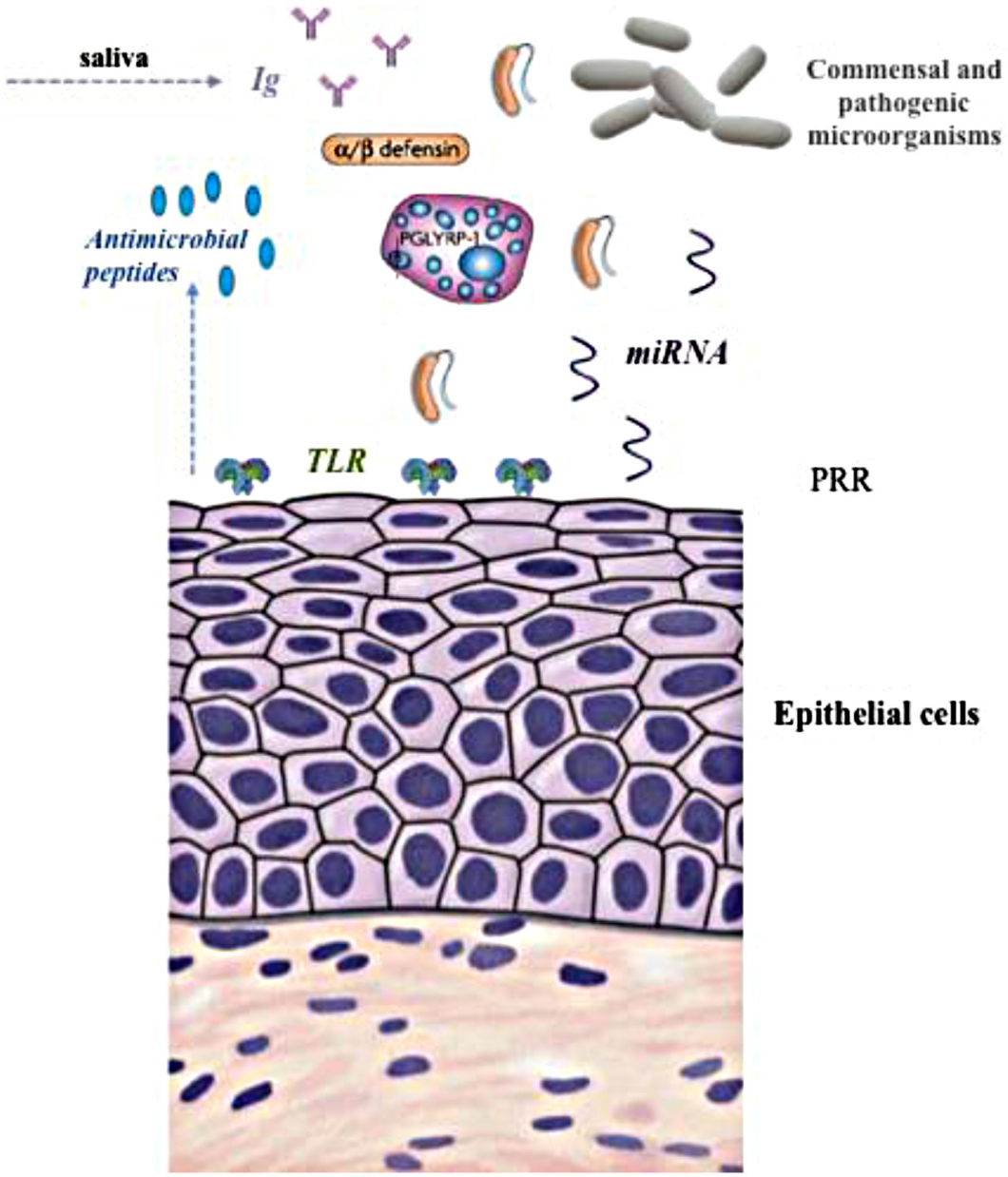

FIGURE 1 | Illustration of different elements of the immune-regulation of the rumen microbiome. TLR, Toll-like receptor; PRR, pattern recognition receptors.

et al., 2015). These studies have confirmed that the major class of immunoglobulin in bovine saliva is IgA and showed that this class of immunoglobulin is also the dominant type in the rumen. In contrast, in serum the major class is IgG. IgA is apparently more resistant to degradation in the rumen compared to IgG, possibly because the secretory component of IgA makes the immunoglobulin more resistant to protease activity in the rumen (Snoeck et al., 2006). Although the research conducted in developing vaccines against specific rumen microorganisms proves that an increase in the titres of Ig in saliva can be achieved, the role of the constant supply of Ig into rumen through saliva in shaping the commensal microbial community and how this innate response functions during rumen development are as yet unknown.

A change in the diet of the animal can result in a shift in the proportion of microbial groups in the rumen (Petri et al., 2013) but little is known as to how the immune system deals with such a shift in ruminants. Some research has been conducted in this area in animals subjected to rumen acidosis challenge. Chen et al. (2012) reported a significant variation of TLR4 gene expression in the rumen epithelium of the animals with different susceptibility to acidosis. The observed correlations between copy number of

total bacterial 16S rRNA genes of epimural bacteria, and ruminal pH, total VFA concentration, and expression of the TLR4 gene, suggested that the innate immune response in the epithelium is associated with the activity of the epimural bacteria. Currently, a total of 10 TLRs have been described in ruminants (Seabury et al., 2010) and two main groups may be distinguished: (i) TLRs1, 2, 4–6, 10, which are expressed in the cell surface and identify bacterial surface associated molecular patterns and (ii) TLRs3, 7–9 that recognize specific nucleic acids from viruses and bacteria (Chang, 2010; Guan et al., 2010; Malmuthuge et al., 2012). In spite of the knowledge available on the innate epithelial-associated response in adult ruminants, very few studies have addressed this in young animals. Malmuthuge et al. (2012) studied the regional and age-dependent expression patterns of TLRs, peptidoglycan recognition protein 1 (PGLYRP1), and antimicrobial proteins (β-defensin) in the rumen, jejunum, ileum, cecum, and colon of 3 weeks and 6-month-old calves. The expression of most TLRs was significantly down regulated throughout the gastrointestinal tract with increasing age. The restricted expression of both β-defensin and PGLYRP1 prior to weaning in calves suggests that significant developmental changes occur in the epithelial immune system of cattle at this time. Malmuthuge et al. (2012) hypothesized that 'newborns may depend on TLRs as a primary innate immune mechanism to monitor commensal microflora and pathogens prior to weaning, but with increasing age it appears that other innate immune effecter mechanisms such as antimicrobial peptides may become more active in providing host defenses and minimizing harmful inflammatory responses'. No studies, however, have been conducted yet on to what extent the expression level of TLR respond to different microbial colonization patterns.

Recently, Liang et al. (2014) studied the potential regulatory role of micro RNAs (miRNAs) in the development of gastrointestinal tract (including the rumen), during the early life of dairy calves. The first finding is that the copy numbers of 16S rRNA gene of *Bifidobacterium* or *Lactobacillus* species or both were positively correlated with miR-15/16, miR-29, and miR-196 expression levels ($P < 0.05$). The authors suggested miRNAs that were expressed differently could be regulators of the differentiation and proliferation of the cells of gastro-intestinal. Indeed Liang et al. (2014) identified three miRNAs as promoters of the gut-associated development at different levels: lymphoid tissues development (miR-196), dendritic cells maturation (miR-29), and of immune cells (miR-15/16). Their results provide novel evidence of gut development mechanisms that are regulated by host-microbiome interactions (Liang et al., 2014).

As stated earlier, the information of the impact of different colonization patterns on the immune system and the long-term effects is scarce. It could be hypothesized that if the colonization of a specific microbial group is prevented in early life, it is likely that the immune system will not recognize that group in later life if it colonizes the rumen later on. As a consequence, the host may mount an immune response against it. This might be a means to control specific populations. However, this is not entirely supported by the research conducted using protozoa-free raised lambs that were inoculated with protozoa later in life (Belanche et al., 2015). Nevertheless, more research is needed to understand the immune response in animals subjected to different microbial colonization patterns and how the animal responses later in life when it is challenged with the inoculation of 'unknown' species.

CONCLUSION AND FUTURE PROSPECT

The development of the rumen needs to be understood at different levels: anatomical, functional and microbial, as they have different temporal sequences in the young animal and the interplay of anatomical/functional rumen development and microbial development is not yet clear.

Notwithstanding the knowledge gaps, from the work described above, we conclude that early life events may be related to the microbial community structure and/or the rumen activity in the animals post-weaning. This would create differences in adaptive capacity due to different early life experiences and leading to the idea of microbial programming. However, the most effective window of time for intervention and the long-term implications are yet to be addressed. Therefore there is a need to perform trials that run long enough to truly assess the impact on the productive life of the animal. In addition, the differences in animal responses in later life need to be adequately assessed. In some cases, there will be no differences between animals reared differently in early life, which nevertheless have different microbiome compositions, if they are tested under 'standard' feeding conditions. The potential different response might become evident when the animals are nutritionally challenged or re-treated with the same pro- or anti-microbial compound as in early life.

Identifying the key immune elements at molecular level involved in early life colonization (with special attention to the rumen epimural population) may help to understand the host-animal response and the extent of persistency of effects in adult life.

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REVIEW
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The Role of Ciliate Protozoa in the Rumen

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First described in 1843, Rumen protozoa with their striking appearance were assumed to be important for the welfare of their host. However, despite contributing up to 50% of the bio-mass in the rumen, the role of protozoa in rumen microbial ecosystem remains unclear. Phylogenetic analysis of 18S rDNA libraries generated from the rumen of cattle, sheep, and goats has revealed an unexpected diversity of ciliated protozoa although variation in gene copy number between species makes it difficult to obtain absolute quantification. Despite repeated attempts it has proven impossible to maintain rumen protozoa in axenic culture. Thus it has been difficult to establish conclusively a role of ciliate protozoa in rumen fiber degradation. The development of techniques to clone and express ciliate genes in λ phage, together with bioinformatic indices to confirm the ciliate origin of the genes has allowed the isolation and characterization of fibrolytic genes from rumen protozoa. Elimination of the ciliate protozoa increases microbial protein supply by up to 30% and reduces methane production by up to 11%. Our recent findings suggest that holotrich protozoa play a disproportionate role in supporting methanogenesis whilst the small *Entodinium* are responsible for much of the bacterial protein turnover. As yet no method to control protozoa in the rumen that is safe and practically applicable has been developed, however a range of plant extract capable of controlling if not completely eliminating rumen protozoa have been described.

Keywords: rumen, protozoa, defaunation, methane, microbial diversity

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INTRODUCTION

First described in 1843, Rumen protozoa with their striking appearance were assumed to be important for the welfare of their host. However, despite the fact that protozoa can contribute up to 50% of the bio-mass in the rumen, the role of protozoa in rumen microbial ecosystem remains unclear (Williams and Coleman, 1992). Here we evaluate recent information on the role of ciliate protozoa in the rumen microbial ecosystem.

DIVERSITY AND PHYLOGENY OF RUMEN CILIATE PROTOZOA

Since they were first discovered by Gruby and Delafond (1843), studies on rumen protozoa have relied on morphologic identification by optical microscopy. There are currently no culture collections of rumen ciliates, so researchers have to use photomicrographs for identification (Ogimoto and Imai, 1981; Williams and Coleman, 1992) or line drawings (Dogiel, 1927; Kofoid and MacLennan, 1930, 1932, 1933; Latteur, 1968, 1969, 1970; Dehority, 1993). It is widely accepted that microscopic identification and counting represents the gold standard for analyzing ciliate

community structure in rumen studies (Williams and Coleman, 1992). However, the polymorphic nature of these microbes (Dehority, 1994, 2006) requires a high level of experience by the researcher to identify rumen ciliates at a genera or species level (Dehority, 2008). Thus, alternative ways to identify protozoa are needed since microscopic techniques are laborious and highly demanding.

Early molecular studies on rumen ciliate protozoa focussed on sequencing of 18S rRNA genes to clarify the internal phylogeny of the class Litostomatea (Embley et al., 1995; Wright et al., 1997). Embley et al. (1995) were the first to describe the monophyletic position of the anaerobic rumen ciliates *Dasytricha ruminantium* and *Polyplastron multivesiculatum* based on 18S rRNA genes and Wright et al. (1997), Wright and Lynn (1997a,b) provided molecular data for *Diplodinium dentatum*, *Entodinium caudatum*, *Epidinium caudatum*, *Eudiplodinium maggii*, *Isotricha intestinalis*, *Ophryoscolex purkynjei*, and *Polyplastron multivesiculatum*.

Numerous qualitative and quantitative studies focused on the diversity of ciliates have been performed since then; these studies have used a variety of techniques, including restriction fragment length polymorphism (RFLP; Regensbogenova et al., 2004a, Tymensen et al., 2012b), denaturing gradient gel electrophoresis (DGGE; Regensbogenova et al., 2004b, McEwan et al., 2005; de la Fuente et al., 2009; Belanche et al., 2010a, Kittelmann and Janssen, 2011), real-time PCR (qPCR; Sylvester et al., 2004; Skillman et al., 2006; Belanche et al., 2010a, Kittelmann and Janssen, 2011), fluorescence in situ hybridization (FISH; Xia et al., 2014) and next generation sequencing (Kittelmann et al., 2013, 2015; Ishaq and Wright, 2014; Moon-van der Staay et al., 2014).

Microscopy holds several advantages over PCR-based molecular methods for studying ciliate protozoa. First, while the vast majority of intestinal ciliates have been characterized morphologically, there is a lack of 18S rRNA gene reference sequences for many of the observed genera and species. Second, as discussed below, copy number variation of ribosomal RNA genes across the different genera or under different growth conditions may skew the observed proportions of these genera in a sample (Medinger et al., 2010). Studies using 18S rRNA gene surveys reveal an apparent higher diversity of ciliates than estimate by conventional morphological methods (Moon-van der Staay et al., 2014).

Like other ciliates, rumen protozoa contain two kinds of nuclei: a micronucleus and a macronucleus. The micronucleus possesses clearly visible chromosomes, is diploid, and synthesizes only a trace of RNA. The macronucleus contains no discernible chromosomes, has many times the diploid amount of DNA, divides amitotically, and provides virtually all of the RNA needed to run the vegetative life of the cell (Prescott and Murti, 1974). The extremely high copy number of rDNA in the macronuclear genome of ciliates, as previously found (Gong et al., 2013), is understandable considering the life history, large cell size and rapid growth of these organisms (Cavalier-Smith, 2005; Zhu et al., 2005; Godhe et al., 2008). In rumen ciliates, while the small protozoa such as *Entodinium*, tended to be under-represented, larger protozoa such as *Epidinium* or *Polyplastron* tended to be over-represented by a pyrosequencing approach compared to microscopic enumeration (Kittelmann et al., 2015). Similarly, *Epidinium caudatum*, which is approximately five times larger by volume than *Entodinium caudatum* (Dehority, 1993) also has approximately five times more 18S rRNA gene copies encoded in its genome (Sylvester et al., 2009). These results agree with the finding that PCR-based methods return lower estimates of abundance of small *Entodinium* spp. (Tymensen et al., 2012a) and overestimates of the abundance of, e.g., *Polyplastron* spp. (Ishaq and Wright, 2014). Knowing the copy numbers and the variations of rDNA sequences within individual eukaryotes is important both for interpreting rDNA-based diversity surveys and when 18s rDNA is used to quantify protozoal biomass. (Crosby and Criddle, 2003; Thornhill et al., 2007; Herrera et al., 2009; Amaral-Zettler et al., 2011). To date no studies addressing the number of copies per cell and variations of rDNA in rumen protozoa have been published. This is especially true as rDNA-based barcoding and microbial diversity studies using high-throughput sequencing are becoming more popular (Amaral-Zettler et al., 2009) and molecular tools based on marker gene surveys are now widely used to study the diversity of other microbes (Schlötterer, 2004; Case et al., 2007; Langille et al., 2013). Studies in non-rumen ciliates have shown that the rDNA copy number variation between and within ciliate species highlighting the difficulty of using the rDNA sequence number-based approach to infer the relative abundance of microbial eukaryotic cells in environmental samples (Wintzingerode et al., 1997; Medinger et al., 2010). Thus, latest methods based on 18S rRNA genes may be unreliable when estimating α-diversity or relative abundances of different genera and species in a given sample, although they can, reliably determine trends in relative abundances of genera and species between different samples (β-diversity). More research comparing molecular and traditional methods is needed (de la Fuente et al., 2009; Tymensen et al., 2012b; Kittelmann et al., 2015).

FUNCTIONAL GENES IN RUMEN CILIATE PROTOZOA

Although there are numerous copies of rDNA in ciliate macronucleus, it is likely that only a small portion of these genes are transcriptionally active in ciliates under any given growth condition, as previously shown for other eukaryotes (Reeder, 1999). Studies in *Tetrahymena thermophila*, *Paramecium tetraurelia*, and *Oxytricha trifallax* have pinpointed the important role of non-coding RNAs in genome rearrangement events (Feng and Guang, 2013). In spirotrichous ciliates, such as *Euplotes*, *Stylonychia*, and *Oxytricha*, more than 95% of the micronuclear DNA is eliminated to form the macronucleus during sexual reproduction (Swart et al., 2013) possible as a mechanism to allow the cell to adapt during times of stress. Moreover, in the macronucleus, the remaining genome is severely fragmented and these fragments are sorted and reordered under the guidance of transcripts from the parental macronucleus to produce protein-coding genes (Nowacki et al., 2011). None of these processes have been studied so far in rumen protozoa, due to the difficulties in getting full length genomes of rumen ciliates.

Despite repeated attempts it has proven impossible to maintain rumen protozoa in axenic culture. Thus it has been difficult to establish conclusively a role of ciliate protozoa in the rumen and

specifically fiber degradation. Early studies sought to isolate and characterize cellulose, hemicellulase and xylanase enzymes from washed protozoal preparations (Howard et al., 1960; Bailey et al., 1962; Clayet et al., 1992), however the presence of both extra and intra cellular symbiotic bacteria in protozoal preparations made it difficult to be sure that the isolated activity was truly of protozoal origin (Delfosse-Debusscher et al., 1979; Thines-Sempoux et al., 1980). We developed techniques to clone and express ciliate genes in λ phage (Eschenlauer et al., 1998), using FISH to confirm the protozoal identity of the expressed genes (Newbold et al., 2005) and developing bioinformatic indices to confirm the ciliate origin of the genes (McEwan et al., 2000). Using these techniques we and others have been able to isolate and characterize genes from a range of rumen protozoa (McEwan et al., 1999; Newbold et al., 2005; Belzecki et al., 2007; Boxma et al., 2007). This includes a wide range of fibrolytic enzymes a number of which have been found to contain multiple domains with binding domains and putative chimeric constructs being observed suggesting a highly evolved fibrolytic capacity in the rumen ciliates (Devillard et al., 1999, 2003; Takenaka et al., 1999, 2004; Wereszka et al., 2004; Bera-Maillet et al., 2005). This observation has been confirmed by recent metagenomic screening of protozoal glucosidases and eukaryotic metatranscriptomes that have confirmed that a diverse range of diverse glycoside hydrolases are present in the rumen protozoa (Findley et al., 2011; Qi et al., 2011).

Based on large-scale construction and analysis of phylogenies of over 4000 Expressed Sequence Tag libraries from the rumen ciliates *Entodinium caudatum*, *Eudiplodinium maggii*, *Metadinium medium*, *Diploplastron affine*, *Polyplastron multivesiculatum*, *Epidinium ecaudatum*, *Isotricha prostoma*, *Isotricha intestinalis*, and *Dasytricha ruminantium*. Ricard et al. (2006) concluded there was extensive evidence of horizontal gene transfer (HGT; 148 out of 3563 non-redundant genes) from bacteria and archaea in rumen ciliate genomes. Among the HGT candidates, they reported an over-representation (>75%) of genes involved in metabolism, specifically in the catabolism of complex carbohydrates (Ricard et al., 2006), suggesting that HGT may have been important in allowing rumen ciliates to adapt to new niches within the rumen and that fibrolytic genes were acquired by protozoan from bacterial sources (Findley et al., 2011).

ROLE OF PROTOZOA IN THE RUMEN

Despite the fact that that protozoa make up a large portion of the rumen biomass, their role in ruminal fermentation and their contribution to the metabolism and nutrition of the host is still an area of substantial controversy (Williams and Coleman, 1992). In the last section of this paper we will review different strategies to manipulate the rumen protozoal density; however most of these dietary interventions also lead to modifications in rumen function making it difficult to assess the effect of the rumen protozoa *per se*. Rumen protozoa are not essential to the animal to survive and defaunation (the removal of protozoa from the rumen using a wide variety of chemicals and physical techniques) and protozoa-free animals have been used to study the role of ciliate protozoa in the rumen function without been affected by dietary interventions (Williams and Coleman, 1992). This paper does not aim to conduct a complete review on the effect of defaunation but compiles the most relevant publications since the excellent and complete review by Williams and Coleman (1992). Thus, a meta-analysis was conducted to study the main effects of defaunation based on 23 *in vivo* studies comprising 48 comparisons (Kreuzer et al., 1986; Vermorel and Jouany, 1989; Ushida et al., 1990; Frumholtz, 1991; Nagaraja et al., 1992; Hegarty et al., 1994, 2008; Faichney et al., 1999; Chandramoni et al., 2001; Machmuller et al., 2003; Eugène et al., 2004; Ozutsumi et al., 2005; Ohene-Adjei et al., 2007; Yáñez-Ruiz et al., 2007a,b; Bird et al., 2008; Morgavi et al., 2008, 2012; Belanche et al., 2011, 2012b, 2015; Mosoni et al., 2011; Zhou et al., 2011). Most of the studies were performed using sheep (87%), while the rest used cattle (13%). Isolation of new born animals from their mothers (40%), use of detergents and other chemicals (35%, using sodium lauryl sulfate, alkanes, synperonic NP9, calcium peroxide, copper sulfate etc.), and ruminal manipulation (25%, emptying and washing of the rumen), were used in order to achieve defaunation of the animals. Trials where the effect of additives, other than defaunation, were significant were discarded from the meta-analysis. Similarly, studies using monofaunated animals or selective defaunation were not included. The majority of the data (75%) were from trials in which animals were fed at maintenance and the remaining trials were from production trials. Animals were mainly fed mixed diets (90%) composed of forages supplemented with concentrate, while 10% of the diets were purely composed of forage. Rumen fermentation data and methane emissions were reported in most trials (69%), while information about digestion (31%), animal performances (12%) and rumen microbial populations (10%) was less abundant. Methane production was measured in chambers (75% in open chambers or respiratory calorimeters) or with the SF_6 (sulfur hexafluoride) tracer method (25% of studies).

Multiple comparisons were included from an individual publication with multiple studies. For each comparison included in the analysis, the effect size was calculated as the natural logarithm of the response ratio (mean value in the defaunated treatment divided by mean value in control treatment) and variance of the ratio calculated based on the reported standard deviation or standard error of the mean for each comparison (Viechtbauer, 2010). All defaunation effects were weighted according to the number of observations (n) in each comparison. The meta-analysis was computed fitting a random-effect model with a DerSimonian-Laird estimator (Dersimonian and Laird, 1989) for assessing heterogeneity (τ^2) in the Metafor package of R for each category separately as follow:

$$\theta_i = \mu + e_i$$

where θ_i = true effect size in the ith study, μ = overall true effect size and e_i = random deviation from the overall effect size [$u_i \sim N(0, \tau^2)$] (Viechtbauer, 2010). For evaluating the response ratio, values below one indicated a negative, while values above one indicated a positive effect of defaunation on that particular parameter.

This meta-analysis confirmed many of the previously reported results (Williams and Coleman, 1992) and helped clarify number of areas in which results were still conflicting. **Figure 1** shows the main effects of defaunation on the rumen function indicating

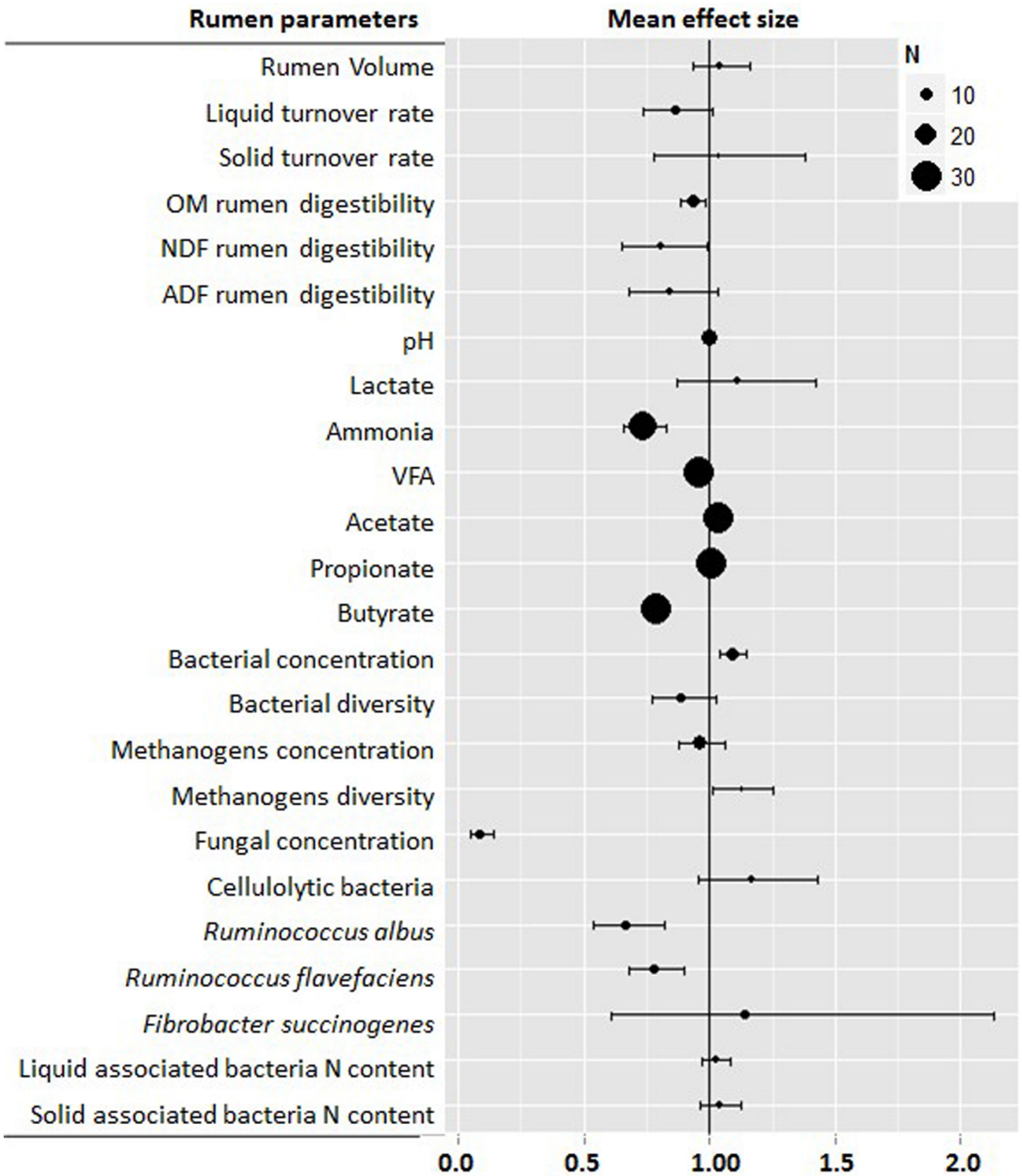

FIGURE 1 | Meta-analysis describing the effects of defaunation on rumen function. For each parameter information is provided about the number of studies, observations (n), and range of values. Graph shows the mean effects and 95% confidential intervals. Values below one indicate a negative effect, while those above one indicate a positive effect of defaunation on that particular parameter.

that the physical and chemical characteristics of the rumen environment were changed by defaunation; although the nature of the observed change was not always consistent. Rumen volume and solid turnover rate were unaffected by defaunation, while the liquid turnover rate tended to decrease (−14%, $P = 0.07$). Elimination of protozoa from the rumen significantly decreased rumen OM digestibility (−7%, $P = 0.008$) and particularly NDF (−20%, $P = 0.040$) and ADF digestibility (−16%, $P = 0.100$), probably as a result of the loss of protozoal fibrolytic activity. This activity seems however to differ across the different protozoal groups: Large *Ophyroscolecidae* such as *Epidinium*, *Polyplastron* and *Eudiplodinium* have greater endoglucanase and xylanase activity (Williams and Coleman, 1992). On the other hand, *Entodinium* spp. have only weak activity (Williams and Coleman, 1992). Similarly, *Dasytricha* has glucosidase and cellobiosidase activity but negligible fibrolytic activity (Takenaka et al., 2004).

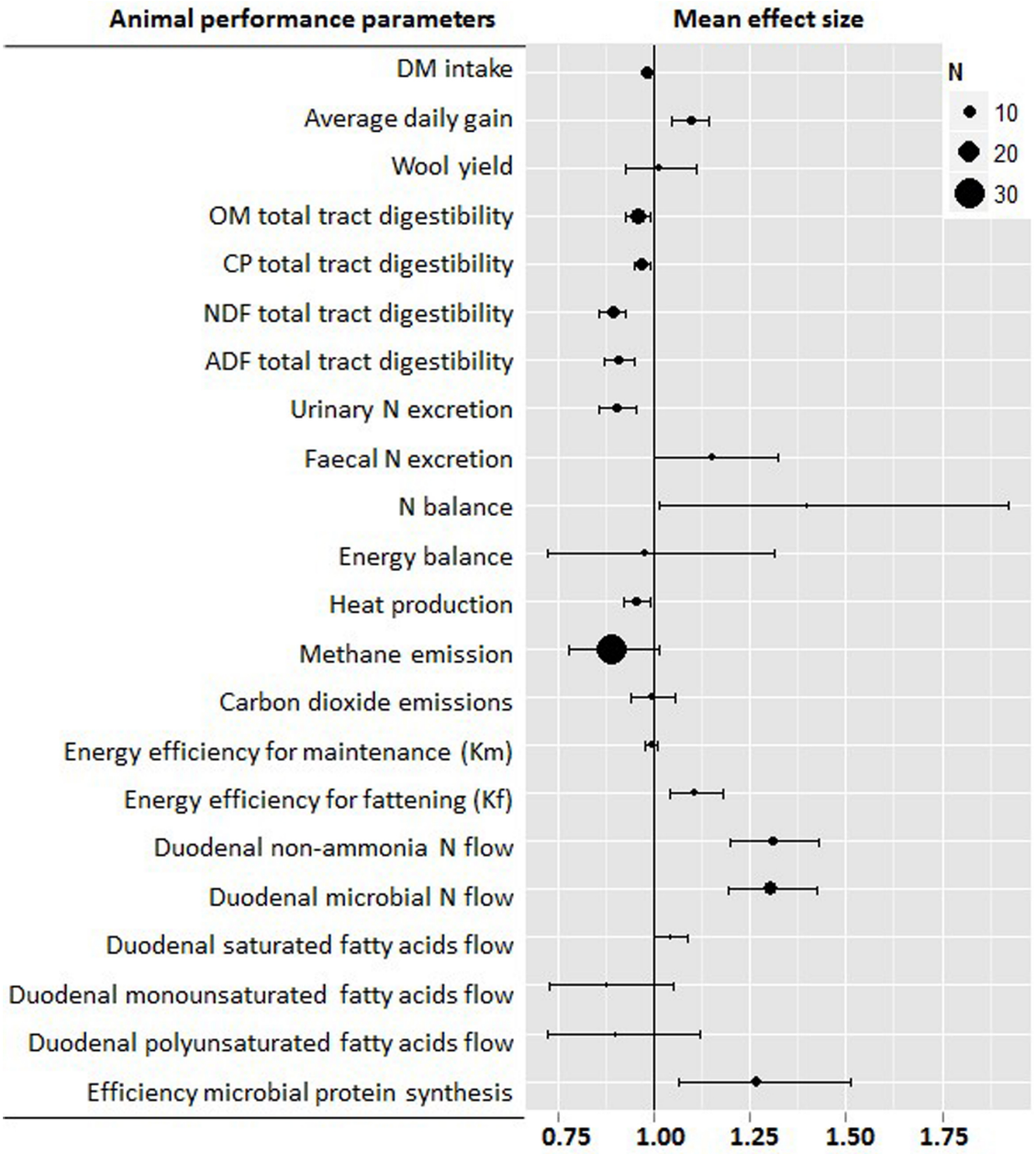

FIGURE 2 | Meta-analysis describing the effects of defaunation animal performance. For each parameter information is provided about the number of studies, observations (n), and range of values. Graph shows the mean effects and 95% confidential intervals. Values below one indicate a negative effect, while those above one indicate a positive effect of defaunation on that particular parameter.

The lower rumen digestibility in defaunated animals is partially compensated by a greater post ruminal digestion resulting in less pronounced differences in terms of total tract digestibility for OM (−4%, $P = 0.089$), CP (−3%, $P = 0.034$), NDF (−11%, $P < 0.001$), and ADF (−9%, $P < 0.001$; **Figure 2**). Another compensatory factor could be a shift toward more energetically efficient reactions in the rumen (less methane emissions) and less metabolic energy required by defaunated animals to eliminate the excess of urea as a result of the lower bacterial protein breakdown and ammonia levels in the rumen. Despite these compensatory mechanism, the decrease in feed digestibility is likely to be the main drawback of defaunation since it could limit the feed intake and efficiency of feed utilization at production levels of intake; this coupled to the lack of a commercially viable approach (see below) to defaunation, mean that defaunation is not recommended as a methane mitigation strategy under farm conditions (Hristov et al., 2013).

The total concentration and production of fermentation products also differs in faunated and defaunated animals. A decrease in rumen ammonia (−26%, $P < 0.001$) is probably

the most consistent of the observed effects of protozoal elimination and seems to be due to decreased bacterial protein breakdown and feed protein degradability in the absence of rumen protozoa (Williams and Coleman, 1992). Moreover it has been demonstrated that although bacterial predation by rumen protozoa is dependent on the protozoal size, holotrich protozoa have a much lower predatory activity than entodiniomorphids (Belanche et al., 2012a) and ultimately a lower impact on rumen ammonia concentration (Belanche et al., 2015) and duodenal microbial protein flow (Ivan et al., 2000a; Ivan, 2009). However, this negative effect of entodiniomorphids on the duodenal protein flow may be overestimated due to the lack of a reliable marker to measure protozoal flow (Broderick and Merchen, 1992). In previous reviews higher lactate levels have been reported in defaunated animals (Williams and Coleman, 1992) because protozoa consume lactate more rapidly than bacteria (Newbold et al., 1986). Our meta-analysis showed a numerical increase in lactate concentration in defaunated animals (+11%) but this increase was non-significant possibly because the dataset used in this meta-analysis was mainly composed by high-forage diets which are less prone to promote these particular nutritional disorders.

Although rumen pH was unaffected, the lower VFA concentration observed in defaunated animals (−5%, $P = 0.013$) seems to highlight the role of protozoa in the synthesis of VFA and feed degradation. The ability of protozoa to engulf exogenous fatty acids (Karnati et al., 2009) may divert more carbon toward VFA production in preference to fatty acid synthesis and ultimately increase VFA production. More interestingly, our data suggested that defaunation substantially decreased butyrate (−22%, $P < 0.001$), slightly increased acetate (+3%, $P < 0.001$) and had no effect on propionate molar proportions. In a previous meta-analysis (Noziere et al., 2011) it has been reported that NDF digestibility positively correlates with acetate molar proportion ($r = 0.95$) but negative with propionate ($r = -0.94$) and butyrate ($r = -0.91$), therefore our observations might indicate that the shift in the molar proportions of VFA induced by defaunation seems to be mainly driven by a decrease in the fiber digestion. On the basis of stoichiometry, such a shift in rumen VFA production should result in a decrease in methane production as less metabolic H_2 will be available as a substrate for methanogenesis (Demeyer et al., 1996). The effect of defaunation on methane production is however still not clear; in various reviews compiling *in vivo* and *in vitro* studies (Hegarty, 1999) or just *in vivo* (Morgavi et al., 2010) it was concluded that removal of protozoa from the rumen would result in a 13 and 10.5% decrease in methane production, which fully agree with our results based on more recent studies (−11%, $P = 0.074$). In line with these observations, in a meta-analysis containing 28 experiments and 91 treatments, it has been reported a significant linear relationship between methane emissions and protozoal concentration ($r = 0.96$) with a decrease in methane yield averaged 8.14 g/kg DMI per each log unit reduction in the protozoal concentration (Guyader et al., 2014), suggesting that protozoa played a catalytic role in rumen methanogenesis. The reasons for the lower methane emissions in defaunated animals are still controversial since the number of rumen protozoa explains only one part (approximately 47%) of the variability in methane emissions indicating that rumen methanogenesis is a complex process in which multiple microbes are involved (Morgavi et al., 2010). A number of mechanisms by which protozoa could enhance methanogenesis are possible based on their ability to produce H_2 in their hydrogenosomes (a mitochondria-like organelle), their ability to host epi- and endo-symbiotic methanogens and to protect them from oxygen toxicity (Fenchel and Finlay, 2006). This interaction is a typical example of interspecies H_2 transfer that favors both the methanogens and the protozoa (Ushida et al., 1997). One hypothesis is that defaunation leads to decreased methanogen numbers, which are the sole producers of methane in the rumen, but our meta-analysis reported that this decrease in methanogens levels was not significant (−3%, $P = 0.48$). An alternative hypothesis suggests that defaunation results in the elimination of protozoa-associated methanogens, which could be considered as one of the most active methanogens communities in the rumen (Finlay et al., 1994), however this later hypothesis based on the substitution of methanogen communities which differ in their methanogenic activity requires further investigation. Several authors have studied the endo-symbiotic archaeal population in protozoa (Sharp et al., 1998; Irbis and Ushida, 2004; Regensbogenova et al., 2004b; Tymensen et al., 2012a) and most of them agree that *Methanobrevibacter* sp. is the predominant genus, while the contribution of *Methanomicrobium* sp. and *Methanomassiliicoccales* to the endosymbiotic methanogen community is variable and could indicate differences among protozoal groups. The development of molecular techniques over the last decades has allowed colleagues to further explore these microbial interactions revealing that ciliate endosymbiotic methanogens differ from rumen free-living methanogens (Tokura et al., 1997; Sharp et al., 1998). In a recent publication it has also been demonstrated that holotrich protozoa have a different endosymbiotic methanogens than entodiniomorphids (Belanche et al., 2014) possibly because either holotrich protozoa have more active hydrogenosomes than entodiniomorphids (Paul et al., 1990) and/or rapid synthesis of glycogen by holotrich protozoa in the presence of excess carbohydrates generate more hydrogen (Hall, 2011; Denton et al., 2015). These differences may explain the greater impact of holotrich protozoa on rumen methanogenesis compared to entodiniomorphids (Belanche et al., 2015). This later observation is based on the successive inoculation of fauna-free sheep with various protozoal groups, thus it should be cautiously interpreted due to possible confounding effects of treatment and period.

The effect of defaunation on the rumen microbial ecosystem is not limited to only the methanogen population and it has been demonstrated that defaunated animals had significantly greater ruminal bacterial populations (+9%, $P < 0.001$) than control animals. The reason for this seems to be based on the availability of an ecological niche for the bacteria when protozoa are not present in the rumen combined with the removal of protozoal predation (Williams and Coleman, 1992). Recent studies using molecular techniques have demonstrated that defaunation also modified the structure of the bacterial community leading to a simplification in population structure and lower bacterial diversity (Belanche et al., 2012b, 2015). Our meta-analysis revealed a drop in the

concentration of fibrolytic microbes such as anaerobic fungi (−92%, $P < 0.001$), *Ruminococcus albus* (−34%, $P < 0.001$) and *Ruminococcus flavefaciens* (−22%, $P < 0.001$) in the absence of protozoa. This observation suggests that fiber digestion in the rumen is a complicated task which requires the symbiotic collaboration of several fibrolytic microbes, including rumen protozoa, to carry out the initial stages of fiber colonization and digestion; therefore the absence of rumen protozoa seems to have a detrimental effect on this fibrolytic consortium and ultimately in fiber digestion. However, Hsu et al. (1991) reported an increase in ruminal fungal zoospores in defaunated animals possibly as a result of the removal of protozoal predation and competition for nutrients (protozoa vs. fungi), clearly more studies are needed to fully understand protozoa interactions with other rumen microbes under different dietary regimes.

The effect of the presence of rumen protozoa on pathogen's survival in the rumen and pathogen shedding is another area of interest. As noted above rumen protozoa engulf and digest a wide range of bacteria (Williams and Coleman, 1992) and can reduce the shedding of potential pathogens from the animal, although the effect is highly dependent on the composition of the protozoal population present (Stanford et al., 2010). However, it has also been shown that rumen protozoa enhance the pathogenicity of certain pathogens leaving the rumen (Rasmussen et al., 2005; Carlson et al., 2007) suggesting that more work is needed in this area.

Figure 2 summarizes the effects of protozoa on animal productivity as the indirect effect of defaunation on the rumen function. Defaunation improved feed conversion rate as a result of the lower DM intake (−2%, $P < 0.001$) and greater average daily gain (+9%, $P < 0.001$). These positive effects of defaunation are particularly obvious with poor quality diets in which the average daily gain was low, possibly as a result of the lower availability of fermentable energy and rumen digestible protein for the rumen microbes (Williams and Coleman, 1992). In a previous meta-analysis the effect of defaunation appeared more pronounced when the ratio N/NDF was below 6 and the percentage of concentrate lower than 40% (Eugène et al., 2004). As defaunated animals have lower feed digestibility, absorbed energy is lower than in conventional animals. Thus better feed conversion rate after defaunation may be mainly attributed to the higher efficiency of utilization of absorbed nutrients. Indeed our meta-analysis showed that defaunation promotes a greater efficiency of energy utilization for fattening (+11%, $P = 0.001$), possibly as a result of a lower heat production (−5%, $P = 0.010$). Other hypothesis to explain the better performance of defaunated animals rely on a more efficient use of dietary protein. There is clear evidence that holotrich protozoa leave the rumen more slowly than bacteria (Abe et al., 1981). The amount and rate of protozoal flow to the lower gut is however the subject of much debate. Firkins et al. (2007), found that post-ruminal flow of protozoa is proportional to rumen protozoal biomass. Although, protozoal counts in the rumen and abomasal fluid indicated that abomasal counts were only 6–64% of rumen fluid counts (Punia et al., 1992), numbers of protozoa in free rumen fluid are unreliable indicators of protozoal biomass and outflow, because the majority of rumen protozoa (63–90%) are found either associated with feed particles or sequestered in the rumen wall (Hook et al., 2012). The use of specific markers of protozoa such as 2-aminoethylphosphonic acid has been questioned because it is present in bacteria and in feed (Ling and Buttery, 1978). Protozoal outflow measured by use of a general microbial markers (e.g., ^{15}N and ^{35}S and purine bases), microbial N minus specific bacterial markers (such as 2,6-diaminopimelic acid, DAPA) yielded variable estimates of protozoal outflow and sometimes even negative values due to methodological limitations for the markers used (Broderick and Merchen, 1992). Recent studies based on the use of 18S rRNA as a novel protozoal marker have reported that although protozoa can represent up to 60% of the rumen microbial biomass, they rarely exceeds 20% of the microbial protein flow into the small intestine (Sylvester et al., 2004, 2005; Yáñez-Ruiz et al., 2006; Belanche et al., 2011, 2012b). However, these new techniques are not free from their own limitations: Sylvester et al. (2005) observed that up to 48% of the protozoal N was actually N from contaminating bacteria which could led to inflate the protozoal N pool in the rumen, particularly when animals have low feed intakes and low particulate passage rate (Dijkstra, 1994). Similarly, Belanche et al. (2010b) observed a greater degradation of protozoal DNA compared to bacterial DNA during abomasal digestion which could result in an underestimation of the protozoal N outflow from the rumen. Despite these limitations, these new findings seem to support that rumen protozoal are partially sequestrated in the rumen. This rumen sequestration is however not equal for all protozoal groups; holotrich protozoa associate to the feed particles after feeding due to their strong chemotaxis toward sugars (Diaz et al., 2014), but rapidly migrate to the ventral reticulorumen to prevent being washed out of the rumen (Karnati et al., 2007), on the contrary Entodiniomorphids also associate to feed due to their moderate chemotaxis toward glucose and peptides but do not show the same affinity to the rumen wall (Diaz et al., 2014) and thus flow out the rumen with the particulate phase (Hook et al., 2012).

Protozoa predate on bacteria as their main protein source (Williams and Coleman, 1992) and as a result, defaunation makes the rumen more efficient in terms of proteosynthesis increasing the duodenal flow of microbial protein (+30%, $P < 0.001$) and total non-ammonia N flow (+31%, $P < 0.001$). Defaunation also increased the efficiency of microbial protein synthesis (+27%, $P = 0.008$) as a result of both a better microbial proteosynthesis and a lower OM digestion. Protozoal generation time is far higher than that of bacteria, thus the energetic requirements for maintenance are higher when expressed as a ratio of protein leaving the rumen (Williams and Coleman, 1992). As a result, the presence of protozoa has a negative impact on the overall energetic efficiency of the rumen ecosystem. In addition, defaunation can also modify the composition of the rumen bacteria (Belanche et al., 2012b) and ultimately the amino acid profile of the duodenal protein supply promoting an increase in specific amino acids such as leucine, threonine and arginine (Ivan, 2009), but not lysine which is considered the main limiting amino acid in high producing animals (Hristov and Jouany, 2005). After a series of *in vivo* experiments in which fauna-free lambs were progressively inoculated with protozoal species

(i.e., *Isotricha intestinalis*, *Dasytricha ruminantium*, *Polyplastron multivesiculatum*, *Epidinium ecaudatum*, *Eudiplodinium maggi*, and *Entodinium caudatum*) in different sequential orders (Ivan et al., 2000a,b; Ivan, 2009), Ivan concluded that holotrich protozoa engulf only a very small number of rumen bacteria and have a small effect on the duodenal NAN flow and protein metabolism. Similarly, holotrich protozoa had no significant effect on the fiber digestion. This together, with the contribution of holotrich's to rumen methanogenesis (Belanche et al., 2015), seems to indicate that presence of holotrich protozoa in the rumen is of little value to ruminant production, unless high-carbohydrate diets are used, in which case the presence of holotrich protozoa could be beneficial as a result of their ability to engulf and accumulate starch grains and soluble carbohydrates (Williams and Coleman, 1992). This engulfment of highly fermentable carbohydrates prevents alternative bacterial fermentation that would otherwise decrease pH and increase the onset of lactic acid acidosis (Mackie et al., 1978). On the contrary cellulolytic protozoa (*Polyplastron*, *Epidinium*, and *Eudiplodinium*) could be beneficial to animals fed with fibrous diets. However, these fibrolytic protozoa and *Entodinium* spp. have a substantial potential to engulf and degrade bacteria (Belanche et al., 2012a) and might be detrimental in terms of protein utilization by the ruminant host. Therefore their presence in the rumen may not be helpful in animals fed low protein diets because their fibrolytic beneficial effects would be counterbalanced by the sensitivity of fibrolytic bacteria and anaerobic fungi to N shortage in the rumen (Belanche et al., 2012c). Thus it seems that defaunation decreased urinary N losses here (-10%, $P < 0.001$) due to a combination of lower dietary CP degradation in the rumen and lower rumen bacterial breakdown. However, the great variability observed in terms of N balance highlights the fact that defaunation may have different effects on the overall efficiency of N utilization depending on the diet consumed by the animal in particular the level of intake and particle passage rate through the tract (Dijkstra, 1994).

The effects of defaunation on ruminal lipid metabolism are less defined. The protozoa contribute to the total microbial lipolytic activity but their role in bio-hydrogenation is less well-understood (Williams and Coleman, 1992) although they do contribute significantly to flow of unsaturated fatty acids leaving the rumen (Yáñez-Ruiz et al., 2006). Using steers fed diets with different chlorophyll levels, it was demonstrated that the high levels of polyunsaturated fatty acids in protozoal cells appears to be associates with ingestion of chloroplasts (Huws et al., 2009). This chloroplasts uptake seems to be specific of Entodiniomorphids since no engulfed chloroplasts have been found in holotrich protozoa (Huws et al., 2012). Thus protozoa appear to protect chloroplast unsaturated fatty acids from the rumen bio-hydrogenation increasing the duodenal flows of mono and polyunsaturated fatty acids. Our meta-analysis agrees with this observation, and defaunation promoted an increase of saturated fatty acids ($+4\%$, $P = 0.046$) and a numerical decrease in mono and polyunsaturated fatty acids (-13 and -10%, respectively) which possibly did not reached significance as a result of diet-dependent effects.

The findings reported in our meta-analysis need to be carefully interpreted since most of the studies had methodological limitations due to the intrinsic difficulty of the defaunation process leading to:

(1) Confusion between the effect of defaunation and period when using the same experimental animals in time,
(2) Nearly all studies estimating protozoal pool size have failed to report duodenal flows of protozoal biomass and *vice versa*, moreover protozoal flow is often underestimated using traditional microbial markers (Broderick and Merchen, 1992),
(3) Studies assessing methane production have not accounted for other variable effects besides protozoal abundance (i.e., NDF digestibility),
(4) Animals are often fed at levels far below the production levels of intake,
(5) In some studies the adaptation period after defaunation/refaunation is too short for the microbial ecosystem to fully adapt,
(6) Elimination of rumen protozoa modifies the ecological structure in the rumen and ultimately alternative microbial groups can take over ecological niche previously filled by protozoa.

Thus, more studies are needed using state-of-the-art technologies to quantify protozoal activity (i.e., fibrolytic and proteolytic) as well as the ruminal protozoal pool size relative to protozoal N outflow for better understanding of the role of protozoa on the ruminant's metabolism. These studies should be done at production levels of intake in order to truly assess the effect of defaunation per unit of product (milk or meat) produced.

MANIPULATION OF RUMEN PROTOZOA

Current concerns regarding the role of livestock in global warning has driven researchers to search for strategies to manipulate rumen protozoa to decrease methane production. As has been previously mentioned, there is a linear relationship between protozoal concentration and methane emissions (Guyader et al., 2014) and it has been estimated that between 9 and 37% of ruminal methane production can be attributed to methanogens associated with protozoa in the rumen (Finlay et al., 1994; Newbold et al., 1995; Machmuller et al., 2003).

Most approaches to defaunation rarely result in the total removal of protozoa from the rumen with their effectiveness largely dependent on diet composition (Hegarty, 1999). Treatments normally used to partially or completely defaunate the rumen include: chemicals that are toxic to protozoa (copper sulfate, dioctyl sodium sulfosuccinate, alcohol ethoxy-late or alkanates, calcium peroxide), ionophores, lipids, and saponins (Williams and Coleman, 1992; Jouany, 1996; Hook et al., 2010).

A recent meta-analysis by Guyader et al. (2014) has shown a concomitant reduction in protozoal numbers and methane emissions in 31% of 70 studies using different methane reduction strategies. Most of the studies used lipids as a protozoal control/methane mitigation strategy. The authors also reported that the antiprotozoal effect of lipids depends on the fatty acid composition with medium chain fatty acids more effective than

polyunsaturated fatty acids in controlling protozoal numbers. Supplements rich in polyunsaturated fatty acids such as linoleic acid (C18:2 from soybean and sunflower) and linolenic acid (C18:3 from linseed) have been shown to have a negative effect on methane production (4.1 and 4.8% decrease per percentage unit of added lipids, respectively, Martin et al., 2010). The antimethanogenic effect of polyunsaturated fatty acids has been related to their toxic effect on cellulolytic bacteria (Nagaraja et al., 1997) and protozoa (Doreau and Ferlay, 1995). Although it was originally suggested that the biohydrogenation of polyunsaturated fatty acids in the rumen could represent an alternative H_2 sink to methanogenesis (Lennarz, 1966), it is now believed that the significance of biohydrogenation to the overall H_2 sink is small (Nagaraja et al., 1997). It has been shown that medium chain fatty acids have potent antiprotozoal effect. Several studies have reported decreased ruminal methanogenesis when supplementing lauric acid (C12:0) and myristic acid (C14:0), either in pure forms or in products rich in these fatty acids (coconut oil) under *in vitro* (Dohme et al., 2001; Soliva et al., 2004) or *in vivo* conditions (Machmuller et al., 2003; Jordan et al., 2006). Martin et al. (2010) reported that medium chain fatty acids, mainly provided by coconut oil, resulted in a decrease in methane of 7.3% per percentage unit of added lipid. However, the methane suppression effect observed was not always related to a decrease in protozoa concentration (Machmuller et al., 2003) which may be due to a direct effect of medium chain fatty acids on methanogens (Dohme et al., 1999; Panyakaew et al., 2013). A recent study (Faciola and Broderick, 2014), found that both coconut oil and lauric acid reduced the number of protozoa by 40% but whereas lauric acid altered fiber digestibility coconut oil did not. However, reductions in methane production and a concomitant decrease in dry matter intake when coconut oil and lauric acid were used as defaunating agents has been reported (Hristov et al., 2013), which would potentially limit their practical on-farm use. Clearly more studies are needed to work out how to use oils and fatty acids to control protozoa in the rumen

The literature suggests that saponins mitigate methanogenesis mainly by reducing the numbers of protozoa, whilst condensed tannins act by both reducing the number of protozoa and by a direct toxic effects on methanogens, whereas essential oils act mostly by a direct toxic effect on methanogens (Cieslak et al., 2013). In agreement with this information, a meta-analysis of the effect of phytochemicals on methanogenesis (Patra, 2010) has shown that changes in protozoa numbers followed a linear relationship with changes in methane production by saponins ($r = 0.69$) and tannins ($r = 0.55$), but this relationship was weaker ($r = 0.45$) with respect to essential oils. Methane inhibition by organosulfur compounds was not associated with changes in the protozoal population (Patra, 2010) as such compounds specifically inhibit methanogenic archaea. For tannin containing plants, the antimethanogenic activity has been attributed to the group of condensed tannins. It has been suggested that tannins have a direct effect on ruminal methanogens and an indirect effect on hydrogen production due to a reduction in fiber digestion (Tavendale et al., 2005). Regarding their effect on protozoa, some studies have reported no effect whereas others have shown a reduction in protozoa numbers in the presence of tannins (reviewed by Patra and Saxena, 2009). This inconsistency is probably due to differences in the structure and dose of the condensed tannin. Saponins, shows a more consistent inhibitory effect on rumen protozoa in the available literature. Goel and Makkar (2012) have suggested that the risk of impaired rumen function and thus reduced animal productivity is greater with tannins than with saponins and when used to decrease methane production, the effective concentration range for tannins is narrower than for saponins. Saponins are glycosylated triterpenes or steroids where the saponin is the aglycone while the glycone is a carbohydrate unit consisting of a monosaccharide or smaller oligosaccharide entity. The most commonly sources of saponins used in ruminant nutrition are *Yucca schidigera*, rich in sterol saponins (10%), and *Quillaja saponaria* which contains triterpene saponins. Lately, other sources of saponins such as tea saponins have being explored (Hu et al., 2005; Guo et al., 2008). The antiprotozoal effect of saponins is related to their interaction with the sterol moiety which is present in the membrane of protozoa (Patra and Saxena, 2009). It has been suggested that saponins with the same aglycone may have a different effect on protozoa depending on the sugar composition and arrangement (Wina et al., 2006). This effect seems to be transitory due to the cleavage of the glycosidic bond by rumen microbes (Newbold et al., 1997). The suppression of rumen protozoa by saponins or saponin-containing plants has been consistently observed in *in vitro* studies (Wina et al., 2005). However, *in vivo* studies have shown that the antiprotozoal effect of saponins tends to disappears after 7–14 days of administration (Wina et al., 2005; Patra and Saxena, 2009). It has been suggested that when saponins are deglycosylated to sapogenins by rumen microbes they become inactive (Newbold et al., 1997). Thus, it can be hypothesized that the combination of saponins with glycosidase inhibitors would avoid deglycosylation, maintaining the intact saponin and, therefore, the activity in the rumen. Preliminary *in vitro* studies (Ramos-Morales et al., 2014) using 2,5-Dihydroxymethyl-3,4-dihydroxypyrrolidine (DMDP) as a glycosidase inhibitor combined with a plant extract rich in saponins have shown the potential of this approach to maintain saponin activity over time. Another approach that is being explored by our research group is the synthesis of a chemically modified saponin without the natural glycoside bonds so the enzymatic cleavage would be structurally prohibited. The effects of saponins on rumen fermentation have not been found to be consistent. These discrepancies appear to be related to the chemical structure and dosage of saponins, diet composition, as well as adaptation of the microorganisms to saponins (Wina et al., 2005; Patra and Saxena, 2009).

Australian researchers have demonstrated the potential of vaccination against methanogens as a method for mitigating methane emissions (Wright et al., 2004). Although this technology is still developing it provides many options for long term methane reduction. Similarly, protozoa, as providers of hydrogen to methanogens, or acetogens which compete for hydrogen with methanogens, could be possible vaccine targets for the reduction of methane emissions. An immunological approach has been explored for defaunation (Williams et al., 2008). Vaccination of sheep with entodinia or mixed protozoal antigens reduced protozoa numbers and IgG antibodies generated against rumen

protozoa remained active and continued to bind target cells for up to 8 h. However, no *in vivo* effect on rumen protozoa has been observed. It has been suggested that the reasons for the lack of effect may be related to insufficient amount of specific Ig delivered in saliva, need of an adjuvant to optimize the production of salivary antibodies, target other antigens of protozoa to generate a greater immune response.

CONCLUSION

Since the landmark publication of the Rumen Protozoa by Williams and Coleman (1992), there has been steady but perhaps not spectacular progress in our understanding of rumen protozoa. The advent of molecular techniques has led to a raft of publications regarding protozoal diversity in the rumen and while as discussed above the techniques used have within themselves limitations in their ability to accurately quantify individual protozoal genera they have provided new insights into the diversity of ciliate protozoa in different ruminant species, in different geographies and under different dietary situations. There has been steady progress in the area of defaunation and whilst at this stage no commercially available defaunation technique has yet been marketed, it seems likely that plant extracts can be used to control protozoa in the rumen; if not completely eliminate them. Work on the consequence of elimination of protozoa has largely focused on their role in methanogenesis, reflecting current concerns regarding the role of ruminants in greenhouse gas production. However the use of meta-analysis of existing data combined with new defaunation studies have help clarify our understanding of the role of protozoa in the rumen as illustrated in **Figures 1** and **2**. It is however perhaps the development of molecular techniques to clone and characterize protozoal genes, originally from single species but more recently from metagenomic and transcriptomic sources, that seems to offer the greatest, but as yet largely unfulfilled, potential to help truly elucidate the role of rumen protozoa in the rumen and in the absence of progress in developing axenic culture of rumen protozoa more effort needs to be put into characterizing rumen protozoa activity through molecular methods.

AUTHOR CONTRIBUTIONS

All authors have equally contributed to the preparation of this publication.

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REVIEW
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Maximizing efficiency of rumen microbial protein production

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Rumen microbes produce cellular protein inefficiently partly because they do not direct all ATP toward growth. They direct some ATP toward maintenance functions, as long-recognized, but they also direct ATP toward reserve carbohydrate synthesis and energy spilling (futile cycles that dissipate heat). Rumen microbes expend ATP by vacillating between (1) accumulation of reserve carbohydrate after feeding (during carbohydrate excess) and (2) mobilization of that carbohydrate thereafter (during carbohydrate limitation). Protozoa account for most accumulation of reserve carbohydrate, and in competition experiments, protozoa accumulated nearly 35-fold more reserve carbohydrate than bacteria. Some pure cultures of bacteria spill energy, but only recently have mixed rumen communities been recognized as capable of the same. When these communities were dosed glucose *in vitro*, energy spilling could account for nearly 40% of heat production. We suspect that cycling of glycogen (a major reserve carbohydrate) is a major mechanism of spilling; such cycling has already been observed in single-species cultures of protozoa and bacteria. Interconversions of short-chain fatty acids (SCFA) may also expend ATP and depress efficiency of microbial protein production. These interconversions may involve extensive cycling of intermediates, such as cycling of acetate during butyrate production in certain butyrivibrios. We speculate this cycling may expend ATP directly or indirectly. By further quantifying the impact of reserve carbohydrate accumulation, energy spilling, and SCFA interconversions on growth efficiency, we can improve prediction of microbial protein production and guide efforts to improve efficiency of microbial protein production in the rumen.

Keywords: rumen microbiology, reserve carbohydrate, glycogen, energy spilling, short-chain fatty acids

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Introduction

Cattle and other ruminants can degrade fibrous feedstuffs owing to the consortium of bacteria, protozoa, fungi, and methanogens inhabiting their rumen and hindgut. This consortium ferments fiber and other feed components to short-chain fatty acids (SCFA) and, in the process, generates ATP that fuels microbial growth (synthesis of cellular protein in particular). This microbial protein supplies 60 to 85% of amino acids (AA) reaching the animal's small intestine (Storm et al., 1983). Maximizing efficiency of its production would consequently improve cattle productivity.

Production of microbial protein is inefficient because microbes do not direct all ATP toward growth. Rather, microbes can direct some ATP toward maintenance functions, synthesis of reserve carbohydrate, or energy spilling (futile cycles that dissipate heat). The impact of maintenance

TABLE 1 | Efficiency of rumen microbial growth.

Organism	Efficiency	
	g microbial DM (mol ATP)$^{-1}$ [a]	% of theoretical maximum[b]
Mixed rumen microbes, *in vivo*	11–21	34–66
Mixed rumen bacteria, *in vitro*	7.5–16.7	23–52
Pure cultures, *in vitro*	10–25	31–78

[a] *Summarized from Russell and Wallace (1997).*
[b] *31.9 g (g microbial DM mol ATP)$^{-1}$; value from Stouthamer (1973) for growth with glucose, amino acids, and nucleic acid bases.*

functions on growth efficiency has been recognized for decades and for both pure and mixed cultures (Pirt, 1965; Russell and Cook, 1995). Only recently, however, have reserve carbohydrate accumulation and energy spilling been accurately quantified in mixed rumen microbes such that their impact on efficiency can be considered (Hackmann et al., 2013a,b; Denton et al., 2015). We will review these recent advances in the context of other factors that depress efficiency of microbial protein production.

For many years, knowledge on the rumen microbiome steadily grew but was viewed myopically—from the context of a relatively few culturable isolates. Researchers used pure cultures or mixtures of a few pure cultures to establish niches, substrates used, growth factors, growth rates, fermentation end-products, and fundamental interactions between those cultures (Krause et al., 2013). Results from mixed cultures and from rumen-cannulated animals expanded those principles. In the past decade, however, the expansion of technology has greatly expanded our view of the rumen microbiome, sometimes from a hyperopic view—from the context of how to integrate extensive metagenomics data on the rumen microbiome with ruminant nutrition (Firkins and Yu, 2015). We will discuss how the growth efficiency of mixed ruminal microbes is affected by metabolic fluxes of anabolic and catabolic reactions, with emphasis on energy spilling.

Improving Efficiency of Microbial Growth

For more than 40 years, we have recognized that microbes grow (synthesize microbial protein) with far from perfect efficiency (Stouthamer, 1973). For mixed rumen microbes *in vivo*, actual growth efficiency ranges from only 1/3 to 2/3 of the theoretical maximum (as calculated from biochemical pathways) (**Table 1**). This implies that microbes spend as little as 1/3 of ATP on growth. Similar efficiencies are reported for mixed and pure cultures of bacteria *in vitro* (**Table 1**).

ATP not spent on growth is instead directed toward non-growth functions such as maintenance, energy spilling, and synthesis of reserve carbohydrate (**Figure 1**). Maintenance functions are those required for cellular "housekeeping" and include (1) re-synthesis of protein following intracellular turnover and (2) maintaining ion balances across the cell membrane (Russell and Cook, 1995). Motility is also a component of maintenance; it is a special case of maintaining ion balances because motility is driven by a proton or sodium

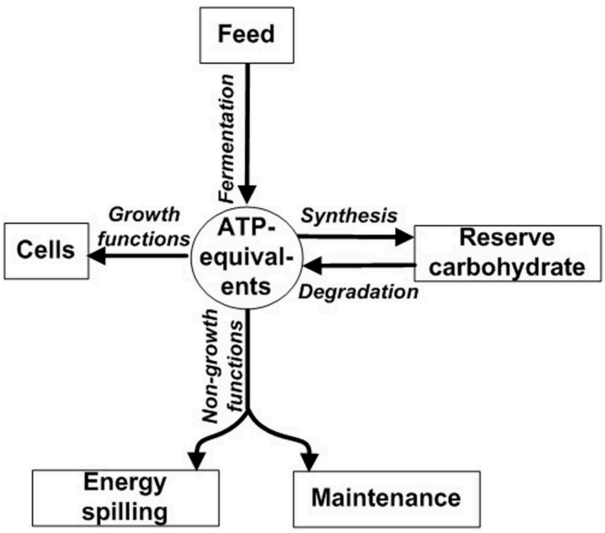

FIGURE 1 | Partitioning of ATP energy toward growth functions, non-growth functions, and synthesis of reserve carbohydrate. ATP-equivalents can include ATP or ATP-yielding carbon compound (e.g., glucose). Modified from Russell and Wallace (1997) and Russell (2007a).

motive force (Russell and Cook, 1995). Reserve carbohydrate synthesis refers to formation of glycogen and other compounds during energy excess (Preiss and Romeo, 1989). Although reserve carbohydrate can be mobilized later for growth (**Figure 1**) (Wilkinson, 1959), some ATP is irreversibly expended during synthesis. Energy spilling (e.g., futile cycling of ions or reserve carbohydrate; Russell and Cook, 1995; Portais and Delort, 2002; Russell, 2007b) refers to energy dissipated as heat when ATP exceeds needs for growth, maintenance functions, and reserve carbohydrate synthesis. It can be analogized to water spilling over the brim of an overfilled bucket (**Figure S1**). It is commonly a response to excess carbohydrate (Russell, 1998), as would occur when the ruminant is fed grain.

Maintenance functions have been long-recognized to be a sink for ATP energy and responsible for inefficient growth (Pirt, 1965). Maintenance energy becomes especially important when growth rates are low. Using the Pirt equation and values for mixed rumen bacteria in chemostats (Isaacson et al., 1975), Russell (2007a) calculated that maintenance energy would account for only 10% of total glucose consumption at the relatively high growth rate of 0.2 h^{-1}. However, it would account for 31% total glucose consumption at the low growth rate of 0.05 h^{-1}.

Because bacteria pass with digesta, their growth rate increases with increasing digesta passage rate in the rumen. Increasing passage rate by nutritional manipulation would be one strategy to decrease the relative impact of maintenance energy and improve growth efficiency. This is a facile strategy, however, because increasing passage rate, such as by grinding forage, decreases feed digestibility (Van Soest, 1994). A more defensible strategy to improving growth efficiency is to target other non-growth functions, such as energy spilling and reserve carbohydrate synthesis.

Occurrence of Energy Spilling in Pure Cultures

Although maintenance functions depress microbial growth efficiency, only energy spilling can explain very low efficiency during carbohydrate excess (and other growth-limiting conditions). Russell (1986) demonstrated energy spilling by pulse-dosing rumen bacterial cultures with glucose. Cultures fermented excess glucose rapidly, produced very little protein (growth efficiency approached 0), and dissipated (spilled) energy by producing heat. Van Kessel and Russell (1996) reported that mixed rumen bacteria fermented glucose 10-fold faster when spilling energy, implying that spilling could be a significant sink for ATP.

Spilling occurs in organisms across all three domains of life (**Table 2**). Spilling has been demonstrated extensively in a few rumen (*Streptococcus bovis*) and non-rumen (*Escherichia coli*, *Klebsiella aerogenes*) bacteria. Though evidence is less extensive, it also appears to occur in protozoa, fungi, and methanogens, suggesting broad importance. In almost all cases, single-species cultures, not mixed communities, have been studied.

Spilling can be evidenced by depressed growth efficiency or elevated heat production in response to excess carbohydrate (**Table 2**). Carbohydrate excesses have been generated by pulse dosing glucose or growing cells under limitation of an anabolic substrate (e.g., N, Mg, P, S, K). Spilling can also be evidenced in response to (1) ammonia-N replacing amino-N and (2) excess H_2 or CO_2 (for methanogens). When measuring changes in growth efficiency and heat production, one must account for any changes in maintenance energy, reserve carbohydrate, and growth (cf. **Figure 1**), though this is sometimes not done (**Table 2**).

The mechanism of spilling is by futile cycles of ions, glycogen, or trehalose (**Table 2**). The best-elucidated mechanism is for *S. bovis*, for which spilling occurs by futile cycling of protons. This cycling results from growth limitation and a cascade of biochemical events (Russell, 2002). Specifically, a growth limitation decreases use of ATP for protein synthesis, increasing fructose-1,6-bisphosphate, decreasing intracellular phosphate, increasing the absolute value of Gibbs energy of ATP hydrolysis, increasing activity of a proton-pumping ATPase, and decreasing membrane resistance to protons. The net result is heat, with no work done by the protons.

For *E. coli*, the mechanism of energy spilling is by cycling of K^+ or NH_4^+ or a combination thereof, depending on the extracellular concentration of K^+ and NH_4^+ (**Table 2**). For many other organisms, cycling of glycogen and trehalose may occur (**Table 2**). Such cycling implies energy spilling can occur, even though spilling often has not been measured directly (growth efficiency and heat production were often not determined; **Table 2**). Some authors have proposed that fructose-6-phosphate/fructose-1,6-bisphosphate (Otto, 1984) or other substrates (Newsholme et al., 1984) can be cycled. However, such cycling is difficult to establish under physiological conditions (Russell and Cook, 1995; Portais and Delort, 2002).

The biological rationale for spilling is not clear: why expend what can be conserved? Some have proposed spilling allows microbes to rapidly catabolize substrate and dissipate energy in order to (1) deprive competitors of energy or (2) hasten re-initiation of growth when a growth limitation is released (Tempest, 1978; Russell and Cook, 1995). Although all futile cycles dissipate energy and thus cause spilling, this spilling may be an unfortunate byproduct, not the primary function, of some futile cycles. Rather, the function of some futile cycles may be to sensitively control the net flux of metabolites (Newsholme and Crabtree, 1975). Regardless of function, energy spilling and futile cycles are wasteful from the perspective of growth efficiency, and their impact on growth of the rumen microbial community needs to be elucidated.

Occurrence of Energy Spilling in Mixed Cultures

Many pure cultures have been demonstrated to spill energy, but until recently, examples of mixed communities that spill energy were few (**Table 2**). In earlier studies, Van Kessel and Russell (1996) suggested that rumen bacteria spilled more energy when grown under ammonia-N vs. amino-N limitation. Their approach assumes constant cell composition, and they did not measure reserve carbohydrate. Some energy may have in fact been directed to reserve carbohydrate synthesis, not spilling. Chen et al. (2000) induced spilling in activated sludge by adding a protonophore, but spilling was not measured under more physiological conditions.

More recently, we quantified spilling for mixed rumen microbes (Hackmann et al., 2013a). When we washed cells with N-free buffer and dosed them with glucose, they consumed glucose rapidly, accumulated reserve carbohydrate, and did not grow (protein remained constant) (**Figures 2A,B**). When we dosed a moderate concentration of glucose (5 m*M* glucose), no spilling was detected, as nearly all heat production (93.7%) was accounted by reserve carbohydrate synthesis and endogenous metabolism (a proxy for maintenance energy) (**Figure 2C**). When we dosed a high concentration of glucose (20 m*M*), energy spilling was not detected immediately, but it accounted for a significant amount of heat production approximately 30 min after dosing (**Figure 2D**). Energy spilling accounted for as much as 38.7% of heat production in one incubation.

Identity and Occurrence of Reserve Carbohydrate

Rumen microbes can accumulate prodigious amounts of reserve carbohydrate. It can exceed 50% of cell weight for pure cultures of both rumen (Russell, 1998) and non-rumen (Preiss and Romeo, 1989) bacteria. Some protozoa (Isotrichidae) accumulate enough reserve carbohydrate to turn opaque (Williams and Coleman, 1992). Net accumulation of glucose into reserve carbohydrate can exceed 50% for mixed microbes (Hackmann et al., 2013a) and protozoa (Denton et al., 2015). The identity of this reserve carbohydrate is glycogen [glucan with (α1→4) and (α1→6) linkages] and appears ubiquitous across rumen bacteria, fungi, and protozoa (Table S1). Synthesis of these prodigious amounts of reserve carbohydrate irreversibly expends ATP, decreasing ATP available for protein synthesis (**Figure 1**). This lowers growth efficiency on a protein basis (g protein/mmol ATP), as it probably does on a dry matter basis (g DM/mmol ATP) (explained later).

TABLE 2 | Occurrence of energy spilling in microbes[a].

Species	Ruminal isolate	Heat production or growth yield evidence	Mechanism (type of cycling)	Notes	References
BACTERIA					
Escherichia coli	N	Lower growth yield per ATP under Mg, P, S, K vs. C-limitation	$NH_3/NH_4^+/H^+$	Mechanism applies during high NH_3/NH_4 and low K^+	Buurman et al., 1991
	N	Lower growth yield per ATP of wild-type vs. K^+ transport mutant	K^+	Mechanism applies during low K^+	Mulder et al., 1986
	N	ND	$NH_3/NH_4^+/H^+/K^+$	Mechanism applies during low NH_3/NH_4^+	Russell and Cook, 1995
Klebsiella aerogenes	N	Lower growth yield per ATP under NH_3, P, S, or K vs. C-limitation	ND		Neijssel and Tempest, 1976; Teixeira de Mattos and Tempest, 1983
Fibrobacter intestinalis	N	ND	Glycogen		Matheron et al., 1998
Fibrobacter succinogenes	Y	ND	Glycogen		Gaudet et al., 1992; Matheron et al., 1998
Prevotella bryantii	Y	Heat production rose rapidly during glucose excess	ND	Growth and reserve carbohydrate not accounted explicitly	Russell, 1986
Selemonas ruminantium	Y	Heat production rose rapidly during glucose excess	ND	Growth and reserve carbohydrate not accounted explicitly	Russell, 1986
Streptococcus bovis	Y	Heat production not accounted by maintenance energy or growth	H^+		Russell and Strobel, 1990; Cook and Russell, 1994; Bond and Russell, 1996, 1998
Mixed rumen bacteria	Y	Lower growth yield per hexose under NH_3-N vs. amino-N	Not defined	Reserve carbohydrate or other cell composition changes not accounted explicitly	Van Kessel and Russell, 1996
PROTOZOA					
Isotricha protostoma	Y	ND	Glycogen		Prins and Van Hoven, 1977
Dasytricha ruminantium	Y	ND	Glycogen		Van Hoven and Prins, 1977
FUNGI					
Saccharomyces cerevisiae	N	Lower growth yield per ATP under glucose excess	ND		Van Urk et al., 1988
	N	Higher heat production and lower growth yield under N vs. glucose-limitation	ND	Reserve carbohydrate not accounted explicitly	Larsson et al., 1993
	N	ND	Trehalose	Mechanism applies under heat shock	Hottiger et al., 1987
ARCHAEA					
Methanobacterium thermoautotrophicum	N	Lower growth yield per CH_4 under H_2- or CO_2- excess vs. limitation	ND	Reserve carbohydrate or other cell composition changes not accounted explicitly	Schönheit et al., 1980; Morgan et al., 1997
MIXED OR UNDEFINED					
Mixed rumen microbes	Y	Heat production not accounted by endogenous metabolism or reserve carbohydrate	ND	Endogenous metabolism used as proxy for maintenance energy	Hackmann et al., 2013a

[a]*N, no; Y, yes; ND, Not determined.*

Dynamics of Accumulation

In the rumen, reserve carbohydrate accumulates immediately after feeding (during carbohydrate excess), and then is mobilized thereafter (during carbohydrate limitation). This is observed for both rumen bacteria (**Figure S2**) and protozoa (**Figure S3**) (Jouany and Thiven, 1972; McAllan and Smith, 1974; Williams and Harfoot, 1976; Leedle et al., 1982). These dynamics are most dramatic for high-grain and low-N diets, which create large carbohydrate excesses after feeding (cf. basal diet + urea vs. basal diet in **Figure S2**).

For *in vitro* batch culture (**Figures 2A,B**), where conditions can be better defined, we observed that rumen microbes showed similar dynamics of accumulation and mobilization as *in vivo* (**Figures S2**, **S3**). When we washed mixed rumen microbes with N-free buffer and dosed glucose, reserve carbohydrate immediately accumulated (**Figures 2A,B**). At peak

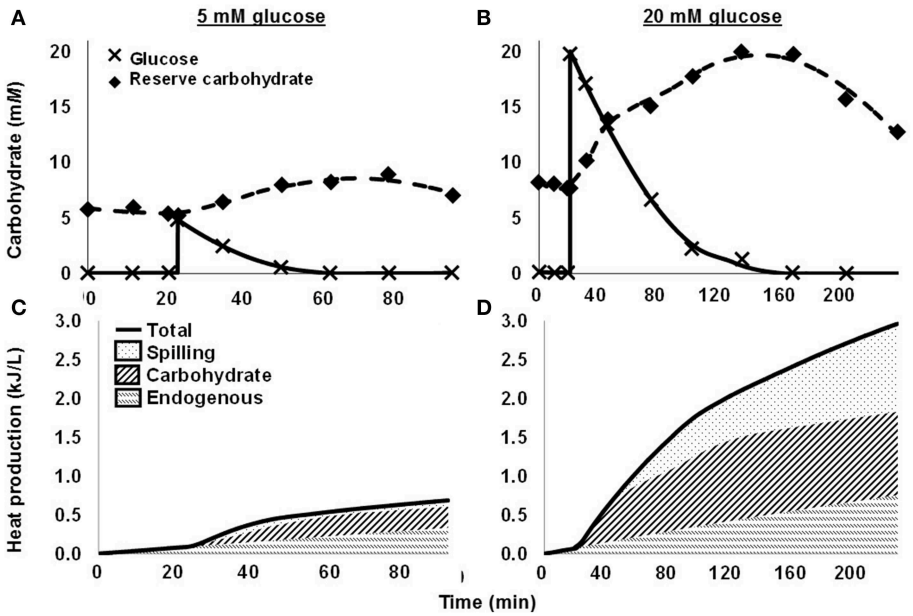

FIGURE 2 | Energy spilling and other responses of mixed rumen microbes to pulse dose of glucose. (A,C) 5 m*M* glucose. **(B,D)** 20 m*M* glucose. **(A,B)** Glucose in media and reserve carbohydrate. Glucose was dosed at 20 min. **(C,D)** Heat production, including heat accounted by endogenous metabolism, synthesis of reserve carbohydrate, and energy spilling. Data are for 1 cow, and each glucose concentration represents a single experiment. Figure adapted from Hackmann et al. (2013a).

accumulation, microbes had incorporated 59.5% of glucose carbon in reserve carbohydrate when we dosed 5 m*M* glucose. The value was lower (52.6%) for 20 m*M* glucose. After glucose was exhausted, reserve carbohydrate quickly declined (**Figures 2A,B**).

In a subsequent batch culture study, we observed that protozoa, not bacteria, were responsible for most glycogen accumulation. In this study, we performed competition experiments in which mixtures of protozoa and bacteria were first dosed with glucose, and then at intervals the two groups were separated for glycogen analysis. When the mixtures were dosed with a moderate concentration of glucose (c. 5 m*M*), protozoa incorporated 58.7% of glucose carbon in reserve carbohydrate at time of peak reserve carbohydrate (**Figure 3A**). Bacteria had incorporated only 1.7%. When we dosed mixtures with a high concentration glucose (20 m*M*), the amounts incorporated were 21.4% for protozoa and 5.0% for bacteria, respectively (**Figure 3B**). Protozoa would thus appear the predominant group accumulating reserve carbohydrate. In sum, rumen microbes display a high capacity for accumulating and mobilizing reserve carbohydrate. As explained later, this can expend ATP and lower growth efficiency.

Relation to Growth Efficiency

At first consideration, synthesis of reserve carbohydrate should not appear to depress growth efficiency; rather, it would seem to improve it. Glycogen, a common reserve carbohydrate, requires fewer ATP for synthesis than all other cellular macromolecules except lipid (**Table 3**). Simple arithmetic would suggest more dry matter could be formed when glycogen vs. most other macromolecules are synthesized—i.e., efficiency of growth on a dry matter basis (g DM/mmol ATP) would be higher.

Simple arithmetic does not consider that reserve carbohydrate accumulation is dynamic. As mentioned, rumen microbes vacillate between (1) reserve carbohydrate synthesis during carbohydrate excess and (2) degradation during carbohydrate limitation. The price paid for such vacillation is expenditure of ATP. For sequential synthesis and degradation of glycogen, 1 net ATP equivalent is expended per glucose (**Figure S4**). Synthesis of glycogen may cost few ATP compared to most other macromolecules (**Table 3**), but this low initial cost may be quickly outweighed by this sequential synthesis and degradation. Reserve carbohydrate synthesis could thus lower growth efficiency on a dry matter basis.

Reserve carbohydrate may have been historically overlooked in growth efficiency measurements because most experiments employ chemostats under steady-state conditions (see references in Russell and Cook, 1995). By design, the steady state input of substrate will prohibit vacillation between glycogen synthesis and degradation. Some experiments employ batch cultures (Russell and Cook, 1995), but they are usually terminated during exponential growth, before reserve carbohydrate degradation typically occurs.

Even when reserve carbohydrate would not lower growth efficiency on a DM basis (g DM/mmol ATP), it would always lower it on an N or protein basis (g N or protein/mmol ATP). No matter its course, reserve carbohydrate synthesis (2 ATP/glucose; **Figure S4**) would reduce ATP available for synthesis of protein and other N-containing macromolecules. This point is indirectly supported by the batch culture experiments of Hall (2011), in

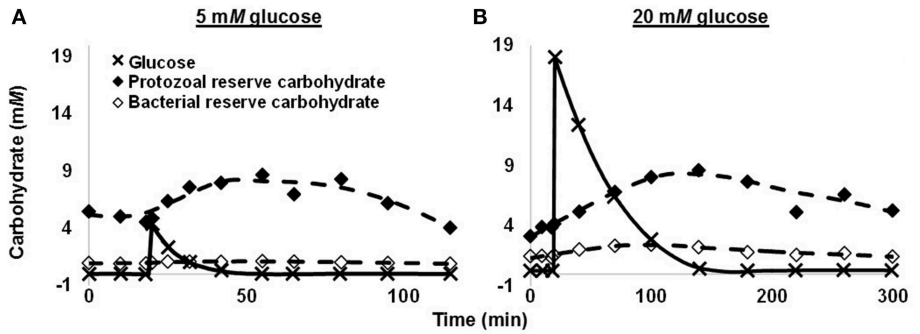

FIGURE 3 | Glucose use and reserve carbohydrate accumulation of a mixture of rumen protozoa and bacteria in batch culture. (A) 5 m*M* glucose. **(B)** 20 m*M* glucose. Data are for 1 cow, and each glucose concentration represents a single experiment. Figure adapted from Denton et al. (2015).

TABLE 3 | ATP required for synthesis of cellular macromolecules.

Macromolecule	ATP requirement (mmol g^{-1})
Protein	36.5
RNA	14.6
DNA	18.0
Lipid	1.5
Polysaccharide (glycogen)	12.4

Calculated from Stouthamer (1973) for growth with glucose, amino acids, and nucleic acid bases, excluding ATP for mRNA turnover and solute transport.

which destroying isotrichid protozoa by blending (1) decreased reserve carbohydrate accumulation by 43% and (2) increased growth efficiency on an N basis (g N/g soluble carbohydrate fermented) by 17%. In ruminant nutrition, growth efficiency is usually expressed in terms of protein or N, reflecting that microbial protein accounts for the majority of AA reaching the animal small intestine (Storm et al., 1983).

Although chemostats are more consistent with *in vivo* conditions than batch cultures because the former permit changes in dilution rate, such changes are simultaneous with increasing substrate supply. In contrast, increasing passage rate from the rumen should increase microbial growth rate, at least in part, independently from substrate supply (Dijkstra et al., 1998). As described in that report, increasing growth rate (decreasing division time) is projected to increase growth efficiency (g bacterial DM/g carbohydrate) and decrease the proportion of ATP used to support non-growth functions. Such models would be more accurate with better understanding of how much more reserve carbohydrate is accumulated under different dietary conditions.

Glycogen Cycling

On the surface, rumen microbes would seem to simply (1) synthesize reserve carbohydrate during carbohydrate excess and (2) degrade it during carbohydrate limitation. However, many rumen and non-rumen microbes have been shown to simultaneously synthesize and degrade (cycle) glycogen (**Table 2**) (Portais and Delort, 2002). This cycling expends ATP, just as sequential synthesis and degradation of glycogen do (**Figure S4**). It would thus depress growth efficiency

Although we have discussed reserve carbohydrate synthesis and energy spilling independently, glycogen cycling would link these two functions because it is a form of energy spilling. Glycogen cycling has not yet been demonstrated for mixed rumen communities (**Table 2**), but we have speculated that it is the mechanism of spilling observed for mixed rumen microbes (Hackmann et al., 2013a).

Occurrence of Carbohydrate Excess in the Rumen

Both energy spilling and reserve carbohydrate synthesis occur primarily under carbohydrate excess. Carbohydrate is in greatest excess in the rumen for animals fed grain, particularly those transitioning to a high-grain diet, and also for animals fed high-sugar diets. For animals transitioning to a high grain diet, glucose can reach high concentrations [c. 5 m*M* (Ryan, 1964; Mackie et al., 1978)]. Even higher glucose concentrations (18 m*M*) have been reported for animals fed dextrose (Piwonka et al., 1994), and soluble sugar concentrations as high as 69 m*M* have been reported for animals fed beet pulp (Clapperton and Czerkawski, 1969). Concentrations in microenvironments (e.g., around starch granules) may also be high (Kajikawa et al., 1997).

For grain-fed animals, N availability can be low (NRC, 2000), also, and intensify carbohydrate excess (NRC, 2000). Availability of N can also be low for dairy rations with corn silage as the sole source of forage (Vandehaar, 2005). For animals in which N is chiefly in the form of ammonia, carbohydrate excess could be further intensified (Van Kessel and Russell, 1996) because rumen microbes grow far slower with ammonia-N than amino-N (Argyle and Baldwin, 1989; Van Kessel and Russell, 1996). Energy spilling and reserve carbohydrate synthesis would likely depress growth efficiency under these conditions, with spilling being more important at large carbohydrate excesses and reserve carbohydrate more important for smaller excesses (Hackmann et al., 2013a). Spilling has previously been suggested to account for low growth efficiency for high-concentrate diets (Clark et al., 1992).

For animals fed high-forage diets or adapted to grain, carbohydrate excess is relatively small, and glucose concentrations rarely exceed c. 2.5 m*M* (Kajikawa et al., 1997; Saleem et al., 2012). Energy spilling may play a minor role under these conditions, given that we did not detect spilling in batch cultures with glucose concentrations of 5 m*M* (Hackmann et al., 2013a). Reserve carbohydrate accumulation may still be important and decrease growth efficiency, however, given that reserve carbohydrate can still be detected even when cattle are provided low-quality grass diets (Van Kessel and Russell, 1997).

Other Factors Depressing Growth Efficiency

Other Responses to Excess Carbohydrate

Rumen microbes may respond to excess carbohydrate in ways other than spilling energy and synthesizing reserve carbohydrate. These other responses include reducing ATP yield by releasing metabolic intermediates (overflow metabolites) and shifting to catabolic pathways that yield less ATP (Russell, 1998). Responses are similar for non-rumen microbes (Tempest and Neijssel, 1984; Preiss and Romeo, 1989; Russell and Cook, 1995; Russell, 2007b).

Recycling of Microbial Protein

Recycling of microbial protein is another factor that depresses growth efficiency. As much as 50% of microbial protein is degraded to non-protein nitrogen in the rumen and recycled (Wells and Russell, 1996; Oldick et al., 2000). Protozoal predation, autolysis, and bacteriophages may all be causes (Wells and Russell, 1996). Most recycling has been thought to be mediated by protozoa predation, based on lysis of pure bacterial cultures in presence and absence of rumen fluid with protozoa (Wallace and McPherson, 1987). However, removing protozoa from the rumen was subsequently shown to have no effect on bacterial N recycling *in vivo* (Koenig et al., 2000). Firkins et al. (2007) reasoned that protozoa-mediated recycling of microbial protein is lessened with increasing passage rates by high-producing animals compared with some of the studies with low intakes or predictions based on measurements *in vitro*.

Exopolysaccharide Synthesis

In addition to synthesizing reserve carbohydrate, rumen bacteria can synthesize exopolysaccharides (Hobson and Macpherson, 1953, 1954; Costerton et al., 1974). One exopolysaccharide, dextran, is synthesized by *S. bovis* when given excess sucrose (Bailey and Oxford, 1958; Cheng et al., 1976), and it forms part of the "slime" observed in grain-fed animals with frothy bloat (Cheng et al., 1976). Up 80% of glucose in sucrose can be directed into its synthesis, but synthesis does not require ATP (Bailey, 1959; van Hijum et al., 2006), and there is no ATP cost of transport because synthesis occurs extracellularly (van Hijum et al., 2006). Thus, dextran formation would not depress growth efficiency on an ATP basis. *Ruminococcus albus* also forms an exopolysaccharide, but synthesis of this exopolysaccharide has been estimated to account for <4% of ATP of total used for synthesizing cell components (Weimer et al., 2006). Many other rumen bacteria form exopolysaccharides (Hobson and Macpherson, 1953, 1954; Costerton et al., 1974), but their formation has not been quantified, and their impact on growth efficiency remains unknown.

Cellodextrin Efflux

At least some cellulolytic bacteria expend ATP on cellodextrin efflux, which should depress their growth efficiency. The cellulolytic *Fibrobacter* spp. synthesize cellodextrins intracellularly, but these can be lost by extracellular efflux (Wells et al., 1995). The cell expends ∼4/3 ATP from combined costs of synthesis (1 ATP) and active transport (∼1/3 net ATP) (**Figure S5**). This ATP expended on cellodextrin synthesis would be recovered by non-cellulolytic bacteria after they take up the cellodextrin (assuming that transport is the exact opposite of efflux). Consequently, cellodextrin efflux should depress growth efficiency of some cellulolytics, not the microbial population as a whole.

Efflux of maltodextrins, not only cellodextrins, has been observed for *F. succinogenes* (Matulova et al., 2001; Nouaille et al., 2005). Maltodextrin efflux would be expected to expend ATP just as does cellodextrin efflux, but the exact expenditure is unknown because the pathway for maltodextrin synthesis is uncertain (cf. Matulova et al., 2001). Consequently, maltodextrin efflux likely depresses growth efficiency of some cellulolytics, but its exact impact on the cellulolytics and microbial population as a whole remains unknown.

Cross-feeding of cellodextrins is often depicted as being beneficial to the non-cellulolytics but also the cellulolytic populations by removing end-product inhibition of cellobiose on cellulases (Russell et al., 2009). As documented by those authors' model, increasing cellulolysis also diverts an increasing proportion of carbon toward cell growth and away from fermentation. However, if growth of the community is uncoupled by limitations of nitrogen or other growth factors, then an increasing proportion of carbon should be directed away from cell growth and toward SCFA, promoting energy spilling. In most studies measuring energy spilling, the medium was buffered. If total SCFA production was fast enough to decrease the ruminal pH below approximately 6.0, as can happen in the rumen, the proton gradient across the cell membrane could inhibit cellobiose transport by cellulolytics and thereby inhibit fiber degradation (Russell et al., 2009).

Cellobiose Hydrolysis or Transport

Russell (2002) described various mono or disaccharide transport mechanisms, including the phosphoenolpyruvate: phosphotransferase systems (PEP-PTS). Active transport also increases the ATP cost, but active transport of a disaccharide can have a decreased ATP charge if it is transported prior to hydrolysis into monosaccharides, and the ATP charge can be further decreased if disaccharide transport is coupled with a phosphorylase. Whether a phosphorylase or a PEP-phosphotransport system phosphorylates the sugar, this ATP charge is recovered by negating ATP required for a hexokinase reaction. Not all pure cultures were shown to express PEP-PTS transport of sugars (Martin, 1994). In mixed ruminal microbes, though, glucose or other sugars were assumed to be transported primarily by the PEP-PTS system (Kajikawa et al., 1997).

Glucose is typically not fed to ruminants, but primarily is the product of cellulose and starch degradation. Some cellobiose was transported into mixed bacterial cells by a PEP-PTS but also by ATP-expending transporters (Kajikawa and Masaki, 1999). Increasing availability of maltose or maltodextrins for transport might lead to increased SCFA production and lower ruminal pH. Cellulolytic bacteria can be inhibited by pH < 6.0 through depressed adherence to cellulose, decreased cellobiose transport, or from inhibitory membrane gradients of anions or protons (Russell et al., 2009).

Genomics-based analyses have revealed a much more complicated mechanism in which genes are expressed as polysaccharide utilization loci (Wang et al., 2013). Di- or oligosaccharides from hydrolyzed cellulose can be transported in gram-negative *F. succinogenes* (Suen et al., 2011), gram-positive *Ruminococcus flavefaciens* (Flint et al., 2008), and from hydrolyzed hemicellulose in gram-positive *Butyrivibrio proteoclasticus* (Dunne et al., 2012), respectively, likely from a combination of ABC-transporters and those linked to phosphorolytic cleavage. Substrate source and availability probably regulates expression of many of these transporters (Bond et al., 2012). Based on metagenomics screening of cellulases and xylanases, many genes were novel, but a relatively high proportion were reputed transporters (Wang et al., 2013). There is likely periplasmic sequestration of oligosaccharides from cellulose (White et al., 2014), hemicellulose (Morgavi et al., 2013), and starch (Rosewarne et al., 2014). Therefore, the net ATP cost of di- and monosaccharide transport into cytosol is not fully known but probably varies with substrate availability.

Rapid growth decreased glycogen concentration of *P. bryantii* B_14 (formerly *P. ruminicola*) (Lou et al., 1997b). However, with slower growth, maltose increased glycogen concentration more than when using glucose as substrate. When using maltose as substrate, maltose phosphorylase activity (which couples transport with phosphorylation of a glucose moiety) was increased and glycogen accumulated even when N was not limiting and growth rates increased. Similar results were detected when grown on cellobiose. In another study, growing *P. bryantii* on maltose or cellobiose increased the activity of UDP-glucose pyrophosphorylase and glycogen synthase compared with growth on sucrose or glucose (Lou et al., 1997a). Thus, transport and metabolism of maltose to glucose-1-phosphate was associated with glucose-1-phosphate polymerization into glycogen. Although poorly studied with mixed microbes, either gene expression of the reversible enzyme phosphoglucomutase or an accumulation of glucose-6-phosphate could help push synthesis of glycogen. UDP-glucose pyrophosphorylase (enzyme prior to glycogen synthase) was activated by fructose-1,6-phosphate in *P. bryantii* (Lou et al., 1997a). Pulse doses of glucose decreased metabolism of cellobiose and activity of cellobiose phosphorylase in *P. bryantii*, and vice versa (Lou et al., 1996). Thus, accumulation of disaccharides from rapid hydrolysis of cellulose or starch could stimulate glycogen synthesis in ruminal bacteria and thereby increase glycogen cycling as a means of energy spilling. *Prevotella bryantii* is well-known for energy spilling among cultivated prevotellas (Russell, 1992), although uncharacterized prevotellas often predominate in the rumen (Firkins and Yu, 2015).

Branched Short-chain Fatty Acids

The primary cellulolytics, most of which were characterized decades ago, have various requirements for growth factors such as branched chain SCFA and phenyl-substituted SCFA that are provided by secondary colonizers, which generally are much more proteolytic (Stewart et al., 1997). Despite the importance of branched chain SCFA required by isolates of cellulolytics, feeding these compounds *in vivo* primarily was associated with post-absorptive rather than ruminal responses (Andries et al., 1987). Given the importance of primary (or "keystone") colonizers (Ze et al., 2013), such a lack of ruminal response can be reconciled with a broader view of how bacteria are stimulated by preformed AA to better balance anabolic and catabolic pathways during growth (Russell and Cook, 1995).

Peptides and Amino Acids vs. Ammonia-N

Although there seems to be little difference between AA and peptides for mixed cultures, many pure cultures of bacteria are stimulated by provision of small peptides rather than free AA (Wallace et al., 1997). For example, *R. albus* was shown to transport peptides but not AA (Kim et al., 2014). Peptides had a minor effect on this strain's growth rate, which was maximized at about 0.9/h. In contrast, *S. bovis*, which is known for rapid growth on starch or sugar, had growth rate of 0.9/h with NH_3 that was stimulated to 1.6/h when AA were provided (Russell, 1993). In that study, incremental growth was synchronized with incremental boluses of glucose compared with a single dose, and the maximal growth available was particularly limited when glucose doses were incrementally staggered and when ammonia replaced AA. In contrast with pure cultures, when AA were provided with a mixture of carbohydrates as substrate, growth of mixed ruminal microbes was still stimulated by approximately 50% compared with providing NH_3 even when the growth rates of the mixed cultures were lower (i.e., from 0.25–0.30 increased to 0.40–0.45/h) (Kajikawa et al., 2002). Because the latter stimulation is from growth rates below the threshold above which AA were expected to stimulate growth (Van Kessel and Russell, 1996), even growth of cellulolytics limited by rate of cellulolysis can be stimulated. In contrast with prior expectations, preformed AA now are considered as potentially stimulatory for consortia of microbes degrading fiber (Newbold, 1999).

Supply and Profile of Preformed Amino Acids

Although energy is required for bacteria to synthesize AA, this energy cost is small; when preformed AA are limiting, though, growth rate is slowed, and the balance of anabolic and catabolic rates leads to increased energy spilling (Russell and Cook, 1995). As depicted in **Figure S6** (only for NH_3 assimilation, not for transamination of other AA), Ala, Glu, and Gln are the primary AA formed from assimilation of ammonia in the rumen. Relative fluxes of these amination reactions depend on the Michaelis constant (K_m) of ammonia for those enzymes but also based on transcription of ammonia-assimilating enzymes (Morrison and Mackie, 1997). Kim et al. (2014) documented

an excellent example of transcriptional control of ammonia-assimilating enzymes in *R. albus*. Although bacteria can make most of their AA, gelatin (which has a poor profile of Leu and the aromatic AA) decreased growth rates of mixed bacteria (Van Kessel and Russell, 1996) and increased energy spilling.

Although AA are stimulatory to growth (Kajikawa et al., 2002), an imbalance of branched or aromatic AA was worse than deletion of the entire group of AA. Bacteria can partially control the flux of AA biosynthetic pathways (**Figure S6**), but congruent pathways likely antagonize availability of closely related AA when they are out of balance. Moreover, pathways for biosynthesis of AA intersect with central metabolic pathways used in fermentation. Most rumen bacteria lack a complete TCA cycle and must use what would be considered both forward and backward reactions of that cycle (if it was complete) to form α-ketoglutarate (Wallace et al., 1997). Thus, the alternating directional flux of this interrupted cycle must be able to provide the mix of intermediates for anabolism while intersecting with catabolic reactions to make ATP to drive anabolism. Because many of these intermediates are produced through dehydrogenases, the NADH/NAD ratio must be resolved with catabolic fermentation reactions. In support, the NADH/NAD ratio can regulate the deamination of reduced AA, in particular the branched chain AA, as this deamination produces NADH (Hino and Russell, 1985).

Part of the difficulty in assessing how AA profile affects growth efficiency lies in how various studies were done. For example, bolus doses of isotopically labeled AA or peptides mostly yielded catabolism for energy, except for Leu, Tyr, and Phe (Armstead and Ling, 1993; Atasoglu et al., 1999). Only deletion of Leu (not deletion of other AA) decreased bacterial growth (Atasoglu et al., 2003), but Atasoglu et al. (2004) noted that Ile, Phe, Lys, and (to a somewhat lesser effect) Leu were incorporated into bacterial protein to a greater extent than were other AA. These results are consistent in that the branched chain and aromatic AA have similar metabolism within their respective groups and are more limiting than other AA.

In some of these types of studies, the peptide or AA were dosed as both the primary nitrogen and carbon source. Most of the pure cultures of predominant saccharolytic bacteria with more moderate deaminative activity did not grow well when peptides or AA provided the sole substrate (Wallace et al., 1997). Those authors reasoned that proteolysis by saccharolytic bacteria might be more important in exposing carbohydrate. Compared with other studies, there was relatively high assimilation of preformed AA in the study of Atasoglu et al. (2004) because they dosed labeled AA concomitantly with carbohydrate substrate. Those authors also noted how preformed AA, after being taken up by cells, can be catabolized if not assimilated. Firkins et al. (2015) postulated that Met is incorporated into cellular protein but that surplus intracellular Met recycles intracellularly and likely exchanges with an extracellular pool. Secretion or leakage of AA is likely when AA are high relative to concentrations needed for protein synthesis or AA are imbalanced.

Some discrepancies among studies evaluating amount or profile of preformed peptides or AA for bacteria also might be a result of inadequate adjustment time upon removal of protozoa. Protozoa appear to contain high deaminase activity (Wallace et al., 1997). However, protozoa might excrete up to half of the degradation fragments from the bacterial protein it consumes (Hristov and Jouany, 2005). Little is known about the AA profile of excreted bacterial proteins (rich in cell wall proteins?), and the excreted peptides might also be mixed with excreted but active protozoal peptidases. Although clearly important, predation of bacteria and lysis rates of protozoa probably have been exacerbated under the *in vitro* conditions in which normal substrate was replaced with various bacterial strains to quantify bacterial predation by loss of planktonic bacterial counts (Diaz et al., 2014). The hyperammonia-producing bacteria make a significant contribution to the total deamination activity in the rumen but are in low numbers (Walker et al., 2005). Thus, effects on growth rate of this group would be masked in mixed cultures.

In studies that have separated protozoal and bacterial fractions, there often is synergistic action when these two groups are added together (Walker et al., 2005). Defaunation (removal of protozoa) consistently decreases ruminal ammonia concentration compared with faunated controls, with explanations typically assuming exclusion of protozoal proteolytic and deaminative enzymes (Hristov and Jouany, 2005). However, an alternative explanation is that defaunation should increase the abundance of bacteria most of which assimilate ammonia, whereas protozoa do not. Ruminal protozoa can limit efficiency of microbial protein synthesis in the rumen through predation of bacteria, but there is a large gap in studies with defaunated animals at production-level intakes (Firkins et al., 2006). Recent improvements in methods allow the extraction of metabolically active protozoa with minimal bacterial contamination (Denton et al., 2015), which is critical because protozoa can degrade endogenous protein (Forsberg et al., 1984).

Asynchrony and Primary and Secondary Colonizers

The rumen microbiome has received considerable attention to optimize fiber degradation and minimize problems with ruminal acidosis (Firkins and Yu, 2015). In contrast with many studies evaluating the synchrony of nutrients for pure cultures or simple communities, the rumen is far more complex. To maintain a balanced consortium, asynchrony of carbohydrate and nitrogen sources can have a profound rippling effect through entire communities of ruminal microbes.

Physical and environmental limitations alter the degradation and usage of carbohydrate by microbes in the rumen. Degradation rates of crystalline cellulose (Weimer, 1996) by cellulases are probably limited by surface area rather than enzymatic capacity (Fields et al., 2000) and therefore are much slower than the potential growth rates by cellulolytics on the resultant cellobiose or cellodextrins (Shi and Weimer, 1997). Secondary degraders cross-feed from the degradation products produced by primary degraders of starch (Cotta, 1992), cellulose (Russell, 1985), and hemicellulose (Cotta and Whitehead, 1998). Excessive degradation of carbohydrate can decrease cellulose degradation by primary degraders; it can do so by decreasing pH or leading to depletion of growth factors (Weimer, 1996; Mouriño et al., 2001).

Role of Short-chain Fatty Acid Interconversions in Mixed Ruminal Communities

The carbon used for substrate should be reconciled with carbon recovered as products, including SCFA and cellular growth. Many SCFA interconversions and usage for anabolism allow anaerobic bacteria to fill intermediates of metabolites. Exchanges of SCFA, especially between acetate and butyrate, are to be expected (Firkins et al., 2006). Therefore, researchers have used radio or stable isotopes to support the integration of anabolic and catabolic fluxes. Indeed, an estimated 28% of [2-^{13}C]acetate infused into cattle was not recovered as absorbed acetate (Kristensen, 2001). Many of these exchanges allow microbes to reoxidize reducing equivalents and have little net effect on growth of the community. For human fecal bacteria, numerous interconversions are possible but with a major conversion of acetate to butyrate (Falony et al., 2006; Morrison et al., 2006). Unfortunately, there is much less known for ruminal bacteria.

Cycling of Acetate During Butyrate Production in Butyrivibrios

Exogenously derived acetate can be used in a cycle to produce butyrate from acetyl coA (Diez-Gonzalez et al., 1999). In that cycle (**Figure S7A**), exogenous acetate would not directly wind up in butyrate but would aid in reactions transferring coenzyme A. The butyrivibrios, which are the main characterized bacteria involved in biohydrogenation, cluster taxonomically by either high or low expression of butyrate kinase (Paillard et al., 2007). The cluster with high butyrate kinase activity (cf. **Figure S7B**) comprised the only stearate producers so far described, whereas the cluster with the lower butyrate kinase activity (cf. **Figure S7A**) could not complete biohydrogenation to stearate. That latter group expressed more butyryl coA-acetyl coA transferase (butyryl coA + acetate → butyrate + acetyl coA). Presumably, the acetyl coA would then produce acetyl-phosphate and then generate ATP as acetyl kinase yields acetate (Diez-Gonzalez et al., 1999), with the acetate then becoming available for another cycle. The two groups also can be characterized based on pyruvate flux. The group expressing butyryl kinase produces little lactate and produces more acetate than the other group. In contrast, the group expressing the butyryl coA-acetyl coA transferase enzyme increases lactate production with increasing concentration of fructose-1,6-phosphate (rapid glycolysis) but takes up considerable acetate. The increasing acetate uptake would indicate that some acetyl coA must be replenishing acetyl coA pools for butyrate production and perhaps other anabolic reactions such as fatty acid biosynthesis.

Although the butyrivibro group expressing the coA transferase was only inhibited by higher concentrations of linoleic acid, when one representative isolate was dosed with linoleic acid above the inhibition threshold, the various acyl coA pools and ATP production dramatically decreased (Maia et al., 2010). Based on that response, those authors proposed a metabolic inhibition rather than disrupted membrane function to explain the toxicity from linoleic acid. This cluster of butyrivibrios produced more lactate in pure cultures, and lactate exacerbated inhibition by linoleic acid (Paillard et al., 2007). A disrupted cycle involving acetate uptake and the butyryl coA-acetyl coA transferase reaction could help explain the depleted acetyl coA pools in the study of Maia et al. (2010).

Increased starch fermentability is well-known to shift biohydrogenation away from the trans-11 pathway used by most butyrivibrios and toward the trans-10 18:1 pathway used by as yet poorly characterized bacteria (Jenkins et al., 2008). To our knowledge, energy spilling has not received much attention with butyrivibrios. We note the commonality for fructose-1,6-bisphosphate accumulation to activate lactate dehydrogenase in both the lactate-producing butyrivibrios (Diez-Gonzalez et al., 1999) and for this stimulation of lactate production to coincide with energy spilling through proton cycling in *S. bovis* (Bond and Russell, 1998). Human gut bacteria closely related to *Butyrivibrio fibrisolvens* were projected to produce butyrate through a scheme projecting a proton motive force for subsequent ATP synthesis (Louis and Flint, 2009). A research question to be tested is whether or not the butyrivibrios (especially the stearate producers) might be inhibited by higher starch fermentability because of their inability to spill energy. In contrast, might the likely candidates to biohydrogenate linoleic acid through the trans-10 pathway (i.e., *Propionibacterium*, *Streptococcus*, and *Lactobacillus*; (Jenkins et al., 2008) be less inhibited by high starch fermentability and more equipped to spill energy.

If bioactive fatty acids accumulate enough to inhibit butyrivibrios, then methanogens also should be affected. Rather than sinking reducing equivalents into biohydrogenation, H_2 is a more favorable and important sink (Jenkins et al., 2008). To our knowledge, detailed studies with polyunsaturated fatty acids have not been done. However, medium chain fatty acids inhibit methanogens by disrupting ion gradients (Zhou et al., 2013). In that report, despite the bolus doses of these fatty acids, many methanogens were stained still active. Firkins and Yu (2015) described the poor relationship between methane production and abundance of methanogens.

De novo Synthesis of Fatty Acids

Exogenous [2-^{13}C]acetate was elongated to butyrate and a variety of longer chain fatty acids in bacterial samples (Kristensen, 2001). Notable recovery was in the odd and anteiso fatty acids needed for bacterial membranes and with minimal isotope recovery in palmitic and stearic acids. Although bacterial long chain fatty acid synthesis was suggested as a mechanism for aerobic bacteria to store excess energy (Bas et al., 2003), the main benefit might be in allowing acetate production to provide ATP in fermentation with subsequent usage of acetate in CoA transferase reactions that consume reducing equivalents while elongating fatty acids during *de novo* synthesis (Duncan et al., 2002). *De novo* fatty acids would clearly be an important sink for carbon diverted from fermentation but also for reducing equivalents derived by fermentation, so hydrogen and carbon recovery models should be reconsidering factors affecting fatty acid biosynthesis vs. fatty acid uptake.

Supplemental fat can improve efficiency of microbial protein synthesis either by inhibition of protozoal predation on bacteria

or by alleviation of the need for *de novo* fatty acid synthesis (Hanigan et al., 2013), which would allow more diversion of carbon toward ATP-generating fermentation rather than anabolism. Ruminal bacteria are thought to lack desaturase enzymes and must rely on methylated long chain fatty acids to maintain membrane fluidity (Russell, 2002). As described in that source, branched chain SCFA are elongated in biosynthetic reactions and are therefore important growth factors for many bacteria. In contrast with bacteria, ruminal protozoa can take up more dietary fatty acids and rely less on biosynthesis (Karnati et al., 2009).

Shifts in Fermentation Pathways Corresponding with Growth Rate

Fermentation of lactate produced by another microbe is an important cross-feeding mechanism to help buffer against ruminal acidosis (Nagaraja and Titgemeyer, 2007). They explained that, when substrate increases, *S. bovis* shifts fermentation toward lactate to increase ATP yield per time while decreasing ATP yield per glucose fermented. With increasing lactate production, *Selenomonas ruminantium* produces propionate via succinate, and *Megasphaera elsdenii* produces propionate via acrylate; these species are regarded as among the most important of the characterized lactate-utilizing bacteria in the rumen (Nagaraja and Titgemeyer, 2007). Exogenously supplied lactate would be labeled as propionate in different ways, depending on the pathway by those two species (Counotte et al., 1983).

Studies have elucidated regulation of lactate metabolism in some pure cultures of bacteria, and enzymatic pathways likely depend on ATP status. The intracellular ATP concentration or some similar energy gauge apparently regulates lactate metabolism in *S. ruminantium* (Asanuma and Hino, 2001). Higher ATP was proposed to allosterically activate pyruvate kinase to stimulate lactate production (which would decrease ATP yield from glucose). In contrast, phosphoenolpyruvate (PEP) carboxykinase (PEPCK; PEP → OAA) would be induced with lower ATP concentration. Using PEPCK allows GTP synthesis and routes carbon to pyruvate through succinate. With the lactate producer *S. bovis,* high ATP concentration represses pyruvate formate lyase (converting pyruvate to acetate) but induces lactate dehydrogenase to produce lactate (Asanuma and Hino, 2002). Along with allosteric activation of lactate dehydrogenase (Bond and Russell, 1996) and energy spilling (Bond and Russell, 1998), *S. bovis* can greatly increase its rate of lactate production when glucose concentration increases.

Lactate fermentation in the rumen is more complicated because lactate is produced in both D and L stereoisomers (Nagaraja and Titgemeyer, 2007). Glucose concentration can influence the fermentation pathway of these stereoisomers (Weimer and Moen, 2013). In that latter study, a strain of *M. elsdenii* fermented lactate to acetate or propionate, but when the lactate was depleted, the strain fermented glucose to butyrate and valerate. Specifically, the strain fermented glucose to acetyl-CoA, then acetate and propionate were elongated to butyrate and valerate by their condensation with acetyl-CoA. CoA transferases appear to allow metabolic versatility in *M. elsdenii* (Prabhu et al., 2012).

Shifts in metabolism are not unique to lactate producers and consumers. In *R. flavefaciens*, the primary route of OAA formation seems to be via PEPCK, but pyruvate carboxylase activity (i.e., pyruvate → OAA) increased moderately with increasing growth rates, even though OAA derived this way would decrease ATP yield compared with pyruvate through PEPCK (Shi et al., 1997). Pyruvate is the route for acetate production (which yields more ATP than succinate considering ATP production by methanogens). Pyruvate carboxylase could also produce OAA as the precursor for Asp and several other AA (**Figure S6**). Thus, catabolic fermentation to yield ATP for anabolic reactions might be better balanced with catabolic fermentative routes by fluxing glucose-carbon through pyruvate.

Exogenous carbon dioxide is used and produced in propionate production through succinate (Mountfort and Roberton, 1978). Those authors also detected label from [2-^{14}C]acetate in succinate. Shi et al. (1997) projected CO_2 conversion to formate in a scheme to help *R. flavefaciens* resolve reducing equivalents during different phases of growth as affected by substrate supply.

Thermodynamic Control of Interconversions

Ungerfeld and Kohn (2006) have elaborated on SCFA interconversions on the basis of thermodynamic principles. These principles need better integration with relative abundance of various microbes, the metabolic pathways expressed by them, and their various approaches to handling asynchronous carbohydrate supply. Those authors discussed that interconversion of SCFA is much more thermodynamically likely for acetate than the more highly reduced propionate.

Multiple approaches have been used to inhibit methanogens to suppress enteric methane emissions from ruminant livestock operations (Hristov et al., 2013). However, many of these efforts were oversimplified because of reputed interacting dietary factors such as increasing forage digestibility and ruminal passage rate (Janssen, 2010). That author presented evidence that increasing aqueous H_2 concentration would be associated with increasing methanogen growth rate but also decrease acetate production by the fermentative microbes based on thermodynamic principles. He acknowledged that the ratio of acetate to methane is not constant, but rather depends on whether H_2 or propionate is formed along with acetate to balance reducing equivalents.

Interconversions of lactate and the SCFA could potentially influence various mechanistic models being derived to associate SCFA stoichiometry and methane production. For example, whether butyrate is produced through butyryl kinase or butyryl coA-acetyl coA transferase can vary the production of the intermediate lactate. If lactate is increased, there should be less of the intermediate H_2 being produced in the first place and the lactate subsequently being fermented to propionate. If expressing butyryl coA-acetyl coA transferase does indeed have an advantage, probably through balancing reducing equivalents and ATP synthesis (Louis and Flint, 2009), then it might also allow a more efficient bacterial growth.

Conclusions

Microbial protein production is inefficient largely owing to maintenance functions, accumulation of reserve carbohydrate, and energy spilling. Reserve carbohydrate is accumulated primarily by protozoa and under even modest carbohydrate excesses, whereas energy spilling occurs under larger excesses. Future work needs to identify microbial groups and biochemical mechanisms for spilling. Interconversion of lactate and SCFA is another potential mechanism for microbes to better manage rates of catabolic and anabolic reactions or, conversely, might be associated with energy spilling.

Principles summarized in this review could improve prediction of microbial protein production by mechanistic models, guiding efforts to maximize efficiency of that production. Some models already represent energy spilling and reserve carbohydrate, but most model parameter values are simple constants or heuristic (not derived directly from data) (Dijkstra et al., 1992; Russell et al., 1992; Dijkstra, 1994; Baldwin, 1995; Hackmann and Spain, 2010). For example, most mechanistic models assume storage of reserve carbohydrate is a constant fraction of microbial biomass (Russell et al., 1992; Baldwin, 1995; Hackmann and Spain, 2010). This review suggests that in order to improve prediction of microbial protein, models need improved representation of energy spilling and reserve carbohydrate, and it also points out experimental data needed to achieve this better representation.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00465/abstract

Figure S1 | Bucket model of energy spilling. The large bucket represents the main pool of ATP-equivalents available to cell functions (maintenance, growth, reserve carbohydrate, energy spilling). The smaller bucket represents pool of ATP-equivalents in reserve carbohydrate, which can be stored from and mobilized to the main pool by pumps. Modified from Russell (2002).

Figure S2 | Dynamics of reserve carbohydrate accumulation for mixed bacteria in the rumen. Diet was fed to calves once daily. Ingredient composition of basal diet 1.15 kg/d hay and straw and 1.26 kg/d flaked maize. Crude protein content was 2.56% DM for basal diet and between 6.56 and 8.32% for basal diet supplemented with urea. Reserve carbohydrate ("α-dextran glucose") was measured as glucose detected by ion-exchange chromatography after hydrolysis in 0.5 *N* H_2SO_4 (100°C, 4 h). Figure redrawn from McAllan and Smith (1974).

Figure S3 | Dynamics of carbohydrate accumulation for the protozoan *Dasytricha ruminantium* in the rumen. Diet was fed to sheep. Ingredient composition was 0.6 kg cubed molassed sugar-beet pulp and 0.3 kg chopped hay. Beet pulp was fed at 07.00 h and chopped hay fed at 16.00 h. Carbohydrate was measured by the phenol-sulfuric acid method after hydrolysis in 1 *N* NaOH (100°C, 5 min). Data from Williams and Harfoot (1976).

Figure S4 | Equations for synthesis and degradation of glycogen.

Figure S5 | Equations for cellodextrin synthesis and efflux. Na^+ transport out of the cell is assumed to require 1/3 ATP (Russell, 2002). Cellobiose synthesis is shown, but synthesis of longer cellodextrins is also observed. After Wells et al. (1995).

Figure S6 | Amino acid (AA) biosynthesis in various bacteria, primarily *Escherichia coli*. PEP, phosphoenolpyruvate; OAA, oxaloacetate; SAM, S-adenosyl methionine; and HMB, 2-hydroxy-4-(methylthio) butanoic acid. Redrawn from Morrison and Mackie (1997), with minor modifications (Paulus and Gray, 1967; Baldwin and Allison, 1983; Or-Rashid et al., 2001; Walker et al., 2005). Numerous reactions are combined in the solid arrows representing enzymatic reactions. Examples of feedback inhibition are indicated by (–) and dashed arrows were excerpted from studies with non-rumen bacteria, again primarily *E. coli* (Gottschalk, 1979; Kalcheva et al., 1994; Hindson, 2003; Lohkamp et al., 2004; Yang et al., 2005; Caldara et al., 2008; Ferla and Patrick, 2014). Most rumen bacteria lack a complete TCA cycle but can make α ketoglutarate by forward or backward reactions from OAA (Wallace et al., 1997).

Figure S7 | Butyrate formation by two groups of ruminal *Butyrivibrio* species as proposed by Diez-Gonzalez et al. (1999) and further elaborated on by Paillard et al. (2007). (A) Butyrate formation by butyryl-CoA/acetate CoA transferase, phosphotransacetylase, and acetate kinase. **(B)** Butyrate formation by butyrate kinase. See those sources for enzymes. The group on the right expresses butyrate kinase but produces more acetate, consumes little acetate, and produces little lactate. This group fully biohydrogenates to stearate. The group on the left expresses butyryl coA-acetyl coA transferase, produces more butyrate and lactate and consumes more acetate. Dashed boxes denote carbon input, although glucose is generalized from disaccharide or other sugars entering the glycolysis pathway and even as a phosphorylated monosaccharide. ATP, adenosine triphosphate; CoA, coenzyme A; GTP, guanosine tri phosphate; OAA, oxaloacetate; P, phosphate; and PEP, phosphoenolpyruvate.

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The requirements for rumen-degradable protein per unit of fermentable organic matter differ between fibrous feed sources

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Ruminant feed evaluation systems use constant minimum requirements of rumen-degradable protein (RDP) and often relate this to apparently degradable organic matter (OM). However, studies with tropical forages indicate that RDP: apparently degraded OM might not be constant across high-fiber diets. This was tested with semi-continuous ruminal cultures (Rusitec) using dried contrasting low-protein fiber sources: brachiaria hay (high in fiber, medium lignified), apple pomace (medium in fiber, highly lignified), and sugar beet pulp (medium in fiber and lignification). Each feed was incubated at 14 g dry matter day^{-1} with 0, 0.85, 1.7, 3.4, 6.8, 13.6, or 27.2 mg g^{-1} urea. The amount of urea needed to reach a similar basal concentration of ammonia in the incubation fluid was tested for each feed in advance. Apparent fiber and OM degradability were determined after 48 h of incubation. Data was evaluated by regressions and analysis of variance. The response curve of incubation fluid ammonia to urea supplementation was similar in slope in all feeds. Plateaus in apparent OM degradability in relation to ammonia concentration were determined. The ammonia concentration where apparent OM and fiber degradability reached 95% of maximum was approached in the order of pomace < pulp < hay. With regard to fiber degradability, a plateau was reached at ≥ 80 g kg^{-1} crude protein only with hay and pomace, whilst a linear relationship existed between RDP and OM degradation for pulp. In hay the ratio RDP: OM degraded was equal to 1.6 but was only 1.0 in the other feeds. There was no obvious lack of branched short-chain fatty acids at low RDP. Thus, the hypothesis was confirmed but the demand for RDP seems even higher in tropical forage compared to food industrial byproducts. The efficiency of urea to promote apparent OM and fiber degradation was also variable. Thus, it seems that minimum thresholds of either RDP or ruminal ammonia concentration may not be reflected appropriately by constants.

Keywords: ammonia, *brachiaria*, sugar beet pulp, apple pomace, nitrogen

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Abbreviations: ADF, acid detergent fiber; ADL, acid detergent lignin; CP, crude protein; NDF, neutral detergent fiber; OM, organic matter; Rusitec, Rumen simulation technique; RDP, rumen-degradable protein; SCFA, short-chain fatty acids.

Introduction

Various tropical grass species and harvest residues are very low in crude protein (CP) and RDP (e.g., Mathis et al., 2000; Schwab et al., 2005). Especially during the dry season, tropical forages have CP values lower than 70 g kg^{-1} dry matter (DM), which is considered limiting for the maintenance of an adequate ruminal fibrolytic activity (Lazzarini et al., 2009; Sampaio et al., 2009). Such sub-optimal ruminal conditions would be associated with low microbial growth and reduced fiber degradation (Sampaio et al., 2010). Increasing the dietary CP concentration close to 100 g kg^{-1} of DM was found by Sampaio et al. (2010) to optimize the use of low-quality tropical forage; ruminal ammonia N concentrations of 3.6–7.2 mmol l^{-1} were therefore considered supplying enough nitrogen to maintain the microbial activity in the rumen. In general, the high importance of RDP as nutrient for fiber degrading microbes in the rumen is known, and most feed evaluation systems for ruminants specify a minimum threshold for dietary RDP (60–80 g kg^{-1} DM) or CP (ranging from >120 to 130 to >160 to 180 g kg^{-1} DM) (reviewed by Schwab et al., 2005). Another approach for determining requirements for RDP is oriented toward minimum rumen fluid ammonia concentrations. Here the recommended concentrations vary extremely, namely between >1.2 (Marini and Van Amburgh, 2003) and >16 mmol l^{-1} (Mehrez et al., 1977). Even when aligning this with the low CP concentrations typical for tropical forages, such forages still were often found to be sufficient to cover at least maintenance requirements of livestock for nitrogen. Consistent with this, feeding tallgrass-prairie hay to cows, containing only 19 g CP kg^{-1} DM, still yielded a digestibility of the neutral detergent fiber (NDF) of 47%, and digestibility was only increased to on average 55% with additional supplemented RDP ranging from 180 to 720 g DM d^{-1} compared to the not RDP-supplemented treatment (Köster et al., 1996). Also, an acceptable NDF digestibility of 59% was found for sheep consuming only brachiaria hay, a wide-spread cultivated tropical grass, with 61 g CP kg^{-1} DM; this, however, at a ruminal ammonia concentration of 3.9 mmol l^{-1} (Tiemann et al., 2008b). Also the fiber of hay prepared from *Brachiaria* containing <40 g CP kg^{-1} DM, was found to be reasonably well fermented in the Rumen Simulation Technique (Rusitec) despite extremely low ammonia concentrations of 0.3–0.6 mmol l^{-1} found in the incubation fluid (Hess et al., 2003; Tiemann et al., 2008a).

Overall, these findings might suggest that less RDP is required to ferment OM and fiber of low-CP (tropical) forages although, based on theoretical considerations, the ratio of RDP: apparently degraded OM needed by the ruminal microbes should be rather static across fibrous feeds sources (Bach et al., 2005). This would imply that RDP requirements might also differ among feeds with similar digestibility, an assumption that goes beyond the known phenomenon that the required concentrations of either ruminal ammonia or RDP differ for feeds with varying digestibility only (e.g., Erdmann et al., 1986; Odle and Schaefer, 1987). Also, the NRC (2001) states, that the requirement for RDP depends on the fermentability of the basal diet. When slowly fermentable roughages are fed, reduced amounts of RDP would be required, whereas when excessive amounts of degradable protein is fed, N losses from the rumen and decreased capacity to recycle N will likely occur (Siddons et al., 1985). A clear indication that the RDP: apparently degraded OM ratio, i.e., the requirement for RDP, could vary among low-CP forages was described by Mathis et al. (2000) comparing the results of three experiments with steers each carried out with different low-CP forages. These forages included Bermuda grass (CP 82 g kg^{-1} DM; RDP 48 g kg^{-1} DM), bromegrass (CP 59 g kg^{-1} DM, RDP 29 g kg^{-1} DM), and forage sorghum (CP 43 g kg^{-1} DM, RDP 25 g kg^{-1} DM). In that study, the proportion of RDP in digestible OM varied for each of the forages between 8.2 and 16.2% for Bermuda grass and from 5.5 and 6.0 to 12.4 and 12.8% for bromegrass and forage sorghum, respectively. Mathis et al. (2000) did not specify the degree of lignification of the forages, and differences were explained by the authors as being an effect of animal-individual concentrations of N recycling within the animals. Still, other factors might be equally important like the cellulose-to-hemicellulose ratio (Griswold et al., 2003) or other fiber properties.

The hypotheses to be tested in the present *in vitro* study were that (i) the requirements for RDP per unit of degradable OM are not static across forages differing in fiber concentration and degree of lignification, and that (ii) differences in RDP requirements per unit of OM degraded are not only the result of variations in N recycling via saliva. For that purpose, the continuously operating Rusitec was employed, a technique excluding the influence of postruminal digestion of OM and fiber as well as of N recycling. Fiber sources were selected which guaranteed a large variation in fiber composition and that were low in crude protein (RDP) to be able to generate low-RDP treatments. In order to obtain information about the status of supply of the ruminal microbes with branched-chain carbon sources under different RDP supply, special attention was paid to the concentration of branched short-chain fatty acids (SCFA) in incubation fluid.

Materials and Methods

Experimental Feeds

Three low-protein fibrous feeds, largely differing in composition of cell wall constituents, were selected for the present study: hay from a tropical grass species (*Brachiaria brizantha* cv. Toledo; CIAT accession no. 26110, Cali, Colombia; high in fiber, medium lignified), obtained from the same batch of hay used in a previous experiment with sheep (Tiemann et al., 2008b), and two dried food industrial by-products, apple pomace (medium in fiber, highly lignified), and sugar beet pulp (medium in fiber and lignification). The analyzed nutrient composition (**Table 1**) slightly differed from that reported for the pooled brachiaria samples across the entire sheep experiment (Tiemann et al., 2008b).

In Vitro Technique

For the present study, an eight-fermenter Rusitec was used. The construction and operation of this equipment is described in detail in Soliva and Hess (2007). The daily portions (14 g of DM) of the experimental feeds, ground to pass a 5-mm sieve, were

TABLE 1 | Analyzed nutrient composition and fermentation characteristics (average of incubation days 6 through 10) of the three experimental feeds ($n = 7$; i.e., across all urea supplementations)[1].

	Brachiaria hay	Apple pomace	Beet pulp	SEM[2]	*P*-value
NUTRIENTS [g kg^{-1} DRY MATTER (DM)]					
DM (g kg^{-1} feed as used)	924	913	911		
Organic matter (OM)	920	989	945		
Crude protein	69	52	91		
Neutral detergent fiber (NDF)	669	474	430		
Acid detergent fiber (ADF)	402	333	234		
Acid detergent lignin (ADL)	55	167	77		
Hemicellulose (NDF-ADF)	267	141	196		
Cellulose (ADF-ADL)	347	166	157		
INCUBATION FLUID PH AND MICROBIAL COUNTS					
Incubation fluid pH	6.85^{a}	6.86^{a}	6.54^{b}	0.043	<0.001
Total bacteria (10^{8} ml^{-1})	2.00^{a}	1.13^{b}	1.98^{a}	0.095	<0.001
Total protozoa (10^{3} ml^{-1})	1.05^{b}	3.05^{a}	0.91^{b}	0.413	<0.001
SHORT-CHAIN FATTY ACIDS					
Total SCFA (mmol day^{-1})	38.5^{ab}	30.7^{b}	48.8^{a}	4.03	0.001
Acetate (%)	65.2^{a}	59.2^{b}	63.2^{a}	1.28	<0.001
Propionate (%)	23.5	22.4	22.8	0.81	0.611
n-butyrate (%)	10.0^{b}	16.2^{a}	11.8^{b}	1.27	<0.001
iso-butyrate (%)	0.283^{ab}	0.520^{a}	0.203^{b}	0.0922	0.022
n-valerate (%)	0.71^{b}	1.23^{b}	1.87^{a}	0.248	<0.001
iso-valerate (%)	0.263^{ab}	0.484^{a}	0.179^{b}	0.0843	0.014
Acetate: propionate ratio	2.79	2.68	2.79	0.337	0.782
APPARENT DEGRADABILITY (%)					
OM	40.9^{b}	35.6^{b}	55.8^{a}	2.12	<0.001
NDF	36.0^{b}	19.3^{c}	47.6^{a}	2.31	<0.001
ADF	32.4^{a}	5.3^{b}	38.3^{a}	2.41	<0.001
RUMEN-DEGRADABLE PROTEIN (RDP) REQUIRED (g g^{-1} FERMENTED NUTRIENTS)					
OM	1.63^{a}	1.08^{b}	1.00^{b}	0.308	<0.001
NDF	1.87^{a}	2.15^{a}	1.18^{b}	0.493	<0.001
ADF	2.09	5.01	1.49	3.954	0.173
METHANE					
ml day^{-1}	127	110	197	21.0	0.055
ml g^{-1} degraded OM	24.6	23.4	26.5	3.00	0.906
ml g^{-1} degraded NDF	33.3^{b}	71.3^{a}	58.6^{ab}	7.17	0.013

[1] *Not including treatment with lowest urea supplementation, i.e., 0.42 g kg^{-1} dietary DM, with apple pomace. Means within the same row carrying no common superscript differ at $P < 0.05$.*

[2] *SEM, standard error of the mean.*

put into nylon bags (70 × 140 mm) with a pore size of 100 μm. Urea was continuously infused into the fermenters after being mixed with the respective buffer solution following the approach of Griswold et al. (2003). Individual buffer storage canisters were used for each fermenter, but buffer flow rate to the fermenters was kept constant at 435 ± 23 ml day^{-1} for all fermenters. The rumen fluid originated from a non-lactating rumen-cannulated Brown Swiss cow that received temperate climate grass hay at *ad libitum* access and about 1 kg d^{-1} of concentrate. The cow was treated in accordance to the Swiss guidelines for animal welfare. For each experimental run, incubations lasted for 10 days with day 6 through day 10 being used for data evaluation. Following this adaptation scheme also ensured that incubation fluid was depleted of the nitrogen compounds initially introduced into the system through the rumen fluid.

Preliminary Assessments

The N source of choice for the present study was urea, as it is completely degradable. However, low-quality highly fibrous roughages may be limited with respect to ruminal fermentation in more than only a lack of degradable N. Therefore, two 10-day preliminary experimental assessments using Rusitec were carried out prior to the main experiment. The first preliminary assessment was carried out to evaluate whether or not fiber degradation by the ruminal microbes in Rusitec is improved by the supplementation of urea and specific microbial growth factors. In addition, changes in incubation fluid ammonia concentration due to urea supplementation were recorded. In the second preliminary assessment the change in incubation fluid ammonia concentration in response to urea supplementation was recorded when incubating the three experimental feeds selected

(brachiaria hay, apple pomace, and beet pulp). This was done to be able to calculate the urea amounts required for balancing differences in ammonia concentration between feeds.

In the first assessment, five treatments tested in one experimental run ($n = 1$), all based on 15 g DM day^{-1} of brachiaria hay but differing in the supplementation of urea and microbial growth factor solution (GF), were tested with Rusitec by providing per g hay DM: (1) zero supplementation; (2) 0.13 ml GF; (3, 4) 4 mg urea, without or with 0.13 ml GF; and (5) 30 mg urea + 0.13 ml GF. The growth factor solution was composed of (μg ml^{-1}) acetate, 1330; iso-butyrate, 66; iso-valerate, 80; valerate, 80; pyridoxine, 2; thiamine, 2; biotin, 0.05; 4-aminobenzoic acid, 0.1 (as described by Scott and Dehority, 1965). The resulting change in the extent of NDF degradability within 48 h of incubation occurring with increasing urea supplementation was as expected (23.9, 27.7, and 36.2% with urea supplementations at 0, 4, and 30 mg g^{-1} feed DM). The addition of the growth factor solution when provided without (23.9 vs. 20.9%, treatment 1 vs. 2) or together with the low urea supplementation (27.6 vs. 27.6%, treatment 3 vs. 4) did not noticeably modify NDF degradability. These findings suggested that, under the present experimental conditions, the rumen fluid used was sufficiently rich in potentially limiting microbial growth factors other than RDP, and no addition of the growth factor solution was required to allow that effects of RDP were fully expressed. Potential limitations given by omitting the addition of preformed, mostly branched-chain AA (Owens and Bergen, 1983) were not tested.

The second assessment, based on the results of the first preliminary assessment and previous, unpublished data, aimed at achieving the same target incubation fluid ammonia concentration of 1.22 mmol l^{-1} for all three feeds (i.e., the basal concentration found with beet pulp), 19.52 mmol l^{-1} for beet pulp and apple pomace (as the concentration approximating the highest one aimed at in the main experiment), and additionally 1.84 mmol l^{-1} for beet pulp (reflecting an intermediate target). The latter two aimed to match the differing recommended minimum rumen fluid ammonia concentrations of >1.2 and >16 mmol l^{-1} specified by Marini and Van Amburgh (2003) and Mehrez et al. (1977), respectively. Without supplementation, the ammonia concentrations assumed from previous, unpublished incubation data were 0.83 and 0.03 mmol l^{-1} for brachiaria hay and apple pomace, respectively. Each of the treatments was tested in the same experimental run ($n = 1$). For the first 5 days of incubation, amounts of urea supplementation were fixed in advance with the goal to add urea in amounts necessary to balance for the differences in ammonia concentrations of the non-supplemented fiber sources. This first attempt did not result in a fully balanced ammonia concentration. Therefore, fine-tuning was performed for the second half of the 10-day incubation period. Apart from the zero control without urea, this finally meant urea supplementations (g kg^{-1} feed DM) of 0.6 for brachiaria hay, 1.3 and 39.0 for beet pulp, and 2.6 and 41.5 for apple pomace. Although the maximum target ammonia concentrations had been approximately reached on average (e.g., maximum values of 18 and 21 mmol ammonia l^{-1} with the high amount of urea in apple pomace and beet pulp, respectively), it was not possible to generate ammonia concentrations being stable across days of incubation. Therefore, for the main experiment it was decided to use the same fixed urea concentrations for strategic increase of incubation fluid ammonia concentrations for all three feeds. Additionally it was considered sufficient to balance only between the feed with the lowest CP concentration, apple pomace, and the other two feeds for the low level control treatments since the latter two feeds had yielded quite similar ammonia concentrations without extra urea (0.9 and 0.7 mmol l^{-1} in brachiaria hay and beet pulp, respectively) even though beet pulp had a higher CP concentration as brachiaria hay.

Main Experiment

The fibrous feeds were tested together with various amounts of urea supplementation. These treatments were randomly allocated to the eight fermenters in three experimental runs ($n = 1$). Urea supplementations chosen were always doubling the previous value with 0.85, 1.7, 3.4, 6.8, 13.6, and 27.2 g kg^{-1} dietary DM. The latter was approximately equivalent to the maximum amount still considered safe, because an urea supplementation level as high as 25 g kg^{-1} DM has been shown previously to be without adverse effects on cattle performance (Duff et al., 2003). Additionally, all feeds were incubated without urea supplementation and an additional urea supplementation was tested with apple pomace at a level of 0.42 g kg^{-1} DM following the decisions made from the results of the second preliminary assessment. Each treatment was tested once in order to generate a regression type of experimental design. The zero urea supplementation treatment with brachiaria hay, however, was repeated in each of the three experimental runs and reflected the control. These data were used to adjust all data across runs based on the calculated proportionate deviations of the control data obtained in the first two runs from the third experimental run. These adjustments mostly did not diverge from the unadjusted values by more than 2–4%.

Laboratory Analysis

Incubation fluid samples were analyzed daily for redox potential (for confirmation of the presence of anaerobic conditions), pH and ammonia concentration using the respective electrodes connected to a pH meter (model 634; Metrohm AG, Herisau, Switzerland). Counts of ciliate protozoa and total bacteria in the incubation fluid were determined as described by Soliva and Hess (2007) daily from experimental days 6–10 by using 0.1- and 0.02-mm depth Bürker counting chambers (Blau Brand, Wertheim, Germany), respectively. Prior to counting, microbes were fixed by the addition of 0.1 ml/ml (protozoa) and 0.99 ml ml^{-1} (bacteria) of Hayem solution (mg ml^{-1}, $HgCl_2$, 2.5; Na_2SO_4, 25.0; NaCl, 5.0). Incubation fluid samples were centrifuged for 5 min at 4000 g (Varifuge® K, Heraeus, Osterode, Germany) and the supernatant was stored at −20°C before being analyzed for the concentration of SCFA using HPLC (System Hitachi Lachrom, Merck, Tokyo, Japan) following the procedure of Ehrlich et al. (1981). After removal from the fermenters, the nylon bags with residues were washed in cold water in a washing machine to remove nitrogen containing particles

originating from ruminal microbes and frozen at −20°C. Later they were lyophilized, weighed, and ground to pass a 1-mm sieve. Feeds and fermentation residues were analyzed following standard protocols (AOAC, 1997). They were analyzed for DM and total ash (TGA-500, Leco Corporation, St. Joseph, Michigan, USA), and N (C/N analyzer, Leco-Analysator Typ FP-2000, Leco Instrumente GmBH, Kirchheim, Germany; with CP defined as 6·25 × N). Further, NDF, acid detergent fiber (ADF), and acid detergent lignin (ADL) (Tecator Fibertec System M, 1020 Hot Extraction, Höganäs, Sweden) were analyzed, with NDF being determined with α-amylase addition but without sodium sulfite as recommended by Van Soest et al. (1991). All fiber values were corrected for residual ash. The fermentation gases were analyzed daily for concentrations of methane with a gas chromatograph (model 5890 Series II, Hewlett Packard, Avondale, PA, USA) equipped with an FID and WLD detector using a 2.34 m × 2.3 mm column (80/100 mesh, Porapak Q, Fluka Chemie AG, Buchs, Switzerland). For more details see Soliva and Hess (2007).

Calculations and Statistical Analysis

The apparent *in vitro* nutrient degradability was estimated from the disappearance of nutrients from the nylon bags during 48 h of incubation. Incubation fluid N turnover was calculated from the N disappearance rates and the daily amounts of ammonia produced. Four N fractions were computed including (i) N recovered as ammonia, (ii) N present in compounds apparently not degraded (representing the rumen-undegraded protein), (iii) RDP calculated as proportion of feed CP not recovered in the fermentation residues (i.e., apparently degraded CP), and (iv) N from compounds apparently degraded (i.e., disappeared from the fermentation residue) but not recovered as ammonia N. The latter fraction was calculated as total feed-N (i.e., inclusive of urea) degraded minus ammonia-N. Assuming that (at low CP supply) RDP is limiting OM degradation, the amount of RDP found with a distinct amount of OM degraded (i.e., ratio between these two variables) was considered to be equivalent to the RDP amount required to achieve this level of degradation.

Data of the main experiment was subjected to regression analysis in SigmaPlot™ (version 12 for Windows, Systat Software GmbH, Germany). Maximizing adjusted R^2 was the criterion used to select either linear or non-linear regression models while unadjusted R^2-values are presented for the selected models in the figures. The equations are characterized in addition with standard errors (SE) and significance for individual coefficients and the overall equation. Significance of difference between the regression coefficients among the three fiber sources was determined using the Proc Reg Data Model described by the Institute for Digital Research and Education (2015) using SAS (version 9.2 for Windows, SAS Institute, Cary, USA). Thereby, all regression coefficients of first order term were compared as a whole to test the null hypothesis: fiber source 1 = fiber source 2 = fiber source 3. A 95% confidence interval was chosen. In case there was a statistical significance, this meant that the null hypothesis for this relationship could be rejected, i.e., the respective trait investigated in the fiber sources (y) did not respond in the same way to the effect (x). In order to further characterize the fiber sources, data averaged across all urea treatments was also evaluated by analysis of variance (procedure GLM) with SAS, where fiber sources, i.e., brachiaria hay, apple pomace, and beet pulp, were considered as the only source of variation. In this context, the N-related variables were not analyzed, as they were directly dependent on urea supplementation. Means were compared with Tukey's method. For all statistical evaluations the level of statistical significance was set to $P < 0.05$.

Results

The three test feeds differed in fiber properties as intended, with a high fiber concentration found in the brachiaria hay, a high lignification in the apple pomace and medium values for fiber and lignification in the beet pulp (**Table 1**). The hemicellulose-to-cellulose ratios were 0.77, 0.88, and 1.25 for brachiaria hay, apple pomace and beet pulp, respectively. Beet pulp had the highest CP concentration, followed by brachiaria hay and apple pomace. On average across all urea concentrations, incubation fluid pH was lowest ($P < 0.05$) with beet pulp. Urea supplementation did not affect incubation fluid pH ($P = 0.92$; data not shown). Apple pomace resulted in the lowest bacteria and the highest protozoa counts ($P < 0.05$). Total SCFA were higher with beet pulp than with apple pomace ($P = 0.001$). Fiber source affected proportions of all individual SCFA except propionate, with apple pomace being different (lower in acetate and higher in most other SCFA proportions) from the two other fiber sources. The OM, NDF, and ADF from apple pomace had the lowest ($P < 0.05$) apparent degradability followed by the brachiaria hay. Brachiaria hay did not significantly differ in OM degradability from apple pomace, and, in case of OM and ADF degradability, from beet pulp. The RDP amount required per unit of OM apparently degraded was highest ($P < 0.05$) with the brachiaria hay with values being similar with the two other feeds. The ratio of RDP-to-fermented NDF was higher with apple pomace and brachiaria hay than with beet pulp, while RDP-to-fermented ADF did not significantly differ between feeds. Absolute methane formation showed a trend to be highest ($P = 0.055$) with beet pulp. Methane per unit of OM degraded did not differ among feeds. When methane formation was related to units of NDF degraded, methane was highest with apple pomace, lowest with brachiaria hay ($P < 0.05$), and intermediate with beet pulp.

The fiber sources responded differently ($P < 0.001$) in incubation fluid ammonia concentration to increasing urea supplementation. The slope of development appeared similar in all three feeds, but with apple pomace this seemed to happen at a generally lower level at any urea supplementation level (**Figure 1A**). These differences were less obvious when relating incubation fluid ammonia concentration to dietary CP (**Figure 1B**), but the response still differed ($P < 0.001$) across fiber sources. The percentage of dietary CP apparently degraded increased with increasing urea supplementation and eventually approached a plateau at around 80% CP degradability (**Figure 1C**).

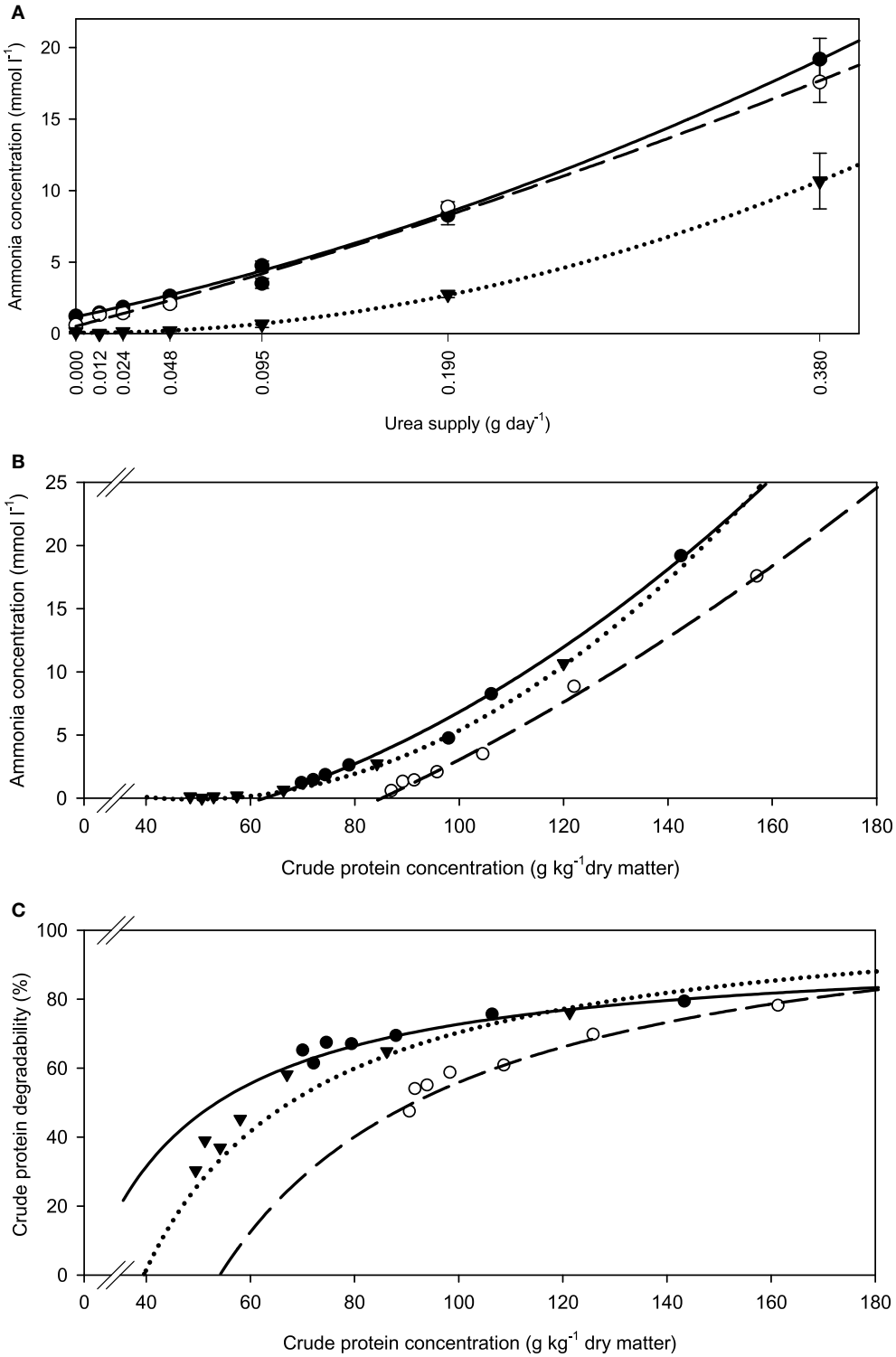

FIGURE 1 | Relationship between (A) incubation fluid ammonia concentration and urea supply, (B) incubation fluid ammonia concentration and dietary crude protein concentration, and (C) crude protein degradability and dietary crude protein concentration. • ▬ brachiaria hay, for **(A)** $y = 1.18$ (±0.134; $P < 0.001$) + 29.4 (±2.47; $P < 0.001$) x + 47.1 (±6.35; $P = 0.0018$) x^2, $R^2 = 1.00$, $SE = 0.217$, $P < 0.001$, **(B)** $y = 4.58$ (±4.56; $P = 0.372$) −0.191 (±0.0922; $P = 0.107$) x +0.00206 (±0.00038; $P = 0.0092$) x^2, $R^2 = 0.994$, $SE = 0.599$, $P < 0.001$, **(C)** $y = 21.4$ (±16.9; $P = 0.2733$) + 0.793 (±0.340; $P = 0.0799$) x −0.00270 (±0.00159; $P = 0.1651$) x^2, $R^2 = 0.928$, $SE = 2.03$, $P = 0.055$; ◦ ▬ ▬ beet pulp, for **(A)** $y = 0.530$ (±0.307; $P = 0.1595$) + 36.5 (±5.66 x; $P = 0.0030$) + 22.9 (±14.6; $P = 0.1911$) x^2, $R^2 = 0.996$, $SE = 0.498$, $P < 0.001$, **(B)** y = −11.5 (±6.06; $P = 0.130$) + 0.0801 (±0.1044; $P = 0.486$)

(Continued)

FIGURE 1 | Continued
x + 0.000676 (±0.00043; $P = 0.190$) x^2, $R^2 = 0.996$, $SE = 0.498$, $P < 0.001$, **(C)** $y = -43.1$ (±32.9; $P = 0.261$) + 1.42 (±0.551; $P = 0.0614$) x − 0.00421 (±0.00223; $P = 0.132$) x^2, $R^2 = 0.956$, $SE = 2.67$, $P = 0.002$; ▼ ..... apple pomace, for **(A)** $y = 0.066$ (±0.036; $P = 0.145$) − 0.253 (±0.667; $P = 0.724$) x + 74.2 (±1.73; $P < 0.001$) x^2, $R^2 = 1.00$, $SE = 0.0592$, $P < 0.001$, **(B)** y = 5.04 (±0.307; $P < 0.001$) − 0.204 (±0.0081; $P < 0.001$) x + 0.00209 (±0.000048; $P < 0.001$) x^2, $R^2 = 1.00$, $SE = 0.0585$, $P < 0.001$, **(C)** $y = -57.6$ (±21.0; $P = 0.0514$) + 2.36 (±0.548; $P = 0.0126$) x − 0.0104 (±0.0032; $P = 0.0319$) x^2, $R^2 = 0.963$, $SE = 3.92$, $P = 0.0013$. The significance of the difference of response across fiber sources was $P = 0.001$, $P < 0.001$, and $P = 0.167$ for **(A–C)**, respectively.

When relating apparently degraded OM to incubation fluid ammonia, curves with mostly pronounced plateaus were found irrespective of the type of regression equation used (**Figure 2**) and the response did not differ across fiber sources. This was different for degraded NDF and ADF in relation to incubation fluid ammonia with trends ($P = 0.099$ and $P = 0.064$, respectively) to differ across the three feeds (**Figures 3**, **4**). Clear but largely differing plateaus in NDF and ADF degradability developed with increasing amounts of urea as had also been the case for OM degradability. The ammonia concentrations where 95% of the maximum was reached ranged between 0.75 and 2.19, 1.73, and 2.54, as well as 0.89 and 3.48 mmol l^{-1} for OM, NDF, and ADF degradability.

A relatively clear plateau in apparent OM, NDF, and ADF degradability was found with brachiaria hay and apple pomace from dietary CP concentrations of about 80 g kg^{-1} DM onwards, whereas no such plateau was found with beet pulp (**Figure 5**). In all relationships, there was a trend ($P < 0.10$) for a difference in response across fiber sources.

Along with increasing OM, NDF, and ADF degradability, the amount of rumen-degradable protein (in dietary CP) was increased for apple pomace and beet pulp (**Figure 6**). In the brachiaria hay no such relationship was apparent. Accordingly, the responses differed ($P < 0.05$) across fiber sources for all groups of nutrients fermented. There was a clear dependence of the amount of RDP required for increasing amounts of OM fermented, but the response across fiber sources differed ($P < 0.001$, **Figure 7**).

When relating the concentration of the two branched SCFA to incubation fluid ammonia concentration, there was a curvilinear response with beet pulp and, less clearly, with brachiaria hay where elevated levels occurred at low (especially with *iso*-butyrate) and at high ammonia concentrations (**Figure 8**). The responses differed ($P < 0.05$) across fiber sources in both SCFA.

Discussion

Composition of the Experimental Feeds

The differences in fiber concentration and degree of lignification among the three feeds were realized as intended. This affected the apparent degradability of OM, NDF, and ADF across all urea treatments with the expected increases from apple pomace to brachiaria hay to beet pulp. The two extremes in this respect, which were represented by apple pomace and beet pulp, were still in the range of the known variation in OM and fiber digestibility for fibrous feeds (DLG, 1997). The CP concentrations of the test batches of apple pomace and beet pulp used were slightly lower than those listed by DLG (1997) and NRC (2001). The apple pomace contained less NDF and ADF at quite similar ADL concentration compared to average values given in NRC (2001). In beet pulp, concentrations of NDF and ADF were as expected, but ADL concentrations was unexpectedly high (77 vs. 16 g kg^{-1} DM as given in NRC, 2001). The extensive fermentation of the beet pulp explains the comparably lower incubation fluid pH and the high SCFA and methane formation. The high protozoa count and the low bacteria count found with apple pomace in comparison to the other feeds might have resulted from its typically substantial residual sugar concentration (Hindrichsen et al., 2004). This fiber source also led to a deviating SCFA profile with a shift from acetate to butyrate (and valerate; inclusive of the branched forms of these SCFA) as is expected from the action of sugar-fermenting microbes. The *Brachiaria* species used (*B. brizantha*) was richer in CP and lower in NDF than other *Brachiaria* species (e.g., *B. humidicola,* also called *B. dictyoneura*; with CP concentrations of <40 g kg^{-1}; Hess et al., 2003; Tiemann et al., 2008a), while ADF and ADL concentrations were comparable to those of other *Brachiaria* species.

Potential Limitations in Organic Matter and Fiber Degradation

The results from the first preliminary assessment demonstrate that the known rumen microbial growth factors were present in the rumen fluid in sufficient amounts to cover the microbial needs for the entire 10 days of incubation. Russell et al. (1992) implicated that the only N source required by microbes to ferment structural carbohydrates is ammonia. However, stimulation of ruminal microbiota by preformed amino acids (AA), especially branched-chain AA and SCFA (Gorosito et al., 1985), has been shown. This is especially true for the cellulolytics, as was shown for three main species, namely *Fibrobacter succinogenes*, *Ruminococcus albus,* and *R. flavefaciens*, by Atasoglu et al. (2001). These branched carbon sources can be provided via rumen-degradable protein, peptides or AA (e.g., Newbold, 1999; Ranilla et al., 2001). Nevertheless, no pre-formed AA or other such stimulants were supplemented in the present main experiment. One reason was that urea would be the least expensive supplementation strategy available to economically disadvantaged tropical farmers and is, therefore, more likely adopted, e.g., in the form of urea-molasses licking blocks or urea-treated straw and hay. Besides this, effects of urea and preformed AA would have inseparably contributed to RDP supply, and it seems that a deficiency of AA, if any, would uncouple carbohydrate degradation from microbial protein synthesis which then would be noticeably impaired (reviewed by Owens and Bergen, 1983). Griswold et al. (2003) noted that a lack

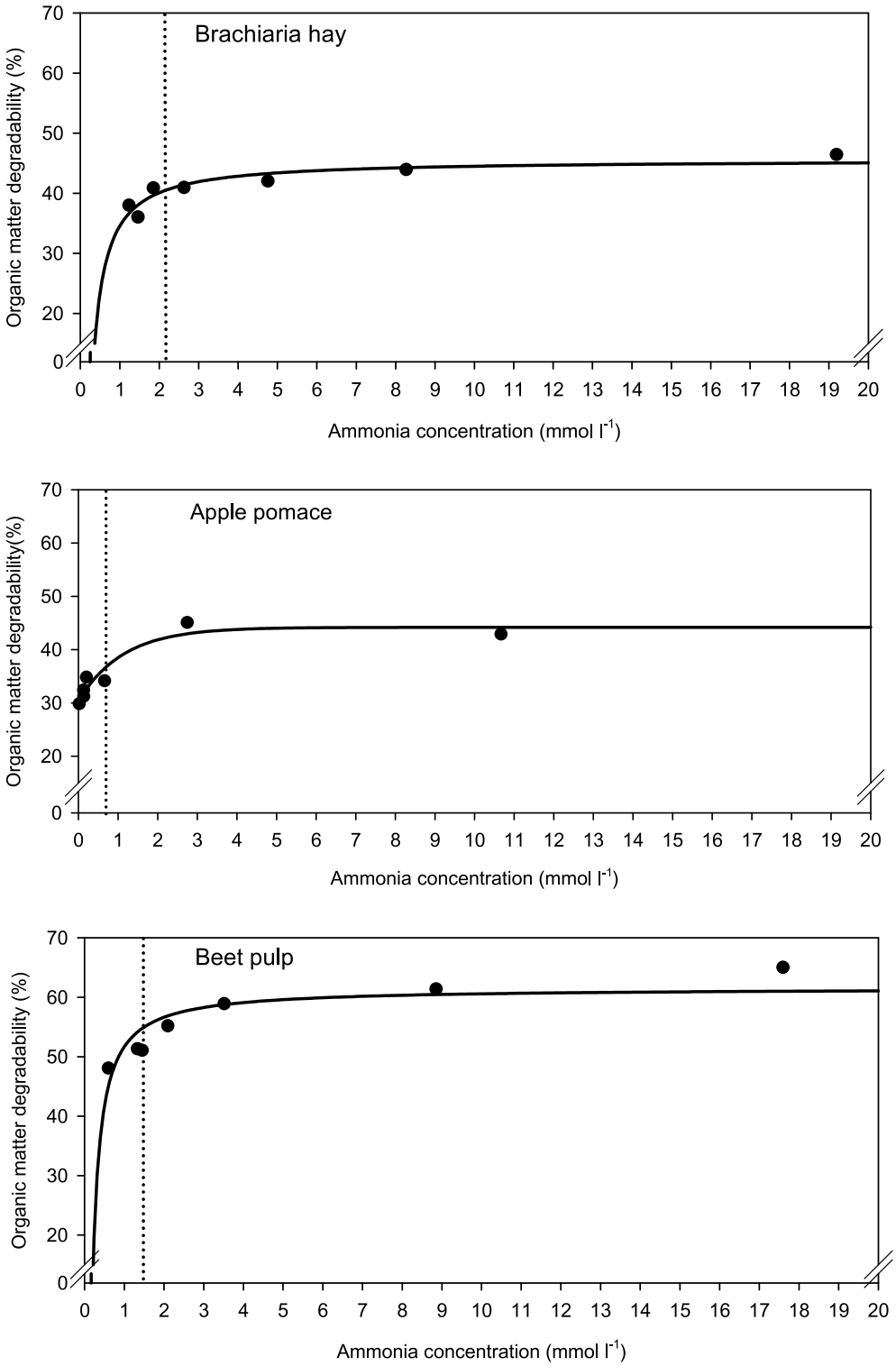

FIGURE 2 | Relationship between apparent organic matter degradability and incubation fluid ammonia concentration. The intersection between the vertical dotted line and the x-axis represents the ammonia concentration at which 95% of the calculated maximal degradability was achieved. For brachiaria hay: $y = 45.6$ (±1.02; $P < 0.001$) − 11.0 (±2.11; $P = 0.0034$) x^{-1}, $R^2 = 0.846$, $SE = 1.50$, $P = 0.0034$; for apple pomace: $y = 30.4$ (±1.46; $P < 0.001$) + 13.8 (±2.12; $P = 0.0029$) [1 − 0.411 (±0.202; $P = 0.1118$)^x], $R^2 = 0.915$, $SE = 2.11$, $P = 0.0073$; for beet pulp: $y = 61.6$ (±1.82; $P < 0.001$) − 9.86 (±2.35; $P = 0.0085$) x^{-1}, $R^2 = 0.787$, $SE = 3.18$, $P < 0.001$. The significance of the difference of response across fiber sources was $P = 0.159$.

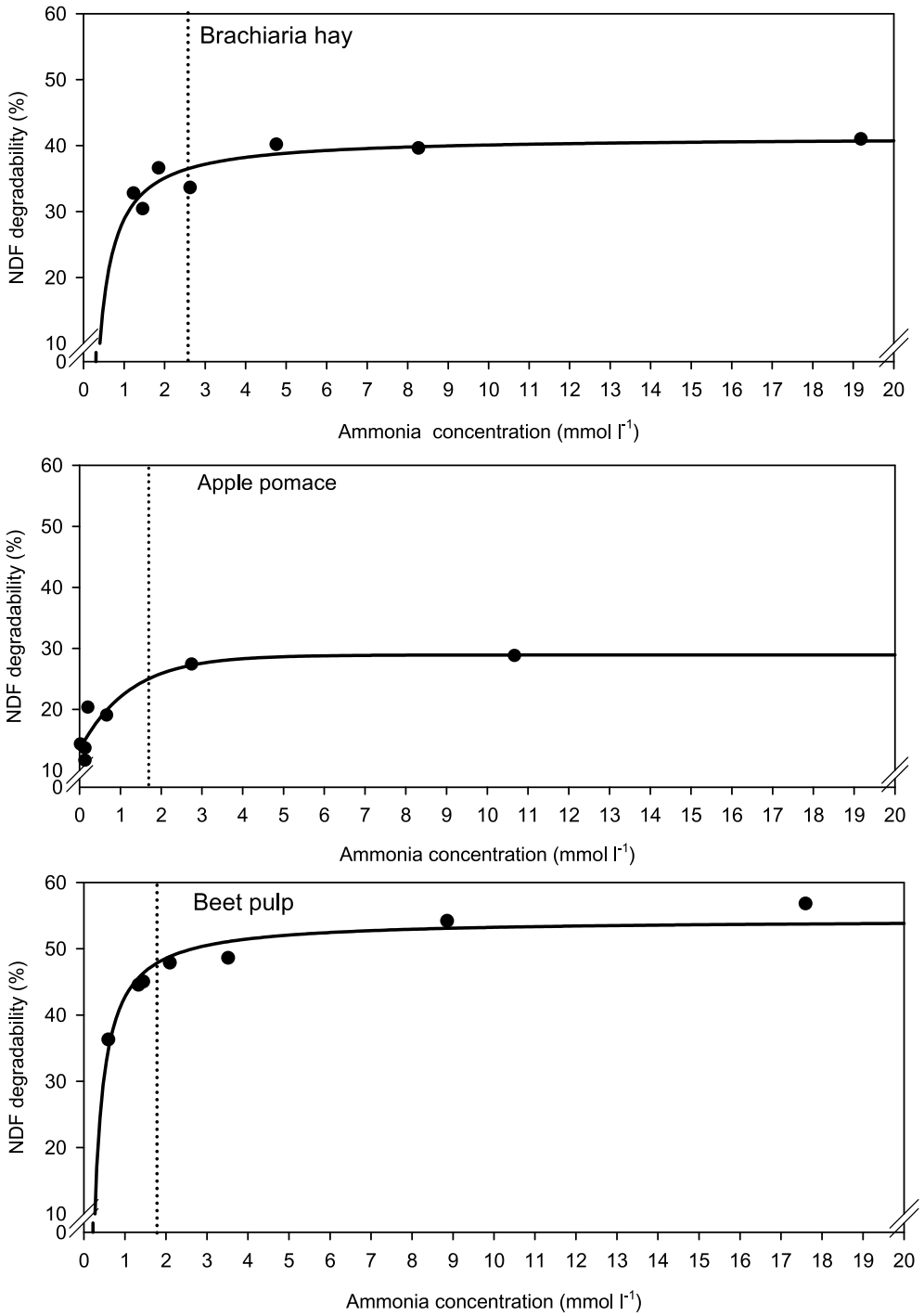

FIGURE 3 | Relationship between apparent neutral detergent fiber (NDF) degradability and incubation fluid ammonia concentration. The intersection between the vertical dotted line and the x-axis represents the ammonia concentration at which 95% of the calculated maximal degradability was achieved. For brachiaria hay: $y = 41.4$ (±1.46; $P < 0.001$) − 12.5 (±3.02; $P = 0.0090$) x^{-1}, $R^2 = 0.775$, $SE = 2.15$, $P = 0.0090$; for apple pomace: $y = 13.4$ (±2.00; $P = 0.0025$) + 15.5 (±3.02; $P = 0.0069$) [1 − 0.444 (±0.251; $P = 0.1522$)^x], $R^2 = 0.870$, $SE = 2.98$, $P = 0.017$; for beet pulp: $y = 54.4$ (±1.22; $P < 0.001$) −11.7 (±1.57; $P < 0.001$) x^{-1}, $R^2 = 0.917$, $SE = 2.13$, $P < 0.001$. The significance of the difference of response across fiber sources was $P = 0.099$.

of urea in the infusate also decreased proteolysis of the feed which would indicate that decreasing ammonia concentration could stress the competition for preformed amino-N vs. ammonia-N in the RDP available. Still, the present result that there is an increase in the concentration of the branched SCFA under the condition of very low ammonia concentration suggests the opposite and

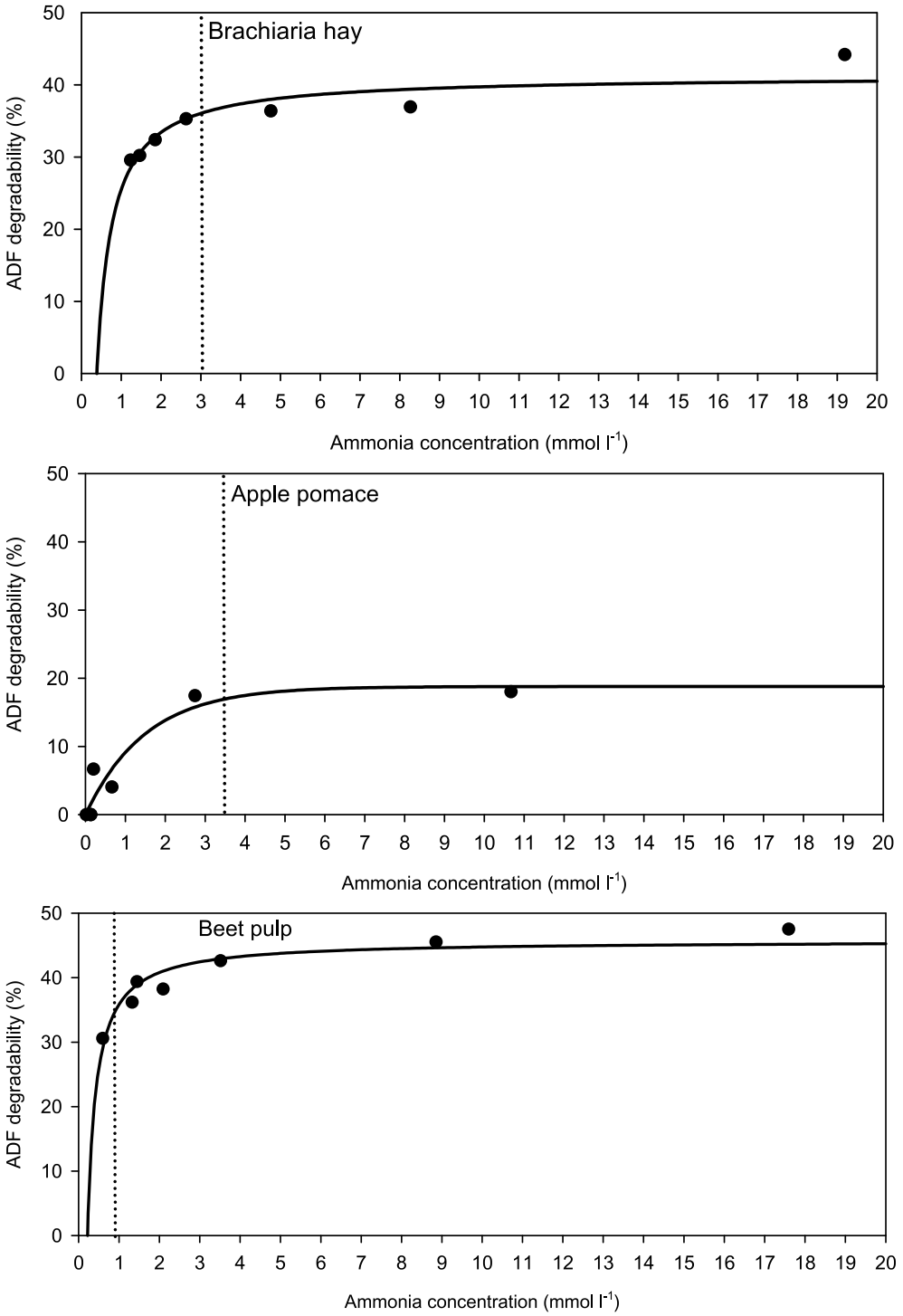

FIGURE 4 | Relationship between apparent acid detergent fiber (ADF) degradability and incubation fluid ammonia concentration. The intersection between the vertical dotted line and the x-axis represents the ammonia concentration at which 95% of the calculated maximal degradability was achieved. For brachiaria hay: $y = 41.3$ (±1.48; $P < 0.001$) − 15.8 (±3.06; $P = 0.0036$) x^{-1}, $R^2 = 0.841$, $SE = 2.18$, $P = 0.0036$; for apple pomace: $y = 18.8$ (±2.49; $P < 0.001$) [1 − 0.514 (±0.141; $P = 0.0147)^x$], $R^2 = 0.911$, $SE = 2.61$, $P < 0.001$; for beet pulp: $y = 45.8$ (±1017; $P < 0.001$) − 9.91 (±1.51; $P = 0.0012$) x^{-1}, $R^2 = 0.896$, $SE = 2.04$, $P = 0.0012$. The significance of the difference of response across fiber sources was $P = 0.064$.

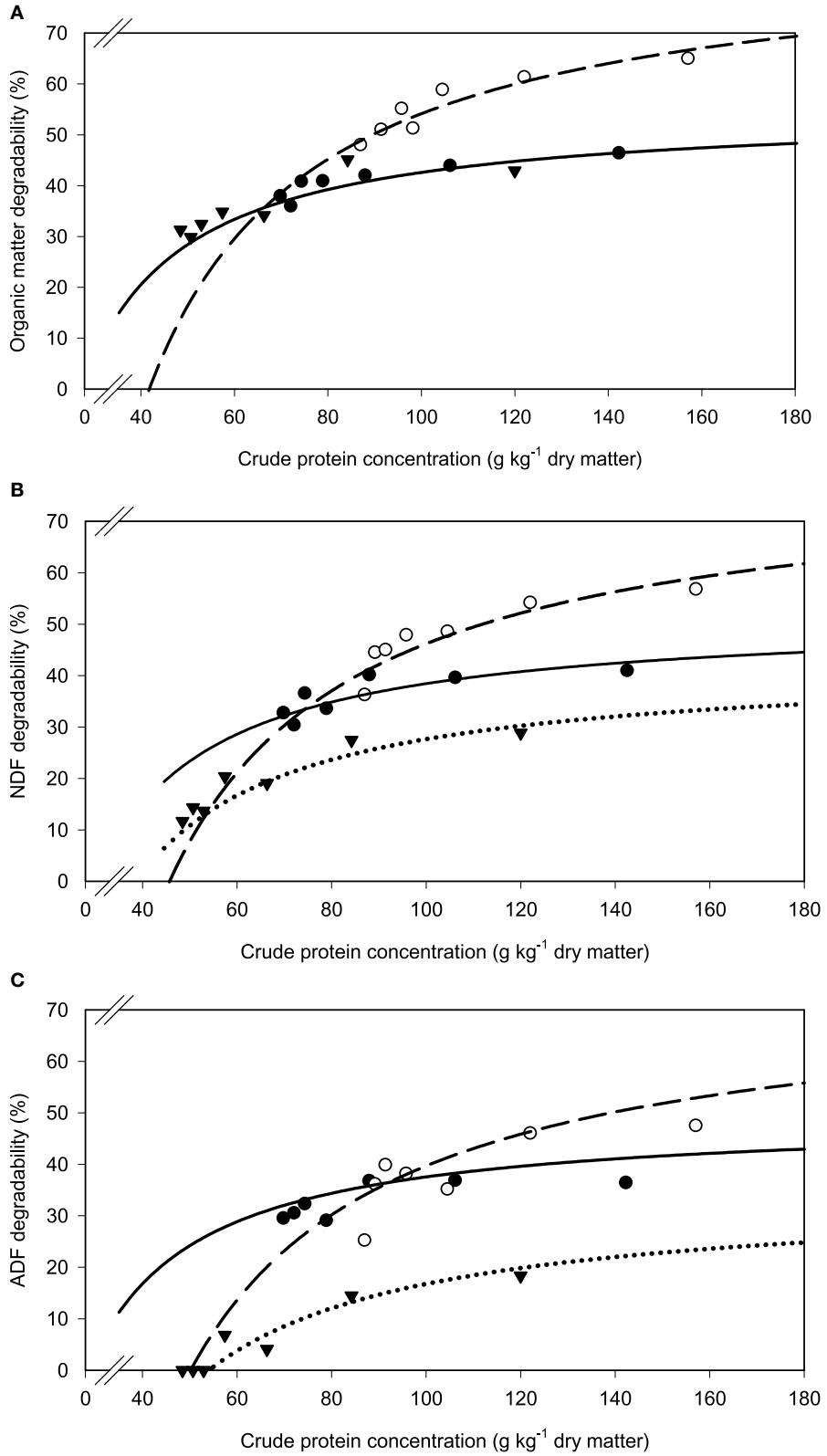

FIGURE 5 | Relationship between apparent degradability of organic matter (A), neutral detergent fiber (NDF) (B) and acid detergent fiber (ADF) (C) and dietary crude protein concentration. • ▬ brachiaria hay, for **(A)** $y = 55.3$ (±2.70; $P < 0.001$) − 1203 (±225; $P = 0.0031$) x^{-1}, $R^2 = 0.851$, $SE = 1.47$, $P = 0.003$, **(B)** $y = 51.4$ (±4.6; $P < 0.001$) − 1283

(Continued)

FIGURE 5 | Continued
(±385; $P = 0.0208$) x^{-1}, $R^2 = 0.689$, $SE = 2.52$, $P = 0.0208$, **(C)** $y = 45.6$ (±4.20; $P < 0.001$) − 1064 (±350; $P = 0.0287$) x^{-1}, $R^2 = 0.649$, $SE = 2.29$, $P = 0.029$; ○ ▬ ▬ beet pulp, for **(A)** $y = 87.4$ (±5.06; $P < 0.001$) − 3289 (±519; $P = 0.0015$) x^{-1}, $R^2 = 0.889$, $SE = 2.26$, $P = 0.002$, **(B)** $y = 79.6$ (±6.72; $P < 0.001$) − 3272 (±679; $P = 0.0048$) x^{-1}, $R^2 = 0.823$, $SE = 3.11$, $P = 0.005$, **(C)** $y = 69.3$ (±10.6; $P = 0.0012$) − 3178 (±1068; $P = 0.0310$) x^{-1}, $R^2 = 0.639$, $SE = 4.90$, $P = 0.031$; ▼ ..... apple pomace, for **(A)** $y = 54.9$ (±3.93; $P < 0.001$) − 1191 (±238; $P = 0.0041$) x^{-1}, $R^2 = 0.834$, $SE = 2.62$, $P = 0.004$, **(B)** $y = 42.2$ (±3.16; $P < 0.001$) − 1430 (±191; $P < 0.001$) x^{-1}, $R^2 = 0.918$, $SE = 2.12$, $P < 0.001$, **(C)** $y = 31.6$ (±3.53; $P < 0.001$) − 1555 (±214; $P < 0.001$) x^{-1}, $R^2 = 0.917$, $SE = 2.37$, $P < 0.001$. The significance of the difference of response across fiber sources was $P = 0.074$, 0.064, and 0.033 for organic matter **(A)**, neutral detergent fiber **(B)**, and acid detergent fiber **(C)**, respectively.

indicates that there was no need for supplementing preformed AA under the conditions investigated.

Responses in Nutrient Degradability to Extra Urea and to Increasing Incubation Fluid Ammonia Concentration

Urea was used to increase the dietary proportion of RDP. The three feeds provided RDP themselves and this at different levels. Therefore, the ammonia concentrations without extra urea, i.e., the starting points, were different with 1.2, 0.1, and 0.6 mmol l^{-1} for brachiaria hay, apple pomace and beet pulp, respectively. However, any of these concentrations were low as is characteristic for RDP-poor feeds. The regression analysis demonstrated the presence of clear responses in apparent OM and fiber degradability to increasing incubation fluid ammonia concentrations. Due to their particularly high demand for ammonia, the fibrolytic microbes obtain a specific advantage with increasing ammonia concentrations because the micro-environment is then better equilibrated with ammonia across the entire rumen (Owens and Bergen, 1983). As a limitation, it has to be stated that, compared to Rusitec, *in vivo* there might be compartmentalization preventing the establishment of a complete equilibration. This was shown by Storm et al. (2012) for the SCFA. In an earlier study (Tiemann et al., 2008a), supplementing 0.167 g urea day^{-1} to 15 g hay day^{-1} of *B. dictyoneura* resulted in an increase in incubation fluid ammonia from 0.6 to 4.4 mmol l^{-1} and in apparent NDF degradability from 24 to 36% in Rusitec. This is comparable with the responses found in the present study with *B. brizantha*. Also in the study by Riemeier et al. (2004), ruminal OM degradability in cows was at a moderate level (66%) at a very low ruminal ammonia concentration of 1.1 mmol l^{-1}, and OM apparently degraded increased to 70% at a ruminal ammonia concentration of about 10 mmol l^{-1}, but even slightly declined when ammonia concentration increased to 20 mmol l^{-1}. Intake of digestible OM was found to increase with supplementary RDP when feeding tallgrass-prairie hay containing <50 g kg^{-1} CP, starting at ruminal ammonia concentration as low as 0.3 mmol ammonia l^{-1} (Olson et al., 1999). Extra urea also increased OM digestibility in sheep in a curvilinear, i.e., more than proportionate manner, starting at a ruminal ammonia concentration of 2.7 mmol l^{-1} (Balcells et al., 1993). Increasing RDP levels in a low-quality grass hay diet of beef steers by the supplementation with sodium caseinate from 0 to 0.64 kg day^{-1} increased ruminal ammonia concentration from 0.62 to 11.22 mmol l^{-1} and concomitantly elicited a positive quadratic response in NDF degradability (Klevesahl et al., 2003). In contrast, Sawyer et al. (2012) did not find an effect on NDF digestibility when supplementing a cattle diet with urea of 0, 40, 80, and 160 g d^{-1} while exhibiting a quadratic response in ruminal ammonia concentration. In the study of Mathis et al. (2000), the response of OM digestibility in steers to RDP (casein) clearly differed among several low-CP forages. Overall, these rather variable findings suggest that extra RDP enhances ruminal ammonia concentration, sometimes in a linear fashion, and sometimes non-linearly. In addition this often, but not always, increases ruminal OM and fiber degradability.

Requirements for Rumen-degradable Protein with Different Feeds

There are various individual ways for describing RDP requirements of ruminal microbes in order to enable them to ferment OM and fiber efficiently. The most direct trait builds on rumen fluid ammonia concentration. Recommendations for minimum ammonia concentration range from slightly above 1 (Satter and Slyter, 1974; Marini and Van Amburgh, 2003) up to 16 mmol l^{-1} (Mehrez et al., 1977) with other recommendations ranging in between (reviewed by Balcells et al., 1993; Schwab et al., 2005). In the present study, when either apparent OM or fiber fermentation approached a plateau, the ammonia concentration was in the lower range of these studies accounting for either 0.75–2.19 or 1.73–2.54 mmol l^{-1}, respectively (depending on the regression equation). Thereby, the threshold for the ammonia concentration required largely differed among test feeds. It was reached first with apple pomace then with beet pulp and finally with the brachiaria hay. Consistent with this, Calsamiglia et al. (2010) stated in their review that the use of ammonia-N as the sole criterion for determining minimum concentration of N for optimal microbial growth should be challenged.

Assessing ruminal ammonia concentration is impractical on farm. Thus, dietary indicators are preferable. The concept of a minimum dietary CP concentration was often applied in the past, with 130–160 g CP kg^{-1} being considered to supply sufficient amounts of RDP (Schwab et al., 2005). Huhtanen and Hristov (2009) even concluded from a meta-analysis that the N fractions currently recommended to be incorporated in feeding systems (RDP and RUP, intestinal digestion, etc.) do not seem to improve our ability to optimize N utilization, and only the CP concentration of the diet appears to be closely related to the efficiency of N utilization. Still minimum requirements for RDP in order to maximize fiber degradation were evaluated by regression analysis of various studies and compiled in the NRC (2001) recommendations. These requirements amount to about

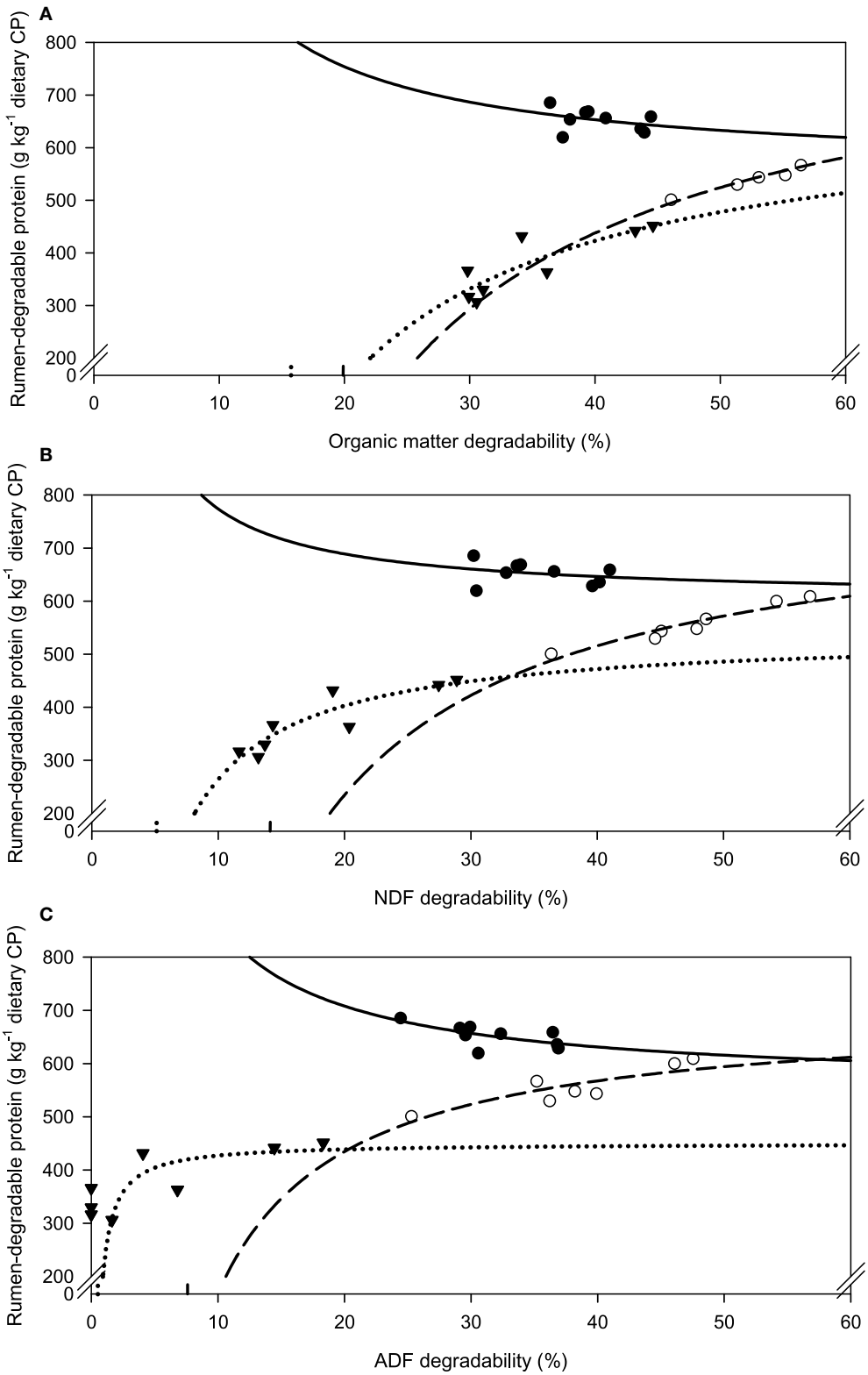

FIGURE 6 | Relationship between apparent degradability of organic matter (A), neutral detergent fiber (ADF) (B) and acid detergent fiber (ADF) (C) and rumen-degradable protein proportion in dietary crude protein. • ▬ brachiaria hay, for **(A)** $y = 552$ (± 101; $P < 0.001$) + 40.3×10^2 ($\pm 40.5 \times 10^2$; $P = 0.3527$) x^{-1}, $R^2 = 0.242$, $SE = 21.0$, $P = 0.3527$, **(B)** $y = 604$ (± 66.3; $P < 0.001$) + 17.0×10^2 ($\pm 23.03 \times 10^2$; $P = 0.4849$) x^{-1}, $R^2 = 0.0721$, $SE = 21.6$, $P = 0.485$, **(C)** $y = 555$

(Continued)

FIGURE 6 | Continued
(±43.2; $P < 0.001$) + 30.7×10^2 (±13.4×10^2; $P = 0.0554$) x^{-1}, $R^2 = 0.429$, $SE = 16.7$, $P = 0.055$. ○ ▬ ▬ beet pulp. for **(A)** $y = 871$ (±19.3; $P < 0.001$) − 17.3×10^3 (±10.6×10^2; $P = 0.0140$) x^{-1}, $R^2 = 0.982$, $SE = 5.68$, $P < 0.001$, **(B)** $y = 797$ (±34.8; $P < 0.001$) -112×10^2 (±16.1×10^2; $P < 0.001$) x^{-1}, $R^2 = 0.9066$, $SE = 12.8$, $P < 0.001$, **(C)** $y = 701$ (±39.8; $P < 0.001$) -53.2×10^2 (±14.4×10^2; $P = 0.0140$) x^{-1}, $R^2 = 0.732$, $SE = 21.7$, $P = 0.014$. ▼ ..... apple pomace, for **(A)** $y = 697$ (±83.6; $P < 0.001$) -11.0×10^3 (±28.3×10^2; $P = 0.0082$) x^{-1}, $R^2 = 0.715$, $SE = 33.8$, $P = 0.0082$, **(B)** $y = 541$ (±33.0; $P < 0.001$) -27.7×10^2 (±5.29×10^2; $P = 0.0020$) x^{-1}, $R^2 = 0.820$, $SE = 26.8$, $P = 0.002$, **(C)** $y = 451$ (±25.9; $P < 0.001$) −231 (±85.3; $P = 0.0735$) x^{-1}, $R^2 = 0.709$, $SE = 38.9$, $P = 0.074$. The significance of the difference of response across fiber sources was $P = 0.018$, 0.018, and 0.008 for organic matter **(A)**, neutral detergent fiber **(B)**, and acid detergent fiber **(C)**, respectively.

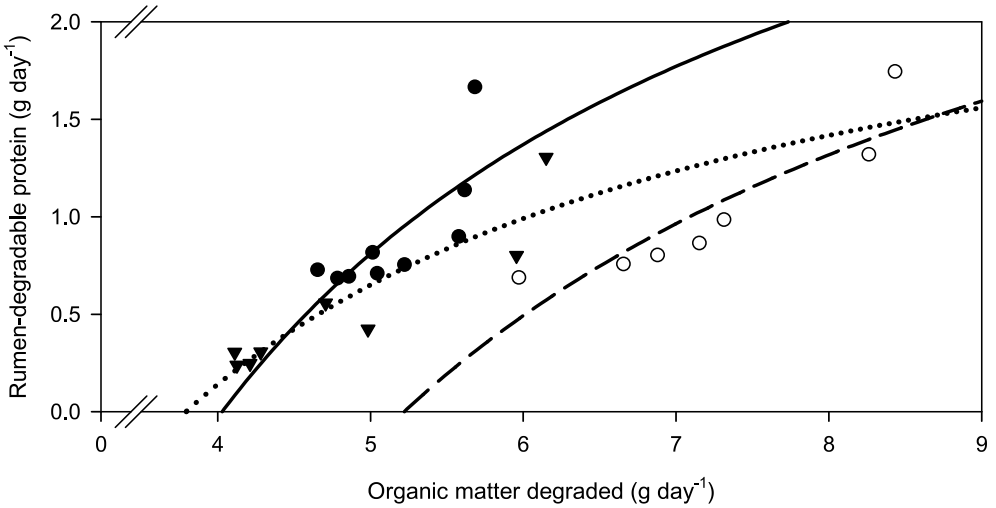

FIGURE 7 | Relationship between the amounts of organic matter degraded and rumen-degradable protein. • ▬ brachiaria hay, $y = 4.18$ (±1.09; $P = 0.0064$) − 16.8 (±5.57; $P = 0.0194$) x^{-1}, $R^2 = 0.566$, $SE = 2.23$, $P = 0.019$; ○ ▬ ▬ beet pulp, $y = 3.80$ (±0.666; $P = 0.0023$) − 19.8 (±4.73; $P = 0.0086$) x^{-1}, $R^2 = 0.778$, $SE = 0.196$, $P = 0.0086$; ▼ ..... apple pomace, $y = 2.70$ (±0.411; $P < 0.001$) − 10.2 (±1.91; $P = 0.0017$) x^{-1}, $R^2 = 0.827$, $SE = 0.166$, $P = 0.017$. The significance of the difference of response across fiber sources was $P < 0.001$.

100 g RDP kg^{-1} (assuming constant DM intake and non-rumen degradable protein levels). Schwab et al. (2005) compiled data indicating that requirements are actually lower with 50–80 g RDP kg^{-1} DM. In the present study, the relatively clear plateau in apparent OM and NDF degradability found with brachiaria hay and apple pomace was starting at a dietary CP level of about 80 g kg^{-1} DM. The CP was widely equivalent to RDP in this context because most of it consisted of supplemented urea. Considering that still part of the CP was undegradable because total CP also included in the test feeds, the present results fall in the range specified by Schwab et al. (2005). By contrast, the situation with the best fermentable fiber source, beet pulp, was different from that found with brachiaria hay and apple pomace, and the results indicated requirements close to the apparently higher amount of RDP recommended by NRC (2001). The increasing proportion of OM degradability with increasing dietary CP supplementation (going up to 180 g CP kg^{-1}) eventually even resulted in the lack of the development of a clear plateau with beet pulp. Schwab et al. (2005) stated that the need for supplemental RDP might be related more to forage CP concentration than to ruminal ammonia concentration. This seems to be true for the beet pulp in the present study as a plateau in apparent OM and NDF degradability was reached even when incubation fluid ammonia concentration further increased.

New concepts of feeding recommendation build on RDP: apparently degraded OM ratios (Bach et al., 2005; Schwab et al., 2005). Others calculate ruminal N balance giving either percentages (NRC, 2001) or the deviation from stated ruminal N MJ^{-1} (metabolizable) energy (DLG, 1997; Riemeier et al., 2004). The latter approaches conceptually do not differ much from the RDP: apparently degraded OM ratio principle. In the present study, the requirements for RDP per unit of OM apparently degraded were similar with apple pomace and beet pulp, but were much higher for the brachiaria hay. Actually, it appeared from the regressions that these requirements did not change at all in the range investigated when rumen degradability of CP was concerned and thus absolute RDP requirements almost linearly increased with the amount of OM fermented. Based on the observations with low-CP tropical diets, the opposite had been expected. Differences in apparent OM and fiber degradability without urea supplementation do not yield an explanation because the brachiaria hay ranged in the middle of the two other feeds in this respect. The observation of a higher RDP: apparently degraded OM ratio with brachiaria hay is, however, consistent with the finding of a particularly high incubation fluid ammonia concentration which was obviously required to approach maximum apparent OM and fiber degradability. In the study by Mathis et al. (2000), most RDP was required to

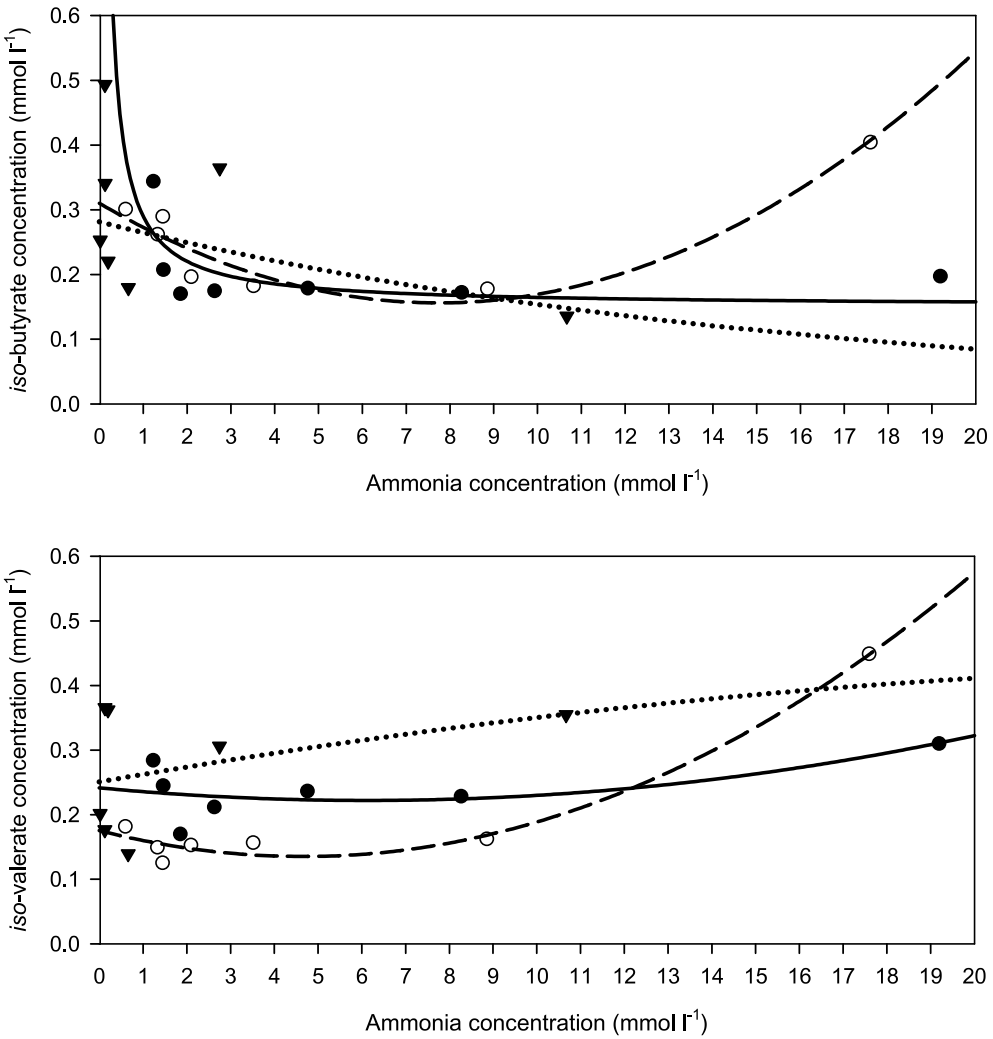

FIGURE 8 | Relationship between the concentrations of incubation fluid *iso*-butyrate and *iso*-valerate and ammonia concentration. • ▬ brachiaria hay, for *iso*-butyrate: $y = 0.151$ (±0.0351; $P = 0.0077$) + 0.139 (±0.0728; $P = 0.1144$) x^{-1}, $R^2 = 0.422$, $SE = 0.0518$, $P = 0.114$, for *iso*-valerate: $y = 0.242$ (±0.034; $P = 0.00021$) − 0.00641 (±0.01193; $P = 0.6213$) x + 0.000520 (±0.00057; $P = 0.4147$) x^2, $R^2 = 0.460$, $SE = 0.0414$, $P = 0.292$, ▼ ..... apple pomace, for *iso*-butyrate: $y = 0.282$ (±0.0483; $P = 0.0021$) exp[0.0602 (±0.0514; $P = 0.2939$) x], $R^2 = 0.303$, $SE = 0.206$, $P = 0.200$, for *iso*-valerate: $y = 0.251$ (±0.0551; $P = 0.0104$) + 0.0119 (±0.0622; $P = 0.8577$) x − 0.000194 (±0.00570; $P = 0.9746$) x^2, $R^2 = 0.157$, $SE = 0.109$, $P = 0.7110$, ○ ▬ ▬ beet pulp, for *iso*-butyrate: $y = 0.310$ (±0.0218; $P < 0.001$) − 0.0398 (±0.0088; $P = 0.0106$) x + 0.00259 (±0.00052; $P = 0057$) x^2, $R^2 = 0.909$, $SE = 0.302$, $P = 0.0082$, for *iso*-valerate: $y = 0.176$ (±0.0143; $P < 0.001$) − 0.0173 (±0.0058; $P = 0.0401$) x + 0.00186 (±0.00031; $P = 0.0040$) x^2, $R^2 = 0.979$, $SE = 0.0198$, $P < 0.001$. The significance of the difference of response across fiber sources was $P = 0.024$ and $P = 0.014$ for *iso*-butyrate and *iso*-valerate, respectively.

digest OM when using bermuda grass, whereas less RDP was needed with bromegrass and forage sorghum. Therefore, it still remains to be investigated whether this phenomenon is typical for at least some tropical grasses or whether feeds like apple pomace and beet pulp require smaller amounts of RDP in order to ferment OM and fiber. Explanations for these clear, although unexpected, findings might be sought in differences in the microenvironment, in addition to variations caused in N recycling as presumed by Mathis et al. (2000). Accordingly, Odle and Schaefer (1987) presumed that the optimum ammonia concentration is affected by chemical or structural characteristics. Definitely, the requirements for RDP for fermenting OM and fiber differed between the fibrous feeds and there was no such phenomenon of a simple overall threshold level to be recommended as it would be desirable and is sometimes practiced.

Conclusion

The hypothesis that the amount of RDP required to ferment OM and fiber differs among fibrous feeds was confirmed by the present findings. However, the results did not give indications for a more favorable situation in case low-CP-high-fiber tropical diets are used. It was further demonstrated that fibrous feeds

differ in their responses to urea addition in apparent OM and fiber degradability. Therefore, using general minimum thresholds for either dietary RDP or ruminal ammonia concentration may be too simplistic. Further screening of fibrous feeds to obtain detailed knowledge of the RDP requirements is needed. This would also help promoting N-efficient ruminant husbandry systems in order to cope with new demands that are increasingly implemented in environmental legislation (e.g., Schwab et al., 2005).

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Electron transport phosphorylation in rumen butyrivibrios: unprecedented ATP yield for glucose fermentation to butyrate

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From a genomic analysis of rumen butyrivibrios (*Butyrivibrio* and *Pseudobutyrivibrio* sp.), we have re-evaluated the contribution of electron transport phosphorylation (ETP) to ATP formation in this group. This group is unique in that most (76%) genomes were predicted to possess genes for both Ech and Rnf transmembrane ion pumps. These pumps act in concert with the NifJ and Bcd-Etf to form a electrochemical potential ($\Delta\mu H^+$ and $\Delta\mu Na^+$), which drives ATP synthesis by ETP. Of the 62 total butyrivibrio genomes currently available from the Hungate 1000 project, all 62 were predicted to possess NifJ, which reduces oxidized ferredoxin (Fd_{ox}) during pyruvate conversion to acetyl-CoA. All 62 possessed all subunits of Bcd-Etf, which reduces Fd_{ox} and oxidizes reduced NAD during crotonyl-CoA reduction. Additionally, 61 genomes possessed all subunits of the Rnf, which generates $\Delta\mu H^+$ or $\Delta\mu Na^+$ from oxidation of reduced Fd (Fd_{red}) and reduction of oxidized NAD. Further, 47 genomes possessed all six subunits of the Ech, which generates $\Delta\mu H^+$ from oxidation of Fd_{red}. For glucose fermentation to butyrate and H_2, the electrochemical potential established should drive synthesis of ~1.5 ATP by the F_0F_1-ATP synthase (possessed by all 62 genomes). The total yield is ~4.5 ATP/glucose after accounting for three ATP formed by classic substrate-level phosphorylation, and it is one the highest yields for any glucose fermentation. The yield was the same when unsaturated fatty acid bonds, not H^+, served as the electron acceptor (as during biohydrogenation). Possession of both Ech and Rnf had been previously documented in only a few sulfate-reducers, was rare in other rumen prokaryotic genomes in our analysis, and may confer an energetic advantage to rumen butyrivibrios. This unique energy conservation system might enhance the butyrivibrios' ability to overcome growth inhibition by unsaturated fatty acids, as postulated herein.

Keywords: rumen microbiology, butyrivibrios, electron transport phosphorylation, ATP yield, energy conservation

Abbreviations: ACP, acyl carrier protein; Bcd-Etf, butyryl-CoA dehydrogenase/electron transferring flavoprotein; COG, clusters of orthologous groups; Ech, *Escherichia coli* hydrogenase-3-type hydrogenase; Eha, energy-converting hydrogenase A; Ehb, energy-converting hydrogenase B; EMP, Embden–Meyerhof–Parnas; Eno, enolase; ETP, electron transport phosphorylation; Fab, fatty acid biosynthesis enzymes; Fd, ferredoxin; Fdh, formate dehydrogenase; Fd_{ox}, oxidized Fd; Fd_{red}, reduced Fd; Glo, glyoxalase; Hyc, hydrogenase 3; Hyd, ferredoxin hydrogenase; Hyl, hydrogenase-like protein; KO, Kyoto Encyclopedia of Genes and Genomes orthology; LA, linoleic acid; Ldh, lactate dehydrogenase; Mbh, membrane-bound hydrogenase; MsgA, methylglyoxal synthase; Mvh/Hdr, methyl viologen hydrogenase/heterodisulfide reductase; NAD, nicotinamide adenine dinucleotide; NAD_{ox}, oxidized NAD; NAD_{red}, reduced NAD; NifJ, nitrogen fixation J; PflD, pyruvate formate lyase D; Por, pyruvate ferredoxin oxidoreductase; Rnf, *Rhodobacter* nitrogen fixation; SA, stearic acid; SLP, substrate-level phosphorylation; VA, *trans*-11 vaccenic acid; $\Delta\mu H^+$, proton electrochemical potential; $\Delta\mu Na^+$, sodium electrochemical potential.

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Introduction

In anaerobic prokaryotes, SLP was long-thought to be the primary mechanism for ATP synthesis and energy conservation. ETP, in contrast, was thought to be minor. It was thought to be coupled only to dissimilatory reduction of inorganic elements or compounds (e.g., sulfate), fumarate reduction, organic acid decarboxylation, efflux of organic acid end-products, and methanogenesis (Gottschalk, 1986; White, 2007). Evidence supported that ETP was coupled to reductive acetogenesis, also, but the full mechanism could not be elucidated (Müller, 2003).

Recently, mechanisms of ETP have been elucidated in more pathways of anaerobic prokaryotes. These pathways include (1) acetate formation during glucose fermentation in *Pyrococcus furiosus* (Sapra et al., 2003); (2) caffeyl-CoA reduction and H_2 oxidation during caffeate respiration in *Acetobacterium woodi* (Imkamp et al., 2007; Bertsch et al., 2013); (3) crotonyl-CoA reduction during butyrate formation in *Clostridium* sp. and *Acidaminococcus fermentans* (Herrmann et al., 2008; Li et al., 2008); (4) H_2 oxidation during reductive acetogenesis in *A. woodi*, *Clostridium ljungdahlii*, and *Moorella thermoacetica* (Schuchmann and Müller, 2014); and (5) CO oxidation during formation of acetate and other end products in *C. autoethanogenum* (Wang et al., 2013a).

In these pathways, ETP involves the transmembrane ion pumps Ech or Rnf. These pumps generate a transmembrane electrochemical potential ($\Delta\mu H^+$ or $\Delta\mu Na^+$) from redox cofactors generated by pathway enzymes. Specifically, Ech is a hydrogenase that generates $\Delta\mu H^+$ from oxidation of Fd_{red} (Welte et al., 2010). Rnf generates $\Delta\mu H^+$ or $\Delta\mu Na^+$ from simultaneous oxidation of Fd_{red} and reduction of NAD_{ox}; (Biegel and Müller, 2010; Schlegel et al., 2012; Tremblay et al., 2013), in a mechanism known as flavin-based electron bifurcation (Buckel and Thauer, 2013). Redox cofactors are generated by pathway enzymes using conventional mechanisms and enzymes recently discovered to generate redox cofactors by the mechanism of electron bifurcation (Buckel and Thauer, 2013). The electrochemical potential established by Ech and Rnf in turn drives F_0F_1-ATP synthase to form ATP by ETP (Welte et al., 2010; Buckel and Thauer, 2013), or, alternatively, it could drive solute transport or motility. Organisms appear to possess either Ech or Rnf, and reports of organisms possessing both are rare (Pereira et al., 2011; Weghoff et al., 2015).

While searching rumen prokaryotic genomes for genes supporting ETP, we found that the rumen butyrivibrios [*Butyrivibrio* and *Pseudobutyrivibrio* sp. (Cotta and Forster, 2006)] uniquely possessed genes for both Ech and Rnf. We suggest Ech and Rnf function in concert with the NifJ protein and Bcd–Etf complex [pathway enzymes generating redox cofactors; (Buckel and Thauer, 2013; Gutekunst et al., 2014)], permitting unprecedented ATP yield during glucose fermentation to butyrate. Activity of Ech and Rnf, if experimentally confirmed, could give members of this group an energetic advantage and help explain their metabolic flexibility.

The butyrivibrios are the best characterized group of bacteria that biohydrogenate LA to VA; *Butyrivibrio proteoclasticus* represents the only known clade that biohydrogenates VA fully to SA through membrane-associated reductases (Jenkins et al., 2008). A hypothesis will be justified that *de novo* synthesis of long-chain saturated fatty acids increases to counteract this fluidization of the outer membrane. Because electron transfer mechanisms reoxidize all NAD_{red} from glucose fermentation to butyrate, though, *de novo* synthesis of fatty acids would be in competition with butyrate production for reducing equivalents, particularly as proposed for transhydrogenation mechanisms generating $NADP_{red}$ from NAD_{red}. This theory attempts to explain why abrupt dosing of LA did not compromise membrane integrity but extended lag time for growth while coenzyme A-esterified intermediates in the butyrate production pathway decreased prior to ATP depletion in a strain of *B. fibrisolvens* (Maia et al., 2010).

Materials and Methods

We analyzed all 62 genomes of *Butyrivibrio* and *Pseudobutyrivibrio* sp. sequenced in the Hungate 1000 project (Creevey et al., 2014) and available in the IMG database (Markowitz et al., 2014). We identified proteins involved in butyrate fermentation and ETP by searching for KO (Kanehisa et al., 2014), COG (Galperin et al., 2015), and pfam protein families (Finn et al., 2014) IDs as indicated parenthetically below. Specifically, we searched for ATPF0ABC and ATPF1ABDEG (K02108 to K02115); Bcd-EtfAB (K00248, K03521, and K03522); EchABCDEF (K14086 to K14091); EhaA-R (K14092 to K14109); EhbA-P (K14110 to K14124); Eno (K01689); the Hyd proteins HydA (K00532), HydA large subunit (K00533), HydB (K00534), HydC (K06441), and HydA1B1G1 (K17997 to K17999); the Fdh proteins FDH (K00122), FdhF (K00123), FdhB (K00125), and FdsD (K00126); the glyoxalase proteins GloA (K01759) and GloB (COG0491); the lactate dehydrogenases LdhA (K03778) and Ldh (K00016); MbhLKJ (K18016, K18017, K18023); MsgA (K01734); MvhADG/HdrABC (K14126 to K14128 and K03388 to K03390); NifJ (K03737); PflD (K00656); PorABDG (K00169 to K00172); and RnfABCDEG (K03612 to K03615, K03617, and COG2878). FdhF was searched as both the Fdh alone and as a complex with (1) Hyl (FdhF-HylABC; K00123, pfam10588, K00334, K00335) or (2) Hyc (FdhF-HycBCDEFG; K00123 and K15827 to K15832). We searched acetyl-CoA carboxylase (AccABCD; K01961, K02160, K01962, K01963), FabD (K00645), FabF (K09458), FabG (K00059), FabH (K00648), FabK (K02371), FabZ (K02372), and acyl transferases (glycerol-3-phosphate acyltransferases, PlsX, K03261, PlsY, K08591; 1-acyl-sn-glycerol-3-phosphate acyltransferase, PlsC, K00655). We searched TCA cycle intermediates (aconitase, AcnA, K01681; citrate synthase, CS, K01647; NADP-isocitrate dehydrogenase, IDH1, K00031; α-ketoglutarate dehydrogenase complex, korABDG, K00174-K00177; malate dehydrogenase, Mdh, K00024; malic enzyme, MaeA, K00027; and succinate dehydrogenase, sdhAB, K00239, K00240) and

transhydrogenase reactions (soluble transhydrogenase, SthA, K00322; pyrimidine nucleotide transhydrogenase, PntAB, K00324, K00325; and hydroxyl-ketoacid transyhdrogenase, ADHFE1, K11173; pyruvate kinase, Pyk, K00873; pyruvate-phosphate dikinase, Ppdk, K01006; phosphoenolpyruvate carboxykinase, PckA, K01610; oxaloacetate decarboxylase, OadAB, K01571, K01572; glutamate dehydrogenase, GdhA, K00262; and glutamate synthase, GltD, K00266). Here and throughout, searches were conducted between 4 and 20 May, 2015.

The KO ID was chosen for searches first, and the COG ID was searched only when the KO ID produced few to no search hits, even for genomes known to possess protein activity. *Prevotella bryantii* B_{14} synthesizes methylglyoxal (Russell, 1992), but GloB was missing when searching for the KO ID (K01069). *A. woodi* has Rnf activity (Biegel and Müller, 2010), but RnfB was missing when searching for K03616. These proteins were found in the respective genomes when searching for the COG IDs (listed above). The pfam ID was searched only when a KO ID was not available for the protein (HylA).

For comparison, we searched for all proteins above for both genomes of non-rumen butyrivibrios that were available in IMG database. Additionally, using the IMG database, we searched for Ech and Rnf in (1) 218 genomes of non-butyrivibrio rumen prokaryotes in the Hungate 1000 project, (2) all 47 of *Desulfovibrio* genomes available, (3) all 451 genomes of *Clostridium* sp. available, (4) 28 genomes of short-chain fatty acid degraders previously analyzed by Worm et al. (2014), and (5) 10 of the 20 genomes of lactate fermenters previously analyzed by Weghoff et al. (2015; the authors did not explicitly identify the other 10 genomes).

Results

Our analysis of rumen butyrivibrio genomes suggests that butyrivibrios possess genes for generating ATP by ETP during glucose fermentation to butyrate. Of 62 total genomes sequenced in the Hungate 1000 project and available on the IMG database, all 62 were predicted to possessed all subunits of the F_0F_1-ATP synthase (ATPF0ABC and ATPF1ABDEG), 62 possessed all subunits of Bcd-Etf (Bcd and EtfAB), 62 possessed NifJ, and 61 possessed all subunits of RnfABCDEG. Additionally, 47 genomes possessed all six subunits of EchABCDEF. These same genomes also possessed Rnf, making 47 genomes (76% of total) that possessed both Ech and Rnf.

EchD and EchF were the subunits of Ech missing most often, with each individually absent in seven genomes. Interestingly, EchD was individually absent in all six *B. fibrisolvens* genomes, and only in one other genome (*Butyrivibrio* sp. TB) was it also absent. EchF was absent over a broader range of genomes; it was individually absent in *Pseudobutyrivibrio ruminis* (two genomes), *Pseudobutyrivibrio xylanivorans* (one genome), unclassified *Pseudobutyrivibrio* sp. (one genome), and unclassified *Butyrivibrio* sp. (one genome). In only one other genome was any Ech subunit absent; *Butyrivibrio* sp. NC3005 lacked all subunits.

No genome possessed genes for PorABDG, a Por similar in function to NifJ. No genome possessed genes for EhaA-R, EhbA-P, HydA (K00532), HydB, HydC, HydA1B1G1, MbhLKJ, or MvhADG/HdrABC, which are similar in function to Ech in the respect that they are hydrogenases and Fd-dependent. Twenty genomes possessed the gene for the HydA large subunit (K00533), part of another Fd-dependent hydrogenase, but they did not possess other subunits (HydB, HydC).

In our proposed pathway of butyrate and H_2 from glucose (**Figure 1**), NifJ generates Fd_{red} during pyruvate conversion to acetyl-CoA. Bcd-Etf generates Fd_{red} and NAD_{ox} during crotonyl-CoA reduction to butyryl-CoA. Ech oxidizes Fd_{red}, and Rnf oxidizes Fd_{red} while reducing NAD_{ox}. In so doing, Ech and Rnf pump H^+ and Na^+, form $\Delta\mu H^+$ and $\Delta\mu Na^+$, and regenerate NAD_{red} and Fd_{ox} to achieve a balanced redox. The electrochemical potential drives ATP synthesis by F_0F_1-ATP synthase to yield ~1.5 ATP/glucose. An additional three

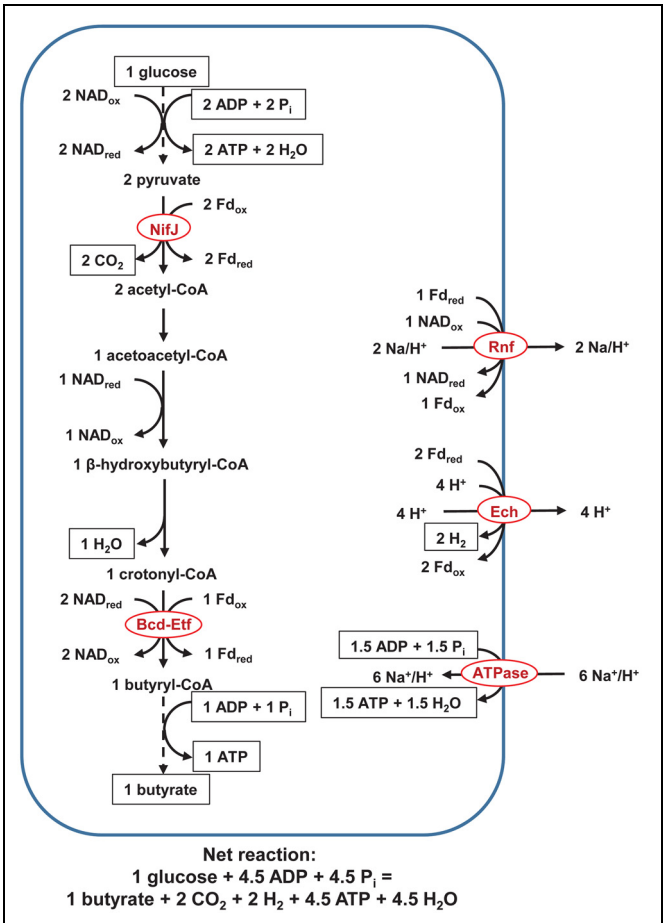

FIGURE 1 | Pathway of fermentation of glucose to butyrate in rumen butyrivibrios with NifJ and with H^+ as an electron acceptor. Three ATP are formed by SLP, as long-recognized, but up to an additional ~1.5 ATP are formed by ETP. Dashed lines represent steps of the pathway condensed for brevity. In the equation of the net reaction, hydrogen atoms and charges are not balanced, following the convention of Alberty (2003) for representing biochemical reactions. ATPase = F_0F_1-ATP synthase. See text for details and additional abbreviations.

ATP is formed by classic SLP. Specifically, two ATP are formed during the EMP pathway, and one ATP is formed either by (1) butyrate kinase or (2) butyryl-CoA/acetate CoA transferase, phosphotransacetylase, and acetate kinase (not shown in **Figures 1–3**). In total, ETP and SLP yield ~4.5 ATP/glucose.

In the pathway above (**Figure 1**), H^+ serves as an electron acceptor. We present a second pathway in which an unsaturated fatty acid serves as an electron acceptor instead (**Figure 2**). In this pathway, fatty acid oxidoreductase couples reduction of rumenate to vaccinate, forming NAD_{ox}. As a consequence of the extra NAD_{ox} formed, Rnf alone, not Ech, is active; only Rnf can reduce the extra NAD_{ox} and regenerate NAD_{red} to achieve a balanced redox. The ATP yield is not affected, with ETP still yielding ~1.5 ATP and SLP yielding 3 ATP/glucose.

The pathways with NifJ are not the only one possible, and an alternate pathway involves a pyruvate formate lyase, PflD (**Figure 3**). It was possessed by 61 of the 62 genomes. If the decarboxylation of acetyl-CoA is catalyzed by PflD instead of NifJ, two fewer Fd_{red} are generated, only Rnf is active, and ATP yield decreases from 4.5 to 3.5/glucose (**Figure 3**).

The pathway with PflD (**Figure 3**) depicts formate as an end product, but if a Fdh were present, formate would be oxidized to CO_2 and H_2. Five genomes possessed FdhF; no other Fdh (FDH, FdhB, FdsD) was present. Of the five genomes with FdhF, none possessed a full Fdh-hydrogenase complex (FdhF-HylABC or FdhF-HycBCDEFG). Formate would indeed be the predicted the end product for the pathway with PflD.

Pathways presented so far presume possession of a full EMP pathway (c.f., **Figures 1–3**). However, one EMP pathway enzyme (the enolase Eno) was missing in 25 genomes (not shown). The methylglyoxal pathway would be an alternate to a full EMP pathway (Supplementary Figure S1). All 62 genomes possessed MgsA, 45 possessed GloA, and 62 possessed GloB. Another methylglyoxal pathway enzyme, D-lactate dehydrogenase (LdhA), was found only in one genome, but it could be substituted by (1) L-lactate dehydrogenase (Ldh)

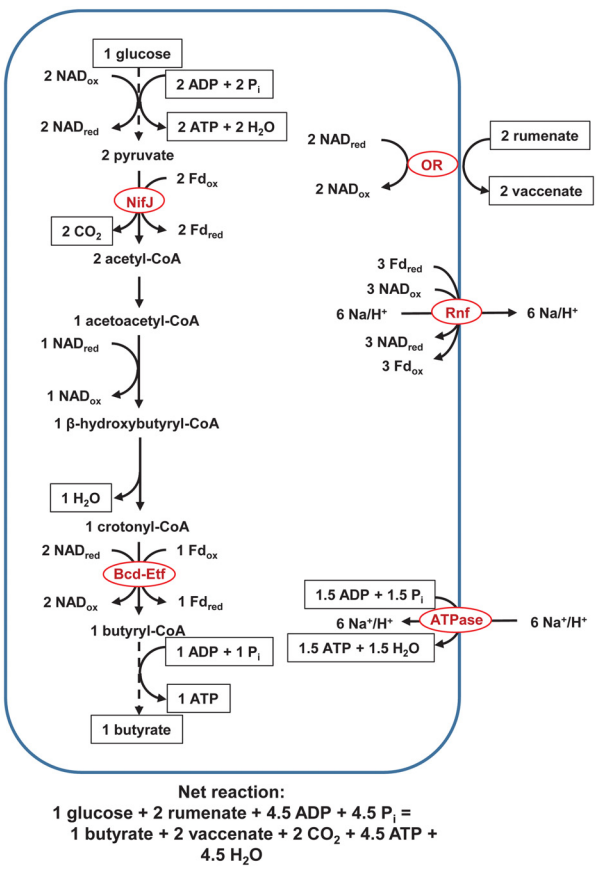

FIGURE 2 | Pathway of fermentation of glucose to butyrate in rumen butyrivibrios with NifJ and with an unsaturated fatty acid bonds as an electron acceptor. Pathway is identical to that in **Figure 1**, except reduction of rumenate to vaccenate by an oxidoreductase is additionally shown. ATPase = F_0F_1-ATP synthase and OR = fatty acid oxidoreductase. Dashed lines represent steps of the pathway condensed for brevity. See text for details and additional abbreviations.

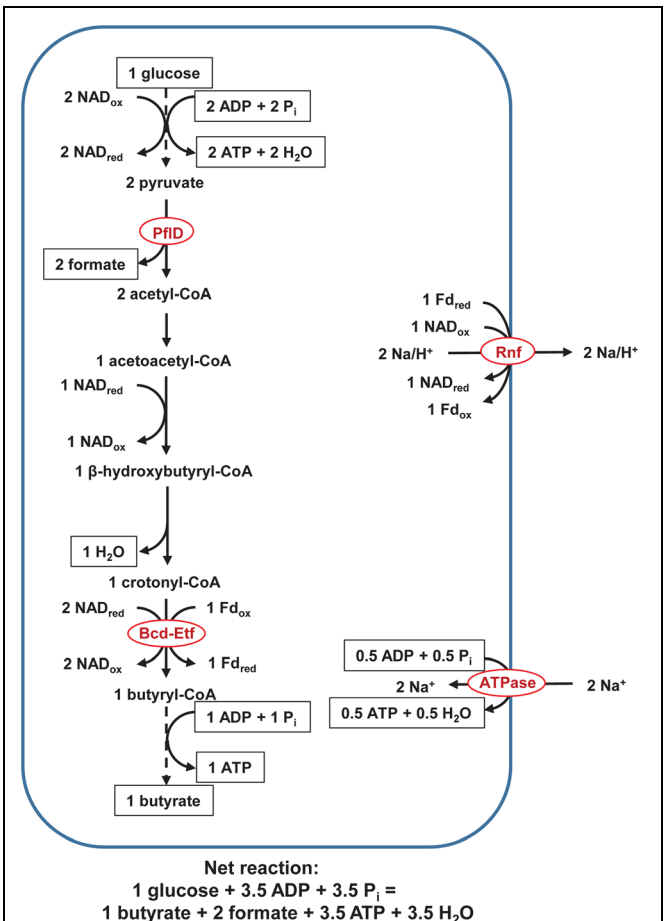

FIGURE 3 | Pathway of fermentation of glucose to butyrate in rumen butyrivibrios with PflD. Pathway is identical to that in **Figure 1**, except PflD replaces NifJ. ATPase = F_0F_1-ATP. Dashed lines represent steps of the pathway condensed for brevity. See text for details and additional abbreviations.

and (2) a lactate racemase (Supplementary Figure S1). Ldh was found in 51 genomes, but possession of a racemase could not be confirmed because it is not in KEGG, COG, pfam or other databases. Presuming that it is complete, the methylglyoxal pathway would yield four fewer ATP than does the full EMP pathway (c.f., Supplementary Figure S1). Consequently, if the methylglyoxal pathway were active, the pathway of butyrate and H_2 from glucose would yield either 0.5 ATP/glucose (with NifJ) or −0.5 ATP/glucose (with PflD).

Outside the butyrivibrios, few rumen prokaryotes possessed both Ech and Rnf. Of the 218 non-butyrivibrio genomes sequenced in the Hungate 1000 project, 134 possessed all subunits of Rnf and 16 possessed all subunits of Ech (c.f., Supplementary Table S1). Of those, 10 possessed both Rnf and Ech. These genomes belonged exclusively to strains of *Desulfovibrio desulfuricans* (one strain) and unclassified Lachnospiraceae (nine strains).

Only two non-rumen butyrivibrio genomes were in the IMG database, and they were sequenced in the Human Microbiome Project. Both genomes (*B. crossotus* DSM 2876, *B. fibrisolvens* 16/4) had all subunits of Bcd-Etf, NifJ, and PflD. One genome (*B. crossotus* DSM 2876) had all subunits of F_0F_1-ATP synthase and all subunits of Rnf. Neither genome had all subunits of Ech.

Of 47 genomes of *Desulfovibrio* sp. available in the IMG database, 17 genomes possessed all subunits of Rnf. Fifteen genomes possessed all subunits of Ech, but only five of those genomes (*D. cuneatus* DSM 11391, *D. desulfuricans desulfuricans* ATCC 27774, *D. desulfuricans* DSM 7057, *Desulfovibrio* sp. 3_1_syn3, *Desulfovibrio* sp. 6_1_46AFAA) also possessed all subunits of Rnf. Of 451 genomes of *Clostridium* sp. available in the IMG database, 392 genomes possessed all subunits of Rnf. One genome (*Clostridium* sp. KLE 1755) possessed all subunits of Ech, and it also possessed all subunits of Rnf. Of the 28 genomes of short-chain fatty acid degraders previously analyzed by Worm et al. (2014), 10 genomes possessed all subunits of Rnf. None possessed all six subunits of Ech. Of the 10 genomes of lactate fermenters previously analyzed by Weghoff et al. (2015), eight possessed all subunits of Rnf. None possessed all subunits of Ech.

Acetyl-CoA carboxylase subunits were predicted with variable results (between 36 and 61 draft genomes), and at least 60 of the 62 butyrivibrio draft genomes predicted FabD, FabF, FabG, FabZ, and FabK, whereas 41 of 62 predicted FabH (**Figure 4**). All 62 butyrivibrio draft genomes predicted the acyltransferases PlsX, PlsY, and PlsC that are needed to synthesize phosphatidic acid, the precursor for fatty acid components in bacterial membranes. No draft butyrivibrio genomes predicted transhydrogenases SthA, pntAB, or ADHFE1. As shown in Supplementary Figure S2, citrate synthase, aconitase, NADP-isocitrate dehydrogenase, malic enzyme, pyruvate kinase, pyruvate-phosphate dikinase, oxaloacetate decarboxylase, glutamate dehydrogenase, and glutamate synthase were predicted in all 62 draft genomes. The following enzymes are not shown in Supplementary Figure S2 because only 10 predicted α-ketoglutarate dehydrogenase complex (for all subunits), only four and five predicted succinate dehydrogenase (A and B subunits, respectively), and none predicted malate dehydrogenase.

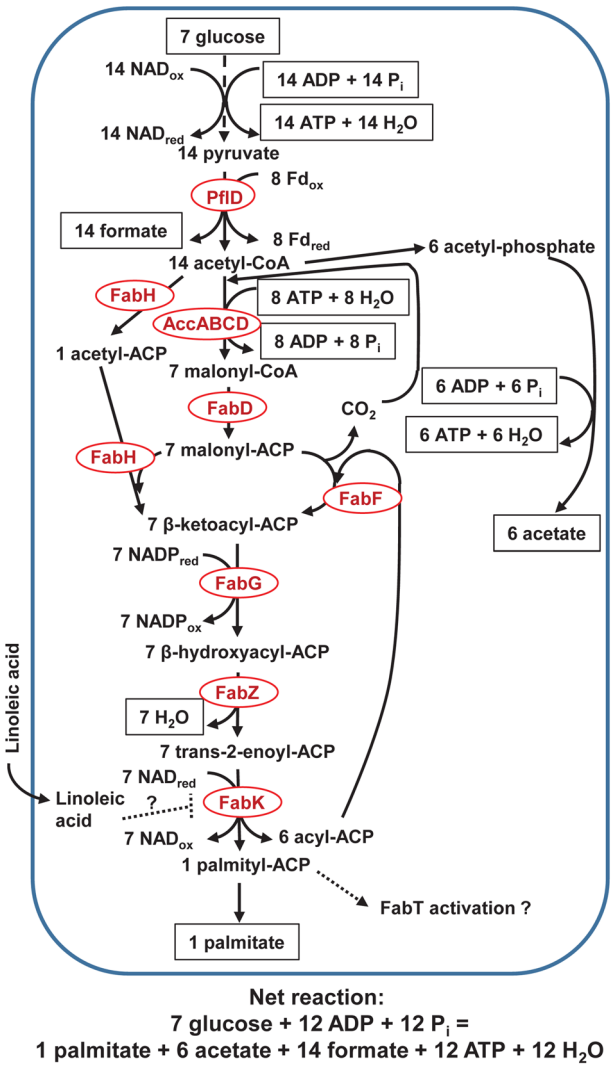

FIGURE 4 | Pathway of palmitate production in rumen butyrivibrios with PflD. Acetate is produced to balance NAD and NADP cofactors, assuming their equivalency (see text and Supplementary Figure S2 for explanation). Only long-chain fatty acids bound to ACP might activate the Fab regulator, FabT, as indicated by a dashed arrow and documented for other non-rumen bacteria. Non-esterified linoleate is assumed to associate with or penetrate the cell membrane and hypothesized (as denoted with a dashed line) to allosterically inhibit the terminal FabK enzyme. See text for details and additional abbreviations.

Discussion

Electron Transport Phosphorylation Involving Both Ech and Rnf

Our genomic analysis suggests that ETP is important to rumen butyrivibrios, and it may enable them to achieve unprecedented ATP yields in fermentation of glucose to butyrate. Glucose fermentation had been long-thought to generate ATP primarily by SLP (Gottschalk, 1986; Russell, 2002; White, 2007). In prokaryotes, the highest yield possible was thought to be four

ATP/glucose; this yield corresponds to fermentation of glucose to acetate and H_2 (Russell, 2002). Higher yields could be possible in eukaryotes expressing pyrophosphate-dependent glycolytic enzymes (Mertens, 1993), but those enzymes appear to be rarely expressed in exclusion to the classic glycolytic enzymes (Müller et al., 2012).

Glucose fermentation to butyrate had been long-accepted to form three ATP by SLP (Gottschalk, 1986), even as thermodynamic evidence suggested additional ATP should be formed (Stadtman, 1966; Ungerfeld and Kohn, 2006). The recent discovery of Bcd-Etf and Rnf in *Clostridium* sp. confirmed that additional ATP could be formed from ETP (Boiangiu et al., 2005; Herrmann et al., 2008; Li et al., 2008). Specifically, Bcd-Etf generates Fd_{red} and NAD_{ox} during crotonyl-CoA reduction to butyryl-CoA, and Rnf generates $\Delta\mu Na^+$ from these cofactors. Louis and Flint (2009) suggested even more energy could be conserved during pyruvate conversion to acetyl-CoA. They suggested the existence of a PFO and transmembrane ion pump that could together produce a $\Delta\mu H^+$, but the PFO and pump were not identified.

In an analysis of rumen butyrivibrio genomes, we have identified a PFO (NifJ) and an ion pump (Ech) that serves the function suggested by Louis and Flint (2009). The presence of NifJ and Ech, along with Bcd-Etf and Rnf, could achieve yields of ∼4.5 ATP/glucose for butyrate and H_2 formation from glucose. This yield rivals or surpasses that for fermentation of glucose to acetate and H_2.

One pathway we propose (**Figure 1**) shows H^+ as an electron acceptor. We propose a pathway with an unsaturated fatty acid as the electron acceptor (**Figure 2**), also, because butyrivibrios are the predominant biohydrogenating organisms in the rumen (Lourenço et al., 2010). If the electron acceptor were an unsaturated fatty acid bond, only Rnf would pump ions, but the ATP yield would be unchanged. Incubating *B. fibrisolvens* in LA decreased its intracellular ATP (Maia et al., 2010), and, all else equal, our analysis suggests that a lower ATP yield due to biohydrogenation is not responsible.

We depict a fatty acid oxidoreductase in the pathway for which unsaturated fatty acid serves as an electron acceptor. Our depiction is based on Hughes et al. (1982), who biochemically characterized and purified part of the oxidoreductase of *B. fibrisolvens* (Hughes et al., 1982). We presume the oxidoreductase is possessed by most genomes, as most butyrivibrios can biohydrogenate unsaturated fatty acids (e.g., LA; Paillard et al., 2007). However, we could not verify this presumption through a genomic search because the oxidoreductase is not found in KEGG, COG, pfam, or other databases [though a sequence of the oxidoreductase has been reported (Fukuda et al., 2007)].

As depicted, the oxidoreductase does not directly contribute to ATP synthesis by ETP. The oxidoreductase likely cannot pump ions because it appears to be a membrane-associated, not an integral, protein (Hughes et al., 1982). The oxidoreductase cannot drive Rnf or Ech to pump ions, either, because it generates only NAD_{ox} and not Fd_{red}; the observed stoichiometry of NAD_{red} oxidation to rumenate reduction is 1:1 (Hunter et al., 1976) and prohibits it from generating Fd_{red}. In sum, the oxidoreductase does not appear to drive ion pumping (either directly or indirectly), supporting that (1) it does not contribute to ATP synthesis and (2) ATP yield is unchanged when an unsaturated fatty acid serves as an electron acceptor in place of H^+.

Several parts of our genomic analysis require qualification. First, although many (76%) of genomes possessed all genes for Ech and Rnf, genes for EchD and EchF were individually absent in 11.3% of butyrivibrio genomes, and they were both absent in one genome (1.6% of total). Even for organisms possessing all genes, the expression and activity of both Ech and Rnf needs to be experimentally confirmed. Some evidence of Ech activity comes from (1) the observation that butyrivibrios produce H_2 (Hungate, 1966) and (2) our finding that most butyrivibrio genomes possessed only Ech and no other Fd-dependent hydrogenase. Some genomes possessed the HydA large subunit, part of another Fd-dependent hydrogenase, but this hydrogenase may not have activity as no other subunits were possessed.

Second, the function we ascribe to NifJ—supplying Fd_{red} to Ech or Rnf during fermentation—is putative. Historically, NifJ has been recognized for supplying Fd_{red} or flavodoxin to nitrogenase during N_2 fixation (Shah et al., 1983; Brostedt and Nordlund, 1991), not to Ech or Rnf during fermentation. In recent experiments comparing wild-type and *nifJ* deletion mutants, however, mutants had low rates of acetate and H_2 production during fermentation (McNeely et al., 2011; Gutekunst et al., 2014). These experiments suggest NifJ has a function in fermentation and can supply Fd_{red} to a hydrogenase (albeit the hydrogenase in those experiments was not Ech). Por, a PFO homologous to NifJ (Kletzin and Adams, 1996), was not possessed by any butyrivibrio genome.

Third, as a pyruvate formate lyase, PflD could serve as an alternative to NifJ, produce formate instead of H_2, and lower ATP yield. ATP yield is reduced because PflD does not generate Fd_{red}, which drives Ech and generates an electrochemical potential. Whereas formate oxidation by a FdhF-Hyl complex could generate Fd_{red} (Wang et al., 2013b), no butyrivibrio genome possessed the full complex.

Pyruvate formate lyase D indeed appears to serve as an alternative to NifJ in many butyrivibrios. Both H_2 and formate are observed fermentation products of butyrivibrios (Hungate, 1966), suggesting that both PflD and NifJ are active. Across strains, large variation exists in formate vs. H_2 production (Hungate, 1966). Within strains, also, large variation exists, with one strain (*B. fibrisolvens* 49) decreasing H_2 and increasing formate production in response to increasing acetate in the medium (Diez-Gonzalez et al., 1999). This variation in formate vs. H_2 production suggests that PflD and NifJ can be differentially expressed or regulated. Because the pathway with PflD yields fewer ATP than that with NifJ, this differential expression or regulation may allow butyrivibrios to modulate ATP yield. More experiments should compare the expression and activity of these enzymes and under different growth conditions.

Fourth, the methylglyoxal pathway, presuming it is complete, could serve as an alternate to a full EMP pathway and lower ATP yield. Kelly et al. (2010) suggested the methylglyoxal pathway could be important in butyrivibrios that appear to be missing

a key EMP pathway enzyme (Eno). We found that 40% of butryivibrios apparently lacked Eno. Though the methylglyoxal pathway could serve as an alternative in these butyrivibrios, the ATP yield would be low (≤0.5/glucose). More work needs to determine if alternatives to the EMP pathway exist besides the methylglyoxal pathway.

Fifth, the stoichiometry of Ech, Rnf, and F_0F_1-ATP synthase is uncertain. Ech has been shown to pump H^+ (Welte et al., 2010), and Rnf pumps either H^+ or Na^+ (Biegel and Müller, 2010; Schlegel et al., 2012; Tremblay et al., 2013). The number of ions pumped has been postulated to be two per Fd_{red} oxidized (Buckel and Thauer, 2013; Schuchmann and Müller, 2014), but this stoichiometry has not been experimentally established. This uncertainty, along with the variable stoichiometry of F_0F_1-ATP synthases (Nicholls and Ferguson, 2013), makes our estimate of ∼1.5 ATP/glucose formed from ETP subject to revision.

Despite its limitations, our analysis still suggests both Ech and Rnf genes are possessed by the majority of rumen butyrivibrios. Possession of both Ech and Rnf genes has been seldom documented for the same organism, much less for a groups of organisms. In their analysis of sulfate-reducing prokaryotes, Pereira et al. (2011) reported that some *Desulfovibrio* sp. possessed both Ech and Rnf. For *C. thermocellum*, Raman et al. (2011) and Rydzak et al. (2012) found some, but not all, subunits of Ech and Rnf were expressed. In their analysis of 28 genomes of short-chain fatty acid degraders, Worm et al. (2014) reported some genomes possessed several subunits of both Ech and Rnf, but their analysis did not examine some subunits (RnfA,E,F). In their analysis of 20 genomes of lactate fermenters, Weghoff et al. (2015) implied both Ech and Rnf were possessed by least one of their genomes.

We analyzed these genomes previously documented to possess both Ech and Rnf, as well as all genomes of non-butyrivibrio rumen prokaryotes. When we analyzed all *Desulfovibrio* genomes available in the IMG database, we found that five genomes (10.6% of total) possessed both Ech and Rnf. When we analyzed all *Clostridium* genomes available, we found only that only one genome (0.002% of total) possessed both Ech and Rnf. Our re-analysis of all genomes of short-chain fatty acid from Worm et al. (2014) revealed none possessed all subunits of Ech and Rnf. In our re-analysis of 10 genomes from Weghoff et al. (2015; the other 10 genomes were not explicitly identified by the authors), we did not find any genomes possessing both Ech and Rnf. In our own analysis of rumen prokaryotes, only 4.6% of non-butyrivibrio genomes were predicted to possess both Ech and Rnf. One genome belonged to the *Desulfovibrio*, and all others belonged to the Lachnospiraceae, of which butyrivibrio are members (Cotta and Forster, 2006). From present analyses, possession of both Ech and Rnf would appear rare outside of the butyrivibrios, related organisms, and a few sulfate-reducers.

Rather than being possessed together, Ech and Rnf are usually possessed separately and appear to substitute in function. This point is made by the reductive acetogens. Three model acetogens (*A. woodi*, *C. ljungdahlii*, *M. thermoacetica*) carry out similar pathways of reductive acetogenesis, except the former 2 possess Rnf and the lattermost possesses Ech (Buckel and Thauer, 2013; Schuchmann and Müller, 2014).

Although most butyrivibrio genomes possessed all subunits of Ech and Rnf, some genomes did not, and these exceptions may help in discriminating between butyrivibrio species. Butyrivibrios make up a genetically diverse group (Cotta and Forster, 2006), but few properties are related to phylogenetic position and can be used to discriminate between different species or phylotypes. Some discriminatory properties previously suggested include butyrate kinase activity, lipase activity, products of linoleate biohydrogenation, and sensitivity to linoleate (Paillard et al., 2007). Other suggested discriminitory properties, such as substrate utilization and cell fatty acid composition, have been criticized as inadequate (Cotta and Forster, 2006; Willems and Collins, 2009). We suggest that absence of Ech subunits may serve as another discriminatory property. Absence of EchD may be a useful property, for example, as all six genomes classified as *B. fibrisolvens* were missing EchD alone; this absence was observed in only one other genome (an unclassified *Butyrivibrio* sp.). Absence of EchD would be even more meaningful if later associated with a phenotype (e.g., absent or altered Ech activity). More discriminatory properties might emerge once our results can be compared to a full a phylogenetic tree of Hungate 1000 strains (full-length 16S rDNA sequences are not yet available).

The possession of both Ech and Rnf by most rumen butyrivibrios suggests a functional importance to this group. Likely, it permits high ATP yields from fermentation and confers an energetic advantage. Such an energetic advantage could help support the metabolic flexibility observed for members of this group. Butyrivibrio strains are capable of degrading an unusually wide range of carbohydrates, and many can degrade protein (Hungate, 1966; Stewart et al., 1997; Cotta and Forster, 2006). Such metabolic flexibility comes at the cost of producing a host of degradative enzymes, but this cost may be offset by a high ATP yield from ETP. If high ATP yields are not required (e.g., during growth limitation), upregulation of PflD, downregulation of NifJ, and upregulation of methylglyoxal pathway enzymes could lower ATP yield (e.g., to prevent energy spilling; Hackmann and Firkins, 2015). The unique combination of genes supporting ETP makes butyrivibrio strains attractive for further study as model anaerobic bacteria. Further, the majority of rumen prokaryotes possess at least Rnf or Ech, suggesting ETP has importance in the rumen beyond just the butyrivibrios and merits further study in itself.

Hypothesis for Varying Linoleic Acid Toxicity by the Butyrivibrios

Figure 4 balances reducing equivalents using PflD (**Figure 3**). Although Fd_{red} produced from NifJ (**Figure 1**) could be reoxidized using a cytosolic FeFe hydrogenase as depicted in Louis and Flint (2009), we could find little evidence for such a role in the butyrivibrio draft genomes. The NAD_{red} needed for synthesis of palmitate required extra acetate production because butyrate production would have reoxidized that NAD_{red} (**Figures 1** and **3**); we note that acetate could be reused for those butyrivibrios producing butyrate through butyryl-CoA acetyl-CoA transferase (Diez-Gonzalez et al., 1999). The ATP loss in **Figure 4** compared with **Figure 1** through **Figure 3**

(if converted to seven moles of glucose) is consistent with the expectation of considerable ATP sparing if exogenous fatty acids are incorporated in rumen bacterial membranes (Wu and Palmquist, 1991; Vlaeminck et al., 2006).

The ATP yield per glucose can be maintained with moderate rumenate reductase activity during biohydrogenation (**Figure 2**), but a high dose of LA increases the lag for growth in *B. fibrisolvens* JW11 (Maia et al., 2010). In contrast with an apparent constitutive expression of the cluster of genes used in butyrate production (Asanuma et al., 2005), unsaturated fatty acids probably increase expression of rumenate reductase (Fukuda et al., 2007) for *B. fibrisolvens*. Biohydrogenation activity was assumed to depend on provision of reducing equivalents from the EMP pathway (Kim, 2003). Our hypothesis builds on the foundations that bolus-dosed LA disrupts synthesis of fatty acids needed to generate membranes during growth of the butyrivibrios because of competition for acetyl-CoA and reducing equivalents.

The butyrivibrios use the standard enzymes for fatty acid (**Figure 4**) and phosphatidic acid synthesis for membrane components (Parsons and Rock, 2013). They discussed that FabH often has species-specific affinity for priming units (often for unsaturated or branched-chain fatty acids), but FabH was only predicted in 66% of the butyrivibrio draft genomes. For mixed rumen bacteria, synthesis of *iso* and *anteiso* fatty acids (which increase fluidity) is thought to be fixed within species, whereas synthesis of even- and odd-chain fatty acids is a function of availability of primers (Vlaeminck et al., 2006).

Jenkins et al. (2008) extended the butyrivibrio taxonomic grouping of Paillard et al. (2007) into three groups. The *B. fibrisolvens* and *Pseudobutyrivibrio* clades stop biohydrogenation of LA at VA (i.e., the VA1 group) and consume acetate to produce butyrate through a butyryl-CoA acetyl-CoA transferase. The *B. hungatei* clade also stops biohydrogenation at VA (i.e., the VA2 group). The *B. proteoclasticus* clade biohydrogenates LA fully to SA (i.e., the SA group). Compared with the VA1 group, the VA2 and SA groups are more sensitive to LA, and both produce butyrate through butyrate kinase. Consistent with this pattern, Kopecný et al. (2003) grouped >40 isolates of butyrivibrios based on fatty acid profile. *B. fibrisolvens*, *P. ruminis*, and *P. xylanivorans* in the VA1 group have higher 16:0 and have no *iso*-16:0 or *anteiso*-17:0; in contrast, *B. hungatei* (VA2 group) and *B. proteoclasticus* (SA group) have much lower 16:0 and higher *anteiso*-17:0, and *B. proteoclasticus* has particularly high *iso*-14:0 and *anteiso*-15:0 concentration. Because of the increased branched (*iso* and *anteiso*) fatty acids, VA2 and especially SA members should be more sensitive to the fluidization from LA permeabilizing their membranes, which are conspicuously thin (Maia et al., 2010). In response, *de novo* synthesis of saturated fatty acids from acetyl-CoA might increase to counter the increased membrane fluidity.

In the rumen, the butyrivibrios must be in close proximity to their substrate, plant hemicelluloses (Kelly et al., 2010). Although not studied specifically with butyrivibrios, mixed particulate-phase bacteria incorporated LA at about double that of fluid-phase bacteria harvested from the rumen after soybean oil was fed, and non-esterified fatty acids were internalized (Bauchart et al., 1990). Both α- or β-oxidation of acyl-CoA is minimal in mixed ruminal bacteria (Wu and Palmquist, 1991). Those authors discussed the likelihood that fatty acids <14 carbons were elongated, but exogenous palmitic acid and SA replaced *de novo* synthesis with little net change in fatty acid composition after dosing fat containing LA. Because butyrivibrios have little 18-carbon fatty acids in their membranes (Kopecný et al., 2003) compared with the much larger percentage (>40% SA) in composites of mixed rumen bacteria (Or-Rashid et al., 2007), the limited β-oxidation of SA to palmitic acid might render dietary 18-carbon fatty acids of little benefit to butyrivibrios.

Our hypothesis assumes that *de novo* synthesis of palmitate would be feedback-interrupted by intracellular LA (**Figure 4**). Because cultures must be growing to biohydrogenate fatty acids at concentrations that are toxic to stationary phase cultures (McKain et al., 2010), fatty acid synthesis presumably is induced in coordination with growth. The series of enzymes in **Figure 4** (from AccABCD to FabK) is the same as that repressed by FabT in other bacteria (Parsons and Rock, 2013). However, they noted that FabT only has a moderating effect. We could not verify if FabT is possessed by butyrivibrios because FabT is not found in KEGG, COG, pfam, or other databases. Non-esterified LA and other long chain unsaturated fatty acids—but not saturated fatty acids—allosterically inhibited FabI (catalyzing the same reaction as FabK) and inhibited ^{14}C-acetate incorporation into membrane lipids of *Staphylococcus aureus* (Zheng et al., 2005). Although FabK is a flavoprotein (unlike FabI), LA inhibition was a result of both its binding to the enzyme and the enzyme-reduced cofactor complex. Because FabI rate-limits fatty acid synthesis, ACP-bound intermediates accumulated from sustained acetyl-CoA carboxylase activity (Cronan, 2014). Consequently, allosteric inhibition by non-esterified LA (i.e., not bound to ACP) of FabK might increase pooling of acyl-ACP intermediates in butyrivibrios after LA concentration at the cell membrane exceeds their membrane-associated biohydrogenation capacity and is internalized.

Although we assumed reduced nucleotide cofactors are interconvertible in **Figure 4**, cellular mechanisms to balance reducing equivalents might explain why lactate accentuated LA toxicity in butyrivibrios (Paillard et al., 2007). Cytosolic SthA or membrane-associated PntAB transhydrogenases (Fuhrer and Sauer, 2009) were not recovered from the butyrivibrio draft genomes, and NfnAB (Buckel and Thauer, 2013) has not yet been annotated by KEGG or COG for our search. Fluxing of acetyl-CoA though NADP-isocitrate dehydrogenase to α-ketoglutarate would produce $NADP_{red}$, but some $NADP_{red}$ might be reoxidized to synthesize glutamate for amino acid biosynthesis (Supplementary Figure S2). *C. thermocellum* produces $NADP_{red}$ through malic enzyme and malate dehydrogenase cycling (Burton and Martin, 2012). All of those enzymes (Supplementary Figure S2) were uniformly in the butyrivibrio draft genomes except for the enzyme critical to complete a cycle, malate dehydrogenase (uniformly absent). However, other enzymes

involved in pyruvate metabolism (especially oxaloacetate decarboxylase and pyruvate-phosphate dikinase) should be investigated for potential to substitute for lack of Eno (see earlier discussion) in production of oxaloacetate for anabolic reactions such as amino acid synthesis. β-hydroxybutyrl-CoA dehydrogenase was proposed as a major supplier of $NADP_{red}$ for anabolic reactions in *B. fibrisolvens* D1 (Miller and Jenesel, 1979). Cycling of forward (oxidizing NAD_{red} when producing β-hydroxybutyrl-CoA) and backward (reducing $NADP_{ox}$ when producing acetoacetyl-CoA) reactions would supply $NADP_{red}$ for fatty acid synthesis and also explain how the acetoacetyl-CoA pools were maintained while other acyl-CoA pools used in butyrate production were dramatically depleted after LA dosing (Maia et al., 2010). Because of reverse Ldh activity after a large dose of lactate, a large increase in NAD_{red} could thermodynamically decrease this cycling to produce $NADP_{red}$ for FabG (**Figure 4**).

Further biochemical characterization is needed to understand how butyrivibrios respond metabolically to high LA challenge coinciding with high carbohydrate availability to circumvent lactate-tolerant bacteria from biohydrogenating LA through alternate *trans*-10 18:1 pathways (McKain et al., 2010). However, the extra ATP yield from mechanisms described in **Figures 1** and **2** likely help the butyrivibrios recover from conditions in which LA exceeds biohydrogenation capacity.

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Supplementary Material

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Toward the identification of methanogenic archaeal groups as targets of methane mitigation in livestock animals

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In herbivores, enteric methane is a by-product from the digestion of plant biomass by mutualistic gastrointestinal tract (GIT) microbial communities. Methane is a potent greenhouse gas that is not assimilated by the host and is released into the environment where it contributes to climate change. Since enteric methane is exclusively produced by methanogenic archaea, the investigation of mutualistic methanogen communities in the GIT of herbivores has been the subject of ongoing research by a number of research groups. In an effort to uncover trends that would facilitate the development of efficient methane mitigation strategies for livestock species, we have in this review summarized and compared currently available results from published studies on this subject. We also offer our perspectives on the importance of pursuing current research efforts on the sequencing of gut methanogen genomes, as well as investigating their cellular physiology and interactions with other GIT microorganisms.

Keywords: methanogens, 16S rRNA analysis, herbivores, rumen microbiology, methane mitigation

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Introduction

In herbivores, fermentation of feed by mutualistic gastrointestinal tract (GIT) communities of microorganisms is essential for proper nutrition of their hosts (Hungate, 1966). These microbial communities consist of a great number of species from phylogenetically diverse groups, mainly bacteria, archaea, protozoa, and fungi, that are mutually dependent through complex trophic relationships (Wolin, 1979). As a result of the collective activities of these microorganisms, polysaccharides, proteins, and lipids are metabolized into end products such as volatile fatty acids (VFAs) that are assimilated by their host to fulfill their energy needs.

Certain products of microbial fermentation, such as carbon dioxide and methane, are not absorbed by the host and are released into the environment. There are two main concerns over methane emissions by livestock animals. First, they have a negative impact on animal productivity, as this process results in lost energy from the host, which can range between 2 and 12% of an animal's energy intake (Johnson and Johnson, 1995). Secondly, methane is a much more potent greenhouse gas than carbon dioxide, thus having a greater effect on climate change (Lashof and Ahuja, 1990). Since the continuous growth of the human population is expected to result in an increase in the number of domesticated ruminants, decreasing methane emissions by livestock has become a priority and an integral part of climate control policies (Thorpe, 2008).

Methane is synthesized by obligate anaerobic archaea that share methanogenesis as part of their energy metabolism (Liu and Whitman, 2008). Many methanogenic archaea, or methanogens, use H_2 and CO_2 as substrates to synthesize methane, with certain species also capable of metabolizing small organic compounds such as formate, methanol, methylamines, or acetate (Thauer et al., 2008). Although they do not contribute to fulfilling their host's energy requirements, methanogens play an important role in the GIT of herbivores by maintaining the fermentative performance of the microbial community. By metabolizing H_2 generated from fermentation of plant polysaccharides, methanogens function as a sink to maintain a low H_2 pressure, which promotes plant fiber digestion by protozoa and bacteria (Wolin, 1982).

As the only producers of enteric methane, methanogens are responsible for the contribution of livestock industries to climate change (Thorpe, 2008), and have thus become the focus of research toward developing mitigation strategies. Variations in methane emissions according to host and/or diet present an important challenge toward achieving this goal. For instance, an early study reported that gray kangaroos emitted less methane than sheep fed the same diet (Kempton et al., 1976). Similarly, lower levels of methane were observed for camelids compared to ruminant livestock (Pinares-Patino et al., 2003; Dittmann et al., 2014), and Franz et al. (2010) found that methane emissions were higher in sheep compared to ponies. Methane production has been found to increase on higher forage/cellulose diets, especially when comparing grass forage to legume forage (McAllister et al., 1996). In growing beef cattle, methane emissions were not affected by the type of grain fed during backgrounding, but they were found to be lower for corn compared to barley during the finishing phase (Beauchemin and McGinn, 2005). In contrast, the addition of high quality feeds, oils, plant secondary compounds, or microbial modifiers can reduce methane emissions (Lovett et al., 2003; Woodward et al., 2004; Carulla et al., 2005; Puchala et al., 2005; Beauchemin et al., 2007, 2009; Grainger et al., 2009, 2010). In most cases, this variation does not appear to be due to differences in methanogen cell density, but rather in the composition of the methanogen community. For instance, it was observed during anti-methanogen vaccination trials that, while methane emissions from immunized animals were decreased in early stages, they returned to control levels after prolonged immunization (Wright et al., 2004). The vaccine was expected to target methanogens that were highly represented in the rumen microbial community, which may have allowed other methanogens that would otherwise be at a disadvantage to increase in abundance in the rumen of immunized animals (Williams et al., 2009). Based on these results, it was hypothesized that GIT methanogen communities may consist of different groups that could vary in their potential for growth and methane production.

Since the composition of a methanogen community represented a likely determinant of its capacity to produce methane, the investigation of mutualistic methanogen communities in the GIT of a variety of host herbivores or in response to different diets has been the subject of active and ongoing research. As with other fields in environmental microbiology, research on GIT methanogens has benefited greatly from the rapid technological improvements of culture-independent experimental approaches. In this review, we have summarized and compared data available from published studies on the composition and representation of methanogens in the GIT of herbivores. While they tend to be distinct, according to a variety of factors including host breed, species, diet and geographical location, and by mechanisms that remain poorly characterized (Kim et al., 2011), the data also indicate that GIT methanogens form phylogenetic clusters that exhibit a certain degree of overlap among different communities.

Prevalent Methanogens in the GIT Communities of Herbivores

Archaea have been identified in a wide range of habitats (Liu and Whitman, 2008), forming a large and diverse prokaryotic domain, not only ecologically but also phylogenetically. The majority of currently known archaeal species have been assigned to the phyla Euryarchaeota or Crenarchaeota, but additional phyla have been proposed to account for the high degree of divergence found in certain archaea, including Thaumarchaeota, Nanoarchaeota, Korarchaeota, Parvarchaeota, and Aigarchaeota (Shin et al., 2004; Allers and Mevarech, 2005; Brochier-Armanet et al., 2008, 2011; Nunoura et al., 2011; Rinke et al., 2013; Petitjean et al., 2014; Raymann et al., 2015). All currently known methanogens belong to the phylum Euryarchaeota, which is divided into seven orders (Methanobacteriales, Methanocellales, Methanococcales, Methanomassiliicoccales, Methanomicrobiales, Methanosarcinales, and Methanopyrales), that include 10 families and 31 genera (Liu and Whitman, 2008; Sakai et al., 2008; Paul et al., 2012; Iino et al., 2013). Methanogens have colonized as a group a wide variety of anaerobic environments, including marine and freshwater sediments, soil, and landfills, and are thus not limited to just the GIT of animals.

The 16S rRNA gene is the most commonly used phylogenetic marker for the characterization of bacterial and methanogen communities (Skillman et al., 2006; Rajendhran and Gunasekaran, 2011). Thus, the data we have selected on methanogen composition from the gut of herbivorous animals was generated using 16S rRNA gene clone libraries or next generation sequencing of amplicons. Typically, a minority of the GIT archaeal 16S rRNA gene sequences identified to date are identical to validly characterized methanogens species, while the remaining majority of sequences exhibit a varying degree of relation to methanogen species. Despite their diversity, GIT methanogens group into very distinct phylogenetic clusters of archaea (Kim et al., 2011). In this section, we aim to present the major groups of methanogens that have been identified in the GIT of herbivores.

Methanobrevibacter-Related Archaea

16S rRNA gene sequences closely related to certain species belonging to the genus *Methanobrevibacter* (order Methanobacteriales) are among the most frequently found

in GIT samples from livestock animals. While representation can vary according to host species, diet, and/or geographical location, dominance of *Methanobrevibacter*-related archaea reported by a number of different studies is quite striking. Indeed, *Methanobrevibacter*-related methanogens represented more than ∼80% of 16S rRNA gene sequences from hosts ranging from birds [hoatzin (Wright et al., 2009)] and marsupials [wallaby-May sample (Evans et al., 2009)] to pseudo-ruminants [alpaca (St-Pierre and Wright, 2012), Bactrian camel (Turnbull et al., 2011)] and ruminants [buffalo – Mediterranean breed (Franzolin et al., 2012), cattle-New Zealand (Seedorf et al., 2015), dairy cattle (Hook et al., 2009, 2011; King et al., 2011), goats (Cunha et al., 2011), impala (Cersosimo et al., 2015), reindeer-Norway (Sundset et al., 2009a), sheep-Venezuela (Wright et al., 2008), sheep-Scotland (Snelling et al., 2014), sheep-New Zealand (Seedorf et al., 2015), and yak (An et al., 2005)]. In other studies, they represented a lower, but well represented proportion (27–60%) of identified clones or sequence reads in cattle (Whitford et al., 2001; Skillman et al., 2006), reindeer-Svalbard (Sundset et al., 2009b), white rhinoceroses (Luo et al., 2013), Chinese roe deer (Li et al., 2014), and Mehsani water buffaloes (Singh et al., 2015). Since characterized species of *Methanobrevibacter* mainly use H_2 and CO_2 as substrates for methanogenesis, it is hypothesized that uncultured *Methanobrevibacter*-related methanogens identified by their 16S rRNA gene sequence are also hydrogenotrophic.

Currently, 15 cultured *Methanobrevibacter* species have been characterized according to the List of Prokaryotic Names with Standing in Nomenclature (LPSN). However, GIT *Methanobrevibacter*-related methanogens from livestock animals tend to be more closely related to either *Methanobrevibacterruminantium*, *Methanobrevibacter millerae*, *Methanobrevibacter gottschalkii,* or *Methanobrevibacter smithii*. While typically found in lower frequency, 16S rRNA sequences with closest identity to either *Methanobrevibacter olleyae*, *Methanobrevibacter thaueri,* or *Methanobrevibacter wolinii* have also been reported in GIT samples. *Methanobrevibacter boviskoreani* has been the latest addition to the list of cultured rumen methanogens from this group (Lee et al., 2013), with *Methanobrevibacter wolinii* as its closest relative. To our knowledge, *Methanobrevibacter woesei* related methanogens have only been reported in chickens, and 16S rRNA gene sequences from the GIT of herbivores that are related to either *Methanobrevibacter curvatus, Methanobrevibacter cuticularis, Methanobrevibacter oralis, Methanobrevibacter arboriphilus, Methanobrevibacter filiformis,* or *Methanobrevibacter acididurans* have only rarely if ever been identified in this environment.

SGMT-RO Population Model for *Methanobrevibacter*-Related Methanogens

While it appears that most *Methanobrevibacter*-related GIT 16S rRNA gene sequences tend to be closely related to a limited number of valid *Methanobrevibacter* species, they exhibit a remarkable level of diversity that has been estimated to be in the 100s of species-level operational taxonomic units (OTUs; Kim et al., 2011). Indeed, the level of sequence identity for *Methanobrevibacter*-related 16S rRNA gene sequences can typically vary between 90 and 100% with their respective closest valid methanogens species. Therefore, although GIT methanogens are from similar phylogenetic groups, they appear to form a continuum of species rather than discrete groups (Janssen and Kirs, 2008). However, only a subset of OTUs are identified in each sample, with typically a few OTUs that tend to be more abundant (Wright et al., 2007, 2009; Sundset et al., 2009a,b; Hook et al., 2011; King et al., 2011; Turnbull et al., 2011; Franzolin et al., 2012; St-Pierre and Wright, 2012; Snelling et al., 2014; Cersosimo et al., 2015; Seedorf et al., 2015). To facilitate the creation of GIT methanogen community structure models from environmental samples, sequence identity cutoffs can be set at specific levels to group 16S rRNA genes from methanogens of the same presumptive species or of the same presumptive genus. The representation of each category in an environmental sample can thus be expressed as a percentage of the total number of clones or sequence reads identified in its corresponding study. Methanogen communities can then be compared between host breeds, species, feed regimens, and/or geographical locations. While there is currently no absolute 16S rRNA gene sequence identity cutoff that has been set to formally distinguish methanogens of the same species or genus from uncultured archaea, it remains a very useful tool to uncover various trends in archaeal community composition.

As a complementary approach, we have also explored the use of phylogenetic analyses of *Methanobrevibacter*-related GIT 16S rRNA gene sequences to create community structure models. While they appear to form a continuum of species, we observed that *Methanobrevibacter*-related GIT 16S rRNA gene sequences are mostly distributed between two large clades. One clade consists of sequences that are closely related to *Methanobrevibacter smithii*, *Methanobrevibacter gottschalkii*, *Methanobrevibacter millerae* or *Methanobrevibacter thaueri*, which we have referred to as the smithii – gottschalkii – millerae – thaurei clade, or simply as the SGMT clade. The other major clade groups *Methanobrevibacter ruminantium* and *Methanobrevibacter olleyae* – like sequences, which we have referred to as the ruminantium – olleyae or RO clade.

After re-examining available data by our research team and other research groups to compare the sequence distribution between the SGMT clade and the RO clade, we were able to group samples from a wide variety of sources into more encompassing categories (**Table 1**). For instance, the SGMT clade was clearly more dominant than the RO clade in impalas (Cersosimo et al., 2015), wallabies (May sample; Evans et al., 2009), in two separate studies involving Holstein dairy cows (Hook et al., 2009, 2011), in alpacas (St-Pierre and Wright, 2012), in water buffaloes (Franzolin et al., 2012), in sheep from Venezuela (Wright et al., 2008), in sheep from Scotland (Snelling et al., 2014), in New Zealand sheep fed two different diets (Seedorf et al., 2015), in Chinese roe deer (Li et al., 2014), and in reindeers (Norway and Svalbard; Sundset et al., 2009a,b). In contrast, the RO clade was distinctively more highly represented than the SGMT clade in the hoatzin (Wright et al., 2009), in an early analysis involving Holstein dairy cows (Whitford et al., 2001), in corn-fed beef

TABLE 1 | Representation of SGMT and R0 methanogens in different hosts and diets.

Host	SGMT[a] (%)	RO[a] (%)	Reference
Alpacas	70.0	17.6	St-Pierre and Wright (2012)
Bactrian camel (Potter sample)	30.2	66.0	Turnbull et al. (2011)
Bactrian camel (Southwick sample)	80.0	18.2	Turnbull et al. (2011)
Beef cattle (corn diet)	4.0	48.0	Wright et al. (2007)
Beef cattle (potato diet)	28.9	21.1	Wright et al. (2007)
Cattle (April 2010)[c]	38.0	49.0	Seedorf et al. (2015)
Cattle (September 2010)[d]	48.0	38.0	Seedorf et al. (2015)
Chinese roe deer (rumen)	77.0	1.0	Li et al. (2014)
Chinese roe deer (cecum)	68.0	1.0	Li et al. (2014)
Dairy cows (Holstein)	0.0	58.5	Whitford et al. (2001)
Dairy cows (Holstein)	65.7	32.5	Hook et al. (2009)
Dairy cows (Holstein)	93.4	5.9	Hook et al. (2011)
Dairy cows (Holstein)[b]	36.0	59.0	King et al. (2011)
Dairy cows (Jersey)	13.3	33.3	Skillman et al. (2006)
Dairy cows (Jersey)[b]	53.0	44.0	King et al. (2011)
Hoatzin	0.0	85.8	Wright et al. (2009)
Horses (pasture)[e]	0.0	66.3	Fernandes et al. (2014)
Horses (forage-grain)[e]	1.4	63.0	Fernandes et al. (2014)
Impalas	93.0	2.9	Cersosimo et al. (2015)
Sheep (lucerne)[f]	42.0	20.0	Seedorf et al. (2015)
Sheep (pasture)[g]	55.0	33.0	Seedorf et al. (2015)
Sheep (Scotland)	75.5–91.6	0.0–1.4	Snelling et al. (2014)
Sheep (Venezuela)	62.5	32.7	Wright et al. (2008)
Reindeer (Norway)	50.0	31.5	Sundset et al. (2009a)
Reindeer (Svalbard)	44.8	2.3	Sundset et al. (2009b)
Wallabies (May sample)	91.6	0.0	Evans et al. (2009)
Water buffaloes	62.5	28.1	Franzolin et al. (2012)

[a] *Representation presented as a percentage of the total methanogen population.*
[b] *Dairy cows of both breeds were maintained as a single herd under the same diet and environmental conditions.*
[c] *Values presented are the median of n = 15, as reported by Seedorf et al. (2015).*
[d] *Values presented are the median of n = 16, as reported by Seedorf et al. (2015).*
[e] *Values presented are the median of n = 6, as reported by Fernandes et al. (2014).*
[f] *Values presented are the median of n = 11, as reported by Seedorf et al. (2015).*
[g] *Values presented are the median of n = 8, as reported by Seedorf et al. (2015).*

cattle (Wright et al., 2007), in Jersey dairy cows (Skillman et al., 2006), and in horses fed a pasture or forage-grain diet (Fernandes et al., 2014). Notably, only a few studies have reported a balanced SGMT:RO, such as from potato-fed beef cattle (Wright et al., 2007).

In some reports, comparative studies have revealed opposite SGMT:RO population composition as a function of breeds or as a function of environmental factors within the same breed. This was observed in Holstein and Jersey dairy cows from the same herd maintained under common environmental conditions (King et al., 2011), as well as in cattle from New Zealand sampled at two different time points (Seedorf et al., 2015). In captive Bactrian camels sampled from zoological parks at two different locations in the USA, the SGMT:RO ratio for hindgut methanogens showed an opposite population structure pattern between the two sampled communities (Turnbull et al., 2011).

Dividing sequences between SGMT and RO clades can also help in uncovering differences in community structure between GIT samples that have a similar representation of *Methanobrevibacter*-related sequences. For instance, while they account for 93.0 and 85.8% of methanogens identified in sheep from Venezuela and in the hoatzin, *Methanobrevibacter*-related sequences have a completely opposite SGMT:RO distribution in these hosts. While additional studies are required to elucidate the respective contributions of host species genetics and environmental factors in the determination of whether the SGMT or the RO clade will be the most highly represented in a methanogen community, they may represent archaeal groups that thrive in different conditions. For instance, factors such as rumen or forestomach pH, tolerance to toxic compounds, and the rate of passage can act as selection agents, either individually or in combination, by promoting the growth of particular groups of methanogens, thereby affecting the population structure of the archaeal community (Janssen and Kirs, 2008). In this context, the natural division of *Methanobrevibacter*-like sequences into the SGMT and RO clades allows a higher level of specificity in developing population structure models for GIT methanogens that take into account phylogeny and representation, which can then be tested for methane production under controlled

conditions *in vivo* or *in vitro*. This strategy could prove to be very valuable in the design of broad range mitigation strategies in the future.

Other Methanogen Groups Commonly Identified in the GIT of Herbivores

In addition to *Methanobrevibacter*-related methanogens, other archaeal phylogenetic groups have also been frequently reported in herbivore GIT samples. Indeed, members of the order Methanomassiliicoccales (Iino et al., 2013), a group of methanogens also referred to as rice cluster III (Kemnitz et al., 2005), rumen cluster C (Janssen and Kirs, 2008) or Methanoplasmatales (Paul et al., 2012), are also a prominent group of GIT methanogens. Not only are they frequently found in GIT samples from livestock animals, they have also been found to be a highly prevalent type of archaea in the rumen environment. This has been the case in wallabies sampled in November (91.7%; Evans et al., 2009), sheep from Australia (80.8%; Wright et al., 2006), yak from China (79.4%; Huang et al., 2012), Svalbard reindeer (47.4%; Sundset et al., 2009b), and in beef cattle fed either a potato (50.0%) or corn (46.1%) diet (Wright et al., 2007). Rumen methanogens from this taxonomic group have been reported to use methylamines as substrates for methanogenesis (Poulsen et al., 2013). Since compounds such as betaine and choline have been shown to be metabolized by rumen bacteria to produce methylamines (Bradbeer, 1965; Neill et al., 1978; Mitchell et al., 1979; Moller et al., 1986; Eklund et al., 2005), their presence in certain feedstuffs such as molasses and wheat derived products, or their use as feed additives, may favor the prevalence of Methanomassiliicoccales methanogens in a rumen environment. Paul et al. (2012) also reported that uncultured Methanomassiliicoccales methanogens could be enriched from the gut of higher termites when methanol was used as a substrate for methanogenesis.

Since they have been found to be highly prevalent in host species that can also have a high representation of *Methanobrevibacter*-related methanogens, this information is necessary to generate more comprehensive models for methanogen populations in the GIT of herbivores, such as perhaps be incorporated with the SGMT-RO model. Interestingly, sequences from specific habitats tend to be associated with certain clades (Paul et al., 2012; Seedorf et al., 2014). However, the limited number of isolates or representative 16S rRNA gene sequences that are available may not currently allow the same level of resolution that can be obtained with *Methanobrevibacter*-related sequences (Seedorf et al., 2014).

While they are in general less abundant than *Methanobrevibacter*-related or Methanomassiliicoccales sequences, 16S rRNA gene sequences that are more closely related to other methanogen species, such as *Methanosphaera stadtmanae* and *Methanomicrobium mobile*, or genera, such as *Methanoculleus* and *Methanosarcina,* have also been identified in the GIT of herbivores. While they are usually detected at a low frequency, they have in some studies been shown to be the most prevalent methanogens under certain conditions. For instance, from studies conducted in India, 94.4% of 16S rRNA gene sequences identified in the rumen of Murrah buffaloes were closely related to *Methanomicrobium mobile* (Chaudhary and Sirohi, 2009), and abundances of 97.1 and 72.3% of the same methanogen group were reported in Surti buffaloes (Singh et al., 2011, 2013). Furthermore, archaea belonging to the order Methanomicrobiales were predominant in the GIT of Japanese local ponies and thoroughbred horses (Lwin and Matsui, 2014). It remains to be determined why these methanogens were so prevalent in these particular conditions while they are usually detected at a much lower frequency. *Methanosphaera stadtmanae* was found to be the most prevalent methanogen in the hindgut of captive orangutans (Facey et al., 2012). This methanogen species has a limited substrate range for methane synthesis, and is notably unable to use H_2 and CO_2 for this purpose. Digestion of fruit pectin in frugivores like the orangutan has been hypothesized to increase GIT concentrations of methanol and acetate, which would provide a favorable environment for *Methanosphaera stadtmanae* methanogens to thrive. Finally, *Methanocorpusculum labreanum* was found to be the most abundant (59.9%) in the hindgut of captive white rhinoceroses (Luo et al., 2013). The identification and predominance of this type of methanogen in a GIT environment is unusual compared to most other reported studies. Predominance of *Methanocorpusculum* has also been reported in the fecal microbiota of Irish Thoroughbred racehorses (O'Donnell et al., 2013), but, as pointed out by the authors of that study, the use of the 16S rRNA gene V4 region may have underestimated archaeal diversity. Another study on equine fecal microbiota found that *Methanocorpusculum*-related methanogens were co-abundant with *Methanobrevibacter*-related methanogens (Fernandes et al., 2014). *Methanocorpusculum* archaea were observed at a median of 17.7% in horses fed a forage-grain diet, and at a median of 31.9% in horses maintained on pasture. They were only found to be more abundant than *Methanobrevibacter*-related methanogens in samples collected 4 days after a transition from a forage-grain diet to pasture had occurred.

Future Perspectives on GIT Methanogen Research in Herbivores

Sequencing of GIT Methanogen Genomes

Progress in biological research is often the result of technological advancements that improve experimental approaches. Numerous investigations of GIT methanogen communities to date have been performed using denaturing gradient gel electrophoresis (DGGE) analyses or Sanger sequencing of clone libraries, which both have intrinsic limitations in scope and resolution. However, next-generation sequencing has greatly improved the scope of microbial ecology studies, providing more comprehensive sequence datasets as well as allowing analysis of more independent samples and replicates (Denman and McSweeney, 2015).

While great strides have been made in characterizing the taxonomic composition of rumen and GIT methanogen communities, there remains a critical need to further our understanding of their metabolism and cellular physiology, particularly for species or candidate species that tend to be the

most abundant. This knowledge would greatly contribute to the development of practical mitigation strategies. By revealing the biochemical potential of an organisms through prediction of its proteome, genome sequencing represents an effective strategy to elucidate the physiology of poorly characterized organisms. In terms of methane mitigation, it could for instance allow the identification of enzymes whose activity may be targeted with chemical antagonists, or surface proteins that may be used as antigens for the production of antibodies. Whether the devised strategies directly target methanogenesis, aim at reducing growth rates of methanogens or antagonize interactions with other microorganisms, they each have the potential to reduce enteric methane production.

Representative genomes of methanogens that have been identified in the GIT of livestock are currently limited in number. For instance, *Methanobrevibacter ruminantium* (Leahy et al., 2010) and *Methanobrevibacter smithii* (Samuel et al., 2007) are the only GIT *Methanobrevibacter* for which genomic data and predicted proteomes have been described in peer-reviewed publications. Permanent drafts for *Methanobrevibacter boviskoreani* and *Methanobrevibacter wolinii* are available, while efforts to complete the genomes of *Methanobrevibacter millerae* and *Methanobrevibacter olleyae* are ongoing. Both *Methanobrevibacter gottschalkii* and *Methanobrevibacter thaurei* have been selected to have their genome sequenced [see the Joint Genome Institute (JGI), Genomes Online Database (GOD)[1]]. As discussed in previous sections, these methanogens together represent the most common or abundant GIT archaea in livestock animals.

For GIT intestinal methanogens belonging to the order Methanomassiliicoccales, three genomes have so far been reported, all from isolates cultured from human feces: *Methanomassiliicoccus luminyensis* (Dridi et al., 2012), *Methanomassiliicoccus alvus* (Borrel et al., 2012), and *Methanomassiliicoccus intestinalis* (Borrel et al., 2013). Once sequence information from Methanomassiliicoccales representatives isolated from livestock become available, it will be of great interest to compare their genome with the human isolates. Available genomes of methanogens that are generally less well represented in GIT environments include species from the genera *Methanosarcina* (Deppenmeier et al., 2002; Galagan et al., 2002; Maeder et al., 2006), *Methanosphaera* (Fricke et al., 2006), *Methanocorpusculum* (Anderson et al., 2009), and *Methanomicrobium mobile* (see JGI-GOD[1]).

Analysis of the *Methanobrevibacter ruminantium* genome is a good example of the information that can be obtained from predicting the proteome of a methanogen (Leahy et al., 2010). For instance, it revealed the ability to use formate in addition to H_2 as a substrate for methanogenesis, showed that this organism is unable to synthesize coenzyme M, and provided a metabolic explanation for the requirement of acetate for growth. It also uncovered a large array of genes encoding putative adhesins, and identified loci related to phage genes. In addition, this genomic information can also be used as a reference for metagenomics and metatranscriptomics analyses in GIT environments.

[1]https://gold.jgi-psf.org/

While this technology is providing an unprecedented capacity for genome sequencing, as attested by the increasing number of published microbial genomes, the complete and accurate determination of a prokaryotic genome is not a trivial undertaking and requires research teams adept in technical and bioinformatic skills. In addition, an important limitation in this process is the isolation and cultivation of methanogens, which remain a challenge for many strains. Therefore, genomes to sequence should be strategically selected considering the wide diversity of methanogens that populate the GIT of herbivores. In this context, population structure studies such as summarized in this review that are based on representation and phylogeny provide a critical basis in the selection of methanogens of interest.

In the long term, providing an increased number of available GIT methanogens genomes is essential for the development of effective and comprehensive mitigation strategies. Since the use of entire genome sequences dramatically improves phylogenetic analysis of archaea compared to only using 16S rRNA gene sequences (Brochier-Armanet et al., 2011), this will allow the accurate identification of phylogenetic nodes that are shared by clusters of GIT methanogens, which can be targeted for mitigation. In addition, comparative genome analyses will reveal conserved proteins within phylogenic clusters of methanogens, such as surface molecules that can be targeted by vaccination or intracellular factors that can be targeted for chemical inhibition. Alternatively, metatranscriptomics can also be used to identify mitigation targets. For instance, Shi et al. (2014) recently reported that transcription of methanogenesis pathway genes was elevated in sheep with high methane emissions.

Culture-Based Investigations of GIT Methanogen Microbiology

As highlighted in the previous section, the available community compositions from gut methanogens in herbivores has revealed that, while there can be some overlap between samples, each so far appears to be unique. By mechanisms that are currently unknown, certain methanogens can be prevalent under particular conditions (e.g., host breed, species, diet, or geographical location), while they are detected at a lower frequency in other cases. In order to gain further insight, there needs to be an increase in culture-based microbiological studies of GIT methanogens, which are better suited for mechanistic studies that require a controlled environment. Due to the limited number of GIT methanogen species that have successfully been isolated and grown *in vitro* (Creevey et al., 2014), direct culturing of GIT samples represents an attractive alternative which would yield valuable insights not only about methanogens, but also of their interactions with other members of the community. The importance of such investigations can be emphasized by reports such as by Popova et al. (2013), where differences in methane production capacity were found between rumen and cecal contents from lambs fed high grain content diets, despite *Methanobrevibacter*-related methanogens being the most abundant archaea in both environments.

Investigation of Intra-Community Interactions Involving Methanogens

The complexity of GIT microbial communities in herbivores is not simply due to their high cellular density and diversity, but is also a result of intricate networks of inter-species trophic relationships. Methanogens depend on other microorganisms for substrates such as H_2 and CO_2 to sustain their energy needs through anaerobic respiration and methane synthesis. While methanogens can acquire substrates from their surrounding environment, some can associate intimately with protozoa or fungi. For instance, it was reported that the free-living (FL) and protozoa-associated methanogen (PAM) populations were composed of the same major groups (*Methanobrevibacter* and *Methanomicrobium*), but that their composition differed between FL and PAM (Tymensen et al., 2012). In addition, the distribution of species-level OTUs within the same subgroups was found to differ as well. A study by Belanche et al. (2014) also reported that PAMs represented a more variable population than FL methanogens. If such interactions contribute to greater methane production, then their disruption could potentially be used as a mitigation strategy. It will also be of interest to investigate the degree of specificity between partner species that is required for these cell–cell interactions to occur.

The potential of specific trophic relationships between methanogens and bacteria should also be further explored. In studies conducted in sheep as a model ruminant (Morgavi et al., 2012), it was reported that the liquid-associated bacterial and methanogens fraction of animals kept without protozoa for more than 2 years produced more methane than the corresponding rumen fractions from faunated animals or animals defaunated for only a few months. Accordingly, the same study found that animals maintained without protozoa for more than 2 years were higher methane emitters than animals that had been defaunated for a few months.

Concluding Remarks

While great strides have been made in the study of rumen methanogen populations in a variety of hosts and environmental conditions, further investigations still are required in order to gain sufficient insight to develop comprehensive methane mitigation strategies targeting methanogens. It will only be through a sustained effort in combining genomics and cellular analyses that this goal may be reached in the near future.

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Insights on Alterations to the Rumen Ecosystem by Nitrate and Nitrocompounds

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Nitrate and certain short chain nitrocompounds and nitro-oxy compounds are being investigated as dietary supplements to reduce economic and environmental costs associated with ruminal methane emissions. Thermodynamically, nitrate is a preferred electron acceptor in the rumen that consumes electrons at the expense of methanogenesis during dissimilatory reduction to an intermediate, nitrite, which is primarily reduced to ammonia although small quantities of nitrous oxide may also be produced. Short chain nitrocompounds act as direct inhibitors of methanogenic bacteria although certain of these compounds may also consume electrons at the expense of methanogenesis and are effective inhibitors of important foodborne pathogens. Microbial and nutritional consequences of incorporating nitrate into ruminant diets typically results in increased acetate production. Unlike most other methane-inhibiting supplements, nitrate decreases or has no effect on propionate production. The type of nitrate salt added influences rates of nitrate reduction, rates of nitrite accumulation and efficacy of methane reduction, with sodium and potassium salts being more potent than calcium nitrate salts. Digestive consequences of adding nitrocompounds to ruminant diets are more variable and may in some cases increase propionate production. Concerns about the toxicity of nitrate's intermediate product, nitrite, to ruminants necessitate management, as animal poisoning may occur via methemoglobinemia. Certain of the naturally occurring nitrocompounds, such as 3-nitro-1-propionate or 3-nitro-1-propanol also cause poisoning but via inhibition of succinate dehydrogenase. Typical risk management procedures to avoid nitrite toxicity involve gradually adapting the animals to higher concentrations of nitrate and nitrite, which could possibly be used with the nitrocompounds as well. A number of organisms responsible for nitrate metabolism in the rumen have been characterized. To date a single rumen bacterium is identified as contributing appreciably to nitrocompound metabolism. Appropriate doses of the nitrocompounds and nitrate, singly or in combination with probiotic bacteria selected for nitrite and nitrocompound detoxification activity promise to alleviate risks of toxicity. Further studies are needed to more clearly define benefits and risk of these technologies to make them saleable for livestock producers.

Keywords: nitrate, nitrocompounds, rumen, methane reduction, nitrate toxicity

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INTRODUCTION

Nitrate and other oxidized nitrocompounds are scientifically pursued due to their toxicity and ability to reduce enteric methane emissions by ruminants. Methane production by microbes within the rumen is recognized as a fermentative inefficiency resulting in the loss of 2–12% of the gross energy consumed by the host (Johnson and Johnson, 1995). Environmentally, methaneis a significant greenhouse gas and strategies are sought to reduce its emission from livestock, which in the United States accounts for 95% of anthropogenic methane emissions arising from enteric fermentation (US EPA, 2012). However, despite its negative association with energy retention and greenhouse gas emissions, methanogenesis plays an important ecological role in the rumen. Archaea consume hydrogen emitted by bacterial and protozoal hydrogenases functioning to reoxidize reduced nucleotides produced during glycolysis and other catabolic pathways (Ellis et al., 1990; Miller, 1995). Methanogenesis functions to maintain a low partial pressure of hydrogen, which at partial pressures above 1 kPa promote end-product inhibition of NADH oxidoreductase thereby disrupting the oxidation of NADH and depleting concentrations of NAD to levels that inhibit fermentation (Miller, 1995; Van Nevel and Demeyer, 1996; Hegarty and Gerdes, 1999).

It is generally recognized that the most effective methane-inhibiting interventions provide alternative mechanisms for maintaining low partial pressures of dihydrogen within the rumen. A variety of electron accepting substrates are available for use as alternative electron acceptors for anaerobic respiration in the rumen, including unsaturated fatty acids, nitrate, sulfate or fumarate (Leng, 2014). From a thermodynamic perspective, however, the use of nitrate is particularly attractive because the dissimilatory reduction to ammonia is energetically more favorable ($\Delta G^{0\prime} = -600$ kJ/mol) than the reduction of carbon dioxide to methane ($\Delta G^{0\prime} = -136$ kJ/mol) and the reduction of other electron acceptors available in an anaerobic environment (Thauer et al., 1977; **Table 1**). Moreover, supplemental nitrate could under certain dietary conditions serve and perhaps even replace nonprotein nitrogen sources such as urea to support microbial protein synthesis in the rumen (Carver and Pfander, 1974; Sophea and Preston, 2011; Li et al., 2012; Silivong et al., 2012; Thanh et al., 2012). Theoretically, the consumption of four electrons with the reduction of nitrate to nitrous oxide, and potential consumption of an additional electron for the reduction of nitrous oxide to dinitrogen (N_2) via denitrification, could also serve as a metabolic route for electron disposal to the reduction of nitrate. However, from an energetic perspective, the reduction of nitrite to ammonia is slightly more favorable thermodynamically than the reduction of nitrite to nitrous oxide (436 kJ/mol vs. 453 kJ/mol), and thus ammonia is the prevalent reduction product in the rumen (**Table 1**).

Early work by Anderson et al. (1993, 1997) revealed that like nitrate and nitrite, the naturally-occurring nitrocompounds 3-nitro-1-propionate and 3-nitro-1-propanol and industrially-produced nitroethane may also serve as electron acceptors within rumen microbial populations. However, in addition to serving as alternative electron acceptors these nitrocompounds also exert a direct inhibition of ruminal methanogenesis (Anderson and Rasmussen, 1998; Gutierrez-Bañuelos et al., 2008). Consumption of electrons, at least with the reduction of naturally occurring nitrocompounds and nitroethane, occurs more slowly than the direct inhibition mechanism and requires *in situ* enrichment of competent nitro-reducing bacteria that are normally present at low numbers. The biological processes involved in the direct chemical inhibition of methane production by the short chain nitrocompounds are ill-defined. It has been speculated that this could occur via inhibition of electron transfer reactions like the nitroethanol-caused inhibition of electron transfer between ferredoxin and hydrogenase (Angermaier and Simon, 1983; Anderson et al., 2008). A number of other short chain nitrocompounds have been tested *in vitro* and while most if not all have been found to effectively inhibit ruminal methane production at present only a few have been found to be suitable electron acceptors for supporting growth of nitro-reducing bacteria. Alternatively, inhibition of methyl-coenzyme M reductase of methanogenic bacteria has been postulated for the recently identified inhibitor, 3-nitrooxypropanol, as well as some other nitro-oxy-compounds (Martínez-Fernández et al., 2014; Prakash, 2014). Thus, these nitro-oxy compounds, which possess an oxygen atom binding the nitro-group at the number 3 carbon, not only differ structurally from the short chain nitrocompounds discussed above, but probably in their mode of action as well.

Multiple literature reviews on the toxicological aspects and methane reducing potential of feeding nitrate to ruminants have been published recently including excellent works by Lee and Beauchemin (2014) and Leng (2008). Consequently, the present work focuses our discusssions on the microbiological response to nitrate and nitrocompound supplementation.

TABLE 1 | Standard molar Gibbs Free energy for reductive processes.

Reaction[a]	Electrons	$E°$ (mV)	$-\Delta G°$ (kJ/mol)
Carbon dioxide reduction to methane	−8	−244	131
Fumarate to succinate	−2	33	86
Oxygen to water	−2	818	228
Nitrate reduction to ammonia	−8	–	599.6
Nitrate reduction to nitrite	−2	433	163.2
Nitrite reduction to ammonia	−6	363	436.4
Nitrate reduction to nitrogen gas	−5	–	1120
Nitrate reduction to nitric oxide	−3	350	147
Nitric oxide reduction to nitrous oxide	−1	1175	306.1
Nitrous oxide reduction to nitrogen gas	−1	1355	341.4

[a] *Adapted from Thauer et al. (1977).*

NITRATE AND NITRITE METABOLISM WITHIN THE RUMEN

Microbial reduction of nitrate can occur by dissimilatory and assimilatory processes. The genes involved, their regulation and the energetics of these pathways substantialy differ (**Table 2**). The assimilatory nitrate reduction pathway consumes energy to

TABLE 2 | Microbial nitrogen metabolism in the rumen[a].

Oxidation state		+5	+3	+2	+1	−3
Dissimilatory nitrate reduction	NO_3	*nar/nap*	NO_2		*nir/nrf*	NH_4
Assimilatory nitrate reduction	NO_3	*nar/nas*	NO_2		*nir*	NH_4
Denitrification	NO_3	*nar/nap*	NO_2	*Nir* NO	*nor* N_2O	

[a] *NO_3, nitrate; NO_2, nitrite; NO, nitric oxide; N_2O, nitrous oxide; NH_4, ammonium.*

reduce nitrate to ammonia as a nitrogen source for microbial protein synthesis and is repressed by ammonia (Moreno-Vivián et al., 1999). Consequently, the functional role of this process is largely unnecessary in environments like the rumen where the availability of ammonia may down regulate this activity. Dissimilatory nitrate reduction, on the other hand, is an energy generating process that is distributed widely amoung obligate and facultative anaerobic bacteria (Thauer et al., 1977). Within the rumen, dissimilatory nitrate reduction occurs primarily via a two-step pathway where nitrate is first reduced to nitrite, which can accumulate as an intermedite before it is ultimately reduced to ammonia. Enzymes involved in dissimilatory nitrate reduction include membrane bound and periplasmc nitrate reductases encoded by *nar and nap* genes and nitrite reductases encoded by *nir* and *nrf* genes (Thauer et al., 1977; Moreno-Vivián et al., 1999; **Table 2**). Dissimilatory nitritereduction occurs on the outer cytoplasmic membrane and depending on the organism, consumes electrons via the oxidation of reduced electron carriers such as NADH or FADH and upon subsequent transfer of these electrons to the respiratory electron transport system they can be used to reduce and thus detoxify nitrite (Thauer et al., 1977; Moreno-Vivián et al., 1999). Certain bacteria may lack a complete functional electron transport chain yet be able to reduce nitrate to nitrite and sometimes to ammonia via reductive reactions with the incomplete chain or other electron carriers without generating ATP. In such cases, these bacteria are thought to still gain an energetic benefit via more effective disposal of electrons and therefore more efficient recycling of NAD via oxidation of NADH produced during glycolysis (Hasan and Hall, 1975).

Denitrification is another pathway for dissimilatory nitrate reduction, yet despite evidence for the presence of the denitrifying genes (*nir, nor*, and *nos*) within the rumen eubacterial and archaeal metagenome (Zumft and Kroneck, 2007; Brulc et al., 2009), this process is not considered to contribute appreciably to ruminal nitrate reduction (Jones, 1972; Kaspar and Tiedje, 1981; Leng, 2008; **Table 2**). In a metagenomic study by Brulc et al. (2009), 85 occurances of genes associated with denifrication and nitrogen fixation were tagged in bovine rumen metagenomics samples compared to the occurance of 636 genes contributing to nitrate and nitrite ammonification and 1233 total genes contributing to nitrogen metabolism. Thus, the authors concluded that denitrification and nitrogen fixation activities were likely inconsequential in the rumen. However, genetically this accounts for 7% of the genes involved in nitrogen metabolism and while clearly not dominant, their contribution cannot be completely ruled out.

Few studies have measured ruminal nitrous oxide accumulation in response to nitrate supplementation, however, in studies that have the amounts produced were found to vary considerably. Nitrous oxide concentrations equivalent to 0.3% the amount of added nitrate or nitrite were measured within nitrate- or nitrite-supplemented *in vitro* rumen fluid incubations and *in vivo* from the rumen of nitrate-supplemented [as 5 $Ca(NO_3)_2NH_4NO_3 10\ H_2O$] sheep (Kaspar and Tiedje, 1981; de Raphélis-Soissan et al., 2014). However, Petersen et al. (2015) found nitrous oxide emissions account for as much as 3.4% of added nitrate (21 g $NO_3 kg^{-1}$ dry matter fed to adapted dairy cows, nitrate type unknown). The later evidence suggests that denitrification may be contributing to nitrous oxide production in the rumen rather than being produced simply as a nonspecific byproduct of dissimilatory nitrite reduction by the nir nitrite reductase as postulated by Kaspar and Tiedje (1981). Potential bacterial denitrifiers are *Pseudomonas aeruginosa* and certain species of *Propionibacterium* and *Nitrosomonas,* which in the absence of added nitrate may be considered transient or minor colonizers of the rumen (Bryant, 1959; Duncan et al., 1999; Mitsumori et al., 2002; Arai et al., 2003). Future meta-transcriptomic analysis and/or RT-qPCR combined with nitrous oxide emission measurements could determine the relative abundance and gene expression of the denitrification pathways within the rumen of animals adapted to different types and amounts of nitrate.

MICROBIAL RESPONSE TO NITRATE

Microbial nitrate and nitrite metabolism in the rumen is a paradox in that it enables detoxification, but also results in the formation of toxicants (**Table 3**). Since these microbes are symbionts to the ruminant host, an understanding of both their response in terms of gene expression and community structure and their susceptibility to added nitrate and nitrite becomes exceedingly important. Exposure of unadapted microbial populations to high intakes of dietary nitrate results in the rapid induction of nitrate reducing activity, as evidenced by

TABLE 3 | Summary of nitrate and nitrocompound toxicity.

Substrate	Source	Intoxication	Organisms involved	Transformation/ metabolism
Nitrate	Feeds	See nitrite	Many groups	Reduced to nitrite
Nitrite	Produced from nitrates in feeds	Methemoglobinemia	Many groups	Further broken down into ammonium
3-Nitro-1-propanol	*Astragulus* and many other plant species: hydrolysis of nitroglycosides in feed	Inhibits succinate dehydrogenase	*Denitrobacterium detoxificans, Coprococcus sp., Megasphaera elsdenii, Selenomonas ruminantium*	Metabolized to aminopropanol in the rumen and 3-nitro-1-propionate in the liver
3-Nitro-1-propionate	*Astragulus* and many other plant species: hydrolysis of nitroglycosides in feed	Inhibits succinate dehydrogenase	*Denitrobacterium detoxificans, Coprococcus sp., Megasphaera elsdenii, Selenomonas ruminantium*	Metabolized to β-alanine in the rumen which is futher metabolized
Nitroethane/ nitroethanol	Synthetic	Unknown (possible respiratory toxicosis)	*Denitrobacterium detoxificans*	Metabolized to ethylamine/possibly to ethanolamine

>14-fold increases in activity within 4 h of first exposure, as well as the gradual selection of highly competent nitrate and nitrite reducing rumen bacteria (Allison and Reddy, 1984). Induction of nitrate and nitrite metabolism by prominent fermentative bacteria in the rumen such as *Selenomonas ruminantium* as well as members belonging to *Butyrivibrio, Clostridium, Peptostreptococcus*, and *Propionibacterium* can readily and rapidly contribute enhanced nitrate reduction capabilities (Alaboudi, 1984; Iwamoto et al., 2002). Subsequent to induction of nitrate and nitrite reducing activity, these bacteria can be enriched in number via exposure to nitrate because of the greater energy yield from electron transport mediated phosphorylation of ADP to ATP or via achieval of more effective electron disposal (Hasan and Hall, 1975; Thauer et al., 1977; Moreno-Vivián et al., 1999). The contribution of propionibacteria to ruminal nitrate reduction is probably atypical, however, as most nitrate-reducing propionibacteria are denitrifiers that produce nitrous oxide as an end product (Kaspar, 1982). *Wolinella succinogenes* may be considered a specialist in that it is nonfermentative and exhibits highly active nitrate and nitrite reducing activity. Nitrate-utilizing *Veillenolla parvula* also contribute to ruminal nitrate and nitrite metabolism however the abundance of these populations appears to be dependent on nitrate concentrations (Asanuma et al., 2002; Iwamoto et al., 2002). A number of other nitrate-reducing bacteria, such as certain species of *Desulfovibrio* and members of family Enterobacteriaceae can inhabit the rumen, albeit at low abundance, and it is reasonable to suspect these bacteria could also be enriched during prolonged exposure to nitrate (Pfennig et al., 1981; Stewart, 1988). Certain members of Enterobacteriaceae, such as entertoxigenic and enterohemorrhagic *Escherichia coli* and numerous *Salmonella enterica* serovars, are important animal or foodborne pathogens and thus their enrichment due to nitrate feeding would be undesirable, although to our knowledge this has not yet been reported.

Evidence for enrichment of nitrate-reducing bacterial populations also comes from studies of Alaboudi and Jones (1985), who reported >3-fold increases in rates of ruminal nitrate and nitrite metabolism in goats adapted to approximately 1.5 g nitrate (as KNO_3) kg^{-1} body weight which coincided with a 3-fold increase in the proportion of nitrate-reducing bacteria. Unfortunately, they did not further characterize their isolated bacteria and the identity of nitrate-reducers was not reported (Alaboudi and Jones, 1985). More recently, Asanuma et al. (2015) reported 2.3–2.8-fold increases in nitrate and nitrite reducing activity following adaptation to a diet supplying approximately 0.18 g nitrate (as KNO_3) kg^{-1} body weight per day to goats. Concomitant with this increase in nitrate and nitrite reducing activity were nearly equivalent increases in the relative abundance of *narG* and *nrfA* gene sequences specific for the nitrate reducing bacterium, *Selenomonas ruminantium*, as well this bacterium's 16S rRNA gene (Asanuma et al., 2015).

Conversely, Lin et al. (2013a) found no differences in abundance of *nar* or of 16S rRNA genes specific to *S. ruminantium* or to the less abundant nitrate-reducers *V. parvula* and *W. succinogenes* in rumen contents from nitrate nitrogen-fed steers (0.22–0.31 g nitrate kg^{-1} body weight, fed as KNO_3) when compared to urea nitrogen-fed steers. Unfortunately, rates of nitrate and nitrite metabolism were not reported so comparison of abundance measurents to activity measurements is not possible. They did, however, observe increases in populations of the nitrate-reducers *Campylobacter fetus,* which was enriched in both liquid and solid fractions of ruminal contents, and *Mannheimia succiniciproducens,* which was enriched only in the liquid fraction collected from the rumen (Lin et al., 2013a). More recently, Zhao et al. (2015) proposed that *Campylobacter* and cyanobacteria were important nitrate-reducing taxa based on results from 16s rDNA sequencing. *Campylobacter fetus* is an important pathogen affecting ruminants and thus its enrichment would be undesirable. Conversely, *M. succiniciproducens* may be an attractive bacterium to enrich in the rumen because it has considerable potential fix carbon dioxide, via carboxylation of phospoenolpyruvate (Lee et al., 2006). Even after enrichment, however, *C. fetus* and *M. succiniciproducens* made up only a small proportion (<0.1%) of the total population (Lin et al., 2013a). Moreover, the study of Lin et al. (2013a) was a cross-over design,

however, with a 12 day wash-out period between nitrate-nitrogen and urea-nitrogen treatments which may have been insufficient to allow populations to re-achieve unperturbed densities. It was not stated if the animals had opportunity to physically contact one another during provision of the respective treatments, but if they had then induction or transfer of nitrate and nitrite reductive capacity could have occurred between groups of animals as has been as reported by Majak and Cheng (1984) and Cheng et al. (1985). In these earlier experiments, animals in treated groups received daily doses of nitrate intraruminally (0.1 g nitrate kg^{-1} body weight, salt unspecified) during the treatment period and were initially kept away from animals in the untreated control group (Majak and Cheng, 1984; Cheng et al., 1985). Then both groups were housed in adjacent pens while the treated group continued to receive the nitrate supplement. Despite receiving no nitrate, the control group of steers showed increased (80–200%) nitrate and nitrite reduction rates during the contact period. The authors hypothesized this transfer may have been mediated via horizontal gene transfer of plasmids containing nitrate reductase (Majak and Cheng, 1984; Cheng et al., 1985). However, this could also originate from other mobile genetic elements such as transposons or bacteriophages, oral transfer of microbes via animal licking, or some other signaling molecule that we are unaware of that would induce the upregulation of nitrate and nitrite reducing genes in the ruminal microbial population.

Exposure of ruminal populations of bacteria to nitrate not only selects for nitrate-reducing bacteria, but also acts as a selection mechanism for a different ecological makeup within the microbial community. Marais et al. (1988), using *in vitro* cultivation techniques, reported that nitrite accumulation resulting from reduction of added nitrate (approximately 26 g nitrate kg^{-1} dry matter; added as KNO_3) decreased ruminal cellulolytic activity. They concluded that the decrease in cellulolytic activity was a result of 64, 25, and 57% decreases in numbers of cellulolytic, xylanolytic and total viable bacteria, respectively, as determined via viable cell count on selective media. Others also have observed toxic effects of nitrite, produced as an intermediate during the reduction of nitrate, on populations of cellulolytic bacteria as well as on other microbial populations including methanogens (Iwamoto et al., 2002; Zhou et al., 2011, 2012; Asanuma et al., 2015). The inhibition of cellulolytic organisms may explain the decreases in dry matter intake sometimes observed in animals feed nitrate-supplemented diets (Newbold et al., 2014; Lee et al., 2015a,b). It is recognized that decreased cellulolysis can decrease rates and extent of neutral detergent fiber digestion thus increasing rumen retention time of a forage and negatively affective rumen fill, both which can cause decreased dry matter intake (Allen, 2000). It is well known that nitrate and nitrite additions cause a shift in volatile fatty acid concentration, sometimes disproportionately against branched-chain volatile fatty acids essential for certain bacterial populations, and this has been suggested as a reason for decreases observed in total bacterial populations and particularly cellulolytic bacteria (Allison and Reddy, 1984) but this was discounted by Marais et al. (1988).

In the case of methanogens, the reduction of nitrate preferentially consumes electrons at the expense of methanogenesis. However, methanogens also appear to be particularly sensitive to the toxic effects of nitrite, with 50% inhibition in cell growth occurring with as little as 0.5 mM nitrite (Iwamoto et al., 2002). Asanuma et al. (2015) similarly found methanogens, as well as total populations of rumen bacteria, protozoa and fungi to be greatly decreased *in vivo* after goats were fed a high nitrate diet (5.4 g nitrateday^{-1}, as KNO_3) for 2 weeks. This total community depression may result from the oxidizing nature of nitrite as it relates to its antimicrobial properties, attributed to inactivation or inhibition of sulfur containing constituents involved in energy metabolism, DNA replication or maintenance of cell wall integrity (Marais et al., 1988; Cammack et al., 1999).

The high reactivity of nitrite could also disrupt the low E°/mV within the rumen as evidenced in the study of Jamieson (1959), who found that sheep intraruminally dosed with 25 g nitrate (as KNO_3) had a higher E_h value pre-dose than 2 h post dose (−225 vs. −70 mV, respectively). As reported by Kalachniuk et al. (1978) and discussed by Zhou et al. (2012), an increase in reduction potential (E_h) has been reported to be inhibitory to some rumen bacteria, notably *S. ruminantium, Bacteroides amylophilus, Fibrobacter* (*Bacteroides*) *succinogenes* and *Streptococcus bovis*. More recently, however, an increased E_h was found to be not particularly inhibitory per se to these same bacterial species (Marounek and Wallace, 1984), but it could potentially perturb thermodynamic control of important oxidation/reduction reactions such as those involved in electron transfer. For instance, Marais et al. (1988) reported that nitrate-caused inhibition of forage digestibility could not be overcome by using the reducing agent cysteine to decrease the nitrite-caused increase in culture E_h. Thus, they proposed a direct effect of nitrite on inhibiting bacterial growth, and while they at the time suggested this inhibition appeared to be most potent against bacteria with electron transport linked phosphorylation capabilities, more recent evidence suggest the toxic effect may not be so specific. In the study of Asanuma et al. (2015) for instance, they found that populations of major the cellulolytic bacteria *F. succinogenes, Ruminococcus flavefaciens,* which contain electron transport capabilities, as well *Ruminococus albus*, which does not contain electron transport capabilities, all decreased in the rumen of goats fed nitrate. Zhou et al. (2012) also reported reductions in abundance of *F. succinogenes, R. flavefaciens*, and *R. albus* as well as in archaeal, but not total bacterial populations when measured by real-time PCR during *in vitro* incubations of mixed populations of ruminal microbes with =12 mM sodium nitrate. They further found evidence of adaption or acquisition of tolerance by populations of *R. albus* as well as *F. succinogenes,* but not *R. flavefaciens* or the archaeal populations following up to 6 consecutive cultures with 12 mM added sodium nitrate (Zhou et al., 2012). Broad activity of nitrite was found in the studies of Iwamoto et al. (2002), who tested 15 different bacterial species and found that growth of all except four were inhibited by concentrations of 3–5 mM nitrite, with many being producers of hydrogen, formate or lactate that can contribute reductants for nitrate and nitrite reduction or methanogenesis (Russell and Rychlik, 2001). Decreases in populations of hydrogen, formate or lactate-producing microbes could potentially limit the availability

of reductant for methanogenesis or nitrate respiration. However, Asanuma et al. (2015) observed significant increases in genetic abundance of the highly efficient sugar-fermenting bacterium, *S. bovis*. It is reasonable to speculate that the sugar-fermentation by *S. bovis* may contribute a pool of electron donating substrates for the nitrate-reducing population thus potentially compensating for the decreased reductant that would be expected by inhibition of the hydrogen, formate or lactate-producing microbes observed by Iwamoto et al. (2002).

In direct opposition to the findings discussed above, Zhao et al. (2015) found that added nitrate *in vivo* (1–2% dry matter, nitrate type unknown) was associated with an increase in many cellulolytic bacterial species including *R. flavefaciens, R. albus*, and *F. succinogenes.* The authors attributed this selection to nitrate-caused increase production and thus availability of branch chain fatty acids required by these bacteria. The authors did not specifically discuss why branched chain fatty acids were increased but it is reasonable to speculate that this may have occurred due to changes in microbial diversity in the rumen population.

The protozoa, which reduce nitrate to ammonia for assimilatory purposes, play an unclear role in the total nitrate metabolism in the rumen. The protozoal fractions showed similar rates of nitrate reduction with less nitrite accumulation without any adaptation period as compared to the whole rumen fraction or bacterial fraction (Yoshida et al., 1982; Iwamoto et al., 2001; Lin et al., 2011). In contrast, Allison and Reddy (1984) reported that the nitrate and nitrite reducing activity was not found in their protozoal fraction. Moreover, they also reported that the nitrate reducing activity in rumen contents was membrane bound and was inhibited by azide and hydroxyl quinolone-N-oxide, each inhibitors of electron carrier mediated respiration. The absence of nitrate-reducing activity when dissimilatory (respiratory) nitrate reduction was inhibited suggests that the assimilatory nitrate-reducing pathway, such as that by protozoa, was inoperative or contributed little to overall nitrate reduction. Future studies are needed to detect changes in the protozoal populations in the rumen from nitrate-fed ruminants as well as additional research exploring their potential, if any, to enhance nitrite removal and reduce methane production.

OTHER EFFECTS OF FEEDING NITRATE

Additions of nitrate to *in vitro* incubations of mixed populations of ruminal microbes have generally resulted in decreased production of methane and propionate while sometimes increasing production of total volatile fatty acids; however, this varies considerably depending on experimental conditions (Bozic et al., 2009; Shi et al., 2012; Zhou et al., 2012; Patra and Yu, 2014). The decreased production of more reduced fatty acids reflects a lesser need for this route of electron disposal because electrons are instead consumed for the reduction of nitrate to nitrite and ultimately to ammonia (Sutherland, 1977; Russell, 2002). Increased acetate production at the expense of butyrate can sometimes occur due to nitrate supplementation (Farra and Satter, 1971; Anderson and Rasmussen, 1998) and this could be due to the stimulation of more thermodynamically favorable pathways for electron disposal (Ungerfeld and Kohn, 2006).

The effects of nitrate feeding ultimately depend on nitrate concentration and its availability within the rumen which is not only affected by the amount fed but also by the type or source of nitrate fed which can markedly affect the ruminal availability of the free nitrate ion. Consequently, *in vivo* additions of nitrate have also resulted in variable effects on volatile fatty acid production and methane emissions as well as on methemoglobin development in studies measuring these responses (**Table 4**). For instance, encapsulated nitrate supplements used in the studies of Lee et al. (2015a,b) would be expected to release nitrate more slowly than the salt forms of nitrate, which themselves can differ in nitrate availability thus affecting their methane reducing potential and their toxicity. In studies using calcium nitrate, amounts consumed ranged from 0.26 to 0.94 g $Ca(NO_3)_2 kg^{-1}$ body weight, an amount sufficient to cause toxicity if an equivalent dose of sodium or potassium nitrate were fed (van Zijderveld et al., 2010, 2011; Hulshof et al., 2012; Li et al., 2012; de Raphélis-Soissan et al., 2014; El-Zaiat et al., 2014; Velazco et al., 2014). Likewise, calcium nitratewas the least effective at decreasing methane, 3.5% reduction when expressed as $mmol^{-1}$ $nitrate kg^{-1}$ body weight, as compared to sodium and potassium nitrate which caused a 14.5 and 9.6% decrease in methane per mmol $nitrate kg^{-1}$ body weight respectively (Takahashi and Young, 1991; Sar et al., 2004, 2006; Nolan et al., 2010; van Zijderveld et al., 2010, 2011; Hulshof et al., 2012; Leng et al., 2012; Li et al., 2012; de Raphélis-Soissan et al., 2014; El-Zaiat et al., 2014; Velazco et al., 2014; Asanuma et al., 2015). Similarly, Phuong (2012) found that 24% less $NaNO_3$ and 12% less KNO_3 as compared to $Ca(NO_3)_2$ resulted in the same amount of methane production. This is most likely due to differences in solubility. Calcium has low solubility at pH above 5.5 and since the rumen pH seldom declines to below 5.5 it is likely that a significant portion of the calcium nitrate is unsolubilized and thus unavailable to exert the full effect of the nitrate. Additionally, the cation themselves (K, Na, and Ca) have important roles in the rumen including motility, osmolality, pH, acid base balance, and are known to influence the dry matter intake. As many of these nitrate salt feeding trials are feeding at level in excess of 2% of intake, there is the possibility that the accompanying cations may alter the microbiome and the animal physiology. Iso-cation diets controls may be necessary in future studies. Overall, it would be prudent to develop standards for nitrate salt feeding with stipulations for salt type, age of the animal, adaptation status, and previous exposure.

In global warming potential (GWP), carbon dioxide is 1, methane is 21, and nitrous oxide is 298 over a 100-year time scale. Therefore, even a small increase in nitrous oxide from nitrate reduction could have large effects on GWP. For example, nitrous oxide emission from sheep was higher when fed nitrate (calcinite at 0.625 g kg^{-1} body weight), which in turn lowered the net benefit of methane mitigation on global warming potential by 18% despite the nitrous oxide being only 0.3% of added nitrate (de Raphélis-Soissan et al., 2014). Likewise, Petersen et al. (2015) found a 28–23% reduction in overall decrease in global warming mitigation effect and Neumeier et al. (2014) found no

TABLE 4 | Effects of nitrate supplementation to ruminants on volatile fatty acid production, metHb, and methane reduction *in vivo*.

Author	Nitrate source[a]	Effects on volatile fatty acids produced ($P < 0.05$)				Administered	Animal	Max MetHb (%)	Methane effect (%)	% Dry matter intake	Highest amount supplemented[b]
		Total acids	Acetate	Propionate	Butyrate						
Alaboudi and Jones, 1985	KNO_3	No effect	Increased	Decreased	Decreased	Top dressed	Sheep	2.0	NM[c]	0.05	0.02480
Asanuma et al., 2015	KNO_3	Decreased	Decreased	Decreased	Decreased	Top dressed	Goats	NM	−89	NM	0.00310
de Raphélis-Soissan et al., 2014	$Ca(NO_3)_2$	No effect	Increased	Decreased	NM	Top dressed	Sheep	45.0	19	2.0	0.00767
El-Zaiat et al., 2014	$Ca(NO_3)_2$:NH_3NO_3	Increased	Increased	No effect	Increased	Encapsulated	Lambs	1.0	−33	4.5	0.02110
Farra and Satter, 1971	KNO_3:$NaNO_3$	No effect	Increased	Decreased	Decreased	Top dressed	Sheep	2.0	NM	6.0	0.00510
Hulshof et al., 2012	$Ca(NO_3)_2$	No effect	No effect	No effect	No effect	Top dressed	Beef	NM	−32	2.5	0.00600
Lee et al., 2015a,b	$Ca(NO_3)_2$	No effect	No effect	No effect	No effect	Encapsulated	Beef	<2.0	18	2.5	0.00844
Leng et al., 2012	KNO_3	NM	NM	NM	NM	Top dressed	Beef	<1.0	−29	6.0	0.01020
Li et al., 2012	$Ca(NO_3)_2$	No effect	Increased	No effect	Decreased	Top dressed	Sheep	<1.0	−35	3.0	0.01150
Lund et al., 2014	$Ca(NO_3)_2$	No effect	No effect	No effect	No effect	Top dressed	Dairy[d]	NM	−31	0.7	0.00120
Newbold et al., 2014	$Ca(NO_3)_2$	NM	NM	NM	NM	Top dressed	Beef	>20	−30	3.0	0.00266
Nolan et al., 2010	KNO_3	Decreased	Increased	Decreased	Decreased	Top dressed	Sheep	<1.0	−23	4.0	0.00680
Pal et al., 2014	KNO_3	No effect	No effect	Decreased	No effect	Top dressed	Sheep[d]	NM	NM	2.0	0.00600
Sar et al., 2004	$NaNO_3$	Increased	Increased	Decreased	Decreased	Via cannula	Sheep	18.4	−50	n/a[c]	0.00580
Sar et al., 2005	$NaNO_3$	No effect	Increased	No effect	Decreased	Via cannula	Sheep	2.0	−50	n/a	0.00580
Takahashi and Young, 1991	$NaNO_3$	NM	NM	NM	NM	Via cannula	Sheep[d]	20.0	−86	n/a	0.00188
van Zijderveld et al., 2010	$Ca(NO_3)_2$	No effect	No effect	No effect	No effect	Top dressed	Lambs	7.0	−32	2.6	0.00805
van Zijderveld et al., 2011	$Ca(NO_3)_3$	NM	NM	NM	NM	Top dressed	Dairy	4.7	−16	1.0	0.00366
Velazco et al., 2014	$Ca(NO_3)_2$	NM	NM	NM	NM	Top dressed	Beef	3.0	−17	2.6	0.00960

[a] *KNO_3, potassium nitrate; $Ca(NO_3)_2$, calcium nitrate; $Ca(NO_3)_2$:NH_4NO_3, 50:50 calcium nitrate:ammonium nitrate; KNO_3:$NaNO_3$, 50:50 potassium nitrate:sodium nitrate; $NaNO_3$, sodium nitrate.*

[b] *mol/kg body weight.*

[c] *NM, not measured; n/a, not applicable.*

[d] *Animal were not previously adapted to nitrate additions.*

net reduction in greenhouse gases when considering the increase in nitrous oxide from feeding nitrate.

Considering the cost and risk to the producers of feeding nitrate, the decrease of greenhouse gases production may not justify the usage of nitrate feeding as a methane mitigation strategy. Toxicity aside, it has been suggested that the limiting factors for the adoption of supplemental-nitrate feeding in beef production are financial in nature (Callaghan et al., 2014).

NITROCOMPOUND METABOLISM WITHIN THE RUMEN

Unlike nitrate and nitrite metabolism, less is known about the mechanisms of nitrocompound metabolism by ruminal microbes. Nishino et al. (2010) isolated 3-nitro-1-propionate-degrading species of strictly aerobic *Cupriavidus* and *Pseudomonas* bacteria from soil and water. Considering, however, that their degradation of 3-nitro-1-propionate proceeded to yield to propionate-3-nitronate and ultimately malonic semialdehyde, nitrate, nitrite, and hydrogen peroxide, it is unlikely these aerobic bacteria contribute to nitrocompound degradation in the anaerobic rumen. In the rumen, the naturally occurring 3-nitro-1-propionic acid and 3-nitro-1-propanol are first hydrolyzed from their glucose conjugates by microbial esterase or β-glycosidase activity thereby liberating the aglycones from their respective esters or ether glycosides (**Table 3**), the latter which are rather innocuous (Anderson et al., 2005). Once liberated, 3-nitro-1-propionic acid and 3-nitro-1-propanol are available to be absorbed or further metabolized by bacteria in the rumen. The nitroacid is absorbed less rapidly but metabolized more rapidly than the nitroalcohol which thus explains why 3-nitro-1-propionic acid is less toxic than 3-nitro-1-propanol to ruminants.

Presently, the ruminal bacterium *Denitrobacterium detoxificans* is the only known anaerobe to exhibit appreciable nitroalkane-reducing activity (Anderson et al., 1996, 1997, 2000). This bacterium conserves energy for growth exclusively via anaerobic respiration, oxidizing hydrogen, formate or lactate in the reduction of nitrate to ammonia and the reduction of 3-nitro-1-propionic acid, 3-nitro-1-propanol, and nitroethane to β-alanine, 3-amino-1-propanol and aminoethane, respectively (Anderson et al., 1993, 1997, 2000). β-alanine was rapidly metabolized to unknown products in mixed populations of ruminal microbes but 3-amino-1-propanol appeared to be a terminal product (Anderson et al., 1993). Early research by Looper et al. (1959) indicated that β-alanine was appreciably deaminated in the rumen but little other information is available on its fate as an endproduct. The reduction of the nitroalkanes to their amines is presumed to consume six moles electrons per mol of amino group reduced and is based on the stoichiometry reported for the reduction of nitroethanol to ethanolamine (Angermaier and Simon, 1983). *Denitrobacterium detoxificans* can also grow on dimethyl sulfoxide and trimethyl amine oxide, reducing these acceptors to dimethylsulfide and trimethyl amine, and can grow, albeit less readily, on nitroethanol, 2-nitro-4-butanol, 1-nitropropane and 2-nitro-1-propanol (Anderson et al., 2000). The fate of these later four substrates has not been investigated. With respect to these nitrocompounds, all except 2-nitro-4-butanol and 1-nitropropane have been tested and found to be potent inhibitors of ruminal methanogenesis (Anderson and Rasmussen, 1998; Anderson et al., 2003, 2008, 2010). Evidence indicates that *D. detoxificans* can effectively contribute to detoxification of 3-nitro-1-propionic acid, 3-nitro-1-propanol, which exist as phytotoxins in certain leguminous plants, notably milkvetchs belonging to the genera *Astragalus* and *Coronilla* (Anderson et al., 2005). However, with little known about the physiology and nutritional requirements more research is needed to make growing this bacterium practical for large scale applications.

More recently, chemically synthesized dimethyl-2-nitroglutarate and 2-nitro-methyl-propionate have also been tested and found to be similarly potent anti-methanogenic compounds (Anderson et al., 2010) as has ethyl nitroacetate (data not shown); however, the fate of these three nitrocompounds within incubations of mixed populations of rumen bacteria has not been determined.

From a thermodynamic perspective, Gibbs free energy values for the reduction of the nitrocompounds to their respective amines have not been determined. However, experimentally, nitrate was preferentially reduced by mixed populations of bovine ruminal microbes compared to 3-nitro-1-propionate or 3-nitro-1-propanol when the compounds were incubated together (Anderson et al., 1997; Zhang et al., 2014). This suggests that the reduction of nitrate may be energetically more favorable than the reduction of the nitrocompounds although the contribution of other mechanisms, such as the presence of more active nitrate-reducing enzymes cannot be excluded.

The two different mechanisms of action of these nitroalkanes, primarily direct inhibition of methanogenesis and secondarily acting as an alternative electron acceptor, are not necessarily incompatible as the process that directly inhibits methanogenesis may promote the redirection of electrons and thereby facilitate the reduction of the nitroalkanes. Subsequent *in vitro* studies have reported significant decreases in methane production by ruminal microbial populations treated with 4–13 μmol nitroethane/mL with modest affects on volatile fatty acid production and with recovery of electrons, expressed as hydrogen recoveries, accounting for 37–52% of the decrease in methane production (Gutierrez-Bañuelos et al., 2008; Bozic et al., 2009). The fate of the remaining hydrogen was undetermined but the authors speculated that other unmeasured sinks such as ethanol, formate or anabolic processes, such as those contributing to cell growth could be functional sinks (Gutierrez-Bañuelos et al., 2008; Bozic et al., 2009) as could reduced products of nitrocompound metabolism or the production of extracellular or intracellular polysaccharide production. Several other studies using a variety of other short chain nitrocompound have supported these findings, with only a few exceptions, the latter possibly resulting from a lesser or even an inability to dispose of electrons onto the nitrocompounds or attributable to differences in cultural conditions (**Table 5**). For instance, dimethyl-2-nitroglutarate and 2-nitro-methyl-propionate were found to effectively decrease ruminal methane production during an *in vitro* batch culture

TABLE 5 | Effects of nitrocompound supplementation on methane and volatile fatty acid production during *in vitro* rumen incubations.

Nitrocompound	Author	Concentration (μmol/mL)[a]	Initial culture gas phase	Methane reduction	Effects on volatile fatty acids produced ($P < 0.05$)			
					Total acids	Acetate	Propionate	Butyrate
3-Nitro-1-propionate	Anderson and Rasmussen, 1998	5–20	50:50 H_2:CO_2	19–69%	Increased	No effect	Increased	Increased
Nitroethane	Gutierrez-Bañuelos et al., 2008	4–9	50:50 H_2:CO_2	89–80%	No effect	No effect	No effect	Increased
Nitroethane	Bozic et al., 2009	13	50:50 H_2:CO_2	99%	Decreased	Increased	Decreased	Increased
Nitroethane	Anderson et al., 2003	2–24	CO_2	58–95%	Variable	Variable	Variable	Variable
Nitroethanol	Anderson et al., 2003	12	CO_2	95%	No effect	No effect	No effect	No effect
2-Nitro-1-propanol	Anderson et al., 2003	12	CO_2	91%	No effect	No effect	No effect	No effect
Nitroethane	Anderson et al., 2010	3–12	CO_2	94–99%	No effect	No effect	Increased	Increased
Dimethyl-2-nitroglutarate	Anderson et al., 2010	3–12	CO_2	92–97%	No effect	No effect	No effect	No effect
2-Nitro-methyl-propionate	Anderson et al., 2010	3–12	CO_2	98%	No effect	No effect	No effect	No effect
Nitroethane	Zhou et al., 2011	12	10:15:85 H_2:CO_2:N_2	None	No effect	No effect	No effect	No effect
Nitroethanol	Zhou et al., 2011	12	10:15:85 H_2:CO_2:N_2	99%	Decreased	Decreased	Decreased	Decreased

[a] *Concentrations have been rounded to nearest whole number.*

with ruminal microbes (Anderson et al., 2010). However, while it was anticipated that hydrolysis of the methyl esters and reduction of the nitrocompounds would yield methionine and alanine as potential endproducts of use to the host, no such evidence was obtained (Anderson et al., 2010). The microbial population had no prior exposure to these nitrocompounds; therefore it would be worthwhile to determine if adapted populations may be able to metabolize the nitrocompounds (Anderson et al., 2010).

With respect to *in vivo* studies, doses of 24 and 72 mg nitroethane and 40 and 120 mg 2-nitro-1-propanol (per kg body weight^{-1} day^{-1}) resulted in methane-reducing activity being 37–69% lower than that in untreated controls, with nitroethane being more potent than 2-nitro-1-propanol (Anderson et al., 2006a). Methane-producing activity was measured via *in vitro* incubation of freshly collected rumen contents with excess methanogenic substrate concentrations and thus conceptually represents an indirect measure of numbers of methanogens, assuming their numbers are correlated with quantities of methane-producing enzymes. When administered to cattle at doses of 30–120 mg nitroethane kg body weight^{-1} day^{-1}, methane-producing activity was decreased by 6 to as much as 44% when compared to controls, although methane production *in vivo,* measured using the sulfur hexafluoride technique, were decreased by no more than 22% (Gutierrez-Bañuelos et al., 2007; Brown et al., 2011). Oral administration of 2-nitro-1-propanol decreased ruminal acetate concentrations from that of controls by about 15% and the high nitroethane dose (120 mg kg body weight^{-1} day^{-1}) decreased rumen acetate concentrations by about 24% in the fed steers of the study of Gutierrez-Bañuelos et al. (2007). When measured, rates of ruminal nitroethane-metabolizing activity were increased more than 30% but these increases were not always significant (Gutierrez-Bañuelos et al., 2007; Brown et al., 2011) thus suggesting that onset and duration of enrichment of competent nitroethane-metabolizing bacteria is highly variable between animals and possibly reflects de-enrichment in steers fed the lower doses due to depletion of ruminal nitroethane concentrations. Similarly, there was evidence of ruminal adaptation of the ruminal population to the lower but not the higher nitroethane dose, with methane-producing activity approaching control levels by 7 days of treatment in steers fed 80 mg nitroethane kg body weight^{-1} (Gutierrez-Bañuelos et al., 2007). This too possibly reflects a depletion of efficacious concentrations of nitroethane in rumen contents of low-dosed steers due to consumption by nitroethane-metabolizing bacteria. In the steers fed 120 mg nitroethane kg body weight^{-1} day^{-1}, for instance, decreases in methane-producing activity persisted to the end of the 15 day administration. Thus, it is likely the higher dose not only allowed consumption of more reductant than the lower dose but also retained sufficient residual nitroethane concentration to maintain inhibitory pressure on methanogens and sustain growth of the nitroethane-metabolizing bacteria.

More recently, other researchers have observed 75–95% decreases in methane production during *in vitro* incubation of mixed populations of ruminal microbes treated with 0.05–0.66 μmol/mL ethyl-3-nitrooxy propionate or 3-nitrooxypropanol that suggests a greater potency of these more oxidized nitrocompounds (Martínez-Fernández et al., 2014; Romero-Pérez et al., 2015a). In the Rusitec study of Romero-Pérez et al. (2015a), hydrogen recovery in volatile fatty acid products was increased approximately 13–14% due to 3-nitrooxypropanol treatment and hydrogen accumulations were increased more than 3-fold yet recovery of all of the reductant spared from methanogenesis was not achieved. These authors also speculated that formate could possibly be an unmeasured hydrogen sink, but the potential metabolism of the nitroxy compounds to reduced products has not been discussed. A number of studies have examined the effect of

3-nitrooxypropanol on ruminal methane emissions from dairy or beef cattle and results have yielded positive results, with methane emissions, based on dry matter intake, being decreased from controls by 6 to nearly 60% (Haisan et al., 2014; Reynolds et al., 2014; Romero-Perez et al., 2014, 2015b; Hristov et al., 2015). Negative effects on animal performance, if observed, were modest. Variability in efficacy between studies possibly reflects differences in administration protocols (intraruminal vs. in feed) and methane measurement techniques. In the study of Hristov et al. (2015), as much as a 64-fold increase in hydrogen emissions was observed in 3-nitrooxypropanol-treated cattle, which was still only about 3% of the hydrogen spared from methanogenesis.

Few studies, as of yet, have determined the microbial response to nitrocompounds. At approximately 4 mM concentration, the naturally occurring nitrocompounds, 3-nitro-1-propionate and 3-nitro-1-propanol, were found to be modestly inhibitory to total culturable anaerobes from the bovine rumen, but the inhibited populations were not characterized (Anderson et al., 1993). These compounds as well as nitroethane, nitroethanol and 2-nitro-1-propanol are also inhibitory to uric acid degrading bacteria of poultry origin (Kim et al., 2005, 2009). Additionally, nitrocompounds, similarly to nitrates, inhibit the growth of many foodborne pathogens including *Listeria*, *Salmonella*, and *Campylobacter* and enteropathogenic *E. coli* (Jung et al., 2003, 2004a,b; Anderson et al., 2006b; Dimitrijevic et al., 2006), a use that has been patented (Anderson et al., 2007).

FACTORS THAT INFLUENCE NITRATE AND NITROCOMPOUND METABOLISM

There is a significant amount of variation between animals with regard to their response to nitrate addition. Part of this response is due to the rumen ecosystem. The largest influencer would be the endogenous microbial populations and their enzymatic capacity. However, there are many other factors that influence the rate of reduction of nitrate, nitrite, and nitrocompounds.

First, the availability and amount of substrate supplying electrons used for the reductive nitrate reactions including carbohydrates, ethanol, mannitol, glycine, malate, citrate, lactate, succinate, pyruvate and formate (Lewis, 1950, 1951; Jones, 1972). Fatty acids, acetic, propionic, and butyric are not electron donors for nitrate reduction under this metabolic process. Thus, supplementing or provision of diets rich with these electron donors can potentially enhance nitrate reduction to ammonia, thereby further enhancing the decrease in methane and reducing the chances of nitrate poisoning as the animal will be exposed to less of the toxic intermediate nitrite which converts hemoglobin to methemoglobin also known as methemoglobinemia (Takahashi et al., 1980).

Dietary interventions such as high starch and cereal grains have been proposed to help mitigate nitrate toxicity (Burrows et al., 1987; Hibberd et al., 1994) purportedly by stimulating the metabolism of ruminal microorganisms thereby promoting production and subsequent availability of electron-donating substrates for nitrate and nitrite metabolism (Lewis, 1950, 1951; Allison and Reddy, 1984). High starch diets, within a pH range of about 6.6–6.8, have been shown to increase hydrogen production by mixed populations of ruminal microbes *in vitro* (Lin et al., 2013b). However, high starch diets are also well established to decrease rumen pH, which could potentially create an environment inhibitory to ruminal nitrate and nitrite metabolism. While enzymatic analysis indicates nitrate and nitrite reductases work optimally at pH of 6.5 or 5.6, respectively (Lewis, 1951; Tillman et al., 1965), nitrate- and nitrite-reducing activity within mixed populations of ruminal microbes was more rapid at neutral pH than at pH 6.0 or lower (Iwamoto et al., 2001). The inhibitory activity of the lower pH on nitrate- and nitrite-reducing activity within mixed rumen populations was attributed to lower availability of electron-donating substrates such as hydrogen, formate, or lactate resulting from an inhibition of fermentation caused by the low pH (Iwamoto et al., 2001). This would suggest that nitrate supplemented to concentrate fed-cattle may be ineffective or at least less effective in decreasing ruminal methane emissions than when supplemented to forage fed cattle. From a methane mitigation perspective, this may be of little consequence as the low ruminal pH in high concentrate-fed ruminants also inhibits methane production (Van Kessel and Russell, 1996; Lana et al., 1998; Russell, 1998) and thus concentrate-fed ruminants emit significantly less methane than do forage-fed cattle (Johnson and Johnson, 1995). From a toxicity perspective, however, it seems likely that supplementing nitrate to animals expected to have low rumen pH may increase risk for nitrite accumulation and subsequent toxicosis due to methemoglobinemia.

The next consideration is adaptation of the rumen microbial population to high nitrate diets. As has been alluded to earlier, the ruminal microbial population can be selected for increased nitrate- and nitrite-reducing activity by gradual feeding of increasing amounts of nitrate. However, little is known as to what might happen to the microbial population if an animal is de-adapted and selective pressure is removed. For instance, it is reasonable to suspect that should an animal go off feed and refuse its meals for a certain period of time a disbalance between rates of nitrate reduction and nitrite reduction may occur, which if favoring high nitrite accumulation may place the animal at greater risk to nitrite intoxication. Establishing or dosing the animal with a population of probiotic bacteria capable of reducing nitrite to non-toxic forms may also reduce the concentration of nitrite in the rumen. Currently, there is one patent, which is sold as a direct-fed microbial additive for the prevention of nitrite toxicity in ruminants fed nitrate. It employs a denitrifying strain of *P. acidipropionici*; however, its nitrate and especially its nitrite reductive capacity have not been proven to remediate nitrate toxicity nor enhance methane reduction (de Raphélis-Soissan et al., 2014). There is also a genetically modified *E. coli* strain that has been developed with enhanced nitrite utilization that has been proven to work *in vitro* and *in vivo* (Sar et al., 2005, 2006). However, its status as a GMO and as a strain of a

pathogenic genus may create a barrier too large for its usage in production.

Adaptation can occur at the animal, or systemic, level as well. Ruminants increase the amount of hemoglobin, red blood cells, and blood volume, thereby increasing the capacity to deliver oxygen and counter the effect of increasing methemoglobin. This process occurs over months (Jainudeen et al., 1964). In addition, NADPH reductase will convert methemoglobin back to hemoglobin (Hibberd et al., 1994). It is reasonable to hypothesize that animals adapted to nitrate have an elevated concentration of NADH reductase, but this has not been documented.

The rate of nitrate reduction also appears to be influenced by sulfur availability and concentrations. The reduction of sulfate to sulphite and then sulfide is less energetically favorable than nitrate to nitrite to ammonia. Opportunistically the enzymes for each reaction generally work for both reductive processes due to the molecule's similarity in structure and charge (Thauer et al., 1977). In addition, sulfur stimulates the growth of sulfide-reducing bacteria. Therefore, the addition of sulfur should hypothetically increase the amount of nitrate and nitrite reduced and the speed of the reaction (Leng, 2008). Experimentally, L-cysteine, which is rich in sulfur, in conjunction with nitrate has been shown to suppress the formation of methemoglobin (Takahashi et al., 1998).

Less is known regarding factors affecting nitrocompound metabolism within the rumen, although protein supplementation enhances *in vivo* rates of ruminal 3-nitro-1-propanol metabolism in cattle (Majak, 1992) and the reducing substrates, hydrogen gas and formate, are known to be stimulatory to reduction of the nitrocompounds *in vitro* (Anderson et al., 1993). Moreover, supplementing ruminal populations with ferrous and sulfide ions markedly increased rates of 3-nitro-1-propionic acid and 3-nitro-1-propanol metabolism (Anderson et al., 1993), but had little if any effect on the metabolism of these nitrocompounds in populations of equine cecal microbes (Zhang et al., 2014). Mechanistically, the ferrous and sulfide ion additions were thought to promote ferredoxin-hydrogenase mediated electron transfer reactions contributing to the reduction of the nitrocompounds.

CONCLUSIONS

Nitrate and nitrite reduction to ammonia in the rumen is a more thermodynamically favorable reaction than the formation of methane with carbon dioxide as an electron acceptor. The effectiveness and risks of toxicity of this strategy are dependent on the nitrate salt type with sodium nitrate being the most biologically available and calcium the least in the rumen environment. A portion, albeit small, of the introducted nitrate appears to be metabolized to nitrous oxide either via dissimilatory nitrate reduction or more likely via incomplete denitrification, which may lessen its net greenhouse gas mitigation. A variety of certain short chain nitrocompounds as well as some nitro-oxy compounds have also been shown to decrease ruminal methane production. The naturally occurring nitrocompound, 3-nitro-1-propionate, is known to be metabolized by ruminal microbes to β-alanine, a non-essential amino acid that may be metabolized by the host and potentially used as a source of carbon, nitrogen and energy making it an attractive candidate. Safe and successful feeding of supplemental nitrate and nitrocompounds requires careful adaptation of the ruminal microbes to prevent risks of toxic intermediates and this practice could benefit via concurrent feeding of appropriate nitrite-reducing or nitrocompound degrading probiotic bacteria. Additionally, combination feeding of 3-nitro-1-propionate, or another appropriate nitrocompound, with subtoxic amounts of nitrate may yield synergistic advantages in inhibition of rumen methanogensis and electron capture in non-methane products.

More research is needed, however, on the pathways involved in nitrate and nitrocompound metabolism, the organisms involved, and the regulation of their enzyme activities in order to mitigate concerns that persist over the risks of toxicities and realize the full potential of these methane-decreasing strategies.

AUTHOR CONTRIBUTIONS

EL: nitrate section and editing, RA: nitrocompound and editing, WP: editing, DN: supervision.

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Shifts in metabolic hydrogen sinks in the methanogenesis-inhibited ruminal fermentation: a meta-analysis

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Maximizing the flow of metabolic hydrogen ([H]) in the rumen away from CH_4 and toward volatile fatty acids (VFA) would increase the efficiency of ruminant production and decrease its environmental impact. The objectives of this meta-analysis were: (i) To quantify shifts in metabolic hydrogen sinks when inhibiting ruminal methanogenesis *in vitro*; and (ii) To understand the variation in shifts of metabolic hydrogen sinks among experiments and between batch and continuous cultures systems when methanogenesis is inhibited. Batch (28 experiments, $N = 193$) and continuous (16 experiments, $N = 79$) culture databases of experiments with at least 50% inhibition in CH_4 production were compiled. Inhibiting methanogenesis generally resulted in less fermentation and digestion in most batch culture, but not in most continuous culture, experiments. Inhibiting CH_4 production in batch cultures resulted in redirection of metabolic hydrogen toward propionate and H_2 but not butyrate. In continuous cultures, there was no overall metabolic hydrogen redirection toward propionate or butyrate, and H_2 as a proportion of metabolic hydrogen spared from CH_4 production was numerically smaller compared to batch cultures. Dihydrogen accumulation was affected by type of substrate and methanogenesis inhibitor, with highly fermentable substrates resulting in greater redirection of metabolic hydrogen toward H_2 when inhibiting methanogenesis, and some oils causing small or no H_2 accumulation. In both batch and continuous culture, there was a decrease in metabolic hydrogen recovered as the sum of propionate, butyrate, CH_4 and H_2 when inhibiting methanogenesis, and it is speculated that as CH_4 production decreases metabolic hydrogen could be increasingly incorporated into formate, microbial biomass, and perhaps, reductive acetogenesis in continuous cultures. Energetic benefits of inhibiting methanogenesis depended on the inhibitor and its concentration and on the *in vitro* system.

Keywords: methanogenesis inhibition, rumen, fermentation, metabolic hydrogen, meta-analysis, volatile fatty acids

INTRODUCTION

Despite its importance in transforming fibrous plant biomass non-edible by humans into useful products such as meat and milk, ruminant production contributes with between 7 and 18% of total anthropogenic greenhouse gas emissions, with CH_4 release from ruminal fermentation being a principal contributor (Martin et al., 2010; Hristov et al., 2013). The release of CH_4 produced in the rumen to the atmosphere is also energetically inefficient to animal production, as it accounts for between 2 and 12% of ruminant gross energy intake (Johnson and Johnson, 1995).

Glucose is mainly metabolized through glycolysis to pyruvate in the rumen (Russell and Wallace, 1997). Glycolysis, and pyruvate oxidative decarboxylation to acetyl-CoA, which is the first step in acetate and butyrate formation, both result in the release of metabolic hydrogen ([H]). Reduced co-factors must then be re-oxidized for fermentation to continue. In the typical ruminal fermentation, methanogenesis is the main route of co-factor re-oxidation, with [H] transferred from the fermentative microbiota of bacteria, protozoa and fungi to methanogenic Archaea mainly as H_2 (Wolin et al., 1997). However, the production of propionate, a useful fermentation product which is the main glucose precursor for ruminants, competes with CH_4 production for [H] (Janssen, 2010) and H_2 (Henderson, 1980). Also, some [H] is incorporated into butyrate production from pyruvate (Miller and Jenesel, 1979); although butyrate production from hexoses results

Abbreviations: ΔH_{gases}, heat of combustion in methane and dihydrogen; ΔH_{VFA}, heat of combustion in total volatile fatty acids; DMD, apparent DM digestibility; *FH* = hexose-equivalent estimated fermented organic matter; [H], metabolic hydrogen; $[2H]_{But}$, metabolic hydrogen incorporated into butyrate production; $[2H]_{CH4}$, metabolic hydrogen incorporated into CH_4 production; $[2H]_{H2}$, metabolic hydrogen incorporated into H_2; $[2H]_{incorporated}$, total incorporation of reducing equivalent pairs into propionate, butyrate, CH_4 and H_2; $[2H]_{produced}$, total production of reducing equivalent pairs; $[2H]_{Pr}$, metabolic hydrogen incorporated into propionate production; $[2H]_{recovery}$, percentage of metabolic hydrogen produced incorporated into methane, propionate, butyrate and dihydrogen; VFA, volatile fatty acids.

in net release of [H], less [H] per mol of glucose is released from butyrate formation compared to acetate (Janssen, 2010).

Because of the negative consequences to the environment and the energetic inefficiency that it represents, much research effort has been directed toward the inhibition of ruminal methanogenesis. Methane production can be strongly decreased using halogenated CH_4 analogs and other chemicals, nitrate, oils, antiprotozoal agents or some plant extracts. Although there has been progress at decreasing CH_4 production *in vitro* and *in vivo*, a problem encountered is the incomplete incorporation of [H] spared from methanogenesis into fermentation products nutritionally useful to the host animal. Although some [H] spared from CH_4 production is redirected to propionate, part is directed to atypical [H] sinks like H_2 (Kung et al., 2003; Mitsumori et al., 2012), and sometimes formate and ethanol (Ungerfeld et al., 2003). Accumulation of H_2 is energetically inefficient and inhibits re-oxidation of co-factors and hence fermentation (Wolin et al., 1997). It would be important to incorporate more [H] spared from methanogenesis into propionate and butyrate production.

The profile of VFA and gases in the normal, non-inhibited ruminal fermentation has been modeled based on thermodynamics and kinetics (Kohn and Boston, 2000; Offner and Sauvant, 2006). Mechanistic explanations have been proposed for the typical range of VFA proportions (Ungerfeld and Kohn, 2006) and the acetate to propionate shift that occurs when ruminal methanogens are inhibited (Janssen, 2010). However, even though the general response in propionate and H_2 to methanogenesis inhibition is well described, shifts in all the main [H] sinks from numerous experiments have not been quantitatively summarized. Furthermore, there is variation among *in vitro* systems and experiments in fermentation shifts when methanogenesis is inhibited, as the present analysis will show. Understanding this variation and identifying the underlying factors that can explain it may allow better manipulation of fermentation to direct [H] toward the most beneficial sinks.

To my knowledge, there have not been *in vivo* studies with simultaneous measurement of actual production of VFA (rather than concentration) and gases. On the other hand, there is a considerable amount of published *in vitro* results on the effects of inhibiting ruminal methanogenesis on net VFA and gases production. A meta-analytical approach can be used to quantitatively summarize results from the existing studies. Meta-analyses can be conducted with the objective of augmenting power in hypothesis testing, modeling responses of studies conducted under dissimilar conditions, establishing new research hypotheses based on aggregated results from many studies, or parameterizing models (Sauvant et al., 2008). Meta-analyses allow integration of studies conducted under disparate conditions, which can allow the emergence of new knowledge or hypotheses not provided by the individual experiments. Rather than establishing cause-effect relationships, meta-analyses can discover associations useful to identify new variables for further research. The objectives of the present meta-analysis are to: (i) Quantitatively summarize the effects of methanogenesis inhibition on [H] sinks in ruminal batch and continuous cultures; and (ii) Understand the underlying variation among *in vitro* systems and experiments in the shifts of [H] to different sinks. Possible implications of the findings from this analysis of *in vitro* experiments to the *in vivo* situation are discussed.

METHODS

DATABASES

Experiments of inhibition of ruminal methanogenesis in batch and continuous cultures were compiled. In order to be included in this meta-analysis, experiments had to meet all of the following criteria:

(i) Production of CH_4, H_2 accumulation, and net production of individual VFA was provided or could be calculated;
(ii) Initial headspace was H_2-free and formate salts or formic acid were not used as substrate;
(iii) The experiment included a methanogenesis-uninhibited control treatment;
(iv) At least one treatment or level within a treatment resulted in a 50% or greater decrease in CH_4 production relative to the control. This ensured sufficient variation in CH_4 production in each individual experiment so as to adequately test the hypotheses that variation in the responses was related to methanogenesis inhibition;
(v) Experiments with very atypical VFA molar percentages in control treatments (Marty and Demeyer, 1973; Hino and Russell, 1985) were not included;
(vi) Treatments within experiments consisting of combinations of methanogenesis inhibitors and fermentation intermediates or their isomers or analogs (malate, fumarate, crotonate, butynoic acid or 3-butenoic acid) were not included, in order to avoid confounding effects of added fermentation intermediates on VFA production unrelated to methanogenesis inhibition;
(vii) In the batch culture study by Nollet et al. (1997), treatments with added reductive acetogen *Peptostreptococcus productus* were not included, as an unknown part of acetate produced when inhibiting methanogenesis could potentially be originated by reductive acetogenesis and hence would not be associated with production of [H].

Incubations including different treatments that were run simultaneously with the same uninhibited control were considered to be one experiment. The batch culture database comprised a total of 193 treatment means from 28 experiments in 14 peer-reviewed published studies (Table S1). The continuous cultures database comprised a total of 79 treatment means from 16 experiments in 13 peer-reviewed published studies (Table S2).

CALCULATIONS

Total production of reducing equivalent pairs ($[2H]_{produced}$) was calculated from the stoichiometry of reducing equivalent-pairs released in acetate (*Ac*), propionate (*Pr*) and butyrate (*But*) production (Marty and Demeyer, 1973) as:

$$[2H]_{produced} = 2Ac + Pr + 4\,\mathrm{But}$$

Production of valerate and caproate was not considered because some experiments reported only the three main VFA acetate, propionate and butyrate.

Total incorporation of reducing equivalent-pairs into the main fermentation products ($[2H]_{incorporated}$) was calculated from the stoichiometry of reducing equivalent-pairs incorporated into propionate, butyrate, CH_4 and H_2(Marty and Demeyer, 1973):

$$[2H]_{incorporated} = 2Pr + 2But + 4CH_4 + H_2$$

Metabolic hydrogen recovery ($[2H]_{recovery}$) was calculated as the ratio between total $[2H]_{incorporated}$ and $[2H]_{produced}$, expressed as percentage (Marty and Demeyer, 1973):

$$[2H]_{recovery} = [2H]_{incorporated} \times 100/[2H]_{produced}$$

Organic matter fermentation on a total fermented hexoses-equivalent basis (FH) was estimated according to Marty and Demeyer (1973) as:

$$FH = \frac{1}{2}Ac + \frac{1}{2}Pr + But$$

All variables in the equations above are expressed in μmol (batch culture) or mmol/d (continuous culture).

Heat of combustion of glucose, acetic acid, propionic acid, butyric acid, CH_4 and H_2 were obtained from Domalski (1972) and Kohn and Boston (2000), and used to calculated heat of combustion output in total VFA (ΔH_{VFA}) and in gases (CH_4 + H_2, ΔH_{gases}).

REGRESSIONS

The incorporation of [H] into propionate ($[2H]_{Pr}$), butyrate ($[2H]_{But}$) and H_2 ($[2H]_{H2}$), $[2H]_{recovery}$, FH, ΔH_{VFA} and ΔH_{gases} were regressed separately against the incorporation of [H] into CH_4 ($[2H]_{CH4}$), for batch and continuous cultures as:

$$response_{ij} = \text{intercept} + exp_i + B_1[2H]_{CH4j} + B_2[2H]^2_{CH4j} + b_i[2H]_{CH4ij} + residual_{ij}$$

where $response_{ij}$ is $[2H]_{Pr}$, $[2H]_{But}$, $[2H]_{H2}$, $[2H]_{recovery}$, FH, ΔH_{VFA} or ΔH_{gases}, exp_i is the fixed effect of the experiment i, B_1 and B_2 are fixed linear and quadratic regression coefficients of $[2H]_{CH4}$, respectively, and b_i is the fixed effect of experiment i on the linear $[2H]_{CH4}$ coefficient (i.e., interaction between the experiment effect and methanogenesis inhibition), with $residual_{ij}$ assumed to be independent and normally distributed. Fixed, rather than random, effect of the experiment, was used because the objective of the analysis was not to parameterize models to predict future *in vitro* results but to use the existing information to understand how methanogenesis inhibition affects the flows of [H] in ruminal fermentation. Regression coefficient estimates were compared to zero with a Student's test. Significance was declared at $p < 0.05$ and tendencies at $0.05 \leq p \leq 0.15$. Non-significant interactions and quadratic effects ($p > 0.15$) were removed and the reduced model re-run.

In meta-analysis, weighting treatment means by the reciprocal of their standard errors (1/SEM) scaled to one is recommended to obtain maximum likelihood estimates while maintaining the original scale of the data (Sauvant et al., 2008). Unfortunately, SEM were not always available for the production of individual VFA, because for many experiments production of individual VFA had to be calculated as the product of total production of VFA by their molar proportions, hence the SEM for the resulting variable is not calculable from the published SEM. Total VFA concentration 1/SEM was instead initially chosen as weighting variable for all regressions (1/SEM of CH_4 production was not chosen because CH_4 production was small or non-detectable in some treatments, which associated with null or very small reported SEM and thus very large 1/SEM). Because there was a linear relationship between total VFA concentration treatment means and their SEM both in batch and continuous cultures ($p < 0.001$) that would have weighted down treatments with greater total VFA concentration, it was decided to use the reciprocal of the coefficient of variation (1/CV) of total VFA concentration as a weighting variable, instead of 1/SEM of total VFA concentration. The studies by Slyter and Wolin (1967) and Chalupa et al. (1980) did not report statistics of variation, and their treatment means were arbitrarily assigned a scaled weighting factor equal to one to maintain the original scale of the data.

Homoscedasticity was examined through the significance of the linear regression coefficient of residuals against predicted values (residual plots) and against the regressor $[2H]_{CH4}$. Normality assumption was examined through residuals normality plots. Outliers and influential treatment means were identified by examining studentized residuals, leverage (hat) values, and Cook's distances. Outliers were identified as treatment means with a studentized residual $> |t_{n-k,\,0.95}|$, with k being the number of parameters and n the number of treatment means used to fit the regression. Influential treatment means were identified as those with a leverage value larger than $2k/n$ (Belsey et al., 1980) or a Cook's distance greater than the 50th percentile of an $F_{k,\,n-k}$ distribution.

Experiments containing treatment means so identified as outliers and/or influential observations were deleted one at a time, and regressions were fitted again in their absence. Treatment means were confirmed to be influential outliers if the conclusions of the analysis changed after the deletion of the experiment containing them, i.e., significant ($p < 0.05$) responses became non-significant ($p > 0.15$) or *vice versa*, or the direction of the response changed. When the presence of influential outliers was confirmed, results are presented both with and without the experiments containing the outliers.

FACTORS AFFECTING THE RESPONSES TO METHANOGENESIS INHIBITION

When the interaction between $[2H]_{CH4}$ and the experiment effect was significant, the experiment effect was replaced by the following co-variables in order to understand possible causes of the variation among experiments in the responses to methanogenesis inhibition:

Batch cultures: ruminal fluid donor species (bovine or ovine), inoculum (ml), substrate (mg), percentage of concentrate in

substrate, duration of incubation (h), and type of methanogenesis inhibitor;
Continuous cultures: substrate (g), percentage of concentrate in the substrate, liquid volume, fractional turnover rate of the liquid phase (h^{-1}), and type of methanogenesis inhibitor.

Methanogenesis inhibitors were classified according to their known or presumed mode of action into: (i) Pure chemical compounds inhibiting or presumed to inhibit methanogens directly; (ii) nitrate and nitrocompounds, which apart from being toxic to methanogens can also decrease CH_4 production by competing as electron acceptors for [H]; (iii) ionophores, which mainly inhibit organisms involved in producing H_2; (iv) oils, which can inhibit methanogens directly, and protozoa that harbor them; (v) antiprotozoal agents; (vi) plant extracts. It is acknowledged that there is overlap between types of methanogenesis inhibitors as grouped herein, and more than one mechanism of action can apply to the same group of compounds, and that mechanisms of action of some inhibitors or additives are not understood. In order to allow analyzing experiments and treatments including more than one type of methanogenesis inhibitor, dummy variable columns were set in the batch and continuous culture databases, indicating, for each treatment mean, the presence or absence of each type of methanogenesis inhibitor.

The backward stepwise procedure was used to identify those co-variables whose interactions with $[2H]_{CH4}$ were potentially important to explain the responses:

$$response_{ij} = \text{intercept} + cov_i + B_1[2H]_{CH4j} + B_2[2H]^2_{CH4j} + b_i[2H]_{CH4ij} + residual_{ij}$$

Where cov_i is the fixed effect of co-variable i, B_1 and B_2 are fixed linear and quadratic regression coefficients of $[2H]_{CH4}$, respectively, and b_i is the fixed effect of co-variable i on the linear $[2H]_{CH4}$ coefficient (i.e., interaction between co-variable *i* and methanogenesis inhibition). The quadratic effect of $[2H]_{CH4}$ was included only if it had not been removed ($p < 0.15$) from the original model including the experiment effect. Final models with the retained co-variables were selected by minimizing the corrected Akaike Information Criterion.

Substrates were classified into roughages (less than 33% concentrate in the dry matter), mixed (between 33 and 66% concentrate in the dry matter), and high concentrate (more than 66% concentrate in the dry matter). Regressions of $[2H]_{Pr}$, $[2H]_{But}$ and $[2H]_{H2}$ against $[2H]_{CH4}$ including the interaction between $[2H]_{CH4}$ by type of substrate and the effect of the experiment were conducted jointly for five batch culture experiments that compared responses between a roughage and a mixed substrate (O'Brien et al., 2013). Experiments in continuous culture conducted with more than one type of substrate (Dong et al., 1997; Machmüller et al., 2001; Klevenhusen et al., 2009) could not be grouped for joint regressions because they were not orthogonal with regard to the type of substrate incubated. Results of regressions per type of substrate in continuous culture are presented for numerical comparison.

Also, the interaction of $[2H]_{CH4}$ with pH and dry matter apparent digestibility (DMD) on the response variables in batch cultures, and the interaction of $[2H]_{CH4}$ with pH, apparent digestibility of organic matter and neutral detergent fiber, and total bacterial and protozoal numbers in continuous culture, were evaluated. These regressions included the experiment effect and its interaction with $[2H]_{CH4}$. In order to provide further insights on the effects of different inhibitors, inhibitors redirection of [H] toward propionate, butyrate and H2 was also numerically compared without adjusting for other co-variables.

In addition, responses to $[2H]_{CH4}$ in individual experiments were visually assessed, and experiments with a response different to the overall response were identified through the *p* value of the interaction between $[2H]_{CH4}$ and the particular experiment.

JMP® 11.0.0 (SAS Institute, Cary, NC, USA) was used for all statistical analyses.

RESULTS

Summaries of descriptive statistics for the batch and continuous culture databases are shown in **Table 1**. Descriptive statistics for main [H] sinks in control treatments, expressed as a percentage of $[2H]_{produced}$, are shown in **Table 2**. There were no issues of heteroscedasticity in any regression. Most residuals normality plots were slightly, but not severely, S-shaped (not shown).

Plots of [H] sinks as a function of $[2H]_{CH4}$ are adjusted by the experiment effect (**Figures 1–7**). In some plots, adjusting by the experiment effect caused some treatment means with low values for the response variable belonging to an experiment with a high positive experiment effect to have negative experiment-adjusted responses.

FERMENTATION AND DIGESTION

In batch cultures, there was an interaction between $[2H]_{CH4}$ and the experiment effect on *FH* ($p = 0.041$; **Figure 1A**), with a tendency to a decrease in *FH* with the decrease in $[2H]_{CH4}$ (i.e., methanogenesis inhibition; $p = 0.12$). Excluding the experiment by Anderson et al. (2010), in which inhibiting methanogenesis was associated with greater *FH*, resulted in a linear decrease in *FH* with the decrease in $[2H]_{CH4}$ ($p < 0.001$; not shown) with an interaction with the experiment effect ($p = 0.044$). Greater inoculum volume ($p = 0.001$) and chemical inhibitors of methanogenesis ($p = 0.001$) were associated with greater inhibition in *FH* as $[2H]_{CH4}$ decreased, in contrast to greater concentrate percentage in the substrate ($p = 0.003$; not shown). Use of chemical inhibitors was in turn positively associated with inoculum volume ($p < 0.001$), which may have caused the unexpected negative association between inoculum volume and *FH*. There was an interaction between $[2H]_{CH4}$ and the experiment effect on DMD ($p < 0.001$), with decreases ($p \leq 0.03$) or tendencies ($p < 0.14$) to decrease DMD with methanogenesis inhibition in three experiments, increases ($p < 0.003$) or tendencies ($p < 0.09$) to increase in two, and lack of effects in one ($p = 0.87$; not shown).

In continuous cultures, there was a linear decrease in *FH* as $[2H]_{CH4}$ decreased ($p < 0.001$), with no interaction with the experiment effect ($p = 0.40$; **Figure 1B**). There was a tendency to decrease apparent digestibility of organic matter ($p < 0.07$), and a decrease in neutral detergent fiber digestibility ($p < 0.001$) with the decrease in $[2H]_{CH4}$, with an interaction with the experiment effect ($p = 0.009$; not shown).

Table 1 | Summary of descriptive statistics[a].

Variable	Batch culture					Continuous culture				
	N	number of experiments	mean	*SD*	min – max	*N*	number of experiments	mean	*SD*	min – max
pH	88	10	6.39	0.29	5.70 – 6.70	66	12	6.69	0.38	5.13 – 7.20
Total gas production (ml or ml/d)	138	20	64.7	27.1	2.7 – 105	43	8	1028	606	76.7 – 2251
CO_2 (ml/100 ml total gas)	67	13	77.8	8.62	61.8 – 97.2	43	8	88.5	5.43	74.7 – 98.7
CH_4 (ml/100 ml total gas)	138[b]	20	10.1	9.10	ND[c] – 42.4	43	8	10.5	6.07	ND – 25.2
H_2 (ml/100 ml total gas)	138[b]	20	6.70	10.2	ND – 38.3	43	8	0.98	1.19	ND – 5.11
Total VFA[c] (mM)	193	28	65.5	26.7	17.6 – 155	79	16	92.5	28.3	30.1 – 130
Acetate (molar%)	193	28	55.3	10.7	33.2 – 88.1	79	16	53.0	7.75	34.4 – 77.4
Propionate (molar%)	193	28	28.9	7.66	8.44 – 51.0	79	16	23.1	7.39	10.4 – 45.5
Butyrate (molar%)	193	28	12.0	5.97	1.47 – 32.4	79	16	15.7	4.91	5.70 - 27.1
Valerate (molar%)	103	16	2.59	1.24	0.29 – 6.59	61	13	5.42	2.62	0 – 10.0
Ac:Pr[c] ratio (mol/mol)	193	28	2.16	1.37	0.84 – 10.4	79	16	2.61	1.15	0.95 – 6.41

[a] *Non-weighted treatment means.*

[b] *There are a total of 193 treatment means for CH_4 and H_2 production, however, the percentage of CH_4 and H_2 in total gas can only be calculated from their production for those experiments that reported CO_2 or total gas production.*

[c] *Abbreviations: ND, not detected; Total VFA, total volatile fatty acids; Ac, acetate; Pr, propionate.*

Table 2 | Metabolic hydrogen sinks in control treatments[a].

	Batch cultures			Continuous cultures		
	Mean	*SD*	Range	Mean	*SD*	Range
$[2H]_{CH4}$ (%$[2H]_{produced}$)[b]	46.6	12.8	12.6 – 86.1	29.1	11.6	10.0 – 64.6
$[2H]_{Pr}$ (%$[2H]_{produced}$)	22.6	4.92	8.52 – 29.5	23.6	10.3	11.8 – 51.3
$[2H]_{But}$ (%$[2H]_{produced}$)	11.3	4.45	2.76 – 21.3	15.5	4.07	7.5 – 22.9
$[2H]_{H2}$ (%$[2H]_{produced}$)	0.043	0.086	0[c] – 0.38	0.26	0.38	0[c] – 1.65
$[H]_{recovery}$ (%)	80.5	17.0	41.6 – 121	68.5	13.3	52.9 – 105

[a] *Non-weighted treatment means.*

[b] *Abbreviations: $[2H]_{produced}$, total amount of reducing equivalents pairs produced; $[2H]_{CH4}$, $[2H]_{Pr}$, $[2H]_{But}$ and $[2H]_{H2}$, percentage of metabolic hydrogen produced incorporated into CH_4, propionate, butyrate and H_2, respectively; $[2H]_{recovery}$, percentage of hydrogen produced recovered in propionate, butyrate, H_2 and CH_4.*

[c] *H_2 reported as non-detected.*

PROPIONATE

In batch cultures, there was a an increase in $[2H]_{Pr}$ as methanogenesis was inhibited ($p < 0.001$) with a tendency to be quadratic ($p = 0.13$; **Figure 2A**; **Table 3**) and no interaction with the experiment effect ($p = 0.26$). In the five experiments in the study by O'Brien et al. (2013), inhibiting methanogenesis resulted in greater increase in $[2H]_{Pr}$ with a mixed than with a roughage substrate ($p < 0.001$; Table S3).

In continuous cultures, there was a quadratic tendency to a decrease in $[2H]_{Pr}$ with methanogenesis inhibition ($p = 0.14$), with a tendency to an interaction with the experiment effect ($p = 0.08$; **Figure 2B**; **Table 3**). Chemical inhibitors of methanogenesis ($p = 0.02$), oils ($p = 0.09$) and greater liquid volume ($p = 0.10$) associated or tended to associate with greater [H] redirection toward propionate when methanogenesis was inhibited.

Both in batch culture and continuous culture, ionophores and linoleic and linolenic acids were numerically associated with a greater response in $[2H]_{Pr}$ than other types of inhibitors. In continuous culture, cashew nut shell liquid resulted in the numerically greater response in $[2H]_{Pr}$ when inhibiting methanogenesis, although these results were provided by one single experiment (Table S4).

BUTYRATE

In batch cultures, there was an interaction between $[2H]_{CH4}$ and the experiment effect on $[2H]_{But}$ ($p < 0.001$), with an overall quadratic tendency to decrease $[2H]_{But}$ with methanogenesis inhibition ($p = 0.13$; **Figure 3A**; **Table 3**). Ionophores ($p = 0.007$) associated with greater decrease in $[2H]_{But}$ with methanogenesis inhibition, whereas greater concentrate percentage ($p = 0.016$) and chemical inhibitors ($p < 0.001$) associated with lesser decrease in $[2H]_{But}$ (not shown). There was a tendency ($p = 0.13$) toward less decrease in $[2H]_{But}$ with methanogenesis inhibition with a mixed than with a roughage substrate in the five experiments of the study by O'Brien et al. (2013) (Table S3).

In continuous cultures, there was an interaction between $[2H]_{CH4}$ and the experiment effect on $[2H]_{But}$($p = 0.006$), with no overall relationship between $[2H]_{But}$ and $[2H]_{CH4}$ ($p = 0.84$;

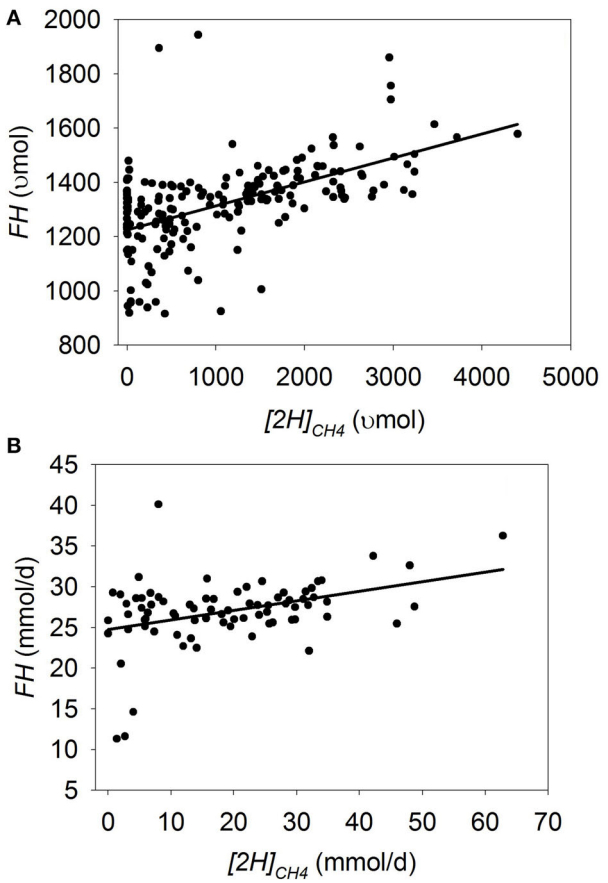

FIGURE 1 | Response of estimated fermented hexoses (*FH*) to metabolic hydrogen incorporated into CH_4 ($[2H]_{CH4}$). Individual responses are adjusted by their experiment effect (*exp*): **(A)** Batch cultures: $y = 1186$ (±44.6; $p < 0.001$) + *exp* ($p < 0.001$) + 0.11 (±0.068; $p = 0.12$) x + *exp* × x ($p = 0.041$); $R^2 = 0.94$ ($p < 0.001$); **(B)** Continuous cultures: $y = 24.4$ (±0.82; $p < 0.001$) + *exp* ($p < 0.001$) + 0.16 (±0.033; $p < 0.001$) x; ($R^2 = 0.74$; $p < 0.001$).

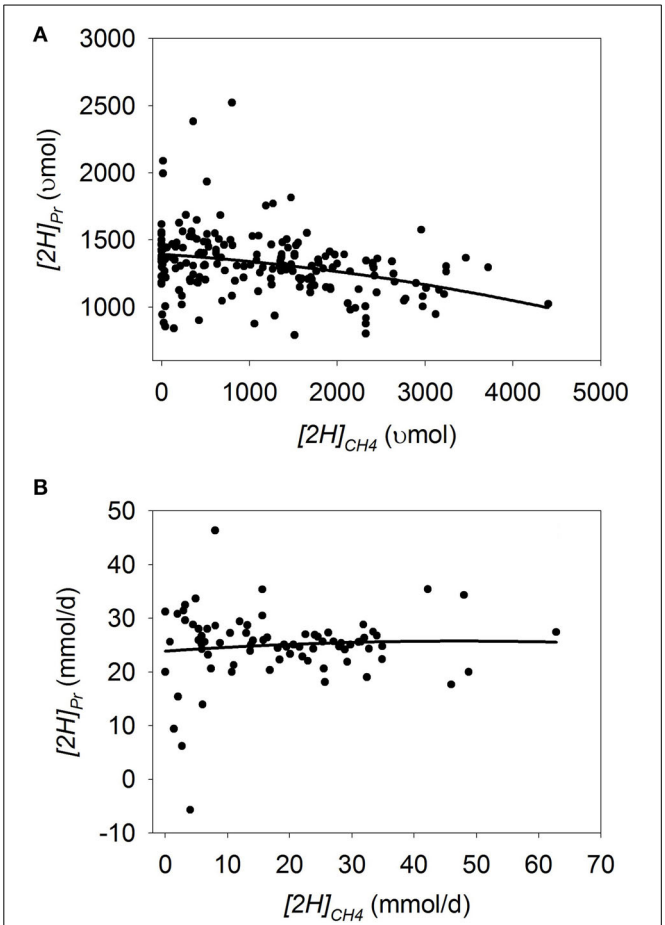

FIGURE 2 | Response of metabolic hydrogen incorporated into propionate ($[2H]_{Pr}$) to metabolic hydrogen incorporated into CH_4 ($[2H]_{CH4}$). Individual responses are adjusted by their experiment effect (*exp*): **(A)** Batch cultures: $y = 1443$ (±42.0; $p < 0.001$) + *exp* ($p < 0.001$) – 0.079 (±0.018; p < 0.001) $x - 2.63 \times 10^{-5}$ ($\pm 1.74 \times 10^{-5}$; $p = 0.13$) $(x - 1366)^2$; $R^2 = 0.82$ ($p < 0.001$); **(B)** Continuous cultures: $y = 26.7$ (±2.58; $p < 0.001$) + *exp* ($p < 0.001$) -0.040 (±0.14; $p = 0.77$) x - 0.0075 (±0.0050; $p = 0.14$) $(x - 18.4)^2$ + *exp* × x ($p = 0.08$); $R^2 = 0.79$ ($p < 0.001$).

Figure 3B; **Table 3**). Removing the experiment by García-López et al. (1996) from the analysis resulted in a decrease in $[2H]_{But}$ with the decrease in $[2H]_{CH4}$ ($p = 0.032$; not shown). Greater turnover time ($p = 0.015$) and concentrate percentage ($p = 0.024$), and use of chemical inhibitors of methanogenesis ($p < 0.001$), positively associated with $[2H]_{But}$ as methanogenesis was inhibited (not shown).

DIHYDROGEN

In batch cultures, there was a quadratic increase in $[2H]_{H2}$ with $[2H]_{CH4}$ decrease ($p < 0.001$), with an interaction with the experiment effect ($p < 0.001$; **Figure 4A**; **Table 3**). Greater amount of substrate ($p = 0.034$), concentrate percentage ($p < 0.001$), and duration of incubation ($p < 0.08$) associated or tended to associate with increased response in $[2H]_{H2}$ to $[2H]_{CH4}$ decrease. Ionophores ($p = 0.038$) and oils ($p < 0.001$) associated with lesser response in $[2H]_{H2}$ to $[2H]_{CH4}$ decrease (not shown). There was a tendency ($p = 0.06$) toward greater increase in $[2H]_{H2}$ with methanogenesis inhibition with a mixed than with a roughage substrate in the study by O'Brien et al. (2013) (Table S3).

In continuous culture, natural log transformation of the response variable $[2H]_{H2}$ was necessary to partially correct for a funnel-shaped residual plot. There was a linear increase in natural log $[2H]_{H2}$ with the decrease in $[2H]_{CH4}$ ($p < 0.001$) with an interaction with the experiment effect ($p = 0.042$; **Figure 4B**; **Table 3**). There was no interaction if the experiment by García-López et al. (1996), with the greatest H_2 accumulation, was eliminated from the analysis ($p = 0.22$; not shown). Greater concentrate percentage in the substrate was associated to greater $[2H]_{H2}$ when methanogenesis was inhibited ($p = 0.031$; not shown).

Linoleic and linolenic acid, and nitrate and nitrocompounds in batch culture, and ionophores in continuous culture, were associated with the numerically smallest increase in $[2H]_{H2}$ with methanogenesis inhibition (Table S4).

Table 3 | Metabolic hydrogen balance predicted[a] for 0 (control treatments)[b] and 100% methanogenesis inhibition.

	$[2H]_{produced}$[c] (μmol or mmol/d)	Metabolic hydrogen in:				$[2H]_{recovery}$ (%)[c, e]
		CH_4(μmol or mmol/d)	Propionate (μmol or mmol/d)	Butyrate (μmol or mmol/d)	H_2^d (μmol or mmol/d)	
BATCH CULTURES						
Control treatments	5248	2570	1201	715	14.4	95.2
100% methanogenesis inhibition	4033	0	1394	592	272	57.6
CONTINUOUS CULTURES						
Control treatments	103	29.0	24.7	14.6	0.087	67.9
100% methanogenesis inhibition	85.5	0	24.2	14.3	1.83	46.1

[a] *Regression equations for metabolic hydrogen incorporated in propionate, butyrate, H_2 and metabolic hydrogen recovery are provided in* ***Figures 2***, ***3***, ***4***, ***5****, respectively.*

[b] *Weighted percentage of $[2H]_{produced}$ incorporated into CH_4 in non-inhibited control treatments was 45.5 and 28.4% for batch and continuous cultures, respectively.*

[c] *Abbreviations: $[2H]_{produced}$, total production of reducing equivalents pairs; $[2H]_{recovery}$, sum of reducing equivalents pairs incorporated into CH_4, propionate, butyrate and H_2 divided by $[2H]_{produced}$, expressed as percentage.*

[d] *For continuous cultures, metabolic hydrogen incorporated into H_2 is presented backtransformed.*

[e] *Because $[2H]_{recovery}$ is the quotient between treatment means for $[2H]_{incorporated}$ and $[2H]_{produced}$ expressed as a percentage, predicted values for $[2H]_{recovery}$ are not equal to the quotient of predicted value of $[2H]_{incorporated}$ divided by predicted values of $[2H]_{produced}$ expressed as a percentage.*

$[2H]_{recovery}$

In batch cultures, there was a quadratic ($p = 0.037$) decrease in $[2H]_{recovery}$ as $[2H]_{CH4}$ decreased ($p < 0.001$) with an interaction $[2H]_{CH4}$ by experiment ($p < 0.001$; **Figure 5A**; **Table 3**). Bovine inoculum ($p < 0.001$) and greater inoculum volume ($p < 0.001$) were associated with greater decrease in $[2H]_{recovery}$ with methanogenesis inhibition. On the contrary, the decrease in $[2H]_{recovery}$ with methanogenesis inhibition was smaller with greater amount of substrate ($p = 0.001$), greater concentrate percentage ($p = 0.006$), and if CH_4 production was inhibited with oils ($p < 0.001$; not shown).

In continuous cultures, there was also a quadratic ($p = 0.002$) decrease in $[2H]_{recovery}$ with methanogenesis inhibition ($p < 0.001$) with an interaction with the experiment effect ($p = 0.017$; **Figure 5B**; **Table 3**). Greater concentrate percentage ($p = 0.04$) resulted in less $[2H]_{recovery}$ when methanogenesis was inhibited, whereas the use of oils to inhibit methanogenesis resulted in less decrease in $[2H]_{recovery}$ ($p = 0.002$; not shown).

HEAT OF COMBUSTION IN VFA

In batch cultures, there was no overall response in ΔH_{VFA} to methanogenesis inhibition ($p = 0.30$), although there was a tendency for an interaction between $[2H]_{CH4}$ and the experiment effect ($p = 0.06$; **Figure 6A**). If the experiment by Anderson et al. (2010) was removed, in which *FH* increased with methanogenesis inhibition, there was a negative association between Δ_{HVFA} and methanogenesis inhibition ($p = 0.016$; not shown). Greater concentrate percentage ($p < 0.001$), duration of incubation ($p < 0.001$) and utilization of chemical inhibitors of methanogenesis ($p < 0.001$) all interacted positively with ΔH_{VFA} when methanogenesis was inhibited (not shown).

In continuous cultures, inhibiting methanogenesis associated with a decrease in ΔH_{VFA} ($p < 0.001$), with no interaction with the experiment effect ($p = 0.52$; **Figure 6B**). However, there was no association of methanogenesis inhibition with ΔH_{VFA} after eliminating from the analysis the experiment by Slyter (1979), which contained two outliers in which methanogenesis inhibition was caused by lowering *pH* ($p = 0.38$; not shown), or the experiment by Slyter and Wolin (1967) ($p = 0.27$; not shown).

HEAT OF COMBUSTION IN GASES

In batch cultures, there was a quadratic decrease in ΔH_{gases} when methanogenesis was inhibited ($p < 0.001$), with an interaction with the experiment effect ($p < 0.001$; **Figure 7A**). Greater amount of substrate ($p = 0.034$), concentrate percentage ($p < 0.001$) and duration of the incubation ($p = 0.08$), associated or tended to associate with smaller decrease in ΔH_{gases} as methanogenesis was inhibited. On the contrary, utilization of ionophores ($p = 0.038$) and oils ($p < 0.001$) associated with greater decrease in ΔH_{gases} with methanogenesis inhibition.

In continuous cultures, there was a linear decrease in ΔH_{gases} with methanogenesis inhibition ($p < 0.001$), with no interaction with the experiment effect ($p = 0.46$; **Figure 7B**).

DISCUSSION

METABOLIC HYDROGEN RECOVERY AND MISSING SINKS

A complete [H] balance for predicted 0 and 100% methanogenesis inhibition in batch and continuous cultures is shown in **Table 3**. In batch cultures, at predicted 100% methanogenesis inhibition, an average of only 0.075 mol [H] per mol of [H] drawn away from CH_4 formation were redirected to propionate production (calculations from **Table 3**), and there was a tendency to less [H] into butyrate. There was no overall re-direction of [H] toward propionate and butyrate in continuous culture when methanogenesis was inhibited (**Table 3**). Inhibiting methanogenesis caused accumulation of H_2 (**Figures 4A,B**), but H_2 accumulation at predicted 100% methanogenesis inhibition accounted for only 10 and 6.0% of [H] in CH_4 in control treatments in batch and continuous cultures, respectively. There was less production of [H] when methanogenesis was inhibited both in batch and continuous cultures (**Table 3**); however, even when accounting for less $[H]_{produced}$, $[2H]_{recovery}$ in the main fermentation products

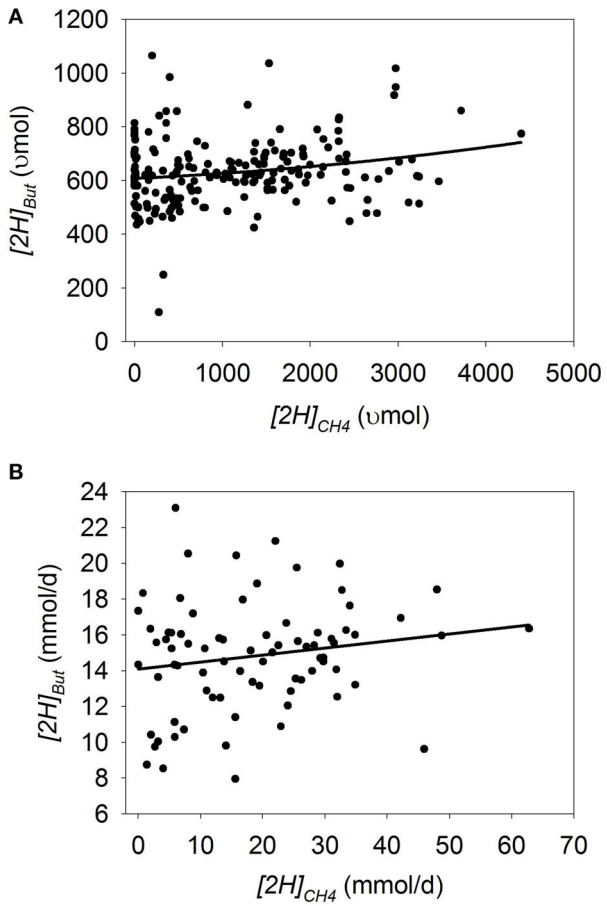

FIGURE 3 | Response of metabolic hydrogen incorporated into butyrate ($[2H]_{But}$) to metabolic hydrogen incorporated into CH_4 ($[2H]_{CH4}$). Individual responses are adjusted by their experiment effect (*exp*): **(A)** Batch cultures: $y = 556$ (±42.4; $p < 0.001$) + *exp* ($p < 0.001$) + 0.0508 (± 0.061; $p = 0.41$) $x + 1.94 \times 10^{-5}$ (±1.29×10^{-5}; $p = 0.13$) $(x - 1366)^2$ + *exp* × x ($p < 0.001$); $R^2 = 0.93$ ($p < 0.001$); **(B)** Continuous cultures: $y = 14.4$ (±1.05; $p < 0.001$) + *exp* ($p < 0.001$) + 0.012 (±0.056; $p = 0.84$) x + *exp* × x ($p = 0.006$); $R^2 = 0.87$ ($p < 0.001$).

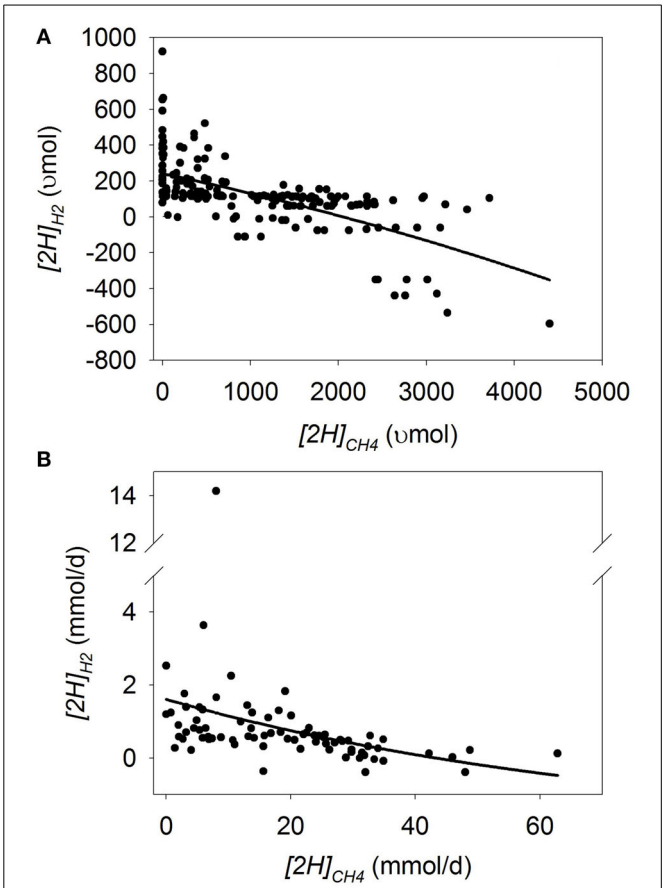

FIGURE 4 | Response of metabolic hydrogen incorporated into dihydrogen ($[2H]_{H2}$) to metabolic hydrogen incorporated into CH_4 ($[2H]_{CH4}$). Individual responses are adjusted by their experiment effect (*exp*): **(A)** Batch cultures: $y = 164$ (±41.8; $p < 0.001$) + *exp* ($p < 0.001$)–0.0907 (±0.0607; $p = 0.14$) $x + 5.77 \times 10^{-5}$ (±1.27×10^{-5}; $p < 0.001$) $(x - 1366)^2$ + *exp* × x ($p < 0.001$); $R^2 = 0.91$; $p < 0.001$; **(B)** Continuous cultures: L_N $(y + 1) = 1.041$ (±0.13; $p < 0.001$) + *exp* ($p < 0.001$) − 0.033 (±0.0070; $p < 0.001$) x + *exp* × x ($p = 0.042$); $R^2 = 0.76$ ($p < 0.001$).

decreased severely both in batch and continuous cultures when methanogenesis was inhibited (**Figures 5A,B**; **Table 3**). Therefore, a major [H] sink or sinks in the methanogenesis-inhibited ruminal fermentation was unaccounted for. Three potential unaccounted [H] sinks will be discussed: (i) fermentation products other than propionate, butyrate and H_2, (ii) microbial biomass, and (iii) reductive acetogenesis.

Other products of fermentation

Most experiments used in this meta-analysis reported valerate production. In batch cultures, the participation of valerate as a [H] sink slightly decreased from 4.8 to 4.5% between predicted 0 and 100% methanogenesis inhibition (not shown). In continuous cultures, valerate increased its participation as a [H] sink from 7.9 to 10.7% of total $[2H]_{produced}$ between predicted 0 and 100% methanogenesis inhibition (not shown). Caproate is an end product of fermentation even more reduced than valerate. Caproate was reported only in the study by Anderson et al. (2010) in batch culture, and tended to increased its participation from 2.2 to 4.8% of total $[2H]_{produced}$ between predicted 0 and 100% methanogenesis inhibition (not shown). Formate was reported in only 6 batch culture experiments, and at predicted 100% methanogenesis inhibition it accounted for an average of 16% of [H] in CH_4 in the corresponding control treatments (not shown). This figure is close to the missing [H] in CH_4 found in formate in recent experiments, which agree with the estimation obtained by Hungate et al. (1970) of 18% ruminal CH_4 formed from formate (S. Muetzel, pers.com). Immig (1996) reported no alterations in major fermentation products when formate was added to methanogenesis-inhibited incubations, suggesting that formate accumulated and was not further metabolized. In the continuous culture experiment by Slyter (1979), there was a slight formate accumulation when methanogenesis was inhibited using dichloroacetamide which accounted for little $[2H]_{incorporated}$. Metabolic hydrogen

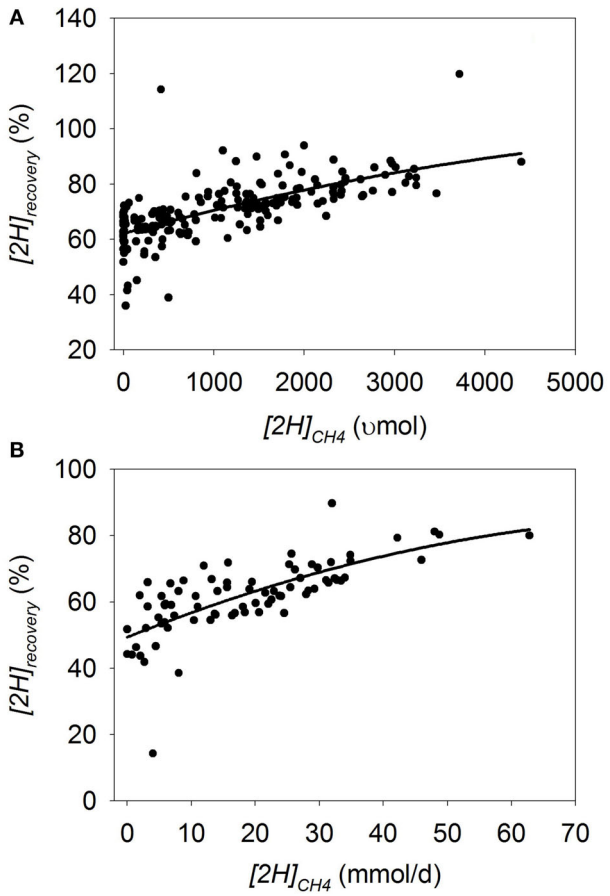

FIGURE 5 | Response of the percentage of metabolic hydrogen produced recovered in CH_4, propionate, butyrate and H_2 ($[2H]_{recovery}$) to metabolic hydrogen incorporated into CH_4 ($[2H]_{CH4}$). Individual responses are adjusted by their experiment effect (*exp*): **(A)** Batch cultures: $y = 59.7$ (± 1.76; $p < 0.001$) + *exp* ($p < 0.001$) + 0.0144 (± 0.00255; $p < 0.001$) $x - 1.12 \times 10^{-6}$ ($\pm 5.32 \times 10^{-7}$; $p = 0.037$) $(x - 1366)^2$ + *exp* × x ($p < 0.001$); $R^2 = 0.91$ ($p < 0.001$); **(B)** Continuous cultures: $y = 51.0$ (± 2.30; $p < 0.001$) + *exp* ($p < 0.001$) + 0.64 (± 0.12; $p < 0.001$) $x - 0.015$ (± 0.0045; $p = 0.002$) $(x - 18.4)^2$ + *exp* × x ($p = 0.017$); $R^2 = 0.87$ ($p < 0.001$).

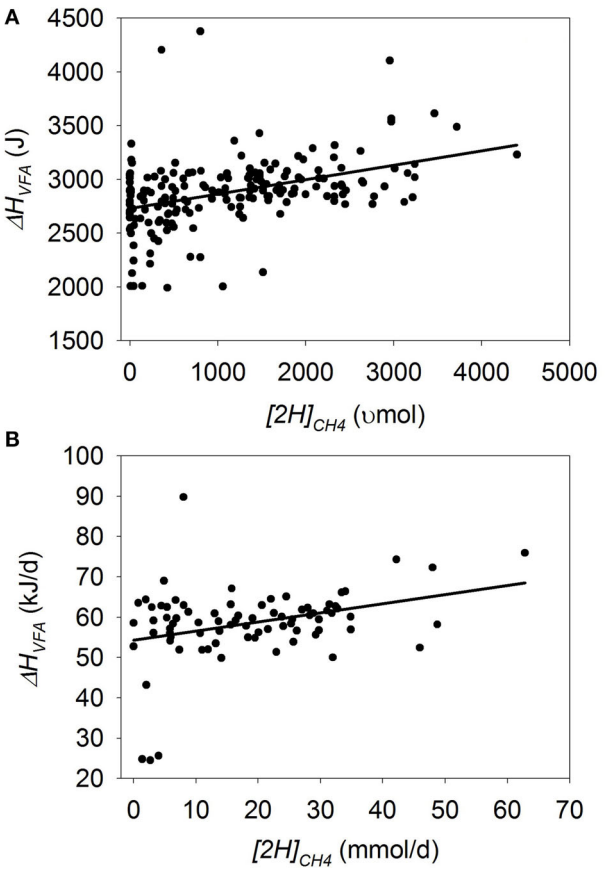

FIGURE 6 | Response of the heat of combustion in volatile fatty acids (ΔH_{VFA}) to metabolic hydrogen incorporated into CH_4 ($[2H]_{CH4}$). Individual responses are adjusted by their experiment effect (*exp*): **(A)** Batch cultures: $y = 2664$ (± 103; $p < 0.001$) + *exp* ($p < 0.001$) + 0.162 (± 0.156; $p = 0.30$) x + *exp* × x ($p = 0.06$); $R^2 = 0.93$ ($p < 0.001$); **(B)** Continuous cultures: $y = 53.9$ (± 1.91; $p < 0.001$) + *exp* ($p < 0.001$) + 0.29 (± 0.077; $p < 0.001$) x; $R^2 = 0.72$ ($p < 0.001$).

incorporation into succinate, ethanol and lactate accounted for little $[2H]_{incorporated}$ both in batch and continuous cultures (not shown). It is important that formate and other atypical reduced end products of fermentation, apart from H_2, as well as the more reduced VFA valerate and caproate, are reported in experiments in which methanogenesis is inhibited.

Microbial biomass

Czerkawski (1986) proposed that inhibiting methanogenesis could favor microbial biomass production as an alternative [H] sink. Chalupa (1977) suggested that [H] incorporated into excess NADH was redirected to fatty acids synthesis and fermentation end products such as lactate and ethanol, although the latter sinks were not quantitatively important in the experiments that reported them in the present analysis (not shown).

Microbial biomass production depends on the rates and efficiencies of ATP generation (catabolism) and utilization for growth (anabolism). Inhibiting methanogenesis decreases the system's reducing potential through greater availability of reducing equivalents in electron donors such as H_2 (Sauer and Teather, 1987) and NADH (Hino and Russell, 1985). If anabolic processes that incorporate reducing equivalents, such as fixation of NH_4^+ into carbon chains for amino acids synthesis, and synthesis of fatty acids, were kinetically limited by the availability of [H] (or were close to thermodynamic equilibrium), increased availability of [H] consequence of inhibiting methanogenesis could stimulate amino acids and fatty acids synthesis and therefore microbial growth. Stimulation of microbial anabolism could then increase the importance of microbial biomass as a [H] sink through two mechanisms: (i) Greater microbial biomass production; (ii) A more reduced microbial biomass composition: if microbial content of lipids increased, that would mean that more [H] was incorporated per gram of microbial organic matter produced.

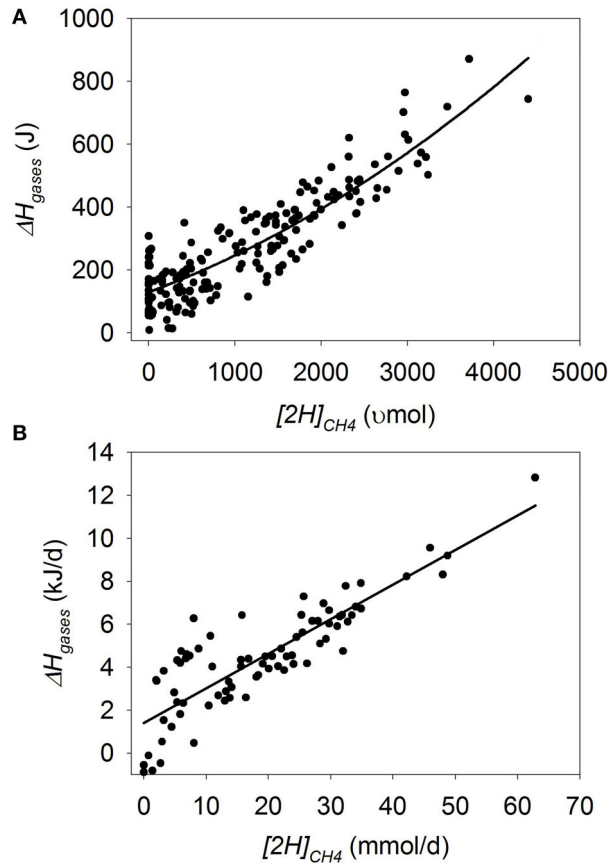

FIGURE 7 | Response of the heat of combustion in gases (ΔH_{gases}) to metabolic hydrogen incorporated into CH_4 ($[2H]_{CH4}$). Individual responses are adjusted by their experiment effect (*exp*): **(A)** Batch cultures: $y = 46.9$ (± 12.0; $p < 0.001$) + *exp* ($p < 0.001$) + 0.20 (± 0.017; $p < 0.001$) x + 1.65×10^{-5} ($\pm 3.63 \times 10^{-6}$; $p < 0.001$) $(x - 1366)^2$ + *exp* × x ($p < 0.001$); $R^2 = 0.98$ ($p < 0.001$); **(B)** Continuous cultures: $y = 0.66$ (± 0.15; $p < 0.001$) + *exp* ($p = 0.057$) + 0.21 (± 0.0062; $p < 0.001$) x; $R^2 = 0.96$ ($p < 0.001$).

Inhibiting methanogenesis could also have consequences on microbial ATP generation. In anaerobic systems like the rumen, part of the negative Gibbs energy change associated to fermentation is used to generate ATP through substrate level and electron transport-linked phosphorylation. An inhibition of *FH*, as in most batch culture experiments in the present meta-analysis, should decrease ATP generation. Apart from being affected by *FH*, ATP generation can be affected by the fermentation profile. It has been shown in experiments with defined cultures that the presence of methanogens alters the pattern of fermentation of bacteria and fungi. A shift from acetate, ethanol, H_2 and CO_2 produced by *Ruminococcus albus* in pure culture, to acetate and CH_4 in co-culture with a methanogen, increased ATP generation by *R. albus* and added the ATP generated by the methanogen (Wolin et al., 1997). Similarly, ruminal anaerobic fungi decreased the production of ethanol and lactate, and increased the production of acetate, when grown with methanogens, increasing ATP generation (Bauchop and Mountfort, 1981; Marvin-Sikkema et al., 1990).

However, when inhibiting methanogenesis in mixed cultures, accumulation of ethanol seems of small importance compared to pure cultures of ethanol producers. In the present meta-analysis, there was an increase in [H] incorporated into propionate production in batch cultures when methanogenesis was inhibited. This agrees with greater production of succinate and propionate by *R. flavefaciens* or *Selenomonas ruminantium*, respectively, when grown in pure culture than when co-cultured with methanogens (Wolin et al., 1997). It is important to understand what could be the consequences of an acetate to propionate shift on microbial ATP generation.

Propionate is produced *via* succinate (randomizing pathway) or acrylate (non-randomizing pathway) in the rumen. Reduction of fumarate to succinate is associated with ATP generation through a transmembrane electrochemical gradient (Russell and Wallace, 1997), but the acrylate pathway does not seem to be associated with ATP generation (Thauer et al., 1977). The proportions of extra propionate produced through the succinate and acrylate pathways when inhibiting methanogenesis are unknown, and therefore constitute an uncertainty of the consequences of an acetate to propionate shift upon ATP generation. Furthermore, the stoichiometry of ATP generation per pair of reducing equivalents incorporated in the reduction of fumarate to succinate is not clearly established as measurements have disagreed (Reddy and Peck, 1978; Kröger and Winkler, 1981).

Generation of ATP in propionate's randomizing pathway is also affected by other metabolic steps. Oxaloacetate (or malate) formation from phosphoenolpyruvate (PEP) entails the utilization of PEP high-energy phosphate bond, which is then not used to generate ATP, but when catalyzed by PEP carboxykinase, PEP carboxylation can still generate ATP or GTP without additional energy input (Atwal and Sauer, 1974). Also, when the entire randomizing pathway takes place in a single cell (i.e., without interspecies transfer of succinate), carboxylation of pyruvate to form oxaloacetate can occur coupled to methylmalonyl-CoA decarboxylation to propionyl-CoA, making oxaloacetate formation from pyruvate energetically neutral (Deborde and Boyaval, 2000).

Therefore, our knowledge about ATP generation by the mixed ruminal microbiota is incomplete due to the difficulties to determine ATP generation associated to propionate production. For the moment, it seems that we will have to rely on the few existing empirical results on the response of microbial growth to methanogenesis inhibition rather than on a deeper mechanistic understanding on the consequences of methanogenesis inhibition on microbial ATP generation and utilization. Improved microbial growth efficiency allowed obtaining increased microbial biomass with methanogenesis inhibition even with decreased true organic matter digestibility (Ungerfeld et al., 2007). Other *in vitro* work also found stimulation of microbial growth by methanogenesis inhibition (van Nevel et al., 1969; Guo et al., 2009). Nolan et al. (2010) reported a numerical increase of 27% in microbial protein production in sheep whose CH_4 production was decreased by 23%. However, other work has found no effect or even a decrease in microbial growth with methanogenesis inhibition (van Nevel et al., 1969, 1971; Russell and Martin, 1984; Lee et al., 2009). Apart from biomass accretion, a more reduced microbial

biomass composition could account for part of the "missing [H]." Changes in microbial biomass amount and composition, shifts in anabolic pathways, and ultimately ATP generation and utilization, as a response to methanogenesis inhibition, need to be studied.

Reductive acetogenesis

If some acetate produced when methanogenesis is inhibited was originated from reductive acetogenesis, part of acetate production would be an unaccounted [H] sink. In the typical ruminal fermentation, methanogens drop H_2 pressure to a level at which reductive acetogenesis has been estimated to be thermodynamically unfeasible (Kohn and Boston, 2000) and as a result the process does not occur (le Van et al., 1998). However, reductive acetogenesis has been shown to occur in batch cultures if methanogenesis is inhibited and reductive acetogens are added (Nollet et al., 1997; le Van et al., 1998). Native reductive acetogens do inhabit the rumen even when methanogenesis is not inhibited (Leedle and Greening, 1988; Henderson et al., 2010), but they seem to rely on substrates other than CO_2 and H_2 (Joblin, 1999). It is possible that reductive acetogenesis could over time become a [H] sink in continuous culture and *in vivo* when methanogenesis is inhibited. It is unknown whether or not some reductive acetogens can adapt to methanogenesis-inhibited continuous culture conditions to conduct reductive acetogenesis. In the experiments used for this meta-analysis, acetate produced through fermentation of carbohydrates would be indistinguishable from acetate produced from reductive acetogenesis, if the latter occurred. Therefore, decreases observed in acetate production with methanogenesis inhibition (data not shown) are not evidence against the possibility of reductive acetogenesis.

VARIATION IN RESPONSES OF [H] SINKS TO METHANOGENESIS INHIBITION

Shifts in [H] sinks when methanogenesis was inhibited varied between batch and continuous cultures, and among experiments within each kind of system. It is of much interest to understand the causes of this variation, especially what the experiments with a greater than average response in $[2H]_{Pr}$ or $[2H]_{But}$, and lower than average response in $[2H]_{H2}$, have in common. Some factors that may affect [H] shifts when methanogenesis is inhibited are discussed below.

Batch vs. continuous cultures

Redirection of [H] toward propionate production when methanogenesis was inhibited was more favored in batch than in continuous cultures. Perhaps, important propionate producers do not survive well, or at least are not stimulated as much by methanogenesis inhibition, in continuous compared to batch culture. Decline in microbial diversity over time in continuous cultures has been reported (Johnson et al., 2009). For example, Broudiscou et al. (2014) reported that the effect of H_2 pressure on propionate production per mol of *FH* in batch cultures depended on the source of inoculum, which were continuous cultures incubated with different substrates. Because they studied the effect of added H_2 on short-term, 6 h batch incubations, the presence of sufficient numbers of propionate producers in their continuous culture inocula may have determined the response of propionate to H_2. In fact, the greatest response in propionate to headspace H_2 in the study by Broudiscou et al. (2014) came from the inoculum obtained from a continuous culture fed a roughage substrate which produced more propionate per mole of *FH* than two other continuous cultures fed different high concentrate substrates.

It is possible that some propionate or succinate producers do not adapt well to continuous cultures to take advantage of the favorable thermodynamic conditions for propionate production occurring when methanogenesis is inhibited. Differences between batch and continuous cultures have been reported for some succinate-producing bacteria with regard to their response to methanogenesis inhibition. For example, inhibiting methanogenesis resulted in an increase in *Fibrobacter succinogenes* in ruminal batch cultures (Guo et al., 2007, 2008; Goel et al., 2009), but no changes in continuous cultures (Goel et al., 2009), although lack of effects in batch cultures has also been reported (González et al., 2006). *R. flavefaciens* was either decreased (Goel et al., 2009) or unaffected (González et al., 2006; Guo et al., 2007, 2008) by inhibiting methanogenesis in batch cultures, and decreased in continuous cultures (Goel et al., 2009).

An alternative explanation to greater response in propionate in batch than in continuous culture could be based on thermodynamics. Most batch culture systems operate with pressurized tubes or bottles, allowing greater H_2 pressure at equal H_2 molar percentage in comparison to continuous cultures, where gas is collected at nearly atmospheric pressure. Elevation of H_2 pressure favors a shift from acetate to propionate production (Janssen, 2010). Based on this technical difference between most batch and continuous cultures, it could be expected that batch cultures favor more a shift to propionate compared to continuous cultures. Indeed, there was a negative quadratic relationship between the estimated final H_2 pressure in batch cultures and the acetate to propionate molar ratio ($R^2 = 0.62$; $P < 0.001$; not shown). Theoretically, batch incubation systems that allow the release of gas to the atmosphere (Wang et al., 2013) should better mimic the *in vivo* situation from a thermodynamics point of view.

Despite most batch culture systems resulting in supra-atmospheric total gas pressures, inhibition of methanogenesis resulted in greater H_2 accumulation in batch than in continuous culture. This could suggest some adaptation over time of continuous cultures to incorporate part of the accumulated H_2 into other pathways not modeled in the present analysis e.g., reductive acetogenesis. In agreement, a progressive decrease in H_2 accumulation in batch cultures from 24 to 48 and 72 h of incubation was observed when methanogenesis was inhibited with 2-bromoethanesulphonic acid (Lee et al., 2009). Still, mid- and long-term H_2 accumulation has been reported to occur in continuous culture with high-concentrate substrates even in the absence of methanogenesis inhibitors (Broudiscou et al., 2014), as well as in various methanogenesis inhibition *in vivo* experiments (Trei et al., 1972; Clapperton, 1974; Kung et al., 2003; van Zijderveld et al., 2011; Mitsumori et al., 2012), and incubations of rumen contents from animals administered methanogenesis inhibitors

for long periods (Czerkawski and Breckenridge, 1975). Therefore, it seems that, although there can be some long term adaptation of microbiota to incorporate [H] into alternative pathways in the methanogenesis-inhibited ruminal fermentation, this is not complete and consistent, and H_2 remains as an end product of fermentation (with the possible exception of the use of some lipids as methanogenesis inhibitors, as discussed below).

It is difficult to conclude on whether the batch or continuous culture system might better reflect changes in fermentation occurring *in vivo* when methanogenesis is inhibited. If there is some long-term adaptation of microbiota to incorporate H_2 into other pathways, the continuous culture systems may better represent the *in vivo* situation in comparison to batch culture. On the other hand, the slight overall increase in propionate production observed in batch culture may better reflect the increase in propionate concentration or molar proportion of most experiments when methanogenesis is inhibited *in vivo* (Clapperton, 1974; Davies et al., 1982; McCrabb et al., 1997; Abecia et al., 2012, 2013; Mitsumori et al., 2012), although actual VFA production, which includes VFA removed by both absorption and passage from the rumen, as well as VFA incorporated into microbial fatty acids and amino acids (Kristensen, 2001), was not quantified in those *in vivo* studies. Simultaneous *in vivo* measurement of VFA and gases actual production when inhibiting methanogenesis would be necessary to conclude which type of *in vitro* system, if any, can better mimic the *in vivo* situation.

Type of substrate

Both in batch and continuous culture, redirection of [H] toward H_2 was greater with more concentrate in the substrate, and in batch culture there was greater H_2 accumulation with greater DMD (not shown). Bauchop (1967) found a 2-fold increase in H_2 accumulation when inhibiting methanogenesis with chlorinated CH_4 analogs in the absence of added substrate, whereas H_2 accumulation was much greater if ruminal solids or formate were incubated. Czerkawski and Breckenridge (1975) reported that when CH_4 production was inhibited, H_2 accumulation was much greater 2 h after the morning feeding, in an actively fermenting rumen, compared to pre-feeding levels. Perhaps, that could be an indication that incorporation of [H] into sinks other than H_2 when fermentation is very active might be limited by the activity of alternative [H]-incorporating pathways i.e., enzyme kinetics. In steers not subjected to methanogenesis-inhibition treatments, Rooke et al. (2014) determined that animals fed a high concentrate diet produced gas with a greater ratio of H_2 to CH_4 compared to those fed a mixed diet, and suggested that rapid H_2 production immediately after feeding can result in methanogens being overloaded in their capacity to utilize H_2. This effect would likely accentuate in the methanogenesis-inhibited fermentation. Slowly degradable diets and uniform feed intake might then be strategies to decrease H_2 accumulation when methanogenesis is inhibited. In that regard, Swainson et al. (2011) suggested that there was a greater spillover of H_2 from fermentation in sheep fed twice a day than 8 times a day. Elevated H_2 associated to rapid fermentation may also been a consequence of methanogens being inhibited by the low ruminal pH associated with the consumption of high concentrate diets (Janssen, 2010).

Interaction with pH

Greater increase in $[2H]_{Pr}$ was obtained by inhibiting methanogenesis at lower pH in batch, but not in continuous culture (not shown). Even though the range in pH was greater in continuous than in batch cultures, the mean (**Table 1**) and median final pH of batch cultures were numerically lower than pH of continuous cultures. Perhaps better buffered continuous cultures precluded detecting interactions between methanogenesis inhibition and pH. With the exception of the Slyter (1979) experiment in continuous culture in which the pH of the buffer was varied in some treatments, for the rest of the treatment means in the batch and continuous cultures databases, pH was a response to treatments, rather than a treatment itself. Controlled experiments of methanogenesis inhibition at different pH would have more power to detect the interaction between methanogenesis inhibition and pH on [H] sinks.

Type of CH_4 production antagonist

Perhaps the most interesting result related to variation in [H] sinks among experiments was the lack of detection of H_2 accumulation with strong methanogenesis inhibition with linoleic and linolenic acids (O'Brien et al., 2013). Other *in vitro* batch (van Nevel and Demeyer, 1981) and continuous (Czerkawski and Clapperton, 1984; Machmüller et al., 1998) culture (Table S4), and *in vivo* (Clapperton, 1974; Czerkawski and Clapperton, 1984) experiments with linseed oil, which is rich in linolenic acid, also found minimal or no H_2 accumulation. On the other hand, Marty and Demeyer (1973) reported considerable H_2 accumulation when inhibiting CH_4 production in batch cultures with linseed oil. Czerkawski and Clapperton (1984) indicated that linseed oil stimulated microbial growth, which would be an alternative [H] sink to CH_4 and H_2, although the opposite result was reported by van Nevel et al. (1969).

It is interesting that linoleic and linolenic acid and linseed oil were strongly propionogenic with mixed (O'Brien et al., 2013) or chemically defined (van Nevel and Demeyer, 1981), but not with roughage (Fievez et al., 2007; O'Brien et al., 2013), substrates (Table S4). Indeed, between about 25 and 50% of [H] spared from methanogenesis was redirected toward propionate production with mixed (O'Brien et al., 2013) and chemically defined substrates (van Nevel and Demeyer, 1981) when methanogenesis was inhibited with linoleic or linolenic acids or linseed oil, which is numerically much higher than [H] redirection to propionate with most of the rest of the inhibitors (Table S4). In continuous culture, linseed supplementation stimulated propionate production disregarding the level of concentrate inclusion (Machmüller et al., 1998). Clapperton (1974) and Yang et al. (2009) reported increases in ruminal propionate concentration *in vivo* with linseed supplementation of mixed diets. Stimulation of propionate production as an alternative [H] sink could therefore partially explain lack of or small H_2 accumulation when methanogenesis was inhibited using linoleic and linolenic acid with mixed and chemically defined substrates, although this explanation seems less likely with roughages. Other unsaturated oils such as some fish (Fievez et al., 2003) and algae (Fievez et al., 2007) oils in batch culture, and sunflower seed (Machmüller et al., 1998) and monolaurin (Klevenhusen et al., 2009) in continuous

culture, were also associated with small H_2 production and were propionogenic.

Research is needed to understand how linolenic acid and linseed oil promoted propionate production, and how that seems to be substrate-dependent. Linseed oil supplementation increased propionate concentration in the rumen, but succinate-producing *F. succinogenes* and *R. flavefaciens* numbers were decreased (Yang et al., 2009). In a batch culture experiment, both linoleic and linolenic acid stimulated propionate production and inhibited *F. succinogenes*, but effects on *R. flavefaciens* were less clear (Zhang et al., 2008). These results are in agreement with pure culture results in which both linoleic and linolenic acid were inhibitory to *F. succinogenes* and *R. flavefaciens*; however, other succinate and propionate producers such as *R. amylophilus*, *Prevotella* spp., *Mitsuokella multiacidus*, *S. ruminantium*, *Veilonella parvula,* and *Anaerovibrio lipolytica* were insensitive to linoleic and linolenic acids at the concentration tested (Maia et al., 2007). Greater toxicity of linoleic and linolenic acids to cellulolytic bacteria and fungi than to other species (Maia et al., 2007), might then explain why linoleic and linolenic acid stimulated propionate more with a mixed than with a roughage substrate (O'Brien et al., 2013). The response of non-cellulolytic succinate and propionate producers in mixed cultures to linoleic and linolenic acid, and linseed oil, would need to be studied.

Unsaturated fatty acids may also decrease H_2 accumulation through biohydrogenation. It is true that the decrease of CH_4 production through competition for [H] by fatty acids biohydrogenation is considered to be small (Nagaraja et al., 1997). However, because H_2 accumulation when methanogenesis is inhibited is smaller than CH_4 production in the typical, methanogenesis-uninhibited ruminal fermentation, the relative biohydrogenation effect on H_2 accumulation would be more important than on CH_4. Furthermore, the effect of biohydrogenation on H_2 would be stoichiometrically greater than on CH_4 by a 4 to 1 ratio. For example, linolenic acid at 1.25 ml/l decreased CH_4 production by 60 and 70% with a mixed and a roughage substrate, respectively (O'Brien et al., 2013). Complete biohydrogenation of linolenic acid at 1.25 ml/l to stearic acid would have incorporated 14 and 18% of $[2H]_{CH4}$ in the control treatment (calculations not shown). This is greater than the average percentage of [H] diverted from CH_4 to H_2 in batch cultures at predicted 100% methanogenesis inhibition (calculation from **Table 3**), and thus, theoretically, it could explain lack of H_2 accumulation. It should be considered, however, that 1.25 ml/l of linolenic acid represented about 11% of the substrate incubated and resulted in decreased DMD (O'Brien et al., 2013); less $[2H]_{produced}$, would then also contribute to explain lack of detection of H_2.

Unsaturated fatty acids have been shown to inhibit lipolysis and biohydrogenation (Noble et al., 1974; Beam et al., 2000; Boeckaert et al., 2007, 2008; Toral et al., 2012), but absolute [H] incorporated of into fatty acids double bonds might increase with oil supplementation, even if the proportion of reduced double bonds relative to the total double bonds available in fatty acids was decreased. The net result would depend on the balance between the limiting [H]-incorporating substrate (fatty acids double bonds) and the effect of the oil supplement on enzyme activity (inhibition of lipases, isomerases and reductases).

The classification of methanogenesis inhibitors into chemical inhibitors, ionophores, oils, and nitrate/nitrocompounds, was inaccurate to describe the effects of the different inhibitors on fermentation shifts. For example, two fish oils differed in the extent they caused H_2 accumulation and stimulated propionate production when inhibiting methanogenesis (Fievez et al., 2003). It has been known that fatty acids differ on their effects on CH_4 production, with polyunsaturated and medium-chain free fatty acids exerting the greatest inhibitory effects on methanogenesis (Beauchemin et al., 2008; Martin et al., 2010); from this analysis, it appears that fatty acids also differ regarding the consequences on [H] incorporation into other pathways when methanogenesis is inhibited. Also, a hexadecatrienoic acid from algae origin (Ungerfeld et al., 2005) caused considerable more H_2 accumulation than a commercial algae product (Fievez et al., 2007). Content of algal secondary compounds such as isoprenoids and small halogenated compounds varies among algae lineages and is associated with the extent of methanogenesis inhibition algae exert *in vitro* (Machado et al., 2014); the type and content of secondary compounds can contribute to explain differences among algae and algae-based products on their effects on shifts in [H] sinks when methanogenesis is inhibited.

Nitrate caused somewhat less H_2 accumulation than did nitrocompounds (not shown). Reduction of NO_3^- to NH_4^+ is both thermodynamically and stoichiometrically very favorable (Thauer et al., 1977). Nitrate reduction would then outcompete H_2 formation for [H], likely explaining the relatively small effect of NO_3^- on H_2 accumulation; however, *in situ* reduction of the nitro moiety in nitrocompounds (Anderson et al., 1993) could be thought to act similarly, and therefore nitrocompounds would be expected to cause equally small H_2 accumulation as NO_3^-.

Inhibiting methanogenesis decreased *FH* in batch cultures. In contrast to this overall response, nitrocompounds increased *FH* in the experiment by Anderson et al. (2010). In other *in vitro* and *in vivo* experiments with nitrocompounds, total VFA concentration was generally not decreased (Anderson et al., 2003, 2006; Brown et al., 2011; Martínez-Fernández et al., 2014), perhaps because nitrocompounds might be partly metabolized to VFA. Nitrocompounds such as 3-nitropropionate may perhaps stimulate propionate production because they were partly converted to propionate. However, 3-carbon units 2-nitropropanol decreased propionate production, and on the other hand 2-carbon unit compounds nitroethane and nitroethanol increased propionate production (Anderson et al., 2003, 2010).

Martínez-Fernández et al. (2014) reported no effects of nitrocompounds on *in situ* apparent digestibility. Inhibiting methanogenesis *in vivo* using 3-nitrooxypropanol resulted in a quadratic tendency to increase dry matter and organic matter digestibility at the highest dose (Romero-Perez et al., 2014). A complete understanding of the effects of nitrocompounds on [H] sinks in the rumen is of much interest to applied animal production.

IMPLICATIONS TO ANIMAL PRODUCTION

In general, the main objective of increasing the output of heat of combustion in VFA through methanogenesis inhibition was

little or not achieved. This was not because of the energetic inefficiency represented by H_2 accumulation, as gaseous energy losses steadily decreased with the decline in CH_4 production (**Figure 7**), but because of the inhibition of fermentation that generally accompanied methanogenesis inhibition. However, there was much variation among experiments and between *in vitro* systems. Methanogenesis inhibition using linoleic and linolenic acid was associated with little or no H_2 accumulation, and no decrease in DMD in case of linoleic acid (although *FH* decreased). Also, nitrocompounds were not associated with a decrease in *FH*. As a result, nitrocompounds, and linoleic and linolenic acids at some concentrations, increased the output of heat of combustion in VFA.

It is generally accepted that elevated H_2 pressure can hinder cofactors re-oxidation and thus inhibit fermentation (Wolin et al., 1997). In the present meta-analysis, H_2 pressure negatively associated with *FH* and neutral detergent fiber digestibility, but not with DMD (not shown). Broudiscou et al. (2014) reported that the effect of headspace H_2 pressure on *FH* in batch cultures was dependent on the source of inoculum. Some *in vivo* studies (Clapperton and Czerkawski, 1971; Cole and McCroskey, 1975; Reynolds et al., 2014) reported negative effects of methanogenesis inhibition on apparent digestibility, although most (Clapperton, 1974; Johnson, 1974; Cole and McCroskey, 1975; Kung et al., 2003; Knight et al., 2011; Mitsumori et al., 2012; Martínez-Fernández et al., 2014; Romero-Perez et al., 2014) did not, despite the elevated H_2 reported in some of these experiments. Johnson (1972) reported a tendency for an interaction between methanogenesis inhibition and intake level on energy digestibility in sheep. Also working with sheep, Sawyer et al. (1974) reported an increase in digestibility of most feed fractions when CH_4 production was inhibited. Neither apparent digestibility nor *FH* consider carbon from substrate digested and incorporated into microbial biomass; if methanogenesis inhibition stimulated microbial biomass production, it may decrease apparent but not true dry matter digestibility in some experiments. Effects of methanogenesis inhibition on true digestibility, and partition of digested and fermented substrate into fermentation products and microbial biomass need to be further studied in order to better understand the relationship between H_2 pressure and fermentation and digestion.

If, as previously discussed, microbial biomass can be one alternative [H] sink to CH_4, the additional microbial protein could benefit those animals not covering their metabolizable protein requirements. Some benefits in N retention in sheep when inhibiting CH_4 production have been reported (Singh and Trei, 1972; Johnson, 1974). A recent experiment did not find effects of inhibiting methanogenesis on N excreted in urine, although the decrease in CH_4 production was relatively small (Reynolds et al., 2014). General effects of methanogenesis inhibition on microbial anabolism and whole animal N metabolism are presently poorly understood, and research is needed to address this problem. It is important that methanogenesis inhibition experiments report formate as a [H] sink, as well as the more reduced VFA valerate and caproate and atypical reduced metabolites such as alcohols. In continuous cultures and *in vivo*, it is also possible that part of [H] when methanogenesis is inhibited was diverted to reductive acetogenesis, where existing reductive acetogens might have a greater opportunity to increase their numbers and/or reductive acetogenic activity over time under elevated H_2 pressure. In agreement, in the present meta-analysis, numerically less H_2 accumulated relative to methanogenesis inhibition in continuous than in batch cultures. It would be interesting if reductive acetogenesis could be stimulated *in vivo* when methanogenesis is inhibited.

With regard to the environmental impact of H_2 emissions in the methanogenesis-inhibited rumen, H_2 has a global warming potential of 5.8 compared to 25 of CH_4 on a CO_2-mass equivalent basis (Rooke et al., 2014). At predicted 100% methanogenesis inhibition, global warming potential of H_2 was 9.8 and 5.8% of global warming potential of control treatments in batch and continuous cultures, respectively (not shown). Therefore, if the *in vitro* conditions analyzed applied *in vivo*, inhibiting methanogenesis would have been environmentally beneficial in spite of H_2 accumulation. From a production point of view however, enthalpy in nutritionally useful fermentation products would have been decreased in many cases.

Batch and continuous cultures differed with regard to redirection of [H] toward propionate and H_2 when methanogenesis was inhibited. The implications of this analysis to the live animal could be quite different depending on which of the *in vitro* systems, if any, better reproduce the *in vivo* situation. Lack of *in vivo* data allowing calculation of complete [H] balances makes it difficult to conclude on whether batch or continuous cultures, or neither of the two, are a better model of the animal with regard to [H] shifts when ruminal methanogenesis is inhibited. This highlights the need for *in vivo* experiments on methanogenesis inhibition where VFA and gases production are simultaneously measured, studying also changes in true digestibility, microbial biomass production and chemical composition, and whole animal N metabolism, and the microbial community structure.

CONCLUSIONS

The main findings of this meta-analysis are:

(i) Inhibiting methanogenesis resulted in a moderate increase in [H] incorporation into propionate production in batch cultures, and no increase in continuous cultures. There was no increase in butyrate in either system;
(ii) Benefit of inhibiting methanogenesis in total energy output in VFA depended on the inhibitor and concentration used and on the *in vitro* system;
(iii) Both in batch and continuous cultures, inhibiting methanogenesis resulted in a consistent decrease in the percentage of $[H]_{produced}$ recovered as the sum of [H] incorporated into propionate, butyrate, CH_4 and H_2.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2015.00037/abstract

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Corrigendum: Shifts in metabolic hydrogen sinks in the methanogenesis-inhibited ruminal fermentation: a meta-analysis

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A Corrigendum on

Shifts in metabolic hydrogen sinks in the methanogenesis-inhibited ruminal fermentation: a meta-analysis
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I wish to point out a mistake I made in the Discussion section of Ungerfeld, E. M. (2015) Shifts in metabolic hydrogen sinks in the methanogenesis-inhibited ruminal fermentation: a meta-analysis. Front. Microbiol. 6:37. doi: 10.3389/fmicb.2015.00037, which was kindly pointed out to me by Dr. Stéphane Duval. When citing the articles by Martínez-Fernández et al. (2014) and Romero-Perez et al. (2014), I mistakenly confused nitrooxycompounds with nitrocompounds. Both Martínez-Fernández et al. (2014) and Romero-Perez et al. (2014) evaluated nitrooxycompounds, not nitrocompounds, in their work. I wish to acknowledge this mistake and thank Dr. Stéphane Duval for contacting me on this.

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Redirection of Metabolic Hydrogen by Inhibiting Methanogenesis in the Rumen Simulation Technique (RUSITEC)

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A decrease in methanogenesis is expected to improve ruminant performance by allocating rumen metabolic hydrogen ([2H]) to more energy-rendering fermentation pathways for the animal. However, decreases in methane (CH_4) emissions of up to 30% are not always linked with greater performance. Therefore, the aim of this study was to understand the fate of [2H] when CH_4 production in the rumen is inhibited by known methanogenesis inhibitors (nitrate, NIT; 3-nitrooxypropanol, NOP; anthraquinone, AQ) in comparison with a control treatment (CON) with the Rumen Simulation Technique (RUSITEC). Measurements started after 1 week adaptation. Substrate disappearance was not modified by methanogenesis inhibitors. Nitrate mostly seemed to decrease [2H] availability by acting as an electron acceptor competing with methanogenesis. As a consequence, NIT decreased CH_4 production (−75%), dissolved dihydrogen (H_2) concentration (−30%) and the percentages of reduced volatile fatty acids (butyrate, isobutyrate, valerate, isovalerate, caproate and heptanoate) except propionate, but increased acetate molar percentage, ethanol concentration and the efficiency of microbial nitrogen synthesis (+14%) without affecting gaseous H_2. Nitrooxypropanol decreased methanogenesis (−75%) while increasing both gaseous and dissolved H_2 concentrations (+81% and +24%, respectively). Moreover, NOP decreased acetate and isovalerate molar percentages and increased butyrate, valerate, caproate and heptanoate molar percentages as well as n-propanol and ammonium concentrations. Methanogenesis inhibition with AQ (−26%) was associated with higher gaseous H_2 production (+70%) but lower dissolved H_2 concentration (−76%), evidencing a lack of relationship between the two H_2 forms. Anthraquinone increased ammonium concentration, caproate and heptanoate molar percentages but decreased acetate and isobutyrate molar percentages, total microbial nitrogen production and efficiency of microbial protein synthesis (−16%). Overall, NOP and AQ increased the amount of reduced volatile fatty acids, but part of [2H] spared from methanogenesis was lost as gaseous H_2. Finally, [2H] recovery was similar among CON, NOP and AQ but was largely lower than 100%. Consequently, further studies are required to discover other so far unidentified [2H] sinks for a better understanding of the metabolic pathways involved in [2H] production and utilization.

Keywords: fermentation, hydrogen, *in vitro*, methane, microbial biomass, reduced product, volatile fatty acid

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INTRODUCTION

In the rumen, metabolic hydrogen ([2H]) is released during the fermentation of feed by bacteria, protozoa and fungi. Metabolic hydrogen transfer to different acceptors ensures the continuity of fermentation by re-oxidizing reduced co-factors. Dihydrogen (H_2) is produced when electrons are transferred to protons in reactions catalyzed by hydrogenases (Hegarty and Gerdes, 1999). Hydrogenase activity can be inhibited by an accumulation of H_2, with bacterial hydrogenases ([Ni-Fe] hydrogenases) being more sensitive than protozoal hydrogenases ([Fe-Fe] hydrogenases) (Fourmond et al., 2013). Metabolic hydrogen can also be incorporated into other pathways, including propionate, methane (CH_4) and microbial protein synthesis (Henderson, 1980; Czerkawski, 1986; Asanuma et al., 1999). The flow of [2H] is then key to energy metabolism, driving most fermentation pathways (Janssen, 2010), which has a major impact on ruminant nutrition.

Decreases in methanogenesis have been proposed to increase ruminant performance by allocating [2H] to fermentation pathways more energetically-beneficial to the animals. However, this assumption has not always been confirmed in animal experiments; inhibition of CH_4 production by up to 30% did not result in greater daily milk production (Haisan et al., 2013; Guyader et al., 2016) or live weight gain (Beauchemin and McGinn, 2005; Brown et al., 2011) in cattle. Consequently, understanding the fate of [2H] spared from decreased CH_4 production may help to develop CH_4-mitigating strategies that could simultaneously improve animal performance.

Ungerfeld (2015) suggested that an increase in ruminal [2H] availability following methanogenesis inhibition enhances fermentation pathways that consume [2H], such as formate, valerate and caproate. *In vivo* studies reported an increase in molar percentages of formate (Olijhoek et al., 2016), valerate (Chung et al., 2011), or isovalerate (Martínez-Fernández et al., 2014) when CH_4 was decreased by 7–29% (expressed as a function of dry matter intake [DMI]). Greater [2H] availability may also stimulate microbial growth or shift biomass composition toward a more reduced fatty acid profile. However, little information has been reported to confirm those relationships and to our knowledge, a simultaneous analysis of the effects of decreasing CH_4 production on both fermentation end products and microbial biomass production and composition has never been conducted *in vivo*. Using *in vitro* techniques, microbial protein synthesis was either not affected (Romero-Pérez et al., 2015) or was increased (Van Nevel et al., 1969; Guo et al., 2009) with CH_4 decrease ranging between 66 and 86%, whereas volatile fatty acid (VFA) profiles were differently modified.

Thus, the objective of this experiment was to provide more detailed information on the fate of [2H] when methanogenesis is decreased in the rumen. To this end, various known chemical inhibitors assumed to have different modes of action on CH_4 production (nitrate, NIT; 3-nitrooxypropanol, NOP; anthraquinone, AQ) were used. In the rumen, NIT decreases methanogenesis by competing for [2H] during dissimilatory nitrate reduction to ammonium (NH_4^+; DNRA) or during denitrification leading to nitrous oxide (N_2O) production (Yang et al., 2016). At the same time, nitrite produced as an intermediary product of DNRA would have a direct toxic effect toward methanogenic *Archaea* (Klüber and Conrad, 1998; Asanuma et al., 2015). Nitrate decreased CH_4 production in lactating dairy cows (21 g nitrate/kg dry matter [DM], −23.4% CH_4; Olijhoek et al., 2016), steers (30 g nitrate/kg DM, −29.4% CH_4; Newbold et al., 2014) and sheep (20 g nitrate/kg DM, −16.5% CH_4; de Raphélis-Soissan et al., 2014). Nitrooxypropanol is a synthetic compound developed by DSM Nutritional Products Ltd (Kaiseraugst, Switzerland). By positioning itself into the active site of the methyl-coenzyme M reductase, NOP inactivates cofactor F_{430} thereby inhibiting the last step of CH_4 production in methanogenic *Archaea* (Duin et al., 2016). It decreased methanogenesis in sheep (100 mg/d, −23.7% CH_4; Martínez-Fernández et al., 2014), lactating dairy cows (2.5 g/d, −6.7% CH_4; Reynolds et al., 2014) and beef heifers (2.0 g/d, −59.2% CH_4; Romero-Perez et al., 2015). Anthraquinones are the largest group of quinones (Thomson, 1971) and are naturally present in a large number of plant-derived drugs (Mueller et al., 1999). Their mode of action as methanogenesis inhibitors in the rumen has not been clearly elucidated although they are known to have antibacterial activity (Odom, 1997) by disrupting bacterial membranes (Chan et al., 2011) and bacterial protein synthesis (Anke et al., 1980). In sheep, 9,10-anthraquinone supplementation caused a decrease in CH_4 production and an accumulation of H_2, suggesting a direct toxic effect toward methanogenic *Archaea* (500 ppm/d, −50% CH_4; Kung et al., 2003).

MATERIALS AND METHODS

Experimental Design and Treatments

The experiment was conducted at the Lethbridge Research and Development Centre (Agriculture and Agri-Food Canada). The Rumen Simulation Technique (RUSITEC) was favored over a batch culture or an *in vivo* experiment as it allows for a more precise control of the system while offering the possibility to evaluate the long-term effect of selected methanogenesis inhibitors. Two RUSITEC apparatuses were used, each one consisting of a water bath maintained at 39°C and 8 fermentation vessels of 900 mL working volume automatically and continuously vertically mixed. The experiment was designed as a randomized block with 4 treatments repeated in duplicate in each RUSITEC apparatus ($n = 4$). The treatments were control diet alone (CON; 60% corn silage and 40% cereals and minerals on a DM basis) or supplemented with NIT, NOP or AQ (**Table 1**). The average organic matter (OM), crude protein (CP), neutral detergent fiber (NDF) and acid detergent fiber (ADF) content of the substrate was 91.1, 21.0, 37.8, and 18.7% of DM. The incubation lasted 19 days. The microbial community was adapted to treatments from day 1 to 7, and measurements started on day 8 until day 19. Supplementation of methanogenesis inhibitors was constant from day 1 to 14 and was then discontinued to study the recovery of fermentation after removal of the methanogenesis inhibitors.

The dose of each additive was selected with an aim of obtaining 80% CH_4 decrease. Calcium ammonium nitrate was

TABLE 1 | Ingredient and chemical composition of the substrates used in the *in vitro* rumen simulation technique; treatments were control (CON), nitrate (NIT), 3-nitrooxypropanol (NOP), and anthraquinone (AQ).

Item	Substrate			
	CON	NIT	NOP	AQ
INGREDIENT (% DM)				
Corn silage	60.0	60.0	60.0	60.0
Barley grain	28.0	28.0	28.0	28.0
Urea	4.26	0	4.26	4.26
Calcium carbonate	4.50	0	4.50	4.50
Calcium ammonium nitrate[a]	0	10.7	0	0
NOP active compound (1,3-propanediol mononitrate)	0	0	0.05	0
NOP carrier (60% SiO_2, 40% propylene glycol)	0.38	0.38	0.38	0.38
Dicalcium phosphate	2.86	0.92	2.81	2.86
CHEMICAL COMPOSITION (% DM)				
OM	89.2	92.3	89.7	93.1
CP	17.9	18.8	23.6	23.6
NPN[b]	2.00	2.00	2.00	2.00
NDF	39.7	35.1	36.5	39.7
ADF	20.3	17.1	17.6	19.8

DM, dry matter; OM, organic matter; CP, crude protein; NPN, non-protein nitrogen; NDF, neutral detergent fiber; ADF, acid detergent fiber.

[a] $5Ca(NO_3)_2$ NH_4NO_3 (75% NO_3^- in DM).

[b] Estimated values based on nitrogen content of urea (46.7% of DM) and calcium ammonium nitrate (18.7% of DM).

used as the NO_3^- source ($5Ca(NO_3)_2{\cdot}NH_4NO_3$, 75% NO_3^- in product DM; Yara International, Oslo, Norway). Given that NO_3^- had never been tested in RUSITEC conditions, the dose was chosen based on a meta-analysis of *in vivo* experiments (Lee and Beauchemin, 2014) to be equal to 803 mg NO_3^- vessel^{-1} d^{-1} (8.0% NO_3^- in substrate DM), equivalent to 1.07 g calcium ammonium nitrate vessel^{-1} d^{-1}. Urea and calcium carbonate were added in CON, NOP, and AQ to compensate for the additional non-protein nitrogen (NPN) and calcium provided with calcium ammonium nitrate in NIT.

Dose of NOP (11.6% 1,3-propanediol mononitrate in product DM; DSM Nutritional Products Ltd., Kaiseraugst, Switzerland) was based on the RUSITEC study by Romero-Pérez et al. (2015) and was equal to 5 mg 1,3-propanediol mononitrate vessel^{-1} d^{-1}, equivalent to 43.1 mg NOP vessel^{-1} d^{-1}. The NOP carrier (a mixture of silica and propylene glycol) was added in equivalent amounts in CON, NIT, and AQ to compensate for any possible effects of the carrier on fermentation.

Dose of 9,10-anthraquinone (97% anthraquinone in product DM; Sigma-Aldrich Corp., Saint Louis, MO) was selected based on the *in vitro* continuous culture incubation by Garcia-Lopez et al. (1996) to be equal to 12.3 mg 9,10-anthraquinone vessel^{-1} d^{-1}. Because of poor solubility in water (Garcia-Lopez et al., 1996), AQ was dissolved in 70% (v/v) ethanol (12.3 g/L). One milliliter of this solution was added every day to AQ vessels, as well as 1 mL pure 70% (v/v) ethanol to CON, NIT, and NOP vessels to compensate for the addition of ethanol in AQ vessels.

Corn silage and barley grain were freeze-dried and ground in a Wiley mill (A.H. Thomas, Philadelphia, PA) through a 4-mm screen. Then, substrates were prepared by mixing all ingredients per treatment except for NOP active compound and carrier. Feed samples were then weighed (10 g) into nylon bags (51 μm mesh opening) sealed with heat and stored at room temperature until further use. The active compound and carrier of NOP were kept in the dark and at 4°C as recommended by the supplier, and were individually weighed daily before being added to the vessels during substrate bag exchange.

Rumen Simulation Technique

On the first day of the experiment, inoculum was collected from 3 rumen-cannulated cows fed for 1 month with a diet comprised of 65% corn silage, 30% dry rolled barley grain and 5% mineral-vitamin supplement on a DM basis. The maintenance of cannulated animals and the rumen fluid collection procedure were approved by the Institutional Animal Care Committee which operates under the guidelines of the Canadian Council on Animal Care (2009). Rumen samples were taken manually before the morning feeding and strained through 4 layers of cheesecloth. Rumen liquid (∼12 L) and solid (∼200 g) samples were stored in separate pre-warmed insulated thermos bottles before being used. Artificial saliva was prepared as described by Romero-Pérez et al. (2015) ($NaHCO_3$, 9.8 g/L; Na_2HPO_4, 3.72 g/L; NaCl, 0.47 g/L; KCl, 0.57 g/L; $CaCl_2.2H_2O$, 0.053 g/L; $MgCl_2.6H_2O$, 0.128 g/L; $(NH_4)_2SO_4$, 0.3 g/L).

To start the incubation, each fermentation vessel was filled under a CO_2 stream with 700 mL strained rumen fluid, 200 mL warm artificial saliva, one nylon bag containing 10 g of solid rumen digesta (wet weight) and one nylon bag containing the substrate. After 24 h, the bag containing the solid rumen digesta was replaced with a new substrate bag. Each following day, the oldest bag was replaced with a new one, so that each bag remained in its vessel for 48 h. During bag exchange process, vessels were flushed with pure CO_2 to maintain anaerobic conditions.

Throughout the experiment, saliva was prepared daily and was continuously infused into each vessel at a rate of 626 mL/d (2.9%/h). The outflows were collected in 1-L volumetric flasks which were connected to 2-L plastic bags for gas collection. Every day, at the time of substrate bag exchange, the gas collection bags were clamped and both flasks and gas collection bags were replaced with new ones.

Fermentation Gases and Dissolved End Products Determination

Every day, at the time of substrate bag exchange, pH was measured in each vessel and volume of gas in collection bags was determined with a gas meter (Alexander-Wright, London) to estimate daily gas production volume. From day 8 to 13 and from day 17 to 19, gas samples (20 mL) were taken in duplicate from gas collection bags for measuring gas composition. Samples were collected with a 26-gauge needle (Becton Dickinson, Franklin Lakes, NJ) and injected into evacuated 6.8-mL Exetainer vials (Labco Ltd, Wycombe, Bucks, UK) for analysis of CH_4, H_2, CO_2, and N_2O with a gas chromatograph equipped with a thermal conductivity detector (Romero-Pérez et al., 2015).

Daily individual gas production volume in each vessel was calculated from daily total gas production and individual gas percentages. Total amount of greenhouse gas (GHG) produced per day, expressed as CO_2-equivalent (CO_2-eq), was calculated by multiplying the amount of CH_4, CO_2, N_2O, and H_2 produced with its 100-year global warming potential ($CO_2 = 1$, $H_2 = 5.6$, $CH_4 = 28$, $N_2O = 265$; Derwent et al., 2006; IPCC, 2014).

From day 8 to 11 and from day 15 to 18, dissolved H_2 (dH_2) concentrations were measured at several time points (0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 7, 10, 13, 17, and 21 h after substrate bag exchange) with a H_2 sensor (H_2-500; Unisense, Aarhus, Denmark) attached to a glass flow-through cell (2 mm internal diameter, 6 mm external diameter). The H_2-sensor was connected to a microsensor multimeter (Unisense) which in turn was connected to a portable computer running SensorTrace Suite software (Version 2.5.0; Unisense). Each day, 4 vessels (one vessel per treatment) were analyzed such that all 16 vessels were measured in 4 days.

The sensor was polarized (1,000 mV) 8 h before starting the measurement period and was calibrated daily before the first measurement point. For calibration, the flow cell was connected to a closed system using H_2-impermeable Masterflex Tygon® Chemical tubing (Cole-Parmer Instrument Company, USA), starting and ending in an Erlenmeyer flask maintained in a 39°C-water bath. The flask was filled with tap water which circulated in the system via a peristaltic pump (Model 1001, Medical Technology Products, Inc., Huntington Station, NY). A 2-point calibration curve was created using water without and with H_2 bubbling (80% H_2-20% CO_2 gas mixture). At the time of substrate bag exchange, vessels were fitted with a Masterflex Tygon® Chemical tubing (Cole-Parmer Instrument Company, USA) closed by a 1-way stopcock with Luer connection. At sampling time points, a 40-mL syringe with Luer-lock was connected to the vessel and a fermentation fluid sample (40 mL) was taken and directly injected into the flow cell of the H_2-sensor. After the measurement, the sample was injected back into the vessel.

From day 8 to 11 and from day 17 to 19, 5-mL samples were taken from the outflow of each vessel for analysis of fermentation end products. Effluent was prevented from further fermentation by adding 4 mL of sodium azide (200 g/L) during sampling days. Samples were mixed with 1 mL of 250 g/L phosphoric acid for analysis of carboxylic acids and alcohols (VFA, formate, lactate, succinate, ethanol and propanol) or 1 mL of 10 mL/L sulfuric acid for NH_4^+ analysis. For nitrate and nitrite analyses, no preservative agent was used. All samples were stored at −20°C until analysis. Concentrations of VFA, lactate, succinate, ethanol and propanol were determined by gas-liquid chromatography (GLC; model 6890; Agilent, Wilmington, DE) with a polar capillary column (30 m × 0.32 mm × 1 μm; ZB-FFAP; Phenomenex Inc., Torrance, CA), a flame ionization detector and helium as carrier gas. For VFA, crotonic acid was used as internal standard with split mode injection. The initial oven temperature was set at 150°C for 1 min before being increased to 195°C for 3 min (5°C/min). The injector and detector temperatures were set at 225°C and 250°C, respectively. For lactate and succinate, malonic acid was employed as internal standard with spotless injections. The injector and detector temperatures were set at 190°C and 250°C, respectively. The oven temperature was maintained at 45°C for 1 min, increased by 30°C/min to 150°C and then by 5°C/min to 190°C, and held at this final temperature for 2 min. Formate concentration was quantified using the GLC method of Richardson et al. (1989) with a Zebron column (30 m × 0.25 mm × 0.5 μm; ZB-1; Phenomenex Inc., Torrance, CA), a flame ionization detector and helium as carrier gas. The oven temperature program started at 50°C for 2 min before increasing to 130°C (5°C/min) and finishing at 240°C (15°C/min) for 4.67 min. The injector and detector temperatures were both set at 275°C. Ammonium concentration was analyzed by the improved Berthelot method (Rhine et al., 1998). Nitrate and nitrite concentrations were determined with water quality test strips (Hach Company, Loveland, CO) on samples taken on days 8, 9, 10, and 11 only, as both components were assumed to be absent from the fermentation liquid during the recovery period.

Substrate Disappearance

One feed sample per treatment was taken before starting the incubation and stored at room temperature for analysis of chemical composition. Dry matter, organic matter (OM), neutral detergent fiber (NDF), and acid detergent fiber (ADF) were analyzed on samples ground in a Wiley mill (A.H. Thomas, Philadelphia, PA) through a 1-mm screen, whereas nitrogen (N) content was determined on ball-ground (mixer mill MM 400; Retsch Inc., Newtown, PA) samples. Dry matter was determined after drying at 135°C for 2 h (method 930.15; AOAC, 2005) and OM after ashing at 550°C for 5 h (method 942.05; AOAC, 2005). Fiber (NDF and ADF) was determined by a sequential procedure (Van Soest et al., 1991) with the ANKOM200 Fiber Analyzer (Ankom Technology Corp., Macedon, NY) after pre-treatment with sodium sulfite and α-amylase and expressed inclusive of residual ash. Nitrogen was analyzed by a combustion method with gas chromatography and thermal conductivity detection (Carlo Erba Instrumentals, Milan, Italy; method 990.03; AOAC, 2005) and crude protein (CP) was calculated as N × 6.25.

Nutrient apparent disappearance (DM, OM, CP) was determined from day 8 to 10 and from day 17 to 19 as described in Romero-Pérez et al. (2015). Briefly, bags collected after 48 h incubation were washed gently under cold running water before being dried at 55°C for 48 h for DM analysis. At the end of the experiment, residues were pooled across days per treatment and chemical composition was analyzed as for feed.

Microbial Protein Synthesis

From day 5 to 13, microbes were labeled using ^{15}N-enriched $(NH_4)_2SO_4$ (Sigma Chemical Co., St. Louis, MO; minimum 10 atom% ^{15}N) in order to estimate microbial protein synthesis from day 11 to 13. Pellets from microbes associated to the liquid phase and weakly attached to the feed were prepared as described in Ribeiro et al. (2015), before being freeze-dried. Pellets from microbes strongly attached to the feed were prepared from feed residues dried at 55°C for 48 h and ball-ground (Ribeiro et al., 2015). The N content of the 3 microbial fractions was determined as described for feed and the enrichment of ^{15}N was

subsequently analyzed with a mass spectrometer (NA 1500, Carlo Erba Instruments, Rodano, Italy; Wang et al., 2000).

Metabolic Hydrogen Balance Calculations

For each treatment, the daily molar production of individual fermentation end products (mmol/d) was calculated. Concentrations of each VFA in each vessel outflow (mM) were multiplied by the daily amount of infused saliva (0.626 L). Volume of CH_4, H_2 and N_2O (mL) was converted to moles using the Ideal Gas Law (1 mol ideal gas = 25.6 L at 39°C and 1.013×10^5 Pa).

Total [2H] produced (mmol/d) was calculated as the sum of [2H] released during daily production of acetate, butyrate and caproate (**Table 2**). Total [2H] consumed (mmol/d) was calculated as the sum of [2H] used in the production of formate, propionate, valerate, caproate, heptanoate, gaseous H_2 (gH_2) and CH_4 (**Table 2**). The following assumptions were made: (i) VFA, except branched-chain VFA (isobutyrate, isovalerate, 2-methylbutyrate), were solely produced from glucose fermentation, with fermentation pathways based on Mills et al. (2001) and Ungerfeld and Kohn (2006); (ii) Heptanoate was produced from the condensation of one propionyl-CoA and 2 acetyl-CoA (Bornstein and Barker, 1948); (iii) Branched-chain VFA mainly derived from branched-chain amino acids (valine, leucine, isoleucine) and the associated [2H] balance were not taken into account. As the proportion of NO_3^- reduced to NH_4^+ and N_2O was unknown, the total [2H] consumed for NIT treatment was not calculated.

Caproate can be synthesized from the condensation of 2 propionyl-CoA or 2 acetyl-CoA generating 1 butyryl-CoA further condensing with a third acetyl-CoA (Barker et al., 1945; Wallace et al., 2004; Kucek et al., 2016). The first pathway requires the incorporation of 4 moles [2H] per mole of caproate whereas the second one releases 2 moles [2H] per mole of caproate. Given that the proportion of caproate produced by either pathway was unknown, 2 scenarios were assessed to estimate total [2H] produced and consumed. The first scenario assumed that 100% of caproate originated from the condensation of propionyl-CoA (SC1), and the second scenario assumed that 100% of caproate originated from the condensation of 2 acetyl-CoA (SC2).

Finally, for treatments CON, NOP, and AQ, and for each scenario of caproate production, the [2H] balance (mmol/d) was calculated as the difference between [2H] produced (mmol/d) and [2H] consumed (mmol/d) and the percentage of [2H] recovered was calculated as the ratio between [2H] consumed (mmol/d) and [2H] produced (mmol/d).

Statistics

All statistics were run with SAS software (Version 2.0.4; SAS Institute Inc., Cary, NC). Data were analyzed using the MIXED procedure, with the vessel used as the experimental unit. Treatment and recovery periods were analyzed separately. For dH_2 concentrations, the model included the fixed effect of treatment. For all other variables, the model included the fixed effects of treatment, day and their interaction, and day was treated as a repeated measure. Variance component was selected as the covariance structure with the lowest Akaike and Bayesian information criteria values for most variables. For all variables, the vessel nested within its treatment was considered as random effect (García-González et al., 2010) and degrees of freedom were adjusted using the Kenward-Roger option. Least-square means are reported throughout the paper and multiple pairwise comparisons between treatments were tested using the PDIFF option. Data were considered significant at $P < 0.05$ and trends were discussed at $0.05 \leq P \leq 0.10$.

Principal component analyses were conducted to illustrate the relationships among variables ($n = 21$; component plots) and treatments ($n = 4$; score plots) using the PRINCOMP procedure. Input data ($n = 16$) consisted of averaged and standardized (STANDARD procedure) data collected during the treatment period. The correlation matrix was prepared with the CORR procedure. Given the large number of computations and to reduce the experimentwise error rate, the raw *P*-values generated from the correlation analyses were subsequently adjusted with the MULTTEST procedure with the false discovery rate (FDR) option. Finally, to compensate for the adjustment of *P*-values, correlations were considered significant at $P \leq 0.10$.

RESULTS

Treatment Period

Fermentation Parameters and Nutrient Disappearance

Nitrate tended to decrease daily total gas production (27% decrease relative to CON), whereas NOP and AQ had no effect ($P = 0.083$; **Table 3**). Overall total GHG produced was different among treatments ($P < 0.001$) with NIT and NOP producing less GHG compared to CON and AQ (−73%). Methane percentage was the lowest with NIT and NOP (75% decrease on average relative to CON), whereas AQ decreased CH_4 by 26% relative to CON ($P < 0.001$). The percentage of gH_2 was similar between CON and NIT, but was greater with NOP and AQ (5.2- and 3.3-fold increase relative to CON, respectively; $P < 0.001$). Carbon dioxide percentage was similar between CON and AQ, but was greater with NIT and NOP (1.18- and 1.05-fold increase relative to CON, respectively; $P < 0.001$). Nitrous oxide was only detected with NIT (0.04% of total gas; $P = 0.011$). Dissolved H_2 concentration also differed among treatments, with the greatest concentration recorded for NOP, followed in descending order by CON, NIT and AQ ($P < 0.001$). Inhibiting methanogenesis did not affect DM and OM disappearance of the substrate, but NIT decreased CP disappearance (77.4 and 69.5% for CON and NIT respectively, $P = 0.028$; **Table 3**).

Medium pH was similar among treatments and averaged 6.95 (**Table 4**). Total VFA concentration tended to differ among treatments ($P = 0.098$), with the lowest concentration for NIT (22.3% decrease relative to CON). Lactate and succinate were not detected for any treatment. Treatments did not modify formate, but tended to change propionate molar percentage ($P = 0.061$), numerically decreasing it with NIT and increasing it with NOP and AQ. Compared to CON, NIT increased acetate molar percentage (+16.0 percentage units) and decreased butyrate, isobutyrate, valerate, isovalerate, caproate and heptanoate molar percentages (−1.8, −0.26, −1.51, −6.77, −3.29, and −1.09

TABLE 2 | Reaction equations used to estimate the production and consumption of metabolic hydrogen ([2H]) during the synthesis of major rumen fermentation end products (presented as underlined in the reaction equations).

Fermentation end product	Reaction equation	Moles [2H] released/mole end product
Formate	$[2H] + CO_2 \rightarrow \underline{HCOOH}$	−1
Acetate	$C_6H_{12}O_6 + 2\ H_2O \rightarrow 2\ \underline{CH_3COOH} + 4\ [2H] + 2\ CO_2$	+2
Propionate	$C_6H_{12}O_6 + 2\ [2H] \rightarrow 2\ \underline{CH_3CH_2COOH} + 2\ H_2O$	−1
Butyrate	$C_6H_{12}O_6 \rightarrow 1\ \underline{CH_3(CH_2)_2COOH} + 2\ [2H] + 2\ CO_2$	+2
Valerate	$C_6H_{12}O_6 + [2H] \rightarrow \underline{CH_3(CH_2)_3COOH} + CO_2 + 2\ H_2O$	−1
Caproate[A]	$3\ C_6H_{12}O_6 \rightarrow 2\ \underline{CH_3(CH_2)_4COOH} + 4\ [2H] + 2\ H_2O + 6\ CO_2$	+2
Caproate[B]	$C_6H_{12}O_6 + 4\ [2H] \rightarrow \underline{CH_3(CH_2)_4COOH} + 4\ H_2O$	−4
Heptanoate	$2\ C_6H_{12}O_6 + 2\ [2H] \rightarrow \underline{CH_3(CH_2)_5COOH} + 2\ CO_2 + CH_3CH_2COOH + 4\ H_2O$	−2
Methane	$CO_2 + 4\ [2H] \rightarrow \underline{CH_4} + 2\ H_2O$	−4

[A] *Acetyl-CoA as intermediate.*
[B] *Propionyl-CoA as intermediate.*

TABLE 3 | Effects of control (CON), nitrate (NIT), 3-nitrooxypropanol (NOP), and anthraquinone (AQ) on gas production and substrate disappearance using a rumen simulation technique.

Item	Substrate				SEM	*P*-value[A]
	CON	NIT	NOP	AQ		
GAS PRODUCTION (L/D)[B]						
Total	0.74	0.54	0.63	0.76	0.066	0.083
Total GHG (CO_2-eq)[C]	1.00[a]	0.16[b]	0.34[b]	0.83[a]	0.122	<0.001
GHG (% OF TOTAL)[B,D]						
Methane	17.1[a]	3.6[c]	5.0[c]	12.6[b]	0.84	<0.001
Hydrogen	2.0[c]	1.0[c]	10.3[a]	6.6[b]	0.99	<0.001
Carbon dioxide	80.9[c]	95.4[a]	84.7[b]	80.9[c]	1.19	<0.001
Nitrous oxide	0.00[b]	0.04[a]	0.00[b]	0.00[b]	0.006	0.011
Dissolved hydrogen (μM)[E]	40.8[b]	28.7[c]	53.7[a]	9.7[d]	3.79	<0.001
SUBSTRATE DISAPPEARANCE (%)[F]						
DM	47.9	43.3	46.6	45.7	2.07	0.446
OM	47.9	45.2	48.1	46.6	1.97	0.731
CP	77.4[a]	69.5[b]	78.6[a]	75.4[ab]	1.97	0.028

CO_2-eq, CO_2-equivalent; DM, dry matter; GHG, greenhouse gas; OM, organic matter; CP, crude protein.
[A] *Within a row, means with different superscripts differ ($P < 0.05$).*
[B] *Average of data collected in all vessels during 6 consecutive days (day 8–13).*
[C] *Sum of CH_4, H_2, CO_2, and N_2O produced corrected for their 100-year global warming potential (CO_2: 1, CH_4: 28, N_2O: 265, H_2: 5.6; Derwent et al., 2006; IPCC, 2014).*
[D] *Gas percentages are based on the sum of CH_4, H_2, CO_2, and N_2O produced.*
[E] *Average of data collected in 4 vessels/day during 4 consecutive days (day 8–11).*
[F] *Average of data collected in all vessels during 3 consecutive days (day 8–10).*

TABLE 4 | Effects of control (CON), nitrate (NIT), 3-nitrooxypropanol (NOP), and anthraquinone (AQ) on fermentation variables using a rumen simulation technique.

Item[B]	Substrate				SEM	*P*-value[A]
	CON	NIT	NOP	AQ		
pH	6.92	6.95	6.97	6.96	0.016	0.200
Total VFA (mM)	65.5	50.9	59.7	63.8	4.02	0.098
VFA PROFILE (mol/100 mol)						
Formate	5.76	5.52	7.21	5.68	0.664	0.293
Acetate (A)	52.1[b]	68.1[a]	44.7[c]	46.5[c]	0.82	<0.001
Propionate (P)	12.6	11.3	13.8	11.3	0.69	0.061
Butyrate (B)	15.3[b]	13.5[c]	17.4[a]	16.3[ab]	0.47	<0.001
Isobutyrate	0.56[a]	0.30[c]	0.53[ab]	0.47[b]	0.022	<0.001
Valerate	5.56[b]	4.05[c]	8.26[a]	5.92[b]	0.187	<0.001
Isovalerate[C]	7.61[a]	0.84[c]	6.03[b]	6.83[ab]	0.268	<0.001
Caproate	4.72[c]	1.43[d]	6.96[b]	9.50[a]	0.671	<0.001
Heptanoate	1.57[c]	0.48[d]	2.31[b]	3.15[a]	0.222	<0.001
A/P (mol/mol)	4.16[b]	6.36[a]	3.28[b]	4.18[b]	0.391	<0.001
(A+B)/P (mol/mol)	5.38[b]	7.57[a]	4.54[b]	5.64[b]	0.431	0.002
Ethanol (mM)	5.89[bc]	9.92[a]	7.68[b]	4.25[c]	0.600	<0.001
n-propanol (mM)	0.19[b]	0.16[b]	0.41[a]	0.16[b]	0.044	0.004
Ammonium (mM)	14.1[b]	11.3[c]	19.2[a]	19.6[a]	0.54	<0.001
Nitrate (mM)	0.00[b]	0.27[a]	0.00[b]	0.00[b]	0.053	0.001
Nitrite (mM)	0.00[b]	0.03[a]	0.00[b]	0.00[b]	0.008	0.036

VFA, volatile fatty acid.
[A] *Within a row, means with different superscripts differ ($P < 0.05$).*
[B] *Average of data collected in all vessels during 4 consecutive days (day 8–11).*
[C] *Co-elutes with 2-methylbutyrate.*

percentage units, respectively; $P < 0.001$). In contrast, NOP and AQ both decreased acetate molar percentage (−7.4 and −5.6 percentage units, respectively) and increased caproate (+2.24 and +4.78 percentage units, respectively) and heptanoate molar percentages (+0.74 and +1.58 percentage units, respectively) ($P < 0.001$). Nitrooxypropanol also increased butyrate and valerate (+2.10 and +2.70 percentage units, respectively), but decreased isovalerate (−1.58 percentage units) molar percentages ($P < 0.001$). Anthraquinone decreased isobutyrate (−0.09 percentage units; $P < 0.001$) without affecting butyrate, valerate and isovalerate molar percentages. Only NIT modified acetate:propionate (+2.2 ratio points relative to CON; $P < 0.001$)

and (acetate+butyrate):propionate (+2.19 ratio points relative to CON; $P = 0.002$).

Ethanol concentration increased with NIT (1.68-fold increase relative to CON; $P < 0.001$) but was not affected by NOP and AQ. No treatment other than NOP affected n-propanol concentration (2.16-fold increase relative to CON; $P = 0.004$). Ammonium concentration was greater for NOP and AQ (1.38-fold increase relative to CON on average) and lower for NIT (19.9% decrease relative to CON) ($P < 0.001$). Finally, nitrate and nitrite were detected in outflow from NIT, showing that a maximum of 94% of the daily amount of NO_3^- supplied could have been fully reduced to NH_4^+.

Microbial N production was modified differently depending upon the treatment (**Table 5**; $P < 0.05$). Treatment NIT increased both total microbial N production and efficiency of microbial protein synthesis (1.17- and 1.16-fold increase relative to CON, respectively), whereas AQ decreased both variables (13.3 and 17.3% decrease relative to CON, respectively). Treatment NOP had no effect on either variable.

TABLE 5 | Effects of control (CON), nitrate (NIT), 3-nitrooxypropanol (NOP), and anthraquinone (AQ) on microbial protein synthesis using a rumen simulation technique.

Item[B]	Substrate				SEM	*P*-value[A]
	CON	NIT	NOP	AQ		
MICROBIAL NITROGEN PRODUCTION (mg/d)						
Total	59.8^{b}	69.7^{a}	54.2^{bc}	51.7^{c}	2.68	0.002
Microbes strongly attached to feed	33.6	36.6	33.8	30.4	1.64	0.084
Microbes weakly attached to feed	5.6	4.8	4.9	4.4	0.58	0.554
Microbes associated to liquid phase	22.4^{bc}	28.8^{ab}	15.6^{c}	16.9^{c}	2.42	0.008
E_{MPS}	15.9^{b}	18.5^{a}	14.1^{bc}	13.3^{c}	0.91	0.001

E_{MPS}, efficiency of microbial protein synthesis (grams microbial nitrogen produced per kilogram organic matter fermented).

[A]Within a row, means with different superscripts differ ($P < 0.05$).

[B]Average of data collected in all vessels during 3 consecutive days (day 11–13).

Variables and Treatments Clustering by Principal Component Analysis

Representation of response variables along components 1 and 2 and along components 1 and 3 is presented in **Figures 1A,B**. The first, second and third principal components explained 83.6% of the total variation of the dataset. The component and score plots between components 2 and 3 are not presented as they did not provide additional information. In **Figure 1**, close variables mean that they are positively correlated whereas variables on opposite directions of the same axis are negatively correlated. Two variables situated on orthogonal axes are not correlated. The correlation matrix between fermentation variables is presented in Supplementary Table 1.

Based on Eigenvalues with absolute value greater than 0.20 (**Table 6**), CO_2 and N_2O percentages, concentrations of NH_4^+, butyrate, valerate and branched-chain VFA as well as efficiency of microbial protein synthesis mostly contributed to the first component. Carbon dioxide was positively correlated with nitrous oxide ($r = 0.83$, $P < 0.001$) and efficiency of microbial protein synthesis ($r = 0.71$, $P = 0.011$), although negatively correlated with butyrate ($r = -0.72$, $P = 0.010$), isobutyrate ($r = -0.83$, $P < 0.001$), valerate ($r = -0.65$, $P = 0.026$), isovalerate ($r = -0.94$, $P < 0.001$) and NH_4^+ ($r = -0.66$, $P = 0.020$). Nitrous oxide was also positively correlated with efficiency of microbial protein synthesis ($r = 0.80$, $P = 0.002$) and negatively correlated with isobutyrate ($r = -0.59$, $P = 0.050$), valerate ($r = -0.50$, $P = 0.100$), isovalerate ($r = -0.79$, $P = 0.003$) and NH_4^+ ($r = -0.66$, $P = 0.023$). Efficiency of microbial protein synthesis was negatively correlated with butyrate ($r = -0.63$, $P = 0.029$), isobutyrate ($r = -0.55$, $P = 0.0681$), valerate ($r = -0.64$, $P = 0.029$), isovalerate ($r = -0.70$, $P = 0.013$) and NH_4^+ ($r = -0.84$, $P < 0.001$). The four VFA were positively correlated together ($r > 0.67$, $P < 0.050$) and with NH_4^+ ($r > 0.55$, $P < 0.068$).

Propionate, caproate, heptanoate, ethanol, and propanol concentrations and OM disappearance were the main contributors of the second component (**Table 6**). Propionate was only positively correlated with propanol ($r = 0.62$, $P = 0.038$) and OM disappearance ($r = 0.80$, $P = 0.002$). Propanol and OM disappearance were positively correlated ($r = 0.55$, $P = 0.068$) as were caproate and heptanoate ($r = 1.00$, $P < 0.001$). Ethanol was negatively correlated with caproate and heptanoate ($r = -0.86$, $P < 0.001$).

Total gas production, CH_4 and H_2 percentages as well as concentrations of dH_2, total VFA, formate and acetate contributed to the third component (**Table 6**). Dissolved H_2 concentration was not correlated with any variable. A positive correlation was observed between total gas production and CH_4 percentage ($r = 0.51$, $P = 0.094$), total gas production and total VFA concentration ($r = 0.50$, $P = 0.100$) and CH_4 percentage and total VFA concentration ($r = 0.52$, $P = 0.089$). Finally, formate was positively correlated with gH_2 percentage ($r = 0.60$, $P = 0.046$) and negatively correlated with acetate ($r = -0.58$, $P = 0.057$), whereas acetate and gH_2 were negatively correlated ($r = -0.53$, $P = 0.078$).

Representation of treatments along components 1 and 2 and along components 1 and 3 is shown in **Figures 2A,B**. Treatment NIT created a distinct group along component 1 because of greater efficiency of microbial protein synthesis and N_2O production and also because of lower gH_2 production and branched-chain VFA. Treatment AQ was separated from other treatments in component 2 as it was characterized by high concentrations of caproate and heptanoate and low acetate concentration. Finally, NOP clustered along component 3 mostly because of high dH_2 and propanol concentrations, but also because of numerically higher formate concentration.

Metabolic Hydrogen Balance

Total [2H] production was not different among treatments, regardless of caproate metabolic pathway used (**Table 7**). However, NIT decreased the amount of [2H] produced from butyrate (30.4% decrease relative to CON; $P = 0.036$), and NOP tended to decrease [2H] production from acetate (21.8% decrease

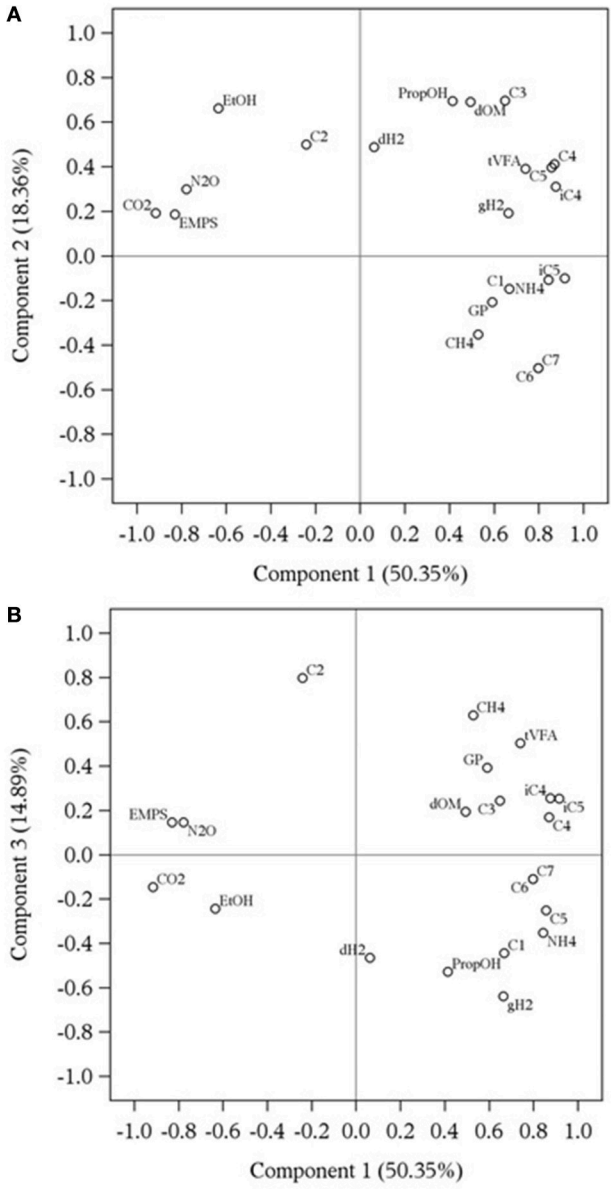

FIGURE 1 | Component pattern plots obtained by principal component analysis describing the relationship among rumen fermentation variables affected by methanogenesis inhibitors using a rumen simulation technique, along components 1 and 2 (A) and components 1 and 3 **(B)**. dH_2: dissolved hydrogen (μM); GP, total gas production (mL/d); CH_4, methane (%); gH_2, gaseous hydrogen (%); CO_2, carbon dioxide (%); N_2O, nitrous oxide (%); dOM, organic matter disappearance (%); E_{MPS}, efficiency of microbial protein synthesis (g microbial N/kg organic matter fermented); tVFA, total volatile fatty acid concentration (mM); C1, formate (mM); C2, acetate (mM); C3, propionate (mM); iC4, isobutyrate (mM); C4, butyrate (mM); C5, valerate (mM); iC5, isovalerate (mM); C6, caproate (mM); C7, heptanoate (mM); EtOH, ethanol (mM); NH_4, ammonium (mM); PropOH, propanol (mM). Gas percentages are based on the sum of CH_4, H_2, CO_2, and N_2O produced.

relative to CON; $P = 0.053$). In SC2, AQ and NIT respectively produced the greatest and the lowest amount of [2H] from caproate ($P < 0.001$) compared to the other treatments.

TABLE 6 | Eigenvalues obtained by principal component analysis describing the contribution to components 1, 2, and 3 of rumen fermentation variables affected by methanogenesis inhibitors using a rumen simulation technique.

Item	Component 1 (50.35%)	Component 2 (18.36%)	Component 3 (14.89%)
Dissolved hydrogen (μM)	0.019	0.249	−0.263
Total gas production (mL/d)	0.182	−0.105	0.222
Methane (%)[A]	0.162	−0.180	0.356
Gaseous hydrogen (%)[A]	0.204	0.098	−0.361
Carbon dioxide (%)[A]	−0.282	0.098	−0.083
Nitrous oxide (%)[A]	−0.239	0.152	0.083
OM disappearance (%)	0.152	0.352	0.110
E_{MPS} (g microbial N/kg OM fermented)	−0.255	0.095	0.083
Total VFA (mM)	0.228	0.199	0.284
Acetate (mM)	−0.074	0.254	0.451
Propionate (mM)	0.200	0.354	0.138
Isobutyrate (mM)	0.270	0.158	0.145
Butyrate (mM)	0.268	0.209	0.096
Valerate (mM)	0.264	0.202	−0.142
Isovalerate (mM)	0.281	−0.051	0.143
Caproate (mM)	0.245	−0.256	−0.062
Heptanoate (mM)	0.245	−0.256	−0.062
Ethanol (mM)	−0.195	0.337	−0.138
Ammonium (mM)	0.259	−0.055	−0.199
Formate (mM)	0.205	−0.075	−0.251
n-propanol (mM)	0.127	0.353	−0.298

OM, organic matter; E_{MPS}, efficiency of microbial protein synthesis; N, nitrogen; VFA, volatile fatty acid.

[A]Gas percentages are based on the sum of CH_4, H_2, CO_2, and N_2O produced.

Treatments modified all pathways of [2H] consumption except propionate (**Table 7**). Nitrate decreased [2H] uptake into formate and valerate (29.2 and 41.7% decrease relative to CON, respectively; $P = 0.001$) whereas NOP increased [2H] diverted toward valerate (1.36-fold increase relative to CON). In SC1, [2H] uptake into caproate was greater with AQ, followed by NOP, CON, and NIT ($P < 0.001$). Both NOP and AQ increased [2H] directed toward heptanoate (0.37- and 1.93-fold increase relative to CON, respectively) whereas NIT decreased [2H] consumption in heptanoate formation (76.7% decrease relative to CON; $P < 0.001$). The amount of [2H] used for methanogenesis was lower for NIT and NOP (86% decrease on average relative to CON; $P < 0.001$) and was not modified with AQ. Nitrate did not modify the amount of [2H] incorporated into gH_2, but NOP and AQ increased it (3.47-fold increase on average relative to CON; $P < 0.001$). Finally, in SC1, NOP presented similar rates of total [2H] consumption as did CON whereas AQ increased total [2H] uptake (1.31-fold increase relative to CON; $P < 0.001$). In SC2, total [2H] consumption was similar among CON, NOP and AQ.

Overall, treatments modified [2H] balance differently ($P < 0.001$; **Table 7**). Expressed as daily millimoles, the [2H] balance in SC1 was similar between NOP and AQ but lower than CON (34.9% decrease on average relative to CON; $P = 0.016$). In SC2, the [2H] balance of NOP was also lower than CON (16.4%

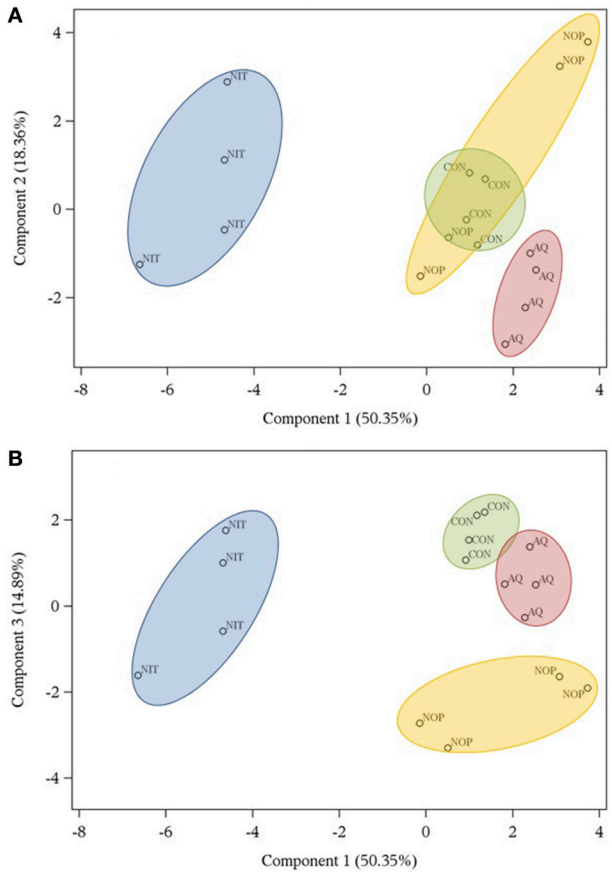

FIGURE 2 | Score plots obtained by principal component analysis describing the distribution of treatments (control, CON; nitrate, NIT; 3-nitrooxypropanol, NOP; anthraquinone, AQ) tested with a rumen simulation technique along components 1 and 2 (A) and components 1 and 3 **(B)**.

TABLE 7 | Effects of control (CON), nitrate (NIT), 3-nitrooxypropanol (NOP), and anthraquinone (AQ) on metabolic hydrogen ([2H]) balance using a rumen simulation technique.

Item[B]	Substrate				SEM	*P*-value[A]
	CON	NIT	NOP	AQ		
[2H] PRODUCTION (mmol/d)						
Acetate	42.7	43.1	33.4	37.2	2.53	0.053
Butyrate	12.5[a]	8.7[b]	13.1[a]	13.1[a]	1.08	0.036
Caproate (SC1)	0	0	0	0	0	–
Caproate (SC2)	3.88[c]	0.92[d]	5.01[b]	7.51[a]	0.302	<0.001
Total (SC1)	55.2	51.8	46.5	50.3	3.54	0.410
Total (SC2)	59.1	52.7	51.5	57.9	3.41	0.351
[2H] CONSUMPTION (mmol/d)						
Formate	2.33[a]	1.65[b]	2.58[a]	2.25[a]	0.157	0.001
Propionate	5.17	3.71	5.25	4.53	0.549	0.219
Valerate	2.28[b]	1.33[c]	3.09[a]	2.35[b]	0.193	<0.001
Caproate (SC1)	7.75[c]	1.83[d]	10.02[b]	15.02[a]	0.605	<0.001
Caproate (SC2)	0	0	0	0	0	–
Heptanoate	1.29[c]	0.30[d]	1.66[b]	2.49[a]	0.100	<0.001
Methane	4.54[a]	0.24[b]	1.00[b]	3.79[a]	0.562	<0.001
Hydrogen	0.16[b]	0.03[b]	0.46[a]	0.65[a]	0.096	<0.001
Total (SC1)	23.5[b]	–	24.0[b]	30.8[a]	1.08	<0.001
Total (SC2)	15.8	–	13.7	16.0	0.89	0.153
[2H] BALANCE[C]						
mmol/d (SC1)	31.7[a]	–	21.4[b]	19.9[b]	2.50	0.016
mmol/d (SC2)	43.3[a]	–	36.2[b]	42.1[a]	1.71	0.034
% (SC1)	42.4[b]	–	54.3[a]	61.2[a]	2.99	0.004
% (SC2)	26.6	–	27.7	27.5	1.14	0.788

[A] *Within a row, means with different superscripts differ ($P < 0.05$).*
[B] *SC1: 100% of caproate is produced from propionyl-CoA; SC2: 100% of caproate is produced from acetyl-CoA.*
[C] *mmol/d = [2H] produced−[2H] consumed; % = [2H] consumed/[2H] produced.*

decrease relative to CON; $P = 0.034$). Total [2H] consumed expressed as a proportion of total [2H] produced was similar between CON, NOP and AQ in SC2, but was greater for NOP and AQ in SC1 (1.36-fold increase on average relative to CON; $P = 0.004$).

Recovery Period

Within treatments, data comparison between measurement and recovery periods presented some differences, most probably because of the microbial evolution of the system throughout time and discontinuation of the treatments. Therefore, data between the two experimental periods were not compared, but comparisons among treatments within each period can still be considered. After removal of methanogenesis inhibitors, total gas production and composition (day 17–19) were similar among treatments, except for gH_2 percentage which remained higher with AQ (2.74-fold increase relative to CON, $P = 0.028$; **Table 8**). Dissolved H_2 still differed among previous treatments ($P = 0.019$) with a lower concentration for AQ (43% decrease relative to CON). However, dH_2 concentrations for AQ recovered to values similar to those of CON on the last day of measurement (day 18; **Figure 3**). Substrate degradation was only affected by NOP, with lower DM ($P = 0.025$) and OM ($P = 0.018$) disappearance and a tendency ($P = 0.104$) for lower CP disappearance (6.3, 6.7, and 4.0 percentage units decrease relative to CON, respectively).

Total VFA concentration and pH were similar among previous treatments but individual VFA molar percentages were still different during recovery (**Table 9**). Compared to CON, NOP and AQ decreased acetate molar percentage (−3.7 and −3.8 percentage units, respectively; $P = 0.001$), AQ decreased propionate molar percentage (−1.3 percentage units; $P = 0.039$) and NIT increased butyrate molar percentage (+2.7 percentage units; $P = 0.023$). Except for isobutyrate, minor VFA molar percentages were also different among treatments. Compared to CON, valerate molar percentage was greater with NIT and NOP (+1.35 and +1.56 percentage units, respectively; $P = 0.005$), isovalerate molar percentage was lower with NIT (−5.85 percentage units; $P < 0.001$) and caproate and heptanoate molar percentages were greater with AQ (+2.78 and +0.92 percentage units, respectively; $P < 0.001$). Volatile fatty acid ratios were

TABLE 8 | Effects of control (CON), nitrate (NIT), 3-nitrooxypropanol (NOP), and anthraquinone (AQ) on gas production and substrate disappearance after treatment withdrawal (recovery period; day 14–19) using a rumen simulation technique.

Item	Substrate				SEM	*P*-value[A]
	CON	NIT	NOP	AQ		
Total gas (L/d)[B]	0.80	1.01	0.92	0.76	0.115	0.399
GAS (% of TOTAL)[B]						
Methane	18.6	16.3	18.1	16.2	1.13	0.336
Hydrogen	1.36^{b}	2.01^{b}	1.31^{b}	3.72^{a}	0.567	0.028
Carbon dioxide	80.0	81.7	80.7	80.1	1.11	0.668
Nitrous oxide	0.00	0.00	0.00	0.00	0.003	0.325
Dissolved hydrogen (μM)[C]	22.7^{a}	23.7^{a}	23.7^{a}	12.9^{b}	2.35	0.019
SUBSTRATE DISAPPEARANCE (%)[B]						
DM	50.0^{a}	49.4^{a}	43.7^{b}	48.8^{a}	1.54	0.025
OM	59.5^{a}	60.6^{a}	52.8^{b}	58.5^{a}	1.55	0.018
CP	74.5	76.1	70.5	73.0	1.48	0.104

DM, dry matter; OM, organic matter; CP, crude protein.
[A]Within a row, means with different superscripts differ (P < 0.05).
[B]Average of data collected in all vessels during 3 consecutive days (day 17–19); gas percentages are based on the sum of CH_4, H_2, CO_2 and N_2O produced.
[C]Average of data collected in 4 vessels/day during 4 consecutive days (day 15–18).

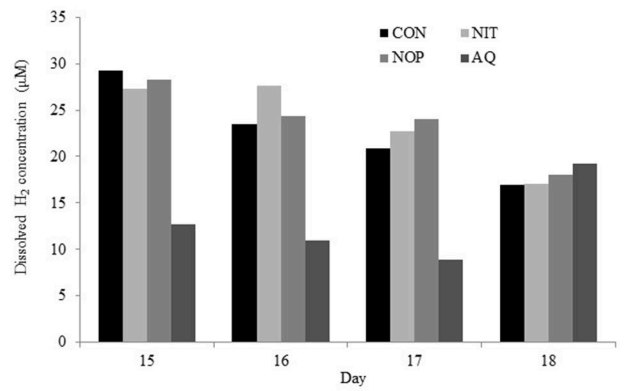

FIGURE 3 | Effects of control (CON), nitrate (NIT), 3-nitrooxypropanol (NOP), and anthraquinone (AQ) on dissolved H_2 concentration after 1 (day 15), 2 (day 16), 3 (day 17), and 4 (day 18) days of treatment withdrawal (recovery period; day 14–19) using a rumen simulation technique.

not modified by treatments (4.01 and 5.18 on average for acetate:propionate and (acetate+butyrate):propionate ratio), as well as ethanol and n-propanol concentrations. Finally, NH_4^+ concentrations were similar among NIT, NOP, and AQ, but lower for all treatments compared to CON (18% decrease on average; $P = 0.040$).

DISCUSSION

Although the high levels of CH_4 production inhibition obtained in this study are rarely achieved *in vivo* mainly due to other undesirable issues related to intake of high doses of tested CH_4 inhibitors, the present approach was useful for understanding redirection of [2H] due to a decrease in methanogenesis. Even though the goal of this study was not to make a direct comparison of the CH_4 efficiency of additives, the choice of treatments provided an interesting contrast, as each methanogenesis inhibitor had a different mode of action on rumen fermentation, as shown by the principal component analyses.

TABLE 9 | Effects of control (CON), nitrate (NIT), 3-nitrooxypropanol (NOP), and anthraquinone (AQ) on fermentation variables after treatment withdrawal (recovery period; day 14–19) using a rumen simulation technique.

Item[B]	Substrate				SEM	*P*-value[A]
	CON	NIT	NOP	AQ		
pH	6.96	6.95	6.94	6.93	0.018	0.641
Total VFA (mM)	54.6	60.0	52.2	59.4	3.28	0.311
VFA PROFILE (mol/100 mol)						
Acetate (A)	52.0^{a}	54.0^{a}	48.3^{b}	48.2^{b}	0.88	0.001
Propionate (P)	13.2^{a}	14.0^{a}	12.9^{ab}	11.9^{b}	0.50	0.039
Butyrate (B)	13.6^{b}	16.3^{a}	15.1^{ab}	14.3^{b}	0.57	0.023
Isobutyrate	0.43	0.37	0.40	0.44	0.020	0.143
Valerate	5.61^{b}	6.96^{a}	7.17^{a}	5.25^{b}	0.362	0.005
Isovalerate[C]	6.75^{a}	0.90^{b}	6.76^{a}	7.71^{a}	0.398	<0.001
Caproate	6.39^{bc}	5.57^{c}	7.00^{b}	9.17^{a}	0.405	<0.001
Heptanoate	2.12^{bc}	1.85^{c}	2.32^{b}	3.04^{a}	0.134	<0.001
A/P	4.00	4.11	3.81	4.13	0.174	0.557
(A+B)/P	5.04	5.31	5.02	5.34	0.219	0.579
Ethanol (mM)	5.65	5.49	4.43	4.71	0.772	0.624
n-propanol (mM)	0.05	0.12	0.02	0.05	0.028	0.127
Ammonium (mM)	27.0^{a}	22.0^{b}	21.5^{b}	23.2^{b}	1.32	0.040

VFA, volatile fatty acid.
[A]Within a row, means with different superscripts differ (P < 0.05).
[B]Average of data collected in all vessels during 3 consecutive days (day 17–19).
[C]Co-elutes with 2-methylbutyrate.

Specific Effects of Methanogenesis Inhibitors on Metabolic Hydrogen Fate

Reduction of Metabolic Hydrogen Availability with Nitrate

Nitrate decreased CH_4 production (mL) by 91%, corresponding to an 11.4% CH_4 decrease per 1% nitrate daily added, assuming a full reduction to NH_4^+. This efficiency is in the range of previous *in vivo* experiments (12.2%, Van Zijderveld et al., 2010; 12.5%, Lund et al., 2014; 7.3%, Lee et al., 2015; 9.2 and 6.8%, Veneman et al., 2015). Nitrous oxide was detected with nitrate, thereby supporting the occurrence of denitrification (Petersen et al., 2015; Latham et al., 2016). However, it can be estimated that only 0.02% of the total amount of nitrate added daily was converted to N_2O, indicating that DNRA must be the major nitrate degradation pathway. As a consequence of this small increase of N_2O, the GHG mitigation efficiency of NIT was only slightly affected.

The numerical decrease in gH_2 production and dH_2 concentration with NIT supports the assumed mode of action

of nitrate acting as a [2H] sink. However, this result contradicts previous batch culture study reporting a numerical increase of gaseous H_2 for nitrate concentrations higher than 4 mM (Guyader et al., 2015b), as well as *in vivo* experiments showing an increase of gaseous (Lund et al., 2014; Veneman et al., 2015) and dissolved (Guyader et al., 2015a) H_2 when nitrate was fed to cows up to 23 g/kg DM. Discrepancy among studies may be related with dosage, which was four times greater in the present study in order to inhibit CH_4 production by at least 80%. One may assume that as the dose of nitrate increases, direct toxicity of nitrite on methanogens gains importance as a mechanism of inhibition of methanogenesis over competition for reducing equivalents. A dose response study to examine effects of nitrate on dH_2 concentration would provide insights into the effect of nitrate on [2H] production and incorporation.

Nitrate increased acetate molar percentage but decreased all other VFA except propionate, which was not modified by any of the treatments. Several studies also reported an increase of acetate molar percentage with nitrate supplementation (El-Zaiat et al., 2014; Guyader et al., 2015a; Veneman et al., 2015). A shift toward acetate, whose formation from carbohydrates results in the release of [2H], could have been caused by lower [2H] availability as shown by the lower gH_2 and dH_2 when NIT was used. In agreement, a negative correlation was observed between gH_2 and acetate supporting the negative relationship reported by Wang et al. (2016) between dH_2 concentration and molar percentage of acetate.

The decrease in isobutyrate, isovalerate and 2-methylbutyrate with NIT strongly suggests a decrease in deamination of branched-chain amino acids. Zhou et al. (2011), Zhou et al. (2012), and Patra and Yu (2013) also observed a decrease in isovalerate and isobutyrate with CH_4 inhibition up to 70% with nitrate addition in batch cultures. Hino and Russell (1985) reported a negative effect of [2H] availability in reduced cofactors and dyes on fermentation of highly reduced amino acids. Because NIT shifted fermentation toward acetate (i.e., a [2H]-releasing pathway), the decrease in deamination of branched-chain amino acids by nitrate may perhaps have been caused by nitrite toxicity on amino acids fermenting organisms, rather than changes in the flow of metabolic hydrogen. A decrease of reduced amino acids concentrations may impact the overall ruminal fermentation of amino acids and increase the proportion of undegradable proteins flowing out of the rumen. This possible decrease in amino acids degradation may be one factor explaining the lower CP degradation and NH_4^+ concentration with NIT, which may also be explained by the partial NO_3^- reduction and, to a lower extent, the greater daily microbial protein production. Guo et al. (2009) also found lower NH_4^+ concentration and higher microbial N concentration in batch cultures supplemented with nitrate, confirming the potential beneficial effect of nitrate on microbial protein synthesis.

Finally, ethanol concentrations increased with NIT supporting the hypothesis that this alcohol may have a role in disposing reducing equivalents spared from methanogenesis (Ungerfeld et al., 2003). Overall, NIT stimulated [2H] producing pathways as shown by the greater acetate molar percentage. Based on the amount of nitrate added daily, the amount of nitrate and nitrite found in the effluent and the production of N_2O, the amount of [2H] diverted toward DNRA and denitrification was estimated to be equal to 51.6 mmol/d assuming that 4 moles [2H] are used within each reaction pathway. Then, [2H] recovery in SC1 and SC2 would be equal to 123.5 and 117.9%, respectively (data not shown). This calculation may be biased due to the use of sodium azide to stop the fermentations in the outflow. Indeed, sodium azide may react with nitrate or nitrite to form N_2O (McIlvin and Altabet, 2005), leading to a potential overestimation of the denitrification pathway. The estimated [2H] balance greater than 100% shows that either some [2H] producing pathways have not been taken into account and/or that the amount of [2H] directed toward DNRA has been overestimated. The use of labeled nitrate to track its decomposition into nitrite, NH_4^+ and N_2O may help understanding its degradation.

Increasing Amount of Reduced Products with Nitrooxypropanol

Nitrooxypropanol is a hydrosoluble compound quickly degradable (Duin et al., 2016). As a consequence, its effects on rumen fermentation probably take place in a limited period of time. However, this property did not prevent NOP from decreasing methanogenesis by 80%, which agrees with similar CH_4-mitigating efficiency observed in a previous RUSITEC study by Romero-Pérez et al. (2015) (76% CH_4 reduction with 5 mg 1,3-propanediol mononitrate $vessel^{-1}$ d^{-1}). The lower CH_4 production with NOP was associated with an increase of gH_2 and dH_2, showing that [2H] spared from decreased methanogenesis was not fully re-allocated toward other fermentation pathways. However, given that NOP decreased CH_4 production by 1.05 mmoles and that 1 mole CH_4 is produced from 4 moles [2H], availability of [2H] increased by 4.21 mmoles. As NOP increased gH_2 by 0.38 mmoles, it means that 9% of reducing equivalents not used in CH_4 formation ended up in accumulated H_2. This percentage is close to a previous meta-analysis estimate (5.8%) based on 100% methanogenesis inhibition in continuous culture (Ungerfeld, 2015).

Another part of [2H] spared from methanogenesis was re-directed toward the production of VFA that requires a net incorporation of [2H] when produced from glucose (valerate, heptanoate, and caproate produced via propionyl-CoA) or VFA whose production results in less release of [2H] per unit of glucose compared to acetate (butyrate and caproate via acetyl-CoA/butyryl-CoA). In agreement with Ungerfeld (2015), [2H] diverted toward propionate was not modified by a decrease in methanogenesis.

Propanol concentration increased during the treatment period, most probably as a result of NOP degradation. Indeed, one mole of NOP is degraded to one mole of propanol through propanediol and propanal (Duin et al., 2016). The calculated moles of additional propanol with NOP (0.14 mmoles/d increase relative to CON) largely exceeded the daily molar supplementation of NOP (0.04 mmoles/d). Assuming that the full amount of NOP was degraded to propanol and that the dilution rate of propanol was similar to the aqueous phase, calculations

showed that the greater propanol concentration was likely related to its accumulation over days (data not shown).

No additional [2H] was directed toward microbial protein synthesis, as microbial efficiency was similar to that of CON, confirming the results of Romero-Pérez et al. (2015). Overall, the [2H] balance was similar between CON and NOP. Depending on the caproate production scenario assumed, between 46 and 72% of [2H] produced was still not accounted for in the balance, indicating that several [2H] sinks were missed in the current experiment.

Upon removal of the NOP treatment, gas production and composition, as well as propanol concentration, recovered to control levels even though acetate molar percentage did not fully recover and remained lower than CON. Similarly, valerate molar percentage was still greater than CON during recovery. These few remaining differences between CON and NOP may indicate changes of microbiota during the NOP treatment period, that require more than 5 days to fully recover.

Increasing Amount of Metabolic Hydrogen Wastage with Anthraquinone

Anthraquinone decreased CH_4 production by only 27%, which was substantially less than expected based on previous experiments. Indeed, in a continuous culture, a lower dosage of anthraquinone decreased methanogenesis by 62% (Garcia-Lopez et al., 1996). Even though reasons for the lower effect observed in the present work are not clear, the decrease in methanogenesis was sufficient to observe several modifications in the fermentation profile.

Anthraquinone increased gH_2 release in agreement with the results of Garcia-Lopez et al. (1996). According to stoichiometry, this increase of gH_2 represented 32% of the total amount of [2H] saved from decreased methanogenesis, showing that at least one third of reducing equivalents spared from CH_4 formation were not incorporated into products with a nutritional value to the host animal. Surprisingly, the increase in gH_2 was not a consequence of greater dH_2, which was actually lowered by 76%. Lower dH_2 concentration may be related to the significant increase in reduced products such as caproate and heptanoate. Thus, dH_2 may have remained at low levels because it was diverted toward reduced VFA and quickly expelled as gH_2.

Despite the lower percentage of CH_4 decrease in comparison to NIT and NOP, AQ had a persistent effect on fermentation as dH_2 concentration remained lower than CON during the recovery period. Moreover, CH_4 production was still numerically lower in AQ compared to CON after treatment removal. This outcome may be the consequence of a strong negative effect of AQ on microbiota, which may have had difficulties recovering after withdrawal. Indeed, this plant extract is known to inhibit bacterial protein synthesis by blocking the first step of RNA translation to protein in the ribosomal A site (Anke et al., 1980). Decreased bacterial activity may also account for the observed decrease in microbial protein synthesis and greater concentration of NH_4^+ during the treatment period. However, similarly to NOP, the lower NH_4^+ concentration observed with AQ during the recovery period compared with CON could be an indication of resumption of microbial activity due to greater NH_4^+ incorporation into microbial amino acids.

How Is Metabolic Hydrogen Used When Methanogenesis Decreases?

Lost as Gaseous Hydrogen

With AQ and NOP, a portion of the [2H] spared from methanogenesis was lost as gH_2. The relationship between gH_2 and CH_4 production seems to be treatment-dependent. Indeed, no overall correlation between variables was observed in the present study in agreement with Wang et al. (2016), whereas positive correlations were observed within treatments (CON: $r = 0.80$, $P < 0.001$; NIT: $r = 0.54$, $P = 0.014$; NOP: $r = 0.74$, $P < 0.001$; AQ: $r = 0.67$, $P = 0.001$; Supplementary Figure 1), highlighting the specificity of each methanogenesis inhibitor on rumen fermentation. Qiao et al. (2015) also observed a positive response in CH_4 production to the replacement of headspace N_2 with H_2 in *in vitro* mixed batch cultures.

Gaseous H_2 was not correlated with dH_2 concentrations, which agrees with Wang et al. (2014) but not with Wang et al. (2016) who reported a positive and quadratic relationship between these two variables. Calculation of the supersaturation factor (S_f, ratio of actual to calculated dH_2 concentration based on gH_2 partial pressure and Henry's law; Wang et al., 2014) indicated variable supersaturation of dH_2 in the medium depending upon the treatment (Sf = 20.0, 77.1, 5.01, and 6.16 for CON, NIT, NOP, and AQ respectively; data not shown). The absence of equilibrium between gH_2 and dH_2 shows that it is not possible to use one to predict the other, and supports the complex theory of mass transfer limitation from the liquid to gaseous phase (Pauss et al., 1990). To foster a more global approach, the relationship between dissolved and gaseous H_2 shows the interest of studying microbial biofilm structures. For instance, treatments may affect parameters involved in metabolite transfer between microorganisms, such as the diffusion coefficients, the concentration of metabolites or the distance between microbes (Leng, 2014).

Finally, it is commonly suggested that ruminal H_2 should be maintained at low concentration to favor hydrogenase and ferredoxin reductase activity, which are two essential enzymes involved in the regeneration of reduced coenzymes produced during routine fermentation processes (Hegarty and Gerdes, 1999). With greater dissolved H_2 concentrations, NOP contradicts this theory as substrate degradability was not modified, suggesting that the availability of oxidized coenzymes was not affected. Similarly, Guyader et al. (2015a) observed an increase of dissolved H_2 concentrations in the rumen of cows supplemented with nitrate, without reduction of diet digestibility. Therefore, the effect of increased H_2 concentration on ruminal fermentation requires further attention.

Changes in Fermentation End Products

Janssen (2010) suggested that [2H] consuming pathways, such as propionogenesis, would be favored with greater [2H] concentrations. However, in our study, no treatment affected propionate production ($P = 0.219$; data not shown) despite increasing H_2 concentrations with NOP or AQ. This result may

indicate that a decrease in methanogenesis would not modify glucose production through gluconeogenesis from propionate, although this effect has yet to be confirmed *in vivo*.

An increase in the average carbon chain length of end products linked to greater [2H] availability has been reported previously with the use of redox mediators as methanogenesis inhibitors (neutral red, methyl viologen, safranin O, and tannic acid; Nerdahl and Weimer, 2015). Similarly, with NOP and AQ, part of [2H] spared from methanogenesis was diverted toward the production of more energy-dense and reduced straight-chain VFA. Caproate may have been mainly produced through the propionyl-CoA pathway given that the [2H] recovery was closer to 100% with SC1.

Another alteration caused by inhibiting methanogenesis, especially with NIT, was a decrease in branched-chain VFA molar percentage. This leads to the hypothesis that amino acid fermentation was inhibited along with methanogenesis, with lower deamination of branched-chain amino acids. However, the amount of [2H] produced or consumed during protein fermentation is difficult to estimate given that several pathways with different [2H] balance can lead to the production of each amino acid (Demeyer, 1991). For instance, there can be net uptake or production of [2H] during glutamate production from glucose (Czerkawski, 1986).

The role of formate in [2H] balance still requires clarification. Leng (2014) stated that formate would be favored as electron carrier in homogenized and aqueous environments whereas H_2 would be more efficient in dense aggregates such as in the ruminal environment. It was estimated that formate may act as an alternative electron carrier accounting for 10–20% of [2H] spared from CH_4 formation (Ungerfeld, 2015) and may be used as a regulator of [2H] balance (Shi et al., 1997). However, despite major modifications in [2H] flow, formate concentration was not affected when inhibiting methanogenesis in the present study. In contrast, an accumulation of formate was reported *in vitro* when using alternative electron sinks to inhibit ruminal methanogenesis (Ungerfeld et al., 2003). Using lactating dairy cows supplemented with up to 21 g nitrate/kg DM, Olijhoek et al. (2016) also reported an increase in formate from 0.08 mol/100 mol VFA up to 0.45 mol/100 mol VFA. Thus, further study of the response of formate concentration in relation to methanogenesis inhibition may help to understand the role of this rumen fermentation intermediate.

Structural Element for Microbial Biomass Synthesis

Greater efficiency of microbial protein synthesis with NIT raises the question of the role of microbial biomass as an alternative [2H] sink. The principal component analysis revealed a negative correlation between E_{MPS} and reduced VFA, suggesting that these pathways compete for [2H] spared from CH_4 production. Mills et al. (2001) estimated that 0.41 moles [2H] would be used per gram of microbial matter produced growing on NPN, which is close to the conditions of the present work. Then, assuming a constant microbial CP content of 54.5 g per gram of dry cell (Reichl and Baldwin, 1975) and a N content in microbial protein of 16%, daily [2H] used for microbial biomass synthesis would average 1.62, 1.89, 1.48, and 1.41 moles for CON, NIT, NOP, and AQ, respectively, representing 2.91, 3.63, 3.16, and 2.78% of [2H] produced (SC1) for the respective treatments. These percentages are in the range of previous estimations (Mills et al., 2001), and support previous suggestions that microbial biomass makes a relatively small contribution to [2H] sinks (Czerkawski, 1986; Mills et al., 2001).

That said, possible changes in microbial composition as a consequence of modified [2H] availability have not been taken into account in previous calculations, and it is possible that this could increase the importance of microbial biomass as an alternative [2H] sink. Indeed, an increase in [2H] availability as observed with NOP and AQ may increase the ratio of saturated to unsaturated microbial fatty acids in cells membrane. As proposed by Ungerfeld (2015), further studies should explore the effect of methanogenesis inhibition on microbial biomass amount and composition. Analysis of changes in the microbial community composition induced by methanogenesis inhibition using molecular techniques is also important (Denman et al., 2015; Martinez-Fernandez et al., 2016).

CONCLUSIONS

Nitrate, nitrooxypropanol, and anthraquinone differently affected the fate of [2H] spared from decreased methanogenesis. Through its role as an electron acceptor, nitrate mostly decreased [2H] availability. Nitrooxypropanol and anthraquinone increased the amount of reduced VFA, but this was not sufficient to incorporate all extra [2H] available. As a consequence, gH_2 increased as a way of evacuating the excess [2H]. Further work is needed to examine ways of efficiently utilizing energy spared from methanogenesis to improve animal performance. Moreover, as [2H] recovery was lower than 60% with CON, NOP, and AQ, future studies should aim at understanding the modifications in metabolic pathways (such as caproate formation) and microbial fatty acid composition resulting from CH_4 decrease in order to improve our understanding of [2H] production and utilization. From a practical point of view, this study also showed the importance of a daily supplementation of NIT, NOP, and AQ to maintain a sustained reduction in CH_4, despite some persistent modifications in VFA profile after removal of the inhibitors.

AUTHOR CONTRIBUTIONS

JG wrote the experimental protocol, ran the experiment including lab analyses, statistically analyzed the data and wrote the paper. EU helped to write the experimental protocol including planning and use of the RUSITEC, and to interprete the data. In addition, EU corrected the manuscript several times. KB helped to write and validate the experimental protocol. She also helped to interprete the data and she revised the manuscript several times too. Moreover, KB provided all the equipment and staff required to run the experiment.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00393/full#supplementary-material

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Limits to Dihydrogen Incorporation into Electron Sinks Alternative to Methanogenesis in Ruminal Fermentation

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Research is being conducted with the objective of decreasing methane (CH_4) production in the rumen, as methane emissions from ruminants are environmentally damaging and a loss of digestible energy to ruminants. Inhibiting ruminal methanogenesis generally results in accumulation of dihydrogen (H_2), which is energetically inefficient and can inhibit fermentation. It would be nutritionally beneficial to incorporate accumulated H_2 into propionate or butyrate production, or reductive acetogenesis. The objective of this analysis was to examine three possible physicochemical limitations to the incorporation of accumulated H_2 into propionate and butyrate production, and reductive acetogenesis, in methanogenesis-inhibited ruminal batch and continuous cultures: (i) Thermodynamics; (ii) Enzyme kinetics; (iii) Substrate kinetics. Batch ($N = 109$) and continuous ($N = 43$) culture databases of experiments with at least 50% inhibition in CH_4 production were used in this meta-analysis. Incorporation of accumulated H_2 into propionate production and reductive acetogenesis seemed to be thermodynamically feasible but quite close to equilibrium, whereas this was less clear for butyrate. With regard to enzyme kinetics, it was speculated that hydrogenases of ruminal microorganisms may have evolved toward high-affinity and low maximal velocity to compete for traces of H_2, rather than for high pressure accumulated H_2. Responses so far obtained to the addition of propionate production intermediates do not allow distinguishing between thermodynamic and substrate kinetics control.

Keywords: methanogenesis inhibition, rumen, dihydrogen, meta-analysis, thermodynamics, kinetics

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INTRODUCTION

Methanogenesis is the main electron sink in ruminal fermentation. Metabolic hydrogen ([H]) released in fermentation is transferred to methanogens mainly as H_2 and incorporated into CH_4. Methanogenesis allows carbohydrates to be fermented to a more oxidized product, acetate, instead of ethanol or lactate, which results in greater microbial ATP generation (Wolin et al., 1997). However, the release of CH_4 to the atmosphere, mainly through eructation and respiration, represents a loss of energy for ruminants, and is also a cause of climate change, as CH_4 is a potent greenhouse gas (Eckard et al., 2010; Martin et al., 2010).

Because CH_4 emissions from ruminants are energetically inefficient and environmentally damaging, research has been conducted on the inhibition of ruminal methanogenesis. There are published reports both of *in vitro* (e.g., Bauchop, 1967; Anderson et al., 2003) and *in vivo*

(e.g., McCrabb et al., 1997; Kung et al., 2003; Mitsumori et al., 2012) experiments in which CH_4 production was dramatically decreased using different chemical inhibitors. Metabolic hydrogen management is central to ruminal energetic efficiency. It is desirable to re-direct reducing equivalents that are not incorporated into CH_4 toward sinks that are nutritionally useful to the host animal, like volatile fatty acids (VFA). Inhibiting methanogenesis diminishes the production of acetate, which is associated with the release of [H], and favors incorporation of [H] into propionate, resulting in a typical acetate to propionate shift (Janssen, 2010). However, in a meta-analysis of methanogenesis inhibition in *in vitro* experiments, incorporation of [H] into propionate production was small for batch culture compared to what the decrease in CH_4 production would stoichiometrically allow, and on average non-existent in continuous culture (Ungerfeld, 2015).

Butyrate production from hexoses results in net release of [H], but, relative to acetate, butyrate production releases less [H] per mol of hexose fermented. Inhibiting methanogenesis could thus be theoretically expected to cause an acetate to butyrate shift. However, there was no overall increase in the incorporation of [H] into butyrate production in batch or continuous cultures associated to the decrease in CH_4 production (Ungerfeld, 2015).

Reductive acetogens inhabit the rumen (Leedle and Greening, 1988; Henderson et al., 2010), but reductive acetogenesis is not a [H] sink in the typical ruminal fermentation because methanogenesis lowers H_2 pressure below the thermodynamic threshold for acetogens to grow (Kohn and Boston, 2000). If methanogenesis is inhibited, addition of reductive acetogens can decrease H_2 accumulation (Le Van et al., 1998; Lopez et al., 1999).

Propionate and butyrate, and acetate formed through reductive acetogenesis, are nutritionally useful [H] sinks to ruminants as energy and carbon sources. Propionate is also the main source of glucose for ruminants. A question of importance is therefore, what are the physicochemical limitations to the incorporation of accumulated H_2 into propionate and butyrate formation, and reductive acetogenesis, in the methanogenesis-inhibited ruminal fermentation. The rate and extent of any biological process can be controlled by enzyme or substrate kinetics, or thermodynamics (**Figure 1**), and understanding the physicochemical control of H_2 incorporation can help in the design of efficient strategies of manipulation of [H] flows. The present analysis calculates thermodynamic limits of the incorporation of accumulated H_2 into propionate and butyrate production and reductive acetogenesis, and discusses possible control mechanisms of these processes in the methanogenesis-inhibited fermentation.

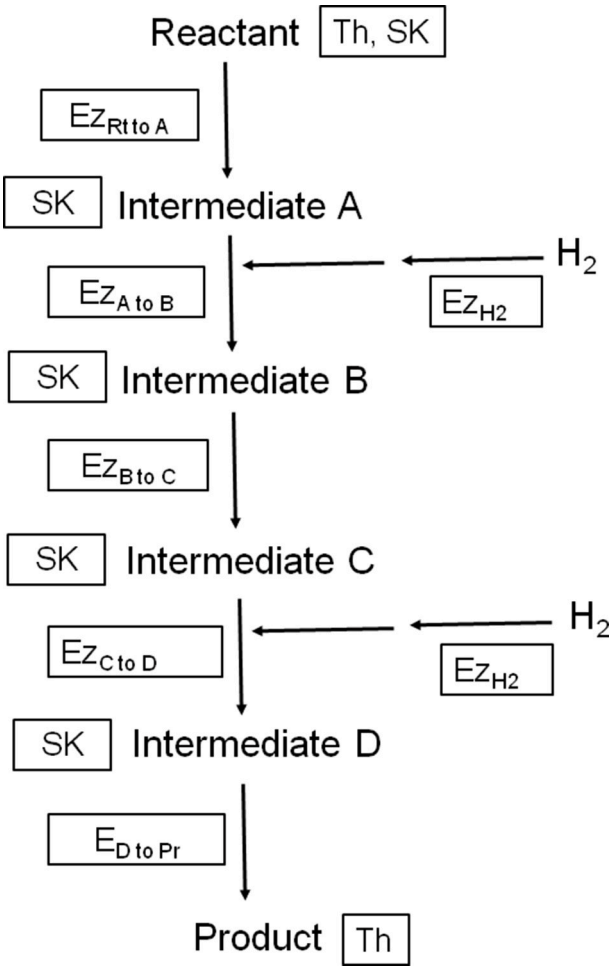

FIGURE 1 | Illustration depicting three different means of physicochemical control of the rate of a fictitious H_2-incorporating biochemical pathway starting with a reactant (Rt) that is converted into four intermediates A, B, C and D, and finishing with a product (Pr). Th, thermodynamic control, which depends on the activities of the reactant and product; Ez, enzyme kinetics control, which depends on the activity of the most limiting enzyme. Depicted in the diagram are two hydrogenases (Ez_{H2}) and five enzymes catalyzing conversions between carbon compounds (Ez_{xtoy}); SK, substrate kinetics control, which depends on the concentration of the reactant or the most limiting intermediate and is not affected by product accumulation.

METHODS

Databases

Databases of *in vitro* mixed ruminal batch and continuous cultures were used to calculate the Gibbs energy change (ΔG) of the incorporation of H_2 into propionate and butyrate production and reductive acetogenesis. The present meta-analysis included part of the batch and continuous culture databases used in Ungerfeld (2015). However, only experiments that reported total gas or CO_2 production were used, in order to calculate CO_2 partial pressure, and eventually ΔG of the H_2-incorporating processes in which CO_2 was involved. If total gas production was reported, but CO_2 production or molar percentage were not, CO_2 molar percentage was calculated as the difference between total gas and the sum of CH_4 and H_2.

The rest of the criteria used for inclusion of experiments were the same as in Ungerfeld (2015):

(i) Initial headspace was H_2-free and formic acid or formate salts were not included as substrate;

(ii) The experiment included a methanogenesis-uninhibited control treatment and results for CH_4, H_2, and VFA in the control treatment were reported;
(iii) At least one treatment or level within a treatment resulted in a 50% or greater decrease in CH_4 production relative to the control. This requirement increases variation in the regressor (methanogenesis inhibition) and decreases the proportion of variation in ΔG associated to the experiment effect, resulting in greater power to test the hypotheses of responses of ΔG to methanogenesis inhibition;
(iv) Treatments within experiments consisting in combinations of methanogenesis inhibitors and fermentation intermediates or their isomers or analogs (malate, fumarate, crotonate, butynoic acid, or 3-butenoic acid) were discarded, as intermediates whose concentration was not reported could affect the thermodynamics of the process being studied;

Some batch culture studies that did not report net VFA production were not used in Ungerfeld (2015) meta-analysis but were included in the present study, because only metabolites final concentrations, but not their net production, is necessary to estimate ΔG.

Incubations including different treatments that were run simultaneously and included the same uninhibited control treatment were considered to be one experiment. The batch culture database comprised a total of 109 treatment means from 17 experiments in nine published studies (Table S1). The continuous cultures database comprised a total of 43 treatment means from eight experiments in six peer-reviewed published studies (Table S2).

Rationale and Calculations

Gibbs energy changes of the incorporation of H_2 into propionate and butyrate production and reductive acetogenesis, were calculated for batch cultures at the end point of the incubations, and for continuous cultures at steady state. Inhibition of methanogenesis *in vitro* generally decreased or did not affect the estimated amount of fermented hexoses (Ungerfeld, 2015). For the present analysis, ΔG of H_2 incorporation into propionate and butyrate production and reductive acetogenesis was estimated assuming that amount of hexoses fermented did not change i.e., accumulated H_2 was not to be incorporated into pathways of additional hexoses being fermented (e.g., additional propionate production from hexoses).

Interconversions among VFA have been experimentally demonstrated (Ungerfeld and Kohn, 2006). Therefore, [H] in accumulated H_2 in the methanogenesis-inhibited fermentation could be incorporated into propionate formation through acetate being converted to propionate:

$$CH_3COO^- + CO_2 + 3H_2 \rightarrow CH_3CH_2COO^- + 2H_2O \quad (1)$$

An acetate to propionate shift with an implied decrease in H_2 accumulation could also occur if there was a decrease in hexoses fermented to acetate compensated with a concomitant increase in hexoses fermented to propionate i.e., a replacement of ½× 4 [2H] in the products of Equation (2) with 2 [2H] in the reactants of Equation (3):

$$\tfrac{1}{2}(C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COO^- + 2H^+ + 2CO_2 + 4[2H]) \quad (2)$$

$$C_6H_{12}O_6 + 2[2H] \rightarrow 2CH_3CH_2COO^- + 2H^+ + 2H_2O \quad (3)$$

The incorporation of two extra pairs of reducing equivalents in half of Equation (2) is stoichiometrically equivalent to incorporation of 2 H_2 into propionate production (Equation 4):

$$2[2H] \rightarrow 2H_2 \quad (4)$$

Such a shift of acetate to propionate could occur intra- and/or extracellularly. Some ruminal bacteria can produce propionate from hexoses, whereas others produce succinate or lactate as intermediates, which are then excreted and converted to propionate by other bacteria (Stewart et al., 1997). An intracellular shift from acetate to propionate or its intermediates occurs when a cell produces less acetate and derives part of fermentation-produced [H] from H_2 and formate to propionate, or succinate or lactate production. For example, the acetate to succinate ratio produced by *Ruminococcus flavefaciens* decreases when grown in pure culture, in comparison to when grown in co-culture with a methanogen (Latham and Wolin, 1977).

Associated to the shift from acetate to propionate occurring when replacing roughages with concentrates (Janssen, 2010), there are changes in the microbial community composition (Petri et al., 2013). The observed shift from acetate to propionate with concentrate supplementation is the result of both changes in the fermentation profile of individual cells and changes in microbial populations, but is currently impossible to determine the contribution of each component.

Production of propionate from hexoses in the typical, uninhibited ruminal fermentation is obviously thermodynamically favorable (ΔG modeled by Kohn and Boston, 2000). Inhibiting methanogenesis results in accumulation of H_2 and reduced cofactors (Hino and Russell, 1985), which would make propionate production from hexoses even more thermodynamically favorable. Therefore, for the present ΔG calculation on the incorporation of accumulated H_2 into propionate production, only incorporation of H_2 into acetate conversion to propionate as depicted in Equation (1) was considered. In any case, because ΔG is a state function, net thermodynamic consequences for a cell or the entire fermentation system of a shift from acetate to propionate through less [H] generated by Equation (2) and more [H] incorporated by Equation (3) as H_2 (Equation 4) would be equal to Equation 1.

Production of butyrate from hexoses results in a net release of [2H], although [2H] release per mol of hexose is lesser compared to acetate production (Equation 2 vs. Equation 5):

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COO^- + H^+ + 2CO_2 + 2[2H] \quad (5)$$

Conversion of acetate to butyrate occurs in the rumen (Ungerfeld and Kohn, 2006) and could theoretically be a mechanism to

incorporate [H] in accumulated H_2 into VFA:

$$2CH_3COO^- + H^+ + 2H_2 \rightarrow CH_3CH_2CH_2COO^- + 2H_2O \quad (6)$$

Alternatively, and analogous to propionate production, a decrease in hexoses fermented to acetate compensated by a concomitant increase in hexoses fermented to butyrate would also result in implicit incorporation of accumulated H_2 into butyrate production, as part of [H] released from acetate production would instead be used to produce butyrate. The net thermodynamic consequences in ΔG that result from a combination of Equations (2, 4, 5) are depicted by Equation (6) above.

For the present analysis, ΔG of incorporation of accumulated H_2 into propionate and butyrate were therefore calculated from the incorporation of H_2 in the conversion of acetate to propionate (Equation 1) or butyrate (Equation 6).

Gibbs energy change for H_2 incorporation into reductive acetogenesis was calculated as:

$$2CO_2 + 4H_2 \rightarrow CH_3COO^- + H^+ + 2H_2O \quad (7;\ \text{Müller, 2003})$$

Gibbs energy changes at standard chemical conditions (ΔG°) and 298 K (25°C) for Equations (1, 6, 7) were calculated from Gibbs energy changes of formation of individual compounds (ΔG_f°) (Kohn and Boston, 2000; Karadagli and Rittmann, 2007). Gibbs energy changes so calculated were then adjusted to a ruminal temperature of 39°C through the Van't Hoff equation (Kohn and Boston, 2000). Gibbs energy changes at standard chemical conditions and 39°C were subsequently adjusted to actual *in vitro* ruminal fermentation conditions using the activities of soluble metabolites and dissolved gases (Karadagli and Rittmann, 2007). Activities were calculated as the product of concentrations by activity coefficients. Activity coefficients of dissolved CO_2 and H_2 were assumed to be equal to unity. Activity coefficients of charged ions in Equations (1, 6, 7) were calculated according to the Davies equation (Hamer, 1968):

$$\log\gamma = z^2 A\sqrt{I}/\left(1+\sqrt{I}\right) + 0.2AI$$

where γ is the activity coefficient (molar scale), z is the charge of the ion, I is the molar ionic strength of the solution, and A is a molar parameter calculated as:

$$A = 1.825 \times 10^6/(T\epsilon)^{3/2}$$

where T is the absolute temperature (312 K) and ϵ is the dielectric constant of the solvent [73.82 for water at 311 K according to Hamer (1968)].

Extracellular ionic strength was calculated as the sum of ionic strength provided by the medium buffer and macrominerals, and VFA. For batch cultures, ionic strength was calculated from the initial ion concentration provided by the buffer and macrominerals and 0.15 M for the inoculum (Kohn and Boston, 2000). It is acknowledged that a net uptake of ions would take place as cultures grew and that release of CO_2 initially present in the buffer as HCO_3^- would consume equimolar amounts of H^+ released from VFA formed in fermentation. In turn, CO_2 released from VFA formed in fermentation would result in H^+ release from carbonic acid. For continuous cultures, ionic strength was calculated as the sum of ionic strength provided by buffer and macrominerals and VFA at steady state.

Total gas pressure was calculated from the ratio of total gas production to headspace volume in batch cultures, and assumed to be equal to 10^5 kPa in continuous cultures. Partial pressure for each gas was calculated from the Ideal Gas Law, and dissolved gases concentrations were calculated from Henry's Law (Kohn and Boston, 2000; Janssen, 2010), assuming equilibrium between the liquid and gas phases. Water concentration was assumed to be 50 M (Kohn and Boston, 2000).

In some control treatments, H_2 was undetectable and reported as equal to zero. In those cases, ΔG was calculated assuming a H_2 concentration equal to the median of controls reporting non-zero H_2 values for batch (2.35×10^{-6} M) or continuous (2.02×10^{-6} M) cultures.

Gibbs energy change for reduction of oxidized ferredoxin (Fd_{ox}) and NAD^+ by H_2 (Buckel and Thauer, 2013) was also estimated for methanogenesis-inhibited and non-inhibited ruminal batch and continuous cultures:

$$Fd_{ox} + NAD^+ + 2H_2 \rightarrow Fd_{red}^{2-} + NADH + 3H^+;\quad \Delta G^\circ = -21\ \text{kJ} \quad (8;\ \text{Buckel and Thauer, 2013})$$

For the estimation of ΔG for Equation (8) in the normal, methanogenesis non-inhibited fermentation, the NAD^+/NADH pair was considered to be 95% in the oxidized form (Bennett et al., 2009), and ferredoxin was considered to be 90% in the reduced form (Buckel and Thauer, 2013). For the estimation of ΔG in the methanogenesis-inhibited fermentation, the ratios of the reduced to oxidized species of both cofactor pairs were multiplied by a factor equal to 1.79 to account for greater concentration of reduced cofactors under methanogenesis inhibition (Hino and Russell, 1985). This factor is equal to the quotient between 0.70/0.39, which are the NADH/NAD^+ ratios reported by Hino and Russell (1985) for methanogenesis-inhibited and control ruminal incubations, respectively.

For the estimation of ΔG for Equation (8), H^+ concentration was assumed to be equal to 10^{-7} M for an intracellular pH of 7. Concentration of H_2 in methanogenesis non-inhibited fermentation was assumed to be equal to the median of controls treatments reporting non-zero H_2 values for batch (2.35×10^{-6} M) and continuous (2.02×10^{-6} M) cultures. Concentration of H_2 under H_2 accumulation was estimated by regressing the response of dissolved H_2 to methanogenesis inhibition in batch and continuous cultures and calculating the predicted H_2 concentration for 100% methanogenesis inhibition.

Regressions

The responses of calculated ΔG for the conversions of acetate to propionate (Equation 1) and butyrate (Equation 6), and for reductive acetogenesis (Equation 7), against the inhibition of methanogenesis expressed as a percentage of CH_4 production in control treatments, were regressed separately for batch and

continuous cultures as:

$$\Delta G_{ij} = \text{intercept} + exp_i + B_1 x_{ij} + B_2 x_{ij}^2 + b_i x_{ij} + residual_{ij}$$

where exp_i is the fixed effect of the experiment i, B_1 and B_2 are fixed linear and quadratic regression coefficients, respectively, of regressor x %CH_4 production inhibition, b_i is the fixed effect of experiment i on the linear coefficient of x (i.e., the interaction between methanogenesis inhibition and the experiment effect), and $residual_{ij}$ is the residual of the ith experiment at the jth level, with *residual* assumed to be independent and normally distributed. Fixed, rather than random, effect of the experiment was used because the objective of the analysis was to understand how methanogenesis inhibition affects ΔG of H_2 incorporation into propionate, butyrate and reductive acetogenesis within the existing analyzable universe of information, rather than building models to predict ΔG for new *in vitro* experiments. Significance was declared at $p < 0.05$ and tendencies at $0.05 \leq p \leq 0.10$. Non-significant interactions and quadratic effects ($p > 0.10$) were removed, although if removing a non-significant term increased corrected Akaike Information Criterion and/or Bayesian Information Criterion, this term was left in the model.

In meta-analysis, weighting treatment means by the reciprocal of their standard errors (1/SEM) scaled to one is recommended to obtain maximum likelihood estimates while maintaining the original scale of the data (Sauvant et al., 2008). However, SEM of ΔG are not calculable from reported data, as they are a logarithmic function of metabolites concentrations, with individual VFA concentrations in turn calculated as the product of total VFA and corresponding molar percentages, each variable with their reported SEM. Instead, reciprocal of SEM of total VFA concentration was chosen as the weighting variable.

Plots of residuals against predicted values were examined. Outliers and influential observations were identified as in Ungerfeld (2015) through examining studentized residuals, leverage (hat) values, and Cook's distances. Potential influential outliers were identified as treatment means with (1) a studentized residual $> |t_{n-k}|$ (=2 SEM), where k was the number of parameters and n the number of treatment means used to fit the regression; (2) a leverage value larger than 2k/n (Belsey et al., 1980); (3) a Cook's distance greater than the 50th percentile of an $F_{k,n-k}$ distribution.

JMP® 11.0.0 was used for all statistical analyses.

RESULTS

Acetate Conversion to Propionate

Both in batch and in continuous culture, inhibiting CH_4 production decreased ΔG for the incorporation of H_2 into the conversion of acetate to propionate ($p < 0.001$; **Figure 2**), with an interaction with the experiment effect ($p < 0.05$). There was a tendency to a quadratic response in batch culture ($p = 0.075$), and a significant quadratic response in continuous culture ($p = 0.011$).

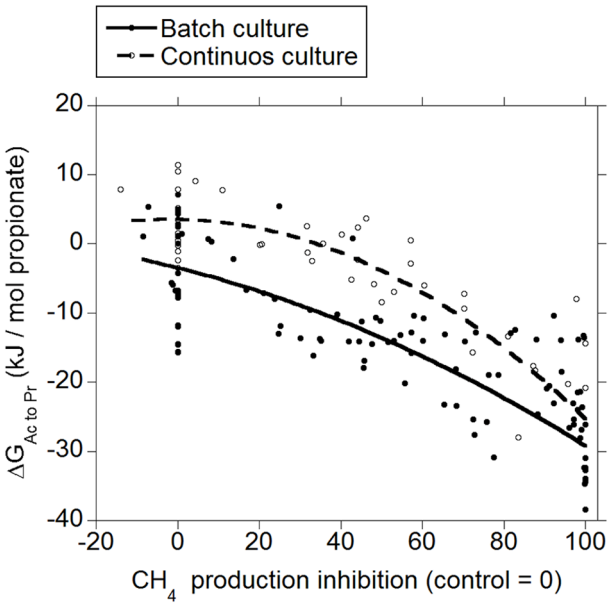

FIGURE 2 | Response of Gibbs energy of acetate conversion to propionate ($\Delta G_{Ac\ to\ Pr}$) to the inhibition of CH_4 production expressed as a percentage of CH_4 production decrease relative to control treatments. Treatment means are adjusted by the effect of their experiment. Batch culture: $y = -1.03$ (±2.12; $p = 0.63$) + *exp* ($p < 0.001$) − 0.25 (±0.029; $p < 0.001$) x − 0.0011 (±0.00062; $p = 0.075$) $(x - 46.6)^2$ + *exp* × x ($p < 0.001$); $R^2 = 0.93$ ($p < 0.001$); Continuous culture: $y = 9.68$ (±2.14; $p < 0.001$) + *exp* ($p < 0.001$) − 0.27 (±0.032; $p < 0.001$) x − 0.0028 (±0.0010; $p = 0.011$) $(x - 46.7)^2$ + *exp* × x ($p = 0.032$); $R^2 = 0.85$ ($p < 0.001$).

Acetate Conversion to Butyrate

Inhibiting CH_4 production decreased ΔG for the incorporation of H_2 into the conversion of acetate to butyrate both in batch and in continuous culture ($p < 0.001$; **Figure 3**), with an interaction with the experiment effect ($p < 0.05$). There was a tendency to a quadratic response in batch culture ($p = 0.074$), and a significant quadratic response in continuous culture ($p = 0.016$).

Reductive Acetogenesis

Both in batch and continuous culture, there was a decrease in ΔG of reductive acetogenesis as methanogenesis was inhibited ($p < 0.001$; **Figure 4**), with an interaction with the experiment effect ($p < 0.05$). In continuous culture, the response was quadratic ($p = 0.011$).

DISCUSSION

In the normal, methanogenesis-uninhibited ruminal fermentation, H_2 production from reduced cofactors is thermodynamically feasible because H_2 concentration is kept very low through rapid removal mainly by methanogenesis (Janssen, 2010). In the methanogenesis-inhibited ruminal fermentation, H_2 removal is smaller but greater ratio of reduced to oxidized cofactors (Hino and Russell, 1985) thermodynamically allows H_2 accumulation. The present

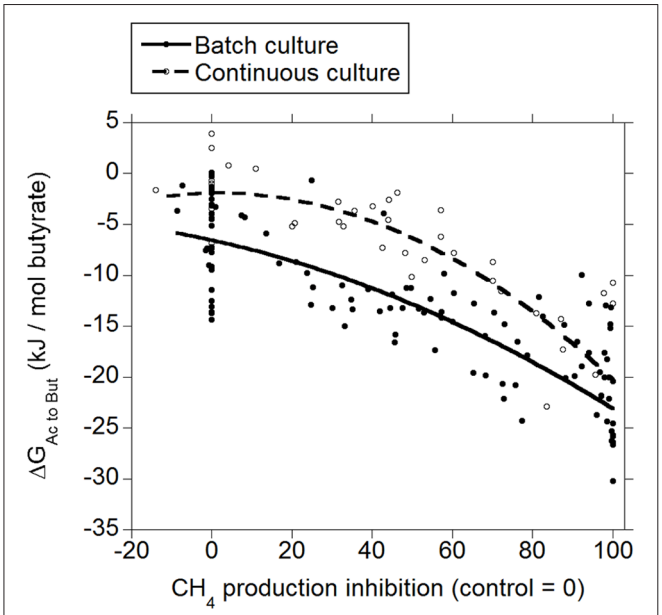

FIGURE 3 | Response of Gibbs energy of acetate conversion to butyrate ($\Delta G_{Ac\ to\ But}$) to the inhibition of CH_4 production expressed as a percentage of CH_4 production decrease relative to control treatments. Treatment means are adjusted by the effect of their experiment. Batch culture: $y = -4.80$ (±1.50; $p = 0.002$) + *exp* ($p < 0.001$) − 0.16 (±0.020; $p < 0.001$) x − 0.00079 (±0.00044; $p = 0.074$) $(x - 46.6)^2$ + *exp* × x ($p < 0.001$); $R^2 = 0.92$ ($p < 0.001$); Continuous culture: $y = 2.21$ (±1.50; $p = 0.15$) − 0.17 (±0.022; $p < 0.001$) x − 0.0019 (±0.00072; $p = 0.016$) $(x - 46.7)^2$ + *exp* × x ($p = 0.030$); $R^2 = 0.87$ ($p < 0.001$).

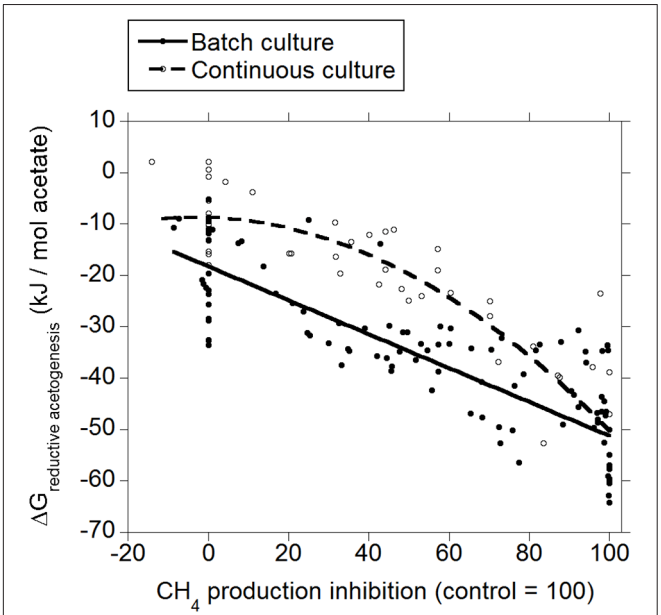

FIGURE 4 | Response of Gibbs energy of reductive acetogenesis ($\Delta G_{reductive\ ace\ to\ genesis}$) to the inhibition of CH_4 production expressed as a percentage of CH_4 production decrease relative to control treatments. Treatment means are adjusted by the effect of their experiment. Batch culture: $y = -18.2$ (±2.43; $p < 0.001$) + *exp* ($p < 0.001$) − 0.33 (±0.038; $p < 0.001$) x + *exp* × x ($p < 0.001$); $R^2 = 0.93$ ($p < 0.001$); Continuous culture: $y = -0.16$ (±2.97; $p = 0.96$) − 0.39 (±0.044; $p < 0.001$) x − 0.0039 (±0.0014; $p = 0.011$) $(x - 46.7)^2$ + *exp* × x ($p = 0.045$); $R^2 = 0.85$ ($p < 0.001$).

analysis addresses the question of what are the physicochemical limitations to the incorporation of [H] in accumulated H_2 into propionate or butyrate production, or reductive acetogenesis, when methanogenesis is inhibited. Three physicochemical potential limitations to H_2 incorporation into propionate and butyrate production and reductive acetogenesis will be discussed:

(i) A thermodynamic limitation would occur if propionate or butyrate production, or reductive acetogenesis, reached thermodynamic equilibrium at the concentrations of metabolites observed in the *in vitro* cultures and according Equations (1, 6, 7), respectively;

(ii) An enzyme kinetics limitation could be one such as insufficient numbers of microorganisms possessing the genes encoding for the necessary enzymes, expression of those genes, enzyme activity regulation, or enzymes k_m and v_{max};

(iii) A substrate kinetics limitation would be an insufficient rate of provision of electron-accepting carbon compounds to incorporate accumulated H_2 into the desired pathways.

TABLE 1 | Predicted ΔG for acetate conversion to propionate and butyrate and for reductive acetogenesis for batch and continues cultures at 0 and 100% methanogenesis inhibition.

Process	System	Methanogenesis inhibition (%)	ΔG (kJ / mol VFA)	95% confidence interval
Acetate to propionate	Batch	0	−3.42	−10.3 to 3.42
		100	−29.2	−42.7 to −15.7
	Continuous	0	3.57	−5.15 to 12.3
		100	−25.3	−41.8 to −8.79
Acetate to butyrate	Batch	0	−6.50	−12.8 to −0.16
		100	−23.1	−34.5 to −11.7
	Continuous	0	−1.93	−7.99 to 4.27
		100	−20.2	−31.8 to −8.59
Reductive acetogenesis	Batch	0	−18.2	−23.0 to −13.4
		100	−51.2	−63.5 to −38.9
	Continuous	0	−8.67	−20.8 to 3.48
		100	−50.2	−73.1 to −27.3

Thermodynamics

The typical range in the profile of VFA in ruminal fermentation has been proposed to result from thermodynamic equilibrium (Ungerfeld and Kohn, 2006). Calculated ΔG for acetate conversion to propionate or butyrate in the ruminal fermentation with functional methanogenesis (zero methanogenesis inhibition) were not different from or very close to zero (**Figures 2**, **3**; **Table 1**), which agrees with the proposition

of thermodynamic control of the VFA profile. Reductive acetogenesis has been estimated to be slightly thermodynamically favorable but close to equilibrium in the typical ruminal fermentation (Kohn and Boston, 2000), in accord to current ΔG estimation in batch culture at zero methanogenesis inhibition; in continuous culture at zero methanogenesis inhibition, reductive acetogenesis ΔG was estimated to be not different from zero (**Figure 4**; **Table 1**).

As CH_4 production was inhibited and H_2 accumulated, H_2 incorporation into reductive acetogenesis, propionate production, and butyrate production, in that order, became thermodynamically more favorable (**Figures 2–4**; **Table 1**). The decreases in ΔG of each process relative to each other reflect their relative stoichiometries of H_2 incorporation. Likewise, the interactions between methanogenesis inhibition and the experiment effect reflect the variation among experiments in the response in H_2 accumulation to methanogenesis inhibition, as H_2 accumulation is the main driving force for ΔG decrease due both to the stoichiometry of H_2 incorporation compared to carbon-containing compounds, and to the relative magnitude of the changes in H_2 concentration in comparison to the rest of the metabolites. Variation among methanogenesis-inhibition experiments with regard to H_2 accumulation has been previously discussed (Ungerfeld, 2015), thus the interactions between methanogenesis inhibition and the experiment effect will not be addressed herein.

Minimal predicted ΔG at 100% methanogenesis inhibition (i.e., with maximal H_2 accumulation) was slightly negative for incorporation of H_2 into propionate and butyrate production and reductive acetogenesis both in batch and continuous cultures (**Table 1**). It has been postulated that bacterial metabolism can occur at ΔG very close to thermodynamic equilibrium (Jackson and McInerney, 2002); however, ΔG calculations herein do not consider the utilization of ΔG in ATP generation. Given a ΔG of ATP hydrolysis of −40 to −50 kJ/ mol, and that thermodynamic efficiency of ATP generation from ADP and phosphate in the energy-conserving process can approach 100% (Thauer et al., 1977; Voet and Voet, 1995), it appears from **Table 1** that if 1 mol of ATP per mol of acetate was generated by reductive acetogenesis, the process would be close to equilibrium, and that incorporation of H_2 into both propionate and butyrate production from acetate could not generate a ΔG negative enough to generate 1 mol of ATP per mol of propionate or butyrate, respectively. However, ATP is generated through transmembrane ion gradient-driven phosphorylation both in reductive acetogenesis and in fumarate reduction to succinate in propionate's randomizing pathway (Russell and Wallace, 1997; Müller, 2003), and less than one ATP may be generated per pair of reducing equivalents incorporated (Reddy and Peck, 1978; Kröger and Winkler, 1981). Furthermore, a minimum ATP generation for maintenance and growth may not be required: reductive acetogens (Joblin, 1999), and propionate producers could obtain most of their energy from sugar fermentation, and perhaps supplement it with a small amount of ATP obtained from ion gradient-driven phosphorylation from reductive acetogenesis and acetate conversion to propionate, in each case. In principle therefore, incorporation of accumulated H_2 into propionate formation or reductive acetogenesis, although close to equilibrium, may perhaps have been thermodynamically feasible in the experiments analyzed. Estimated thermodynamic feasibility of reductive acetogenesis under H_2 accumulation *in vitro* agrees with a decrease in H_2 verified when reductive acetogens have been added to methanogenesis-inhibited ruminal incubations (Nollet et al., 1997; Le Van et al., 1998; Lopez et al., 1999).

Predicted ΔG of H_2 incorporation into butyrate production at 100% methanogenesis inhibition was also slightly negative (**Table 1**). However, butyrate production involves the generation of 1 mol of ATP per mol of butyrate through substrate level-phosphorylation. This requirement seems to make the incorporation of additional H_2 into butyrate production with acetate as the carbon donor thermodynamically unfeasible. However, ATP generation associated to butyrate production through ion gradient-driven phosphorylation recently proposed for the genus *Butyrivibrio*, may allow these bacteria greater ATP generation than what has been previously thought for this pathway (Hackmann and Firkins, 2015), and perhaps enable incorporation of H_2 in the conversion of acetate to butyrate.

One should be cautious about the thermodynamic calculations herein presented. Limitations to estimations of ΔG have been discussed by Maskow and von Stockar (2005). Metabolite concentrations, and most significantly H_2, are not uniform in microbial ecosystems and gradients exist (Boone et al., 1989). In particular, lack of equilibrium between gaseous and dissolved H_2 can result in large differences between ΔG estimated assuming equilibrium and actual ΔG calculated from the aqueous phase concentration of gases (Hackmann, 2013). Working with ruminal batch cultures, Wang et al. (2014) reported that H_2 was supersaturated in the liquid phase with respect to the headspace, and there was no relationship between concentration of dissolved H_2 and gaseous H_2. Thus, actual ΔG are likely lower than ΔG estimated assuming equilibrium between gaseous and dissolved H_2, making the processes discussed likely more feasible than has been herein estimated.

Rumen contents can be described as a more heterogeneous environment compared to *in vitro* cultures, due both to greater solid particle size and solids to fluid ratio. Greater heterogeneity likely creates more microenvironments with H_2 gradients *in vivo* than *in vitro*, implying that H_2-incorporating pathways could be more thermodynamically favorable *in vivo* close to H_2 production microenvironments compared to the present estimations from *in vitro* results. On the other hand, gases accumulate to supra-atmospheric pressures in batch cultures compared to continuous cultures and the rumen. This could give batch cultures a thermodynamic edge compared to continuous cultures and *in vivo* fermentation with respect to H_2 incorporation; perhaps this causes ΔG of H_2 incorporation into propionate and butyrate production and reductive acetogenesis to be slightly more favorable in batch than in continuous culture (**Figures 2–4**), due to numerically greater response in H_2 accumulation to methanogenesis inhibition (Ungerfeld, 2015).

Enzyme Kinetics

Propionate Production

Enzyme-limited capacity of the mixed ruminal microbiota to produce propionate can be caused by insufficient activities of enzymes catalyzing conversions between carbon compounds or by insufficient activity of hydrogenases (**Figure 5**). With regard to enzymes catalyzing conversions between carbon compounds, Denman et al. (2015) recently found an increase in the genes encoding for most of the enzymes of propionate's randomizing pathway when methanogenesis was inhibited in goats.

With respect to hydrogenases activity, H_2 incorporation in the reduction of fumarate to succinate has been demonstrated in ruminal bacteria (Henderson, 1980; Asanuma et al., 1999), but H_2 incorporation into the other reduction steps in **Figure 5** has not been shown for ruminal fermentation. The question that will be discussed next is if the mixed ruminal microbiota could potentially incorporate H_2 at a fast enough rate to avoid H_2 accumulation when CH_4 production is inhibited, if H_2 incorporation into additional propionate production from acetate is indeed thermodynamically feasible.

Recent research efforts have focused on understanding *in vivo* changes in the ruminal microbial community composition when methanogenesis is inhibited. Mitsumori et al. (2012) reported an increase in *Prevotella* sp. and *Clostridium aminophilum*. *Prevotella* sp. produce propionate (Stewart et al., 1997) and are often the most abundant organisms in the rumen (Stevenson and Weimer, 2007; Bekele et al., 2010; Stiverson et al., 2011). Moreover, *P. ruminicola* can incorporate H_2 into propionate production (Henderson, 1980). The increase in the abundance of *Prevotella* sp. agreed with an increase in propionate concentration (Mitsumori et al., 2012). Shinkai et al. (2012) also found an increase in *P. ruminicola*, along with propionate producer *S. ruminantium* when methanogenesis was inhibited, and variable changes in other propionate and succinate producers. However, along the increases in propionate concentration and abundance of propionate producers (Mitsumori et al., 2012; Shinkai et al., 2012), and genes encoding for enzymes of propionate's randomizing pathway (Denman et al., 2015) observed with *in vivo* methanogenesis inhibition, there still has been persistent long-term H_2 accumulation (Trei et al., 1971, 1972; Kung et al., 2003; Mitsumori et al., 2012). Therefore, the changes occurring in these microbial populations and presumably the kinetic capacity to produce propionate, do not seem to be sufficient to take up all of the accumulated H_2.

Broudiscou et al. (2014) inoculated batch cultures with continuous cultures vastly differing in H_2 content in their headspaces. Adding H_2 to the batch cultures headspace actually decreased propionate molar proportion when the inoculum came from a low-H_2 continuous culture, and did not affect it when the inoculum came from high-H_2 continuous cultures. These results suggests no long-term adaptation of the mixed microbiota growing in those continuous culture fermenters to incorporate elevated H_2 into propionate production, both because of H_2 accumulation occurring in the continuous culture fermenters themselves, and because of lack of response in propionate to H_2 in batch cultures inoculated with high-H_2 continuous cultures. It should be noted though that lack of adaptation to elevated H_2 in this experiment may partially reflect loss of microbial diversity in continuous cultures (Johnson et al., 2009).

Changes in the ruminal microbial community composition and metabolism as a response to supplementation with intermediates of propionate production have also been studied. Mao et al. (2008) reported that feeding disodium fumarate to goats induced *Succinivibrio dextrinisolvens*-related organisms, which would likely reduce the added fumarate to succinate. Supplementation with disodium fumarate did not affect the abundance of a *P. ruminicola*-related organism. Zhou et al. (2012) found that feeding disodium fumarate to sheep decreased succinate-producers *Fibrobacter succinogenes* in ruminal solids, and *R. flavefaciens* in ruminal fluid in one trial and caused erratic results in a longer term trial. However, the abundance of organisms such as *Prevotella* spp., *Selenomonas* spp., or *Succinivibrio* spp., was not evaluated in that study. In pure culture, several succinate and propionate-producing bacteria increased fumarate reductase activity when grown in a fumarate-containing medium (Asanuma and Hino, 2000).

Several ruminal bacteria produce formate as a means of disposing [H] (Stewart et al., 1997). In the mixed ruminal fermentation, formate is an intermediate whose concentration is kept low because formate produced is rapidly consumed mainly in CH_4 formation (Hungate et al., 1970; Asanuma et al., 1998). If methanogenesis is inhibited, formate often accumulates (Asanuma et al., 1998; Ungerfeld, 2015). Therefore, it is important to find ways to incorporate also formate into [H] sinks useful to the host animal. Most bacterial species that were able to use H_2 as [H] donor in fumarate reduction to succinate could also use formate, although their affinity for formate was generally lower than for H_2 (Asanuma et al., 1999).

Addition of a direct-fed microbial would enhance the enzyme kinetics capacity of pathways carried out by the added microbes. For example, Henning et al. (2010) successfully decreased lactate concentration in the rumen of acidotic lambs and steers by dosing a strain of lactate utilizer *Megasphaera elsdenii*, demonstrating that utilization of accumulated lactate was being limited by enzyme kinetics in the control treatments. Mamuad et al. (2014) decreased CH_4 production in mixed ruminal batch cultures by adding the fumarate-reducing bacterium *Mitsuokella jalaludinii*. Competition for [H] with methanogenesis was therefore in their experiment controlled by enzyme kinetics, but because propionate production was not affected by addition of *M. jalaludinii* and succinate increased only temporarily, it is difficult to conclude which was the competing [H] sink or sinks. On the other hand, addition of propionibacteria *in vivo* was unsuccessful to decrease CH_4 production or increase propionate molar proportion (Aikman et al., 2011; Vyas et al., 2014a,b, 2015). Vyas et al. (2014a) quantified the added propionibacteria strains throughout the day and concluded that added propionibacteria did not persist long enough in the rumen to cause effects on CH_4 and propionate. It is also possible that enhancement of enzyme kinetics without accumulated H_2 did not result in extra propionate if additional propionate production was not enzyme-, but substrate- or thermodynamically limited. Evaluation of the effect of the addition of a propionate or succinate producer in

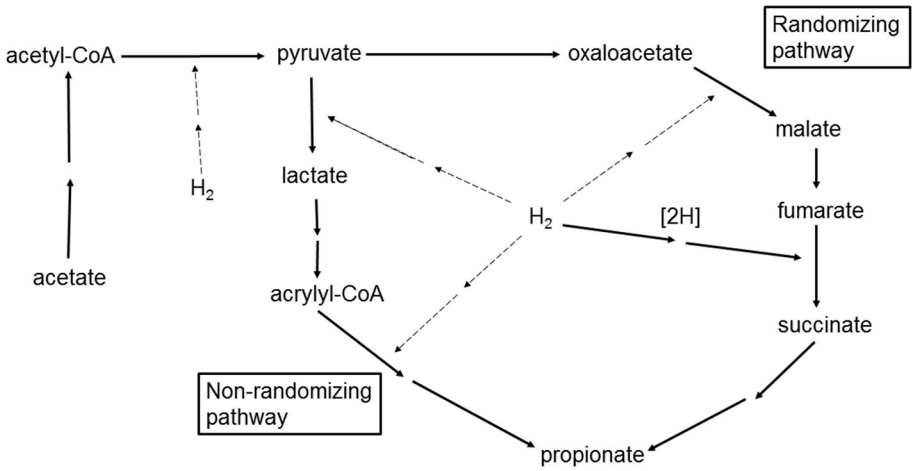

FIGURE 5 | Incorporation of H_2 in the conversion of acetate to propionate through the randomizing and non-randomizing pathways. Two sequential arrows represent multiple reactions condensed for simplicity. Carbon dioxide, water, and HS-CoA are omitted for simplicity. Solid arrows represent known or demonstrated reactions. Dashed arrows represent putative pathways of H_2 incorporation.

methanogenesis-inhibited *in vitro* systems or *in vivo* would allow understanding if and under which conditions incorporation of accumulated H_2 into propionate production can be limited by enzyme kinetics.

Butyrate Production

Overall, there was no re-direction of [H] toward butyrate production when methanogenesis was inhibited in batch or continuous culture (Ungerfeld, 2015). The same as propionate, H_2 incorporation into butyrate production could potentially be limited by insufficient activities of enzymes catalyzing the conversion between carbon compounds or by insufficient activities of hydrogenases to incorporate elevated H_2 (**Figure 6**). A first question is then if the mixed ruminal microbiota can incorporate H_2 into butyrate production. Non-ruminal *Clostridium kluyveri* was shown to use H_2 as [H] donor for the reduction of acetoacetate to ß-hydroxybutyrate in butyrate production (Cohen and Cohen-Bazire, 1950). In ruminal *B. fibrisolvens* D1 (Miller and Jenesel, 1979) and *Megasphaera* (formerly *Peptostreptococcus*) *elsdenii* (Baldwin and Milligan, 1964), the [H] donors for butyrate production were NADH and NADPH. Also in *M. elsdenii,* Whitfield and Mayhew (1974) showed that the reduction of crotonyl-CoA to butyryl-CoA was coupled to the oxidation of D-lactate to pyruvate. In the above described findings in ruminal bacteria, the reductions of acetoacetate to ß-hydroxybutyrate and of crotonyl-CoA to butyryl-CoA would therefore be coupled to intracellular electron transfer reactions rather than to incorporation of H_2. Baldwin and Milligan (1964), however, showed that [H] could be transferred from H_2 to NAD^+ using ferredoxin in *M. elsdenii.*

In a recent comprehensive analysis of the energy metabolism of the genus *Butyrivibrio*, Hackmann and Firkins (2015) reported that 47 out of 62 *Butyrivibrio* spp. genomes possessed genes encoding for all six subunits of *Escherichia coli* 3-type hydrogenase (Ech), a transmembrane ion pump that transfers electrons in reduced ferredoxin to protons to form H_2. A question of interest would be if under elevated H_2, Ech could catalyze the reverse reaction and reduce oxidized ferredoxin using H_2 (**Figure 6**). Interestingly, all 47 *Butyrivibrio* spp. genomes that possessed genes encoding for Ech also had genes encoding for ion pump *Rhodobacter* nitrogen fixation Rnf, which catalyzes the reduction of ferredoxin by NADH (Hackmann and Firkins, 2015). Perhaps, if H_2 could donate electrons to reduce ferredoxin when H_2 accumulation made this reaction thermodynamically favorable, reduced ferredoxin could then reduce NAD^+ in a reaction catalyzed by Rnf. NADH thus generated would be available as a direct [H] donor for butyrate production from acetate, with the net result of [H] indirectly donated by H_2 for butyrate production (Equation 10, **Figure 6**):

$$\begin{aligned} &Fd_{ox} + NAD^+ + 2H_2 \rightarrow Fd^{2-}_{red} + NADH + 3H^+; \\ &\qquad \Delta G^\circ \quad = -21\ \text{kJ} \qquad (8;\ \text{Buckel and Thauer, 2013}) \end{aligned}$$

$$\begin{aligned} &\text{crotonyl} - \text{CoA} + Fd_{ox} + 2NADH \rightarrow \text{butyryl} - \text{CoA} \\ &\quad +2NAD^+ + Fd^{2-}_{red}\ (9;\ \text{Buckel and Thauer, 2013}) \end{aligned}$$

$$\begin{aligned} \text{crotonyl} - \text{CoA} + 2Fd_{ox} + 2H_2 + NADH \rightarrow \text{butyryl} - \text{CoA} \\ + 2Fd^{2-}_{red} + 3H^+ + NAD^+ \qquad (10) \end{aligned}$$

Equation 10 = Equation 8 + Equation 9

It can be estimated that the reduction of ferredoxin and NAD^+ with H_2 as [H] donor (Equation 8; Buckel and Thauer, 2013) may be thermodynamically favorable even in the non-methanogenesis inhibited ruminal fermentation without H_2 accumulation (**Table 2**).

It remains to be studied how gene expression and activity of enzymes involved in the conversion of acetate to butyrate

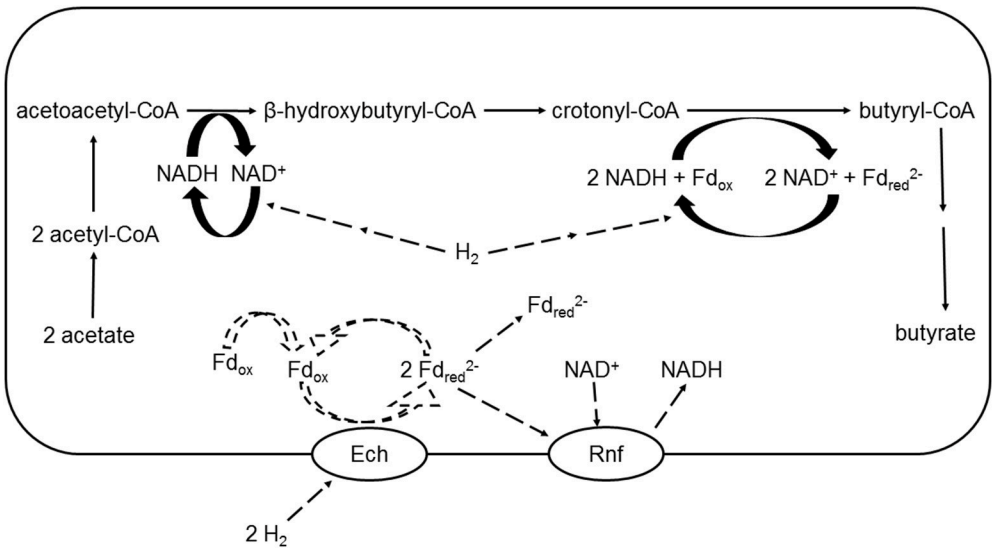

FIGURE 6 | Upper part: putative incorporation of H_2 in the conversion of acetate to butyrate in a ruminal *Butyrivibrio* spp. cell; lower part: putative mechanism of electron transfer from H_2 to NAD^+ as described by Buckel and Thauer (2013) for non-ruminal organisms: $Fd_{ox} + NAD^+ + 2\ H_2 \rightarrow Fd_{red}^{2-} + NADH + 3\ H^+$. Ech, *Escherichia coli* hydrogenase-3-type hydrogenase transmembrane ion pump; Rnf, *Rhodobacter* nitrogen fixation transmembrane ion pump; Fd_{ox}, oxidized ferredoxin; Fd_{red}, reduced ferredoxin; Solid arrows represent known or demonstrated pathways. Dashed arrows represent putative H_2 incorporation and electron transfer reactions. For simplicity, reactions are not balanced for H^+, and extruded cations, water and HS-CoA are omitted. Adapted from Hackmann and Firkins (2015).

TABLE 2 | Estimated Gibbs energy changes for the reduction of oxidized ferredoxin and NAD^+ with dihydrogen (Equation 8).

In vitro system	Methanogenesis	Predicted dissolved H_2 (M)	ΔG (kJ)
Batch	Non-inhibited	2.35×10^{-6}	−60,151
Batch	100% inhibited	1.11×10^{-4}	−77,112
Continuous	Non-inhibited	2.02×10^{-6}	−59,366
Continuous	100% inhibited	2.54×10^{-5}	−69,468

is affected by H_2, but results so far on the response to acetate addition indicate that the operon encoding for enzymes involved in butyrate production in *B. fibrisolvens* (not including butyryl-CoA/acetyl-CoA transferase and crotonase) is constitutively transcribed (Asanuma et al., 2005). If the expression of the operon encoding for enzymes involved in butyrate production does not respond to H_2 (or to the ratio of reduced to oxidized cofactors), this would be an indication that an important group of ruminal butyrate producers did not evolve to utilize elevated H_2 (or perhaps even to incorporate H_2).

Reductive Acetogenesis

Reductive acetogenesis is functional and even predominates over methanogenesis in the gastrointestinal tract of some humans and termites, rodents, pigs, the rumen of newborn lambs (Joblin, 1999) and kangaroos (Godwin et al., 2014). Reductive acetogens inhabit the rumen (Leedle and Greening, 1988; Le Van et al., 1998) and, potentially, could utilize H_2 to reduce CO_2 to acetate as a source of energy and carbon if the process was thermodynamically feasible. However, the fact that the enzyme kinetics capacity to conduct reductive acetogenesis is present in the rumen does not mean that it is non-limiting in the short- or long-term to incorporate atypically high H_2 concentrations. An important question would be if the abundance of native ruminal reductive acetogen populations can be increased by favorable thermodynamic conditions in the long term. In such a case, a long term decrease in H_2 accumulation would be expected. The few *in vivo* results reporting the long-term evolution of accumulated H_2 in methanogenesis-inhibited rumens over time are contradictory, with some long-term decrease in H_2 accumulation (Clapperton, 1974; Hristov et al., 2015) or steady state gaseous H_2 concentration (Kung et al., 2003). Knight et al. (2011) reported a decrease in reductive acetogen numbers in the rumen when CH_4 production was inhibited with chloroform, which was interpreted as a possible consequence of chloroform toxicity. Mitsumori et al. (2014) reported that, although the total number of reductive acetogen sequences was unaffected by methanogenesis inhibition, the phylogeny of reductive acetogens changed. Recently, Denman et al. (2015) found that methanogenesis inhibition in goats resulted in a decrease in the reads of reductive acetogenic enzymes CO dehydrogenase/acetyl-CoA synthase complex and methyltetrahydrofolate:corrinoid/iron-sulfur protein. Their results do not support previous speculation on reductive acetogenesis partially accounting for the observed decrease in [H] recovery in *in vitro* methanogenesis-inhibited ruminal fermentation (Ungerfeld, 2015).

An alternative to stimulation of native populations of reductive acetogens would be the daily or frequent dosing of a

reductive acetogen to rumens also receiving a potent inhibitor of methanogenesis. If the reductive acetogenic microbial additive adapted to the rumen environment and used accumulated H_2 to reduce carbon dioxide to acetate, a decrease in H_2 accumulation would be expected, albeit to a H_2 pressure higher than in the ruminal fermentation with active methanogenesis, due to the higher H_2-threshold of reductive acetogenesis compared with methanogenesis (Thauer et al., 1977; Cord-Ruwisch et al., 1988). That said, reductive acetogens are not obligate hydrogenotrophs and can ferment sugars (Joblin, 1999), therefore, the actual decrease in H_2 would depend both on the extent of mixotrophy of the dosed reductive acetogen and the availability of alternative substrates. This approach would require screening of reductive acetogens with the highest v_{max} and lowest k_m for H_2 and suitable to be used as a microbial additive mixed with the feed. The ideal reductive acetogen additive would be an obligate hydrogenotroph, if that could exist naturally, or developed as a genetically-engineered organism with deleted carbohydrate metabolism genes.

A situation somewhat analogous to methanogenesis inhibition occurs in experiments in which gnotobiotic animals are inoculated with methanogens-free microbial communities. Gnotobiotic lambs inoculated with reductive acetogens had more reductive acetogens than control lambs. Also, batch incubations under H_2 and CO_2 inoculated with methanogen-free ruminal fluid from gnotobiotic lambs produced more acetate than control incubations inoculated with ruminal fluid from conventional lambs (Fonty et al., 2007). This result could indicate that the reductive acetogenic capacity in gnotobiotic lambs had developed enough to contribute to H_2 incorporation. However, because *in vivo* H_2 accumulation was not reported in that study, the extent to what reductive acetogenesis could decrease H_2 accumulation in these methanogens-free rumens is unknown.

Substrate Kinetics

Tatsuoka et al. (2008) and Ebrahimi et al. (2011) could decrease H_2 accumulation in methanogenesis-inhibited ruminal batch cultures by adding fumarate or malate. Whilst the addition of a substrate or a fermentation intermediate might remove or decrease the substrate kinetics limitation of a process, it could at the same time make it thermodynamically feasible. This approach, therefore, does not allow distinguishing between substrate kinetics and thermodynamic control.

End product accumulation does not affect substrate kinetics but decreases thermodynamic feasibility. Therefore, adding a fermentation end product could be a more suitable approach to distinguish between substrate kinetics and thermodynamic controls. One could envision an experiment in which the rate of a process responded positively to the addition of an intermediate. If this response was negated by the addition of an equimolar amount of the corresponding end product, we could conclude that the process was thermodynamically limited before any addition; if the response remained unchanged when adding the fermentation end product along with the intermediate, we would conclude that the process had been under substrate kinetics control before any addition.

In principle, being acetate and CO_2 plentiful in ruminal fermentation, it seems unlikely that the availability of these substrates could limit the incorporation of accumulated H_2 into propionate or butyrate production from acetate, or reductive acetogenesis.

FINAL REMARKS

The present analysis suggests that the incorporation of accumulated H_2 in the methanogenesis-inhibited ruminal fermentation into acetate conversion to propionate and reductive acetogenesis, and perhaps into acetate conversion to butyrate, may have not be thermodynamically limited in most of the *in vitro* experiments analyzed. Limitations of the ΔG calculations presented include the low number of treatment means used in the continuous culture analyses, and the fact that more than half of the treatment means in the batch culture analyses belonged to one study. Also, H_2 equilibrium between aqueous phase and headspace was assumed, and extracellular, rather than intracellular, concentrations of metabolites and ionic strength were used in the calculations. Incomplete knowledge on actual ATP generation in the processes considered also adds uncertainty to this conclusion.

Even if the incorporation of accumulated H_2 into propionate and butyrate production from acetate, and into reductive acetogenesis, was thermodynamically feasible, the capacity to incorporate elevated H_2 resulting from methanogenesis inhibition may be enzyme-limited because of insufficient v_{max} of non-methanogenic H_2-incorporating enzymes to take up H_2 at the rates it is produced, even at the lower H_2 production rate of the methanogenesis-inhibited fermentation. One must also bear in mind that in processes close to equilibrium, the reverse reactions may proceed fast enough so as to decrease the net rate of the forward reaction substantially (Ungerfeld and Kohn, 2006).

The present analysis was conducted based on *in vitro* results, and one aspect of enzyme kinetics in which *in vitro* cultures differ from the rumen is microbial diversity. In *in vitro* cultures, possible lack of enough capacity of non-methanogenic hydrogenotrophs to take advantage of elevated H_2 may be magnified because of lack of adaptation of key organisms incorporating H_2 into alternative pathways, as loss of microbial diversity has been reported for both batch (Weimer et al., 2011) and continuous (Johnson et al., 2009) cultures. That said, and as discussed, inhibition of methanogenesis *in vivo* has also consistently resulted in long-term H_2 accumulation (Trei et al., 1971; Kung et al., 2003; Mitsumori et al., 2012; Hristov et al., 2015).

It may be that ruminal microbiota can shift and adapt to dietary changes, and even unphysiological changes such as ruminal contents exchange between animals, to maintain a similar fermentation pattern (Weimer et al., 2010), but perhaps not to incorporate all [H] spared from methanogenesis into VFA production. If some of the H_2-incorporating processes analyzed have not reached thermodynamic equilibrium, it could be that ruminal hydrogenases that incorporate H_2 into processes such as propionate (and perhaps butyrate) production, and reductive

acetogenesis, have evolved toward high affinity to compete for H_2 traces, but at the same time toward low v_{max}, in the typically low-H_2 ruminal environment. If the ruminal microbiota has not evolved to respond to methanogenesis inhibition by incorporating H_2 at high rates, there might be a niche for high-rate H_2–incorporating microbial additives to engineer fermentation toward desired products when CH_4 production is inhibited. If the limitation is thermodynamic, there would be no responses to exogenously added microorganisms.

A metabolomics approach could give insights on the physicochemical control of the dynamics of H_2 incorporation in the rumen, given that *in vivo* studies on methanogenesis inhibition have often measured VFA concentrations, but not actual flows of VFA production. Transcriptomic and proteomic analyses of functional genes involved in fermentation pathways can be valuable approaches to gain insights on enzyme kinetics beyond what has been found so far using genomics, but the ultimate answers about enzyme kinetics control would be provided by enzyme activity assays. *In vitro* and *in vivo* experimental work on understanding the physicochemical control of H_2 incorporation in ruminal fermentation is recommended.

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SUPPLEMENTARY MATERIAL

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Metagenomic analysis of the rumen microbial community following inhibition of methane formation by a halogenated methane analog

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Japanese goats fed a diet of 50% Timothy grass and 50% concentrate with increasing levels of the anti-methanogenic compound, bromochloromethane (BCM) were investigated with respect to the microbial population and functional shifts in the rumen. Microbial ecology methods identified species that exhibited positive and negative responses to the increasing levels of BCM. The methane-inhibited rumen appeared to adapt to the higher H_2 levels by shifting fermentation to propionate which was mediated by an increase in the population of H_2-consuming *Prevotella* and *Selenomonas* spp. Metagenomic analysis of propionate production pathways was dominated by genomic content from these species. Reductive acetogenic marker gene libraries and metagenomics analysis indicate that reductive acetogenic species do not play a major role in the BCM treated rumen.

Keywords: rumen, methane, H_2, microbial community, metagenomics

Introduction

Enteric fermentation in ruminants generates methane, which can represent an energy loss of between 2–12% for the animal (Johnson and Johnson, 1995) and significantly contributes to greenhouse gas emissions (Hristov et al., 2013). Significant efforts are being made to develop nutritional strategies, based on the use of antimethanogenic compounds, to reduce methane production in ruminants (Benchaar and Greathead, 2011). In this metabolic process, H_2 obtained from the conversion of feedstuffs into various fermentation end products is mainly consumed by methanogens to produce methane in the rumen (Janssen, 2010). Therefore, H_2 produced in the rumen has to be considered when developing strategies to control ruminant methane emissions since H_2 accumulation can impair digestion and fermentation if it accumulates (Wolin et al., 1997). Furthermore, redirecting rumen H_2 production toward alternative energy yielding pathways could improve efficiency of energy utilization from feed in ruminants.

Ungerfeld and Kohn (2006) provided an excellent overview of the thermodynamics of ruminal fermentation and identified several strategies for utilizing H_2 in the rumen as an alternative to methanogenesis. Fumarate and malate have been used by several investigators to stimulate succinate/propionate producers in the rumen which compete with methanogens for H_2. Many of these organisms use the succinate–propionate (randomizing) pathway as a major route for

propionate synthesis in the rumen (Baldwin et al., 1963). In this pathway, malate is dehydrated to fumarate, and the reduction of fumarate to succinate is coupled to ATP synthesis. Succinate is either an intermediate or an end-product in the pathway of different rumen bacteria. However, several researchers have shown in mixed ruminal cultures that fumarate (and malate) is converted to propionate and acetate in varying proportions (Ungerfeld et al., 2007). The relative amounts of propionate and acetate formed from fumarate will impact on the H_2 pool available to methanogens. Stoichiometrically, propionate production from fumarate consumes one pair of reducing equivalents while acetate production from fumarate releases two pairs of reducing equivalents. Therefore, the production of acetate from fumarate is counterproductive when the objective is to reduce H_2 available for methane production. It is important therefore to identify the microorganisms involved in these pathways and determine the physiological and biochemical conditions which favor propionate rather than acetate production from fumarate.

Bromochloromethane-cyclodextrin (BCM-CD) has been used in numerous studies to reduce methane production in ruminants and it is considered one of the more effective methane inhibitors (Denman et al., 2007; Goel et al., 2009; Tomkins et al., 2009; Abecia et al., 2012; Mitsumori et al., 2012). The responses to the BCM-CD of rumen fermentation, methane production, H_2 flux and microbial abundances have been previously published (Mitsumori et al., 2012), showing a dose-dependent inhibitory effect on methane production and an increase in H_2 production, with no detrimental effects on rumen fermentation. In line with other studies, there was an observed fermentation shift toward more propionic production, which could be due to a reduction in competition for H_2 in the rumen. Real-time PCR quantification of microbial groups showed a decrease in abundance of methanogens and fungi, whereas there were an increase in *Prevotella* spp. and *Fibrobacter succinogenes* (Mitsumori et al., 2012). It was concluded that the methane-inhibited rumen adapted to the higher H_2 levels by shifting fermentation to propionate via *Prevotella* spp., but the majority of metabolic H_2 was expelled as H_2 gas (Mitsumori et al., 2012). However, the effect on microbial populations has not been studied in depth, particularly in those microorganisms that play an important role in hydrogenotrophy pathways when methanogenesis has been impeded in the rumen. New molecular techniques, such as next-generation sequencing (NGS), have been adopted to study rumen microbiology, providing a higher resolution observation of the rumen microbial populations with respect to metabolic activity, abundance and facilitating the analysis of an increased volume of data (Callaway et al., 2010; Hess et al., 2011; Lee et al., 2012; Pope et al., 2012; Ross et al., 2012; St-Pierre and Wright, 2013).

Therefore, we have studied the effect of different levels of the antimethanogenic compound, BCM-CD, on the rumen microbial community in goats using metagenomic sequence analysis. The aim of the present study was to identify and characterize the microbiology and genetics underpinning the alternative hydrogenotrophic pathways in ruminants, using metagenomics.

Materials and Methods

Animals and Experimental Design

Three ruminally fistulated Japanese native goats (*Capra aegagrus hircus*, female) 35.7 ± 4.85 kg were treated with three levels of the antimethanogenic compound BCM.

The experimental design, treatments and fermentation parameters have been described by Mitsumori et al. (2012). Animals were initially adapted to the basal diet for 14 days and measured as a control period. Within the sampling period, goats were placed into the respiration chambers for a period of 3 days for analysis of rumen gas production. Animals were then adapted to the increasing doses of BCM for 8 days before sampling in the respiration chambers for a further 3 days. Rumen samples were collected at the end of each respiration-sampling period (3 days) for DNA extractions. The animal experiments were carried out in accordance with a protocol approved by the Guide for the Care and Use of Experimental Animals (Animal Care Committee, NILGS).

DNA Extractions

DNA extractions were performed on rumen samples collected from the goats using the FastDNA kit and FastPrep instrument (MP Biomedicals, Cleveland, OH, USA; Mitsumori et al., 2012).

Functional Gene Analysis

Functional gene libraries were constructed from DNA samples collected from the control and high BCM dosing periods. One, targeting methanogenesis using the methyl coenzyme A reductase (mcrA), using mcrA forward primer 5′-GG TGGTGTMGGATTCACACARTAYGCWACAGC and mcrA reverse primer 5′-TTCATTGCRTAGTTWGGRTAGTT (Luton et al., 2002). Two libraries targeting reductive acetogenesis genes: the formyl tetrahydrofolate synthase (FTHFS), using the FTHFS forward primer 5′-TTYACWGGHGAYTTCCATG C-3′; and FTFHS reverse 5′-GTATTGDGTYTTRGCCATACA-3′ (Leaphart and Lovell, 2001) and acetyl CoA synthetase (ACS), using the ACS forward primer 5′-CTBTGYGGDGCIGTIWSMT GG and ACS reverse 5′-AARCAWCCRCADGADGTCATIGG (Gagen et al., 2010). Amplified PCR products were gel extracted from a 1.5% agarose gel and cloned using the pGEM-Teasy vector system (Promega). Transformed *Escherichia coli* cells harboring the cloned products were selected and sequenced using BigDye sequencing reagents (ABI). Sequence data was analyzed and placed in phylogenetic trees in the ARB software environment (Ludwig et al., 2004) and clustering to a defined operational taxonomic unit (OTU) analysis performed using MOTHUR (Schloss et al., 2009).

16S rDNA Analysis

16S rRNA gene pyrotagging was performed using modified universal bacterial primers (27f and 515r; Lane, 1991; Felske et al., 1997). Specific sequences matching the Roche 454 sequencing adaptor B were added to the 27f primer, while adaptor A was added to the 515r. In addition between the

adaptor A sequence and the 16S 515r sequence a 10 bp barcode was inserted. Each individual DNA sample was amplified using the 27f primer and a uniquely barcoded 515r primer. After, amplification products were visualized by performing gel electrophoresis. Product quantities were calculated and an equal molar amount of each product was pooled. The pooled products were run in a 1.5% agarose gel and the product gel extracted and purified prior to submission for 454 pyrosequencing.

Short read sequence data generated using 454 sequencing was analyzed using the QIIME: Quantitative Insights Into Microbial Ecology software package (Caporaso et al., 2010). Raw sequences were passed through Acacia for 454 error correcting (Bragg et al., 2012). Error corrected sequences were then de-multiplexed in QIIME based on their unique barcode, clustering of sequences to OTUs of 97% similarity were performed using uclust (Edgar, 2010). Chimeric sequences were identified using chimera slayer (Haas et al., 2011) and removed. Taxonomic assignment of sequences was performed against the Greengenes database (McDonald et al., 2012) using the RDP classifier software (Wang et al., 2007).

Additional analysis of OTU's was performed in the R packages ade4 and Phyloseq (Chessel et al., 2004; McMurdie and Holmes, 2013). The sequences obtained in this paper have been deposited in the European Nucleotide Archive (ENA) under the accession number PRJEB10560.

Meta-genomic Analysis

Metagenomic assessment of the goat microbiome from the control and high BCM dosing using 454 pyro-sequencing was undertaken. DNA extracted from the three goats for control and high BCM samples were pooled based on treatment, control and BCM respectively and nebulized and 454 adapter fragments were added. One half plate of sequencing was performed on each library, data generated from the 454 sequencing run was initially passed through CD-HIT for de-replication of the 454 data (Niu et al., 2010). De-replicated reads were analyzed for the occurrence of ribosomal DNA reads using hidden Markov models implemented in the software package hmm_rRNA (Huang et al., 2009). Phylogenetic analysis of metagenomics samples for the comparison of community structures was performed using PhyloSift (Darling et al., 2014). Normalization of read data for functional abundance profiling was performed by calculating reads per kb per genome equivalent after accounting for the estimated average genome size of the microbial communities in each sample using MicrobeCensus (Nayfach and Pollard, 2014). Assembly of metagenomic sequences into larger contiguous sequences (contigs) was implemented with Newbler (Roche ver. 2.6). Contigs and orphaned reads were annotated using the MG-RAST server (Meyer et al., 2008), phylogenetic placement of reads and contigs was performed using both PhyloPythiaS (Patil et al., 2012) and RAIphy (Nalbantoglu et al., 2011). Sequence data has been deposited at MGRAST under project number 14718 (MG-RAST ID for BCM sample 4452826.3 and Control 4452824.3).

Results

16S rDNA "Pyrotag" Monitoring of Microbial Populations

As previously described, a dose mediated response was observed for goats that were administered increasing concentrations of the anti-methanogenic compound BCM (Mitsumori et al., 2012). With increasing concentrations of BCM, a decrease in the measured methane output was detected with an increase in H_2 release and fermentation shifts toward propionate production. Preliminary analysis of the rumen microbial populations using denaturing gradient gel electrophoresis (DGGE) and quantitative PCR (qPCR) found changes in fibrolytic species and an increases in certain *Prevotella* OTUs (Mitsumori et al., 2012).

An extensive analysis of the rumen microbiota showed that total microbial species richness of the goat rumen microbiome was not impacted with the administration of the low and mid doses of BCM. With no significant changes to alpha diversity [Shannon 6.56 $\pm$ 0.15, 6.49 $\pm$ 0.18, 6.57 $\pm$ 0.10 for Control, low and mid dose, respectively (mean $\pm$ SEM)]. While the highest dose of BCM caused a contraction in observed and estimated species richness (Shannon 6.10 $\pm$ 0.22) with an ~14–20% decrease, respectively (**Figure 1**). The structure of the microbiomes as assessed by beta diversity measures (unifrac) clearly showed alterations between the control, low and mid, high dose groups, with 28% of the variance being explained between the control and highest dose of BCM (**Figure 2**). Variation in the rumen microbiome between animals explained the next level of variance with one animal possessing a divergent microbial population compared to the other two animals (**Figure 2**). The variance between the animals was less at the highest dose of BCM compared to all other treatments. Co-inertia analysis applied to the microbiome and biochemical measures of the rumen samples placed the high BCM microbiomes on the axis coupled with increasing BCM, propionate and H_2 with a concurrent decrease in methane, while the control and low BCM dose were on the opposite axis (**Figure 2**). The goat rumen microbiome for the control diet was dominated by OTUs assigned to the Bacteroidetes and Firmicutes phylum (60 and 24%, respectively; Supplementary Figure S1). Both the Synergistetes and Lentisphaerae phylum contributed ~4% each to the microbiome of control animals. An increase in the proportion of sequences assigned to the Bacteroidetes phylum was observed for the increasing doses of BCM with a concomitant decrease in Firmicutes, Synergistetes, and Lentisphaerae. Seventy eight OTUs were positively correlated ($r > 0.6$, $p < 0.05$) to the increased concentration of BCM administered to the animal, accounting for 27.88% of the sequence data at the high BCM samples with 52 of these assigned to the *Prevotella* genus level (Supplementary Figure S2). The OTUs that corresponded to the previously described *Prevotella* groups 1 and 7 from DGGE analysis (Mitsumori et al., 2012) were identified and were positively correlated with the increase in BCM concentration $r = 0.997$ and 0.999 respectively. The observed fold change for the sequence data was generally consistent with that of the qPCR data as previously

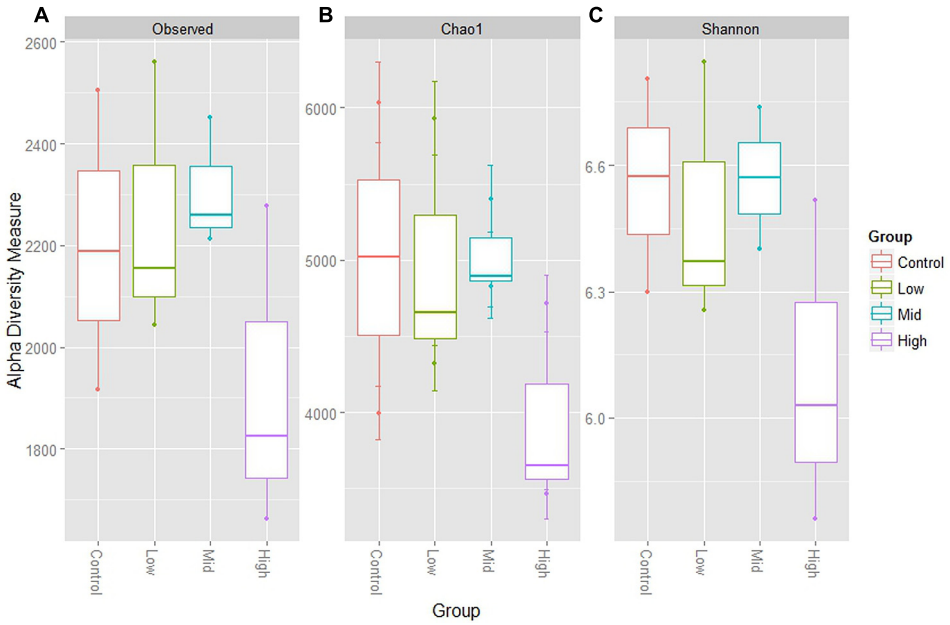

FIGURE 1 | Alpha diversity measures for goat rumen microbiomes at control, low, mid, and high doses of BCM. (A) Shows total observed taxonomic units, **(B)** the Chao1 estimates and, **(C)** the Shannon diversity index. Boxplots indicate the first and third quartiles with the median value indicated as a horizontal line the whickers extend to 1.5 times the inter quartile range.

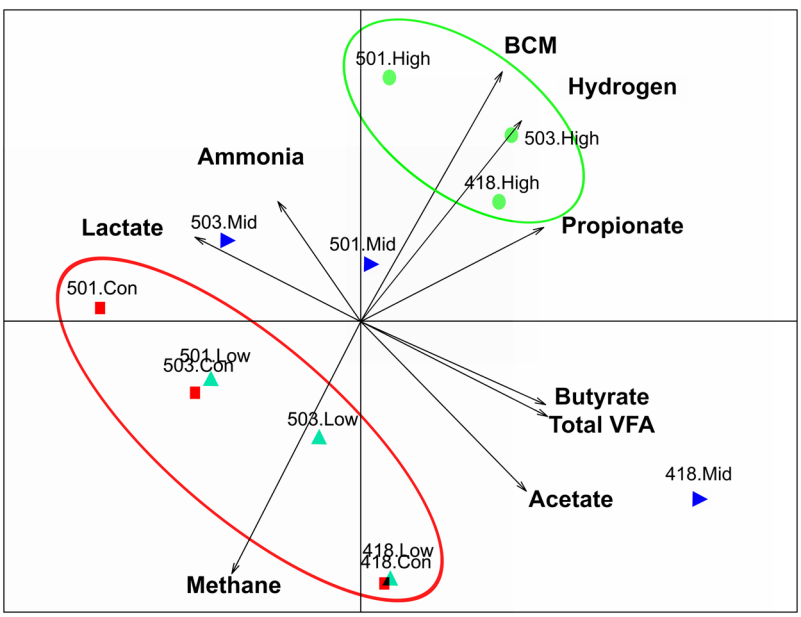

FIGURE 2 | Principal coordinate analysis co-inertia plot coupling rumen microbiome Euclidean analysis and biochemical measures, with arrows indicating increasing values of biochemical measures. Rumen microbiomes for animals on varying levels of BCM for control (red square), low dose (turquoise triangle), mid dose (blue right triangle), high dose (green circle), labels indicate animal number and treatment.

reported for these two *Prevotella* groups (Mitsumori et al., 2012). Sequence abundance data found that these species were observed on average at 6.5 and 9.4% of the species sequenced respectively for the high BCM samples. Nine OTUs of lower abundance from the family Veillonellaceae were positively associated with increasing BCM concentrations (r = 0.61–0.79), of which six were further characterized to belong to the genus *Selenomonas*. These OTUs accounted for 0.78% of the sequence data in the high BCM samples. A single OTU accounting for 0.9% of the sequence data in the high BCM

samples was associated with the genus *Succiniclasticum* and was moderately associated with the change in BCM levels ($r = 0.52$). Sequence data classified to OTUs closely associated to the fiber degrading species *F. succinogenes*, although observed to significantly increase at the mid and high dose of BCM, were only moderately correlated with a dose response of BCM ($r = 0.30$–0.59).

The increasing level of BCM in the rumen was negatively associated ($r < -0.6$, $p < 0.05$) with 27 OTUs of which six were assigned to the TG5 genus of the phylum Synergistetes (1.49% abundance of control sample), all of which were not observed at the highest BCM level (Supplementary Figure S2). A further five from the BS11 family from the Bacteroidetes phylum and three associated with Victivallaceae family from the Lentisphaerae phylum were also not detected at the high BCM levels.

Hydrogenotrophic Functional Gene Analysis

Gene libraries encoding functional genes associated with methanogenesis (methyl coenzyme-M reductase, mcrA, Supplementary Figure S3) and reductive acetogenesis (FTHFS, Supplementary Figure S4; ACS, Supplementary Figure S5) were constructed from control and high BCM samples.

Diversity of methanogens from the control goat rumen were dominated by members of the Methanobacteriales family. The administration of BCM produced a marked decrease in the methanogen diversity compared to the control period with only 27% of the sequence data shared between the two samples. The predominant OTU in the control and high BCM were different, however the dominant species in both were found to be affiliated with *Methanobrevibacter* species.

Investigations related to the diversity of the FTHFS amino acid sequences from the *fhs* gene for the control and high BCM samples showed a similar species richness for the libraries, however, the populations that make up the libraries were significantly altered between samples (libshuff $p < 0.001$). Sequences with a homoacetogen similarity score (HSS) >80 were found to lie in the acetogenic bacteria clustering of the FTHFS phylogenetic tree. From this region the predominant FTHFS OTU's for the control sample were positioned close to *Ruminococcus obeum,* while for BCM the most predominate OTU grouped closest to *Clostridium magnum*. Four OTU's comprising 43% of the sequences in the BCM sample were associated with the mixotrophic acetogen *Sporomusa* spp., while only a single representative from the control library was observed in this group.

Acetyl CoA synthase libraries were also generated using recently designed primers that exclude sulfate reducing bacteria and archaeal *acsB* genes (Gagen et al., 2010). A greater diversity of sequences was observed from the control samples and the composition of the BCM sample tended to be a subset of the control sample (libshuff $p = 0.33$), but the control sample was different from the high BCM due to its increased diversity (libshuff $p < 0.003$). The most abundant OTU was the same for both samples being associated closest to *Acetitomaculum ruminis,* while, the rank abundance of the remaining OTU's was different between the samples.

Metagenomic Data Analysis of Goat Microbiome

Genomic DNA from all animals was pooled together based on sampling period and subjected to 454 titanium shot gun sequencing for the control and high BCM samples. Replicated sequences due to construction of the 454 sequencing libraries were identified and accounted for 19% of the control and 22% of the high BCM metagenomic datasets. Assembly of metagenomic reads produced over 6500 contigs greater than 500 bp for the control sample, while in excess of 9000 contigs were generated for the High BCM metagenome. The largest assembled contig for the BCM library was close to 40 kb in length, while the largest contig for the control was just over 6.4 kb. Annotation of the 40 kb high BCM contig identified the coding sequences belonging to previously described genes from *Prevotella* species, while the largest contig for the control sample could only be accurately classified to the Firmicutes phylum level.

Hidden Markov models were used to identify sequences containing 16S rDNA data, resulting in 435 and 700 sequences for the control and high BCM samples, respectively. Clustering of the metagenomic 16S sequences in association with the 16S amplicon data to a Greengenes 97% reference set captured 402 and 619 of the metagenomic reads for the control and high BCM samples. Similar to the amplicon data, the control and high BCM samples were divergent in their composition (**Figure 3**). The control metagenomic 16S rDNA data, although separated from the high BCM meta 16S rDNA data did not group closely

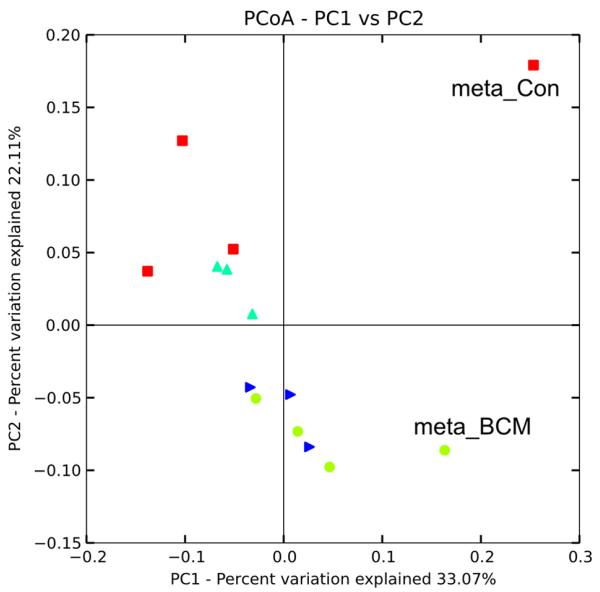

FIGURE 3 | Weighted Unifrac diversity principal coordinate analysis of 16S rDNA amplicon generated rumen microbiomes and 16S rDNA sequences extracted from metagenomic sequencing for animals on varying levels of BCM for control (red square), low dose (turquoise triangle), mid dose (blue right triangle), high dose (green circle). Point's labeled meta_con and meta_BCM indicate 16S rDNA sequences obtained from metagenomic sequencing of pooled animal samples for the control and high BCM period, respectively.

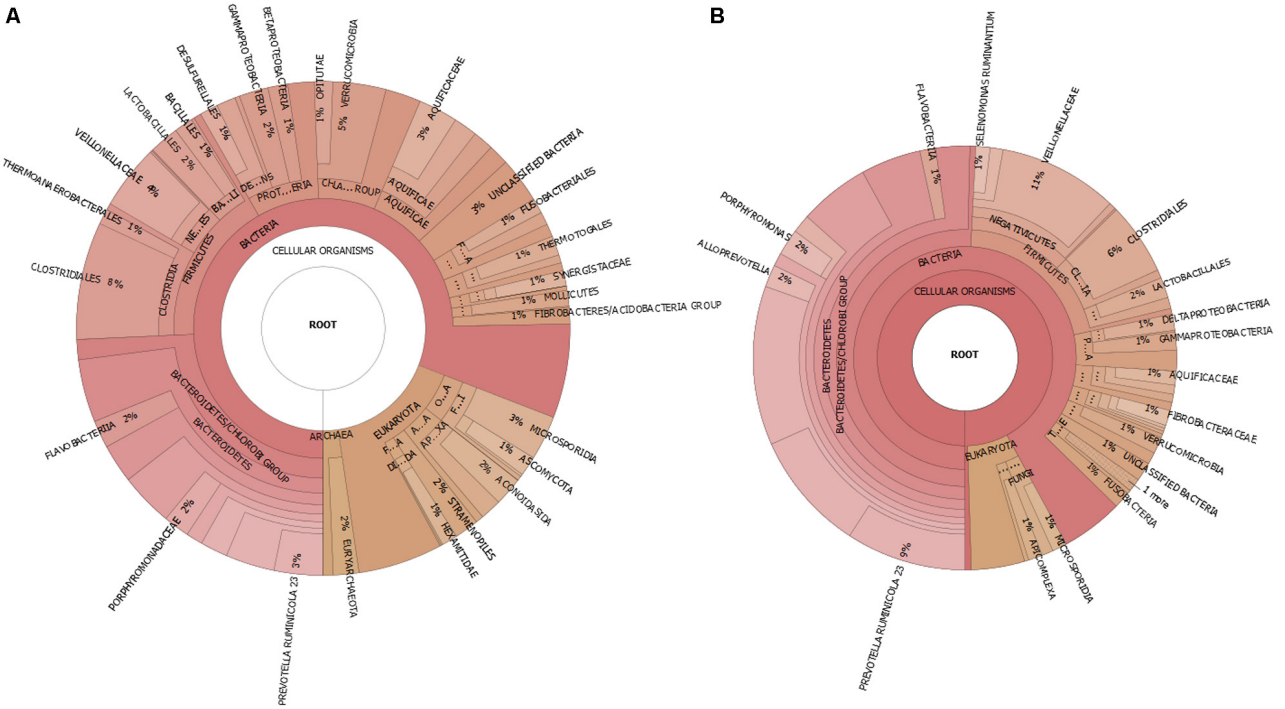

FIGURE 4 | Taxonomic visualization of goat rumen microbiome for (A) control and (B) high BCM metagenomics sequence data.

with the control 16S rDNA amplicon data, while the high BCM meta 16S rDNA data was more closely related to the high BCM amplicon data (**Figure 3**). The rank abundance of phyla for the control were in agreement for the most abundant populations, those being from the Bacteroidetes and Firmicutes, however, the 16S rDNA sequences from the metagenomic data also detected high levels of Verrucomicrobia and Actinobacteria which where only observed as a minor proportion of the amplicon sequences. The high BCM samples were in closer agreement with each other and had a similar rank abundance, but again the amplicon data only detected a small contribution of Verrucomicrobia compared to the metagenomic 16S rDNA data (Supplementary Figure S6).

Further analysis of the microbial community using PhyloSift based on the single-copy 37 "elite" gene families (Darling et al., 2014), also concluded a similar rank abundance and variance for the major phyla between the control and high BCM samples (**Figure 4**). Although the absolute values were not similar, the changes for the abundance of the phyla due to BCM was consistent with increases in Bacteroidetes and decreases in Proteobacteria and no real change to the Firmicutes. Archaeal sequences were only identified within the control metagenomic sample, as were sequences associated with Synergistetes and Tenericutes phyla. These were found to be reduced in abundance in the high BCM amplicon datasets.

Phylogentic classification of the assembled and unassembled reads using PhyloPythiaS assigned ∼52% of the data to the phylum level or lower. RAIphy classification assigned 96–94% of the reads to the phylum level or below for the high BCM and control data respectively. The observed number of phyla was greater in the metagenomic binning methods than for the 16S rDNA and PhyloSift approaches and included reads assigned to phyla that are not expected to be present in the rumen environment. These were predominantly thermophilic phyla and only represented a small proportion of the taxonomic assignment. For both metagenomic samples there was a high proportion of reads assigned to the Bacteroidetes and Firmicutes as was observed for the 16S rDNA and PhyloSift analysis.

Annotation and functional assignment of coding genes was performed at the MG-RAST server (http://metagenomics.anl.gov/). Approximately 380,000 and 350,000 protein coding regions were identified for the control and high BCM samples of which 36.5 and 46.5% could be assigned a known function. Enzymes involved in the biochemical pathway for the production of propionate in the rumen utilizing the randomizing (succinate) pathway were identified within the metagenomic data (**Figure 5** and Supplementary Figure S7). There was an absence of lactyl-CoA dehydratase and acryloyl-CoA-reducatse reads for the non-randomizing (acrylate) pathway from either metagenomic dataset. The abundance of reads associated with the enzymes of the randomizing pathway from pyruvate, was increased approximately twofold on average for the high BCM rumen compared to the control sample. Representatives from six families contained the entire pathway for the assignment of the genes involved in the decarboxylation of succinate to propionate: *Chlorobiaceae, Desulfovibrionaceae, Fibrobacteraceae, Hyphomicrobiaceae,*

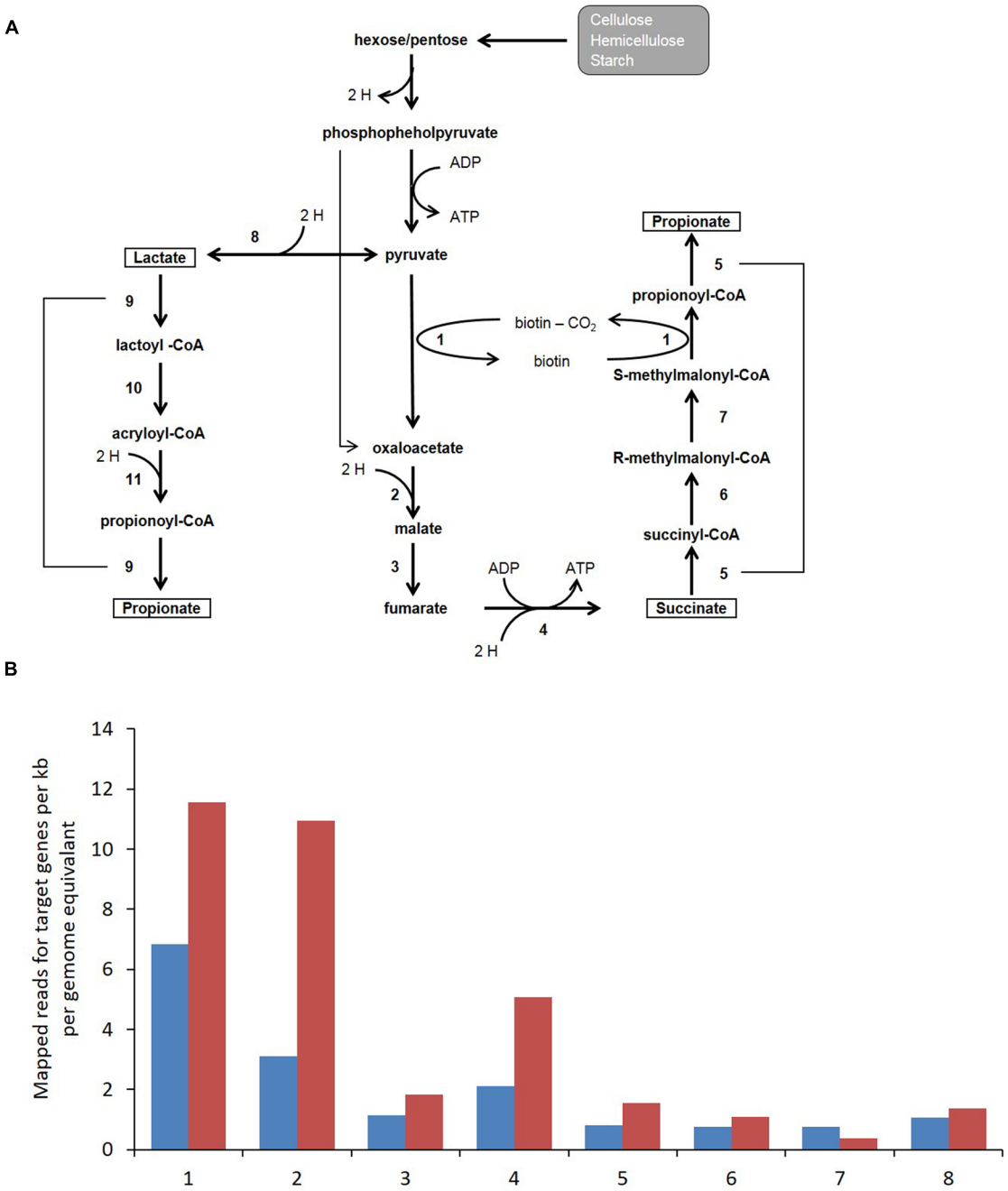

FIGURE 5 | (A) Microbial fermentation pathway for propionate production which consumes H_2 via the randomizing (succinate) or non-randomizing (acrylate) pathways. Numbers refer to enzymatic conversion catalyzed by 1, transcarboxylase (pyruvate carboxylase, methylmalonyl-CoA decarboxylase or methylmaloynyl-CoA carboxytransferase); 2, malate dehydrogenase; 3, fumarase; 4, fumarate reductase; 5/9, propionyl-CoA transferase; 6, methylmalonyl-CoA mutase; 7, methylmalonyl-CoA epimerase; 8, lactate dehydrogenase; 10, lactoyl-CoA dehydratase; 11, acryloyl-CoA reductase. **(B)** Bar charts indicates metagenomic reads assigned to a given target gene per kb per genome equivalent for control (blue) and High BCM (red). Numbers on the x axis correspond to the same enzymatic steps as indicated in **(A)**.

Porphyromonadaceae, Prevotellaceae. The *Prevotellaceae* group contained the highest representation of genes for the propionate pathway. A further ten families contributing at a lower abundance were identified to contain all but one enzyme for the succinate to propionate pathway and included *Acidobacteriaceae, Bacteroidaceae, Bifidobacteriaceae, Clostridiaceae, Coriobacteriaceae, Corynebacteriaceae, Geobacteraceae, Rikenellaceae,* and *Veillonellaceae*. Reads assigned to *Propionibacteriaceae* showed an incomplete pathway with no hits to either fumarase or methylmalonyl-CoA

epimerase genes and were only observed in the BCM sample at low abundance.

Enzymes involved in the reductive acetogenic pathway that possibly provide the best evidence for reductive acetogenic bacteria including the carbon monoxide dehydrogenase/Acetyl CoA synthase (CODH/ACS), corrinoid/Fe–S:methyl-transferase and a corrinoid/Fe–S protein were identified. Only limited sequence data was mapped for these key enzymes from both metagenomic libraries emphasizing the relatively low abundance of bacteria capable of reductive acetogenesis (**Figure 6**). All genes were assigned to the clostridiales class of bacteria. The data, although limited, shows a reduction in the identification of these key enzymes in the BCM treated rumen.

Discussion

The administration of BCM-CD at 5 g/100 kg live weight lead to a 91% reduction in methane production with a concurrent increase in ruminal H_2 levels with no apparent negative effect on dry mater intake (Mitsumori et al., 2012). Initial investigations into the microbial population's shifts due to the introduction of BCM identified reductions in the methanogen population, shifts in fibrolytic populations and the increase in *Prevotella* spp. (Mitsumori et al., 2012). Further analysis presented here, using extensive sequencing of the rumen microbiome through targeted 16S rDNA amplicons and metagenomic sequencing were in agreement with these previous results and provide greater insight into the complexity of the microbiome shifts.

The increasing concentration of BCM administered to goats altered the microbial diversity through a reduction in the richness and overall diversity of the bacterial species observed. At the highest level of BCM the rumen microbiomes showed the least divergence. The alteration to the rumen environment with increased levels of H_2 affected the various goat rumens to an equivalent extent, thus overcoming the original animal to animal variance observed in the control and lower doses

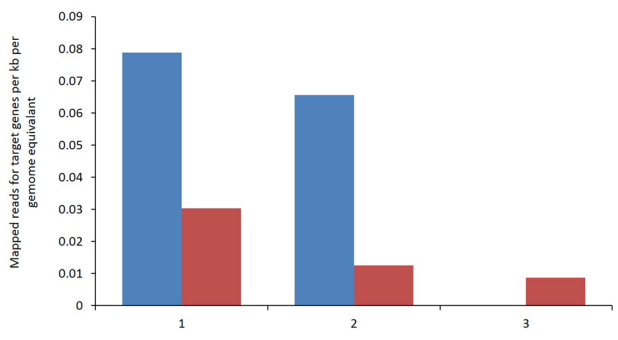

FIGURE 6 | Metagenomic reads assigned to a given target gene per kb per genome equivalent for control (blue) and High BCM (red). (1) CO dehydrogenase/acetyl-CoA synthase complex, (2) methyltetrahydrofolate:corrinoid/iron–sulfur protein and (3) corrinoid/iron–sulfur protein.

of BCM. Animal 418 exhibited a higher level of microbial richness for alpha diversity measures and was more divergent from the other two animals as measured through beta diversity metrics (unifrac analysis), but this was reduced at the highest level.

Many bacterial OTUs were positively associated with the increasing concentration of BCM in the rumen, with the most dominant species being two *Prevotella* spp. that were previously identified through DGGE and qPCR analysis to be closely related to *Prevotella ruminicola* (Mitsumori et al., 2012). They also exhibited some of the highest correlation values for the increasing BCM concentration, indicating a true dose response. The increase in these OTUs was primarily responsible for the overall decrease in alpha diversity observed in the high BCM animals. In addition seven OTUs classified in the *Selenomonas* genus were also positively associated with the increase in BCM. Both *P. ruminocola* and *Selenomonas ruminatium* are known propionate producers (Paynter and Elsden, 1970; Strobel, 1992). Co-culturing of *S. ruminatium* with the fiber degrading succinate producer *F. succinogenes* on cellulose illustrated how interspecies interactions are possible in the rumen with the non-cellulolytic *S. ruminantium* decarboxylation of succinate to propionate (Scheifinger and Wolin, 1973). In line with the observed increase in *S. ruminantium* an increase in *F. succinogenes* was also noted in the 16S rDNA sequence data and quantified previously with qPCR. *F. succinogenes* is not negatively affected by increases in H_2 concentration like the *Ruminococcus* species (Wolin et al., 1997). Interestingly, both the qPCR data and the sequencing data show the highest level for *F. succinogenes* at the mid BCM dose with a slight reduction at the highest BCM level. This plateau in abundance for *F. succinogenes* explains the moderate association to the changing concentration of BCM.

Bacteria that were negatively correlated by the increase in BCM and subsequent increases in H_2 concentration within the rumen were dominated by species from two groups; those from the Dethiosulfovibrionacaea family within the Synergistes phylum and those from the Victivallaceae family from the Lentisphaerae phylum. *Victivallis* spp. OTUs were found to be distantly related to *Victivallis vadensis*, a human fecal isolate that can grow on cellobiose or glucose producing acetate, ethanol, H_2 and bicarbonate. However, like most H_2 producers, when grown syntrophically with a methanogen species, *V. vandensis* converted glucose exclusively to acetate and H_2 (Zoetendal et al., 2003). A feature similar was observed for the fibrolytic species *Ruminococcus flavefaciens* (Wolin et al., 1997). Likewise, studies of members of the Dethiosulfovibrionacaea family revealed stimulation of growth through removal of high H_2 concentrations (Surkov et al., 2001). These groups of bacteria would seem to be consistently down regulated due to the higher H_2 concentrations being generated in the BCM treated rumens.

Metagenomic sequencing of the control and high BCM goat rumen microbial communities provided evidence of a reduction in reads assigned to archaeal genomes in the BCM sample and supports the previous findings of the reduction of measured methane and methanogen numbers (Mitsumori et al., 2012). In addition a decrease in eukaryotic reads associated with fungal

and protozoal genomes in the BCM sample likely reflects the negative impact of the higher H_2 concentrations in the treated rumen (Bauchop and Mountfort, 1981; Ushida and Jouany, 1996). Increased H_2 concentrations are known to alter the fermentative end products of H_2 producing bacteria, fungi, and protozoa toward less energy yielding reduced compounds like lactate, ethanol, and butanol (Wolin, 1974). The presence of methanogen species allows for the reduction of the H_2 partial pressure and the favorable conditions for the reoxidation of nicotinamide adenine dinucleotide (NADH) and redirection of the reducing equivalents toward more energy yielding products like acetate for the fermentative species (Wolin, 1974; Bauchop and Mountfort, 1981; Hillman et al., 1988; Teunissen et al., 1992; Ushida et al., 1997; Wolin et al., 1997). Although previous observations of these rumen samples confirmed a reduction in fungal numbers through qPCR, the protozoal numbers as determined by microscopic counts were not different (Mitsumori et al., 2012). As the current genomic databases are limited for representatives from rumen protozoal and anaerobic fungal this is likely to cause inaccuracies in these assignments. Further work to quantify the protozoal numbers using a qPCR assay should be undertaken to confirm this reduction in their contributions to the rumen microbiome. The reduction in eukaryotic reads lead to a concurrent increase in reads being assigned to bacterial origin and reflects the constraining of the diversity due to the administration of BCM. Moreover a shift in the GC% for the sequences was observed in the BCM sample with an increase in reads with a GC% range of 40–70%. This would be in agreement with the observed increase in Bacteroidetes numbers, especially *Prevotella* groups in the 16S OTU data. Most phylum level classification of the metagenomics reads was also in agreement with the OTU data for the samples not only in abundance levels, but similar in the changes to the percentage of the data set represented between the samples.

Phylogenetic binning and functional assignment of metagenomics reads resulted in two major genera, *Prevotella* and *Selenomonas,* being confidentially assigned all enzymes involved in the randomizing succinate pathway. Both of these groups were also assigned lactate dehydrogenase activity, which converts pyruvate to lactate for use in the non-randomizing acrylate pathway (Prins and Van Der Meer, 1976). Neither lactyl-CoA dehydratase or acryloyl-CoA reductase were found in either of the metagenomic datasets, indicating that the non-randomizing acrylate pathway is not the major route for the production of propionate in the rumen or at least not contained within the abundant bacterial groups. As lactate dehydrogenase is a reversible reaction, it is capable of converting lactate to pyruvate for use in the randomizing succinate pathway (Paynter and Elsden, 1970). Based on these data, the major shift in bacterial population abundance in response to BCM can be attributed to *Prevotella* and *Selenomonas* sp. utilizing pathways for propionate production which consume H_2 via the randomizing (succinate) pathway through the fermentation of sugars and lactate (Bryant et al., 1958; Strobel, 1992; Purushe et al., 2010). It is likely that these pathways were the primary routes for consumption of H_2, which accumulated as a consequence of reduced methanogenesis. The development of qPCR primers to monitor two of these *Prevotella* clusters (Mitsumori et al., 2012) confirmed the observations of the DGGE analysis and 16S OTU data, in that these were dominant bacterial populations that increased in abundance at high BCM dosing levels. However, other bacteria such as *Megasphaera elsdenii*, *S. ruminantium*, *Succinimonas amylolytica*, *Propionibacterium acnes* and *Veillonella parvula*, were observed to change and may also be involved (Hungate, 1966; Marounek et al., 1989; Stewart et al., 1997; Wolin et al., 1997). Further studies involving deeper sequencing and tracer experiments with C-labeled sugars and lactate are required to determine the relative contribution of these pathways as the rumen microbiota adapts to high H_2 concentration in the rumen.

With a decrease in methanogenesis and subsequent increase in ruminal H_2 concentrations, reductive acetogens are able to compete successfully with methanogens for H_2 within the rumen (Ungerfeld and Kohn, 2006). In this regard shifts in bacterial populations carrying key functional genes from the reductive acetogenesis pathway such as *acsB* and *fhs* should identify changes to these populations. The administration of high doses of BCM-CD (5 g/100 kg live weight) significantly altered and restricted the diversity of the FTHFS sequences. The restriction in diversity is in contrast to a previously reported study for cattle supplemented with BCM-CD at a dose rate of 1 g/100 kg live weight, which produced marked changes in the populations that carry the *fhs* gene but produced an increase in diversity (Mitsumori et al., 2014). The restriction in diversity in the goats is likely a reflection of the higher doses of BCM and reveals a negative impact on certain acetogenic populations. BCM is a halogenated methane analog that's mode of action is through reacting with reduced vitamin B_{12} and inhibiting the cobamide-dependent methyl transferase step of methanogenesis (Wood et al., 1968; Chalupa, 1977). The B_{12} dependent methyl transferases also play an important role in one carbon metabolism in acetogenic bacteria (Banerjee and Ragsdale, 2003), and therefore BCM may have an effect on reductive acetogenesis, which has not been investigated. One of the groups that was prevalent in the BCM treated goats was associated closely to *Sporomusa ovata*. *S. ovata* is unique in its requirement for phenolyl cobamides as opposed to the more common benzimidazolyl cobamides associated with acetogenic and methanogenic methyl transferases (Stupperich et al., 1988; Kaster et al., 2011; Mok and Taga, 2013). This preference for phenolyl ligand cobamides and the ability of *Sporomusa* species to grow more productively on organic substrates such as methanol and lactate in conjunction with H_2 oxidation to produce acetate gives these species an advantage over purely reductive acetogens (Breznak and Blum, 1991). Experiments to investigate the effect that BCM may have on acetogenic bacterial isolates need to be performed to ascertain any negative mode of action.

Existing tools targeting the FTHFS sequence as a marker for reductive acetogenesis are compromised by lack of specificity due to the involvement of FTHFS in other pathways (Drake et al., 2008; Pierce et al., 2008). ACS is unique to the acetyl-CoA pathway and is an excellent marker gene for detecting acetogenic bacteria. However, as this pathway is also used by some

methanogens and sulfur reducing species for generation of cell carbon and/or acetoclastic growth some caution in interpretation is still required (Ragsdale, 1991). A recently designed primer set that excludes, or at least minimizes these non-acetogenic populations has proven to target acetogenic populations from gut environments (Gagen et al., 2010). Similarly, the only ACS sequences recovered from the goat rumen using this primer set showed affiliation to groups between the Lachnospiraceae and Clostridiaceae acetogen families. Within the BCM treated animals the majority of the ACS sequences were associated with *A. ruminis*. This grouping was not strictly identified using the FTHFS gene analysis possibly due to the limitations of the FTHFS primers to amplify the gene from these species (Henderson et al., 2010). However, the dominant FTHFS OTU closely located with *C. magnum* is in the same position in the tree that *A. ruminis* is located (Gagen et al., 2010). It is likely that this group of bacteria are the dominant acteogens in the BCM treated goat rumen. In addition, the lack of congruency for the grouping of FTHFS genes associated with the *Sporomusa* species and ACS sequences can be explained in that these grouping are not supported by bootstrapping for these species when comparing tree placements between FTHFS and ACS libraries and is therefore likely to be placed inaccurately within the ACS tree (Gagen et al., 2010).

Conclusion

The dose-dependent inhibitory effect of BCM on methanogens in the goat rumen with measured reductions in methane and increases in H_2 has both directly and indirectly effected the rumen microbiome. Rumen fermentation end products were mostly unchanged with respect to the addition of BCM, but significant increases in propionate and iso-valerate were detected at the mid and high doses. In depth microbial ecology and metagenomic analysis allowed high-resolution observations into the changes in rumen microbial populations with respect to abundance and presumed level of metabolic capacity within the system. Metagenomic analysis was dominated by genomic content from *Prevotella* and *Selenomonas* species and showed potential to produce propionate through the randomizing (succinate) pathway. Reductive acetogenic populations were also affected significantly by the changes in the rumen environment. Both markers gene studies and metagenomics data suggest that they provide minor contributions to the redirection of H_2 in BCM treated animals.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.01087

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Methane Inhibition Alters the Microbial Community, Hydrogen Flow, and Fermentation Response in the Rumen of Cattle

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Management of metabolic hydrogen ([H]) in the rumen has been identified as an important consideration when reducing ruminant CH_4 emissions. However, little is known about hydrogen flux and microbial rumen population responses to CH_4 inhibition when animals are fed with slowly degradable diets. The effects of the anti-methanogenic compound, chloroform, on rumen fermentation, microbial ecology, and H_2/CH_4 production were investigated *in vivo*. Eight rumen fistulated Brahman steers were fed a roughage hay diet (Rhode grass hay) or roughage hay:concentrate diet (60:40) with increasing levels (low, mid, and high) of chloroform in a cyclodextrin matrix. The increasing levels of chloroform resulted in an increase in H_2 expelled as CH_4 production decreased with no effect on dry matter intakes. The amount of expelled H_2 per mole of decreased methane, was lower for the hay diet suggesting a more efficient redirection of hydrogen into other microbial products compared with hay:concentrate diet. A shift in rumen fermentation toward propionate and branched-chain fatty acids was observed for both diets. Animals fed with the hay:concentrate diet had both higher formate concentration and H_2 expelled than those fed only roughage hay. Metabolomic analyses revealed an increase in the concentration of amino acids, organic, and nucleic acids in the fluid phase for both diets when methanogenesis was inhibited. These changes in the rumen metabolism were accompanied by a shift in the microbiota with an increase in Bacteroidetes:Firmicutes ratio and a decrease in Archaea and Synergistetes for both diets. Within the Bacteroidetes family, some OTUs assigned to *Prevotella* were promoted under chloroform treatment. These bacteria may be partly responsible for the increase in amino acids and propionate in the rumen. No significant changes were observed for abundance of fibrolytic bacteria, protozoa, and fungi, which suggests that fiber degradation was not impaired. The observed 30% decrease in methanogenesis did not adversely affect rumen metabolism and the rumen microbiota was able to adapt and redirect [H] into other microbial end-products for both diets. However, it is also required dietary supplements or microbial treatments to capture the additional H_2 expelled by the animal to further improve rumen digestive efficiency.

Keywords: rumen, microbial community, metabolites, methane, hydrogen, 16S sequencing

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INTRODUCTION

The principal greenhouse gas emitted from livestock is enteric CH_4 which represents between 7 and 18% of total anthropogenic emissions (Hristov et al., 2013). Methane produced as an end product of fermentation constitutes an energy loss from digested feed (estimated between 2 and 12% of gross energy intake; Johnson and Johnson, 1995). If methanogenesis was inhibited and the available [H] was redirected into alternative energy-yielding metabolic pathways increased productivity could be expected. Thus, reducing CH_4 production could potentially improve productivity for the same energetic intake by the animal provided rumen metabolism is not compromised.

The fermentation of feedstuff in the rumen by bacteria, protozoa, and fungi produces H_2 which is used by some bacteria and methanogens to obtain energy while generating metabolic end products through this process. Methanogenic archaea are the main consumers of the H_2 in this ecosystem, producing CH_4 as the end product (Ungerfeld and Kohn, 2006; Janssen, 2010). It has been assumed that H_2 accumulation resulting from the inhibition of methanogenesis will impair fiber digestion and fermentation (Wolin et al., 1997; McAllister and Newbold, 2008; Janssen, 2010). However, Mitsumori et al. (2012) found that inhibition of methanogenesis by bromochloromethane (BCM) in goats (around 80% CH_4 decrease) dramatically increased H_2 expelled without affecting dry matter intake (DMI) and feed digestibility. BCM has been used in numerous studies to decrease CH_4 production in ruminants and it is considered one of the most effective inhibitors of methanogenesis (Denman et al., 2007; Goel et al., 2009; Abecia et al., 2012; Mitsumori et al., 2012). The restrictions on use of BCM due to its ozone depleting capacity have led to other halogenated methanogenesis inhibitors such as chloroform and bromoethanesulfonate (BES) being used in ruminant research as experimental models (Dong et al., 1999; Ungerfeld et al., 2004; Knight et al., 2011). Chloroform appears to decrease rumen CH_4 production to the same extent as BCM with little or no adverse effect on rumen fermentation in dairy cows and *in vitro* (Knight et al., 2011; Hwang et al., 2012). Chloroform, like BCM, interferes with the transfer of the methyl group to methyl-coenzyme M (CoM) at the cobamide-dependent methyl transferase step of the methanogenesis pathway (Gunsalus and Wolfe, 1978; Graham and White, 2002).

Management of H_2 in the rumen was identified as an important consideration when inhibiting ruminant CH_4 emissions since it is assumed that accumulation of H_2 would inhibit the re-oxidation of NADH and adversely affect fermentation (Wolin et al., 1997; Joblin, 1999). However, H_2 is rarely measured while much effort is expended on quantifying the amounts of CH_4 formed in animal studies. Recently three studies have measured both the expelled CH_4 and H_2 simultaneously in cattle and dairy cows. Rooke et al. (2014) showed significant differences on CH_4 and H_2 emissions between diets when cattle were fed a high-concentrate or a mixed forage-concentrate diet. Veneman et al. (2015) observed an increase in expelled H_2 when methanogenesis was inhibited in dairy cows treated with nitrates or linseed oil and fed with a total mixed ration. Significant increases in expelled H_2 occurred when methanogenesis was inhibited in dairy cows fed with a total mixed ration and treated with 3-nitrooxypropanol with non-detrimental effects on feed intake or apparent total tract digestibility (Hristov et al., 2015). In fact, a recent meta-analysis (Ungerfeld, 2015) of *in vitro* trials observed a greater accumulation of H_2 when inhibiting methanogenesis in incubations with increasing concentrate substrate. However, little is known about rumen fermentation and microbial population responses to CH_4 inhibition when animals are fed with slowly degradable diets, such as low quality roughage hay. With the exception of a study conducted by Vyas et al. (2016), there is scarce information about the effects of inhibiting methanogenesis with different types of diet in the same *in vivo* study.

The aims of the present study were to analyze the effect of chloroform on CH_4 production, [H] flux, and subsequent responses in rumen fermentation and microbial community composition of cattle on diets of varying quality. Three dose levels of chloroform were administered into the rumen of steers fed *ad libitum* on two different diets of roughage:concentrate (60:40) or roughage hay. It was hypothesized that lesser amounts of H_2 relative to intake would accumulate in the roughage fed animals compared to those receiving a roughage:concentrate diet due to a shift in fermentation to reductive processes that will consume more reducing equivalents, resulting in less energy lost to the animal.

MATERIALS AND METHODS

The experimental protocol complied with the Australian Code for the Care and Use of Animals for Scientific Purposes (eighth edition, 2013) and was approved by the local Animal Experimentation and Ethics Committee (A18/2013).

Experimental Design and Sampling

Eight rumen-fistulated Brahman (*Bos indicus*) steers (mean, live weight LW, 288 $\pm$ 7 kg) were used in the experiment. Animals were randomly allocated to two groups (four animals per group), each group receiving a different *ad libitum* diet twice per day. One group was fed a roughage hay diet (Rhode grass hay; chemical composition: DM, 881 g/kg fresh matter; in g/kg of DM: OM, 802; CP, 50; NDF, 765: ADF, 454; ADL, 64; ash, 65; and GE 16.5 MJ/kg) and the second group received a roughage hay:concentrate diet (60:40; Ridley AgriProducts Pty Ltd, Brisbane, QLD, Australia. Concentrate ingredients (g/kg): barley (574), sorghum (200), molasses mixer (30), cotton hull pellet (100), urea (5); concentrate chemical composition: DM, 906 g/kg fresh matter; in g/kg of DM: OM, 906; CP, 116; NDF, 263; ADF, 120; ADL, 30; fat, 34; ash, 74; and GE 17.2 MJ/kg) being the hay the same for both diets. Animals were adapted to each diet over an initial 17 days period. After that initial period, experimental animals were maintained in individual pens in an animal house for the measurement of individual intakes (10 days) and were treated with cyclodextrin (CD; 3 g/100 kg LW). On days 9 and 10 animals were confined in open-circuit respiration chambers for measurement of CH_4 and H_2 production and collection of rumen samples (control period). Following the

initial adaption/control period animals received a low dose of chloroform-cyclodextrin (CCD; 1 g/100 kg LW) for 10 days with the last 2 days being confined in open-circuit respiration chambers for direct measurement of CH_4 and H_2 production. Doses were then increased to a mid level (1.6 g/100 kg LW) for 10 days with rumen fluid collection and CH_4/H_2 measurements, and then to a high level (2.6 g/100 kg LW) for 10 days with a similar sampling regime for the final 2 days. The CCD doses were split up in two shots and administered through the rumen cannula at 0 and 3 h after feeding. After a 15 days period without CCD animals were returned to open circuit respiration chambers during two consecutive days with a similar rumen sampling regime (post-treatment period). Rumen fluid samples (60 mL per animal) were collected using a probe covered with two layers of cheesecloth at 3 h post feeding and just before dosing with CCD during each confinement period in respiration chambers. Rumen samples were stored at −20°C for short chain fatty acids (SCFA) and NH_3-N analyses. Additionally, 20 mL were kept at −80°C for DNA extraction and metabolite analyses.

Antimethanogen Formulation

The antimethanogen was an halogenated hydrocarbon (chloroform) entrapped in a β-CD matrix (May et al., 1995). The formulation was prepared by CSIRO Manufacturing (Clayton, VIC, Australia) in 3-kg batches and contained 6–7% w/w chloroform. Chloroform was encapsulated within a CD matrix, to increase its stability and slow its rate of release in the rumen. Similar halogenated analogs (such as bromochloromethane) entrapped in a CD matrix have been previously used in ruminants, to delay the release of the antimethanogenic compound in the rumen (Denman et al., 2007; Abecia et al., 2012; Mitsumori et al., 2012).

Gas Measurements

Four open circuit respiration chambers were used to determine CH_4 production from individual steers. Each chamber had an internal volume of 23.04 m^3 and was equipped with a water trough and feed bin containing the daily ration. Each chamber was maintained at 2°C below ambient air temperature, approximately −10 Pa, and the relative humidity for the two, 24 h measurement periods varied from 50 to 75%. Air was drawn through a 250 mm diameter duct into each chamber at a rate of 3000 L/min. Exact flow rates, corrected to measured conditions for temperature and pressure for each chamber were used in calculations for CH_4 and H_2 production (Takahashi et al., 1999; Williams et al., 2007). Flow rate through each chamber was measured using thermal flow sensors (SS20.500 SCHMIDT® Flow Sensor). The air sample for the analysis of gas composition was drawn from a point in the exhaust duct through polyurethane tubing at 4.5 L/min using a micro diaphragm pump located between a multiport gas switching unit (SW & WS Burrage, Ashford Kent UK) and membrane drier (Perma Pure LLC). Air samples from each chamber initially passed through particulate filters (AF30-02 SMC Pneumatics Aust. Pty Ltd) and a four port fridge drier prior to the multiport gas switching unit which was programmed to cycle through each chamber and two outside air ports. Air samples passed through a chemical drier and were metered through independent rotameters before compositional analysis for CO_2 and CH_4 (Servomex 4100 Servomex Group Ltd. Crowborough, UK) and H_2 (Servomex Chroma, Servomex Group Ltd, Crowborough, UK; and Dräger X-am 5000, Draeger Safety Pacific Pty. Ltd., Notting Hill, VIC, Australia). Data for flow rate, temperature and chamber pressure, and CH_4/H_2 content of the exhaust air for the final 315 s of each sampling event was used to calculate CH_4 and H_2 flux.

Chemical Analysis

The feed samples were dried in a forced-air oven at 105°C prior to grinding. Feed samples were ground through a 1-mm sieve before analysis. DM, ash, NDF, ADF, lignin, fat, gross energy (adiabatic calorimeter), and total nitrogen contents were analyzed by Symbio Alliance (Eight Mile Plains, QLD, Australia) following the accredited methods CF006.1, CF007, CF038.3, CF038.3, CF038.6, CF004.1, CF237, and CF003.2, respectively (Association of Official Analytical Chemists [AOAC], 2005 official methods: 925.60, 923.03, 920.39, 990.03, 2002.04, and 973.18). The nitrogen values were converted to CP by multiplying by 6.25.

Concentrations of SCFAs (acetate, propionate, *n*-butyrate, iso-butyrate, iso-valerate, and *n*-valerate) were measured by gas chromatography (GC) as described by Gagen et al. (2014). Iso-valerate (3-methyl butyrate) includes 2-methylbutyrate, which co-eluted.

The NH_3-N concentration was determined by a colorimetric method following Chaney and Marbach (1962).

An UltiMate® 3000 HPLC system (Dionex, Sunnyvale, CA, USA) with a dedicated Photodiode Array Detector and an Autosampler was used to determine the presence of formic acid in samples supernatants as described by Gagen et al. (2014).

Calculation of [H] Redirection and Non-carboxyl SCFA Carbons

As actual flows of metabolites formation were not measured, concentrations were used as a proxy to estimate changes in the incorporation of [H] into SCFA (HUSr) and formate (HUFr). The stoichiometry was calculated as follows (Goel et al., 2009):

$$\mathrm{HUSr} = 2\,x\,C3 + 2\,x\,C4 + C5$$

$$\mathrm{HUFr} = C1$$

The CH_4 gas production (GP; mol/day) and H_2 gas production (GP; mol/day) was used to calculate the ratio between [H] redirected to H_2 expelled/CH_4 decrease ((H_2 GP)/(CH_4 GP decrease × 4)).

Total non-carboxyl SCFA carbon concentration were calculated using the following formula:

$$\text{Total non-carboxyl SCFA carbon concentration} = [\mathrm{ACL} - 1]\ \text{x mM total SCFA}$$

Average chain length (ACL) = [mM Formate + (2x mM Acetate) + (3x mM Propionate) + (4x mM Butyrate) + (5x mM Valerate)]/[Total mM Formate + Acetate + Propionate + Butyrate + Valerate]

The calculation removes from consideration the carboxyl group of the SCFA and provides a standard for comparison for SCFA energy available to the animal (Ungerfeld, 2013).

The non-carbohydrate contributions to SCFA were assumed to be unimportant and were not considered in the calculations described above.

Rumen Metabolomics Analyses

Samples from rumen fluid were prepared and metabolites quantified by Metabolomics Australia, University of Melbourne.

Amines quantification: Sample volumes of 10 μL of rumen fluid supernatant were placed in 2 mL Eppendorf tubes under cold conditions (4°C). Methanol (100% MeOH, 250 μL) containing four internal standards [^{13}C-sorbitol (0.5 mg/mL), $^{13}C_5$-^{15}N-Valine (0.5 mg/mL), 2-aminoanthracene (0.25 mg/mL), and pentafluorobenzoic acid (0.25 mg/mL)] was added to the sample tubes and the samples were vortexed for 5 min until uniform. The samples were then incubated in a Thermomixer (Eppendorf brand, distributed by Quantum Scientific, Australia) at 70°C with a mixing speed of 850 rpm for 15 min, followed by a 15 min of centrifugation at 4°C at 13,800 × *g* in an Eppendorf benchtop centrifuge. The MeOH supernatant was transferred into a new 2 mL Eppendorf tube and set aside. Water (250 μL, Milli-Q grade) was added to the remaining sample pellet in the initial tube and vortexed for 5 min before being centrifuged at 13,800 x *g* at 4°C for 15 min. The H_2O supernatant was transferred to the Eppendorf tube containing the MeOH supernatant and vortexed to mix. A 10 μL aliquot was transferred to a fresh Eppendorf tube in preparation for derivatisation with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (Aqc) followed by LC-MS analysis as per Boughton et al. (2011). In summary, 70 μL borate buffer was added to the sample aliquot, vortexed to mix and then centrifuged at 13,800 × *g* at 4°C for 1 min. Then, 20 μL Aqc reagent was added to the sample aliquot, vortexed immediately to mix and then centrifuged at 13,800 × *g* at 4°C for 1 min. The Aqc-treated samples were incubated in a Thermomixer at 55°C with a mixing speed of 1150 rpm for 10 min and then centrifuged at 13,800 × *g* at 4°C for 5 min. The derivatized samples were then transferred to HPLC vials for LC-MS analysis as described by Boughton et al. (2011).

Sugars, organics, and fatty acids quantification: Sample volumes of 240 μL of rumen fluid supernatant were placed in 2 mL Eppendorf tubes under cold conditions (4°C) and 320 μL of 100% methanol, containing 2% $^{13}C_6$-Sorbitol as a quantitative internal standard, was added. The samples were then vortexed for 1 min and incubated with shaking (950 rpm) at 30°C for 15 min prior to centrifugation at 13,800 × *g* for 15 min at room temperature in a bench-top Eppendorf centrifuge. The supernatant was transferred to a clean tube while the remaining pellet was re-extracted with 640 μL of $CHCl_3$ and vortexing for 1 min. After re-centrifugation as before, 60 μL of the polar upper phase was removed and dried under vacuum without heating prior to preparation for sugars and organic acids quantitation as described by Dias et al. (2015). The lower $CHCl_3$-phase was dried under vacuum without heating then reconstituted in 320 μL of $CHCl_3$ before a 160 μL aliquot transferred to a glass insert and re-dried under vacuum prior to preparation for fatty acid quantitation described by Dias et al. (2015).

DNA Extractions

DNA extractions were carried out on rumen samples using the cetyltrimethylammonium bromide (CTAB) method of Brookman and Nicholson (2005) with minor modifications as follows: samples were centrifuged (13,000 × *g* for 5 min), and the supernatant was removed before DNA extraction. Cells were homogenized with 200 mg of silica–zirconium beads (1:1 mixture of 0.1- and 1.0-mm beads; Biospec, Bartlesville, OK, USA) and 800 μl of CTAB buffer in a Mini-Beadbeater-8 (Biospec) on maximum speed for 2 min, twice. Samples were incubated at 70°C for 20 min and centrifuged at 10,000 × *g* for 10 min, and the supernatant was mixed with 500 μl of 25:24:1 phenol–chloroform–isoamyl alcohol (Fluka BioChemika, Buchs, Switzerland). The yield and purity of the extracted DNA were assessed with a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

Real-Time PCR Analysis

The DNA samples were used as templates for quantifying the abundance of anaerobic rumen fungi, protozoa populations, and mcrA gene for methanogens. The primers and assay conditions used were previously published (Sylvester et al., 2004; Denman and McSweeney, 2006; Denman et al., 2007). Real-time PCR (qPCR) analyses were run in triplicate from one DNA extraction on an Applied Biosystems™ ViiA™ 7 Real-Time PCR System (Thermo Fisher Scientific Inc.). Assays were set up using the SensiFAST SYBR® Lo-ROX (Bioline). Optimisation of assay conditions was performed for primer, template DNA, and $MgCl_2$ concentrations. An optimal primer concentration of 400 nM, a final $MgCl_2$ concentration of 3 mM and DNA template concentration of 50 ng were used for each assay under the following cycle conditions: one cycle of 50°C for 10 s and 95°C for 2 min 30 s for initial denaturation, forty cycles at 95°C for 15 s and 60°C for 1 min for primer annealing and product elongation. Fluorescence detection was performed at the end of each annealing and extension step. Amplicon specificity was performed via dissociation curve analysis of PCR end products by raising the temperature at a rate of 0.05°C/s from 60 to 95°C. Changes in targeted populations were calculated using a relative quantification calculation and the $2^{-\Delta\Delta C_t}$ method, with the control period used as the calibrator and total bacterial (Denman and McSweeney, 2006) C_t (cycle threshold) values used as the reference value (Livak and Schmittgen, 2001). Estimation of abundance of target populations to indicate their contribution to the sample was also calculated using standard curves for each target gene generated from cloned PCR products (Mitsumori et al., 2012).

16S rDNA Analysis

Using high throughput sequencing platforms and barcoded primer sets, phylogenetic based methods targeting the 16S rDNA gene were used to deeply characterize the microbial populations present in the rumen for the control and treatment periods. The V4 region of the 16S rRNA gene was targeted using specific primers (Kozich et al., 2013). Each individual DNA sample was amplified using the specific primers and a unique barcode combination. Afterward, amplification products were visualized by performing gel electrophoresis. Product quantities were calculated and an equal molar amount of each product was pooled. The pooled products were run in a 1% agarose gel and bands were visualized and excised under blue light transillumination. The amplicons were gel purified with QIAquick Gel extraction Kit (Qiagen, Hilden, Germany) prior to submission for Illumina Miseq.

Short read sequence data generated was analyzed using QIIME: Quantitative Insights Into Microbial Ecology software package (Caporaso et al., 2010). Sequences were clustered as operational taxonomic units (OTUs) of 97% similarity using uclust (Edgar, 2010). Taxonomic assignment of sequences was performed against the Greengenes database (McDonald et al., 2012). Alpha and beta diversity and significant fold change of OTU's were performed in the R packages ade4, Phyloseq, and DESeq2 (Chessel et al., 2004; McMurdie and Holmes, 2013; Love et al., 2014). The sequences obtained in this paper have been deposited in the European Nucleotide Archive (ENA) under the accession number PRJEB13653.

Statistical Analyses

The effect of dose, diet, and their interaction were analyzed for the CH_4/H_2 production, DMI and fermentation variables as a univariate repeated-measures analysis of variance using the GLM procedure of SPSS (IBM, version 21.0), with animal as the experimental unit. Linear and quadratic components of the response to incremental dose of CCD were evaluated using polynomial contrasts. Effects were considered significant at $P \leq 0.05$ and considered as tendencies toward significance at $P \leq 0.10$. When significant differences were detected, differences among means were tested by pairwise comparisons (LSD test). The model used was:

$$y_{ijtk} = \mu + d_j + f_k + (d \times f)_{jk} + \varepsilon_{ijtk}$$

Where:

y_{ijtk} is the dependent variable measured at time t on the i-th steer treated with the j-th dose and the k-th feed,

μ the overall mean,

d_j is the fixed dose effect,

f_k is the fixed feed effect,

$(d \times f)_{jk}$ the interaction between dose and feed,

ϵ_{ijkt} the residual error associated with the i-th steer within the j-th dose and k-th feed at time t.

RESULTS

Ruminal Fermentation and Gas Production

There was an interaction ($P = 0.040$) between dose and diet on DM intake (DMI), with a decrease in DMI with CCD dose increase in the hay:concentrate diet, and an increase in DMI with CCD dose increase in the hay diet (**Table 1**). Methane production (g/kg DMI) was decreased significantly ($P < 0.01$) for mid and high doses compared with the control on both diets. Conversely H_2 expelled by treated animals increased significantly ($P < 0.05$) as CH_4 production was decreased, with the greatest amounts of H_2 release (g/kg DMI) occurring in animals supplemented with the roughage hay:concentrate diet. Both effects were linear, showing a dose-dependent response. In addition the amount of H_2 expelled relative to the decrease in CH_4 (mol H_2/mol CH_4 decrease) was greater in animals fed the concentrate diet showing a significant ($P < 0.05$) diet effect, although no diet–dose interaction was observed. Fourteen days after the CCD treatment was terminated, the CH_4 and H_2 production were no significantly different to the control period for both diets (data not shown).

Rumen SCFA analysis showed a shift in the fermentation pathways (**Table 1**) toward a higher propionate profile when CCD was linearly increased on both diets. This was reflected by a significant decrease in acetate molar percentage ($P < 0.001$) and an increase in propionate molar percentage ($P < 0.05$) for both diets. As a result, a significant linear and quadratic decrease of the acetate:propionate ratio was observed at all doses for both diets. A diet–dose interaction ($P = 0.005$) was observed for the total SCFA concentration, with a significant decrease with the highest dose of CCD for the hay:concentrate diet. The hay diet, however, showed a significant ($P < 0.001$) increase in total SCFA concentration when animals were treated with the low and mid dose of CCD and a decrease with the high dose. Butyrate molar percentage linearly increased with CCD with both diets ($P < 0.011$). The branched-chain SCFA linearly increased with both diets, showing a diet–dose interaction effect ($P < 0.05$), with the greatest increase observed for the hay diet. Rumen pH increased linearly with CCD ($P < 0.001$) treatment for both diets showing a diet–dose interaction ($P = 0.01$). Rumen ammonia concentration was unchanged compared with the control period for both diets. A significant linear increase in formate concentration was observed for all CCD doses for the hay:concentrate diet and only with the hay diet at the highest CCD dose, showing a diet and a diet–dose interaction ($P < 0.001$). Regarding the diet effect, it was observed for formate, pH, acetate, propionate, butyrate, and acetate:propionate ratio. Interestingly, the total non-carboxyl SCFA carbons linearly decreased ($P = 0.002$) for the hay:concentrate diet and increased for the hay diet at low and mid doses, showing a diet-dose interaction ($P = 0.01$).

Rumen metabolite profiles for control and treated animals at mid dose of CCD are shown in Supplementary Table S1. Some amino acids, organic acids and sugars were significantly increased on both diets in animals treated with CCD, although the profile observed was different for each diet. A greater number

TABLE 1 | Control and CCD doses (low, mid, and high) effects on DMI, CH_4, and H_2 production, and rumen fermentation variables from samples collected 3 h after feeding of animals fed with hay:concentrate or hay diet.

	Hay:concentrate diet				Hay diet					*P*-value			
Item	**Control**	**Low**	**Mid**	**High**	**Control**	**Low**	**Mid**	**High**	**SEM**	**Dose**	**Diet**	**Dose × Diet**	**Contrast**
DMI kg	5.9	5.8	5.6	5.0	4.2	4.6	4.4	4.7	0.28	0.494	0.005	0.040	
CH_4 (g/kg DMI)	24^{a}	19^{b}	15^{bc}	10^{bc}	27^{a}	21^{b}	17^{bc}	12^{bc}	1.40	0.001	0.160	0.998	*L*
H_2 (g/kg DMI)	0.00^{d}	0.97^{c}	1.43^{b}	3.16^{a}	0.00^{c}	0.33^{b}	0.83^{a}	1.73^{a}	0.18	0.001	0.029	0.144	*L*
mol H_2/molCH_4 decrease[1]	–	1.7	1.4	1.6	–	0.66	0.63	0.89	0.41	0.813	0.020	0.898	
pH	6.4^{b}	6.6^{a}	6.6^{a}	6.7^{a}	6.6^{c}	6.7^{ab}	6.8^{a}	6.7^{b}	0.04	0.001	0.026	0.010	*L, Q*
Formate (mM)	0.0^{d}	4.2^{c}	8.5^{b}	12^{a}	0.0^{b}	0.0^{b}	0.0^{b}	4.2^{a}	1.06	0.001	0.001	0.001	*L*
NH_3–N (mg/100 mL)	4.4	2.8	3.1	4.2	2.3	4.7	4.8	5.2	0.54	0.337	0.137	0.063	
Total SCFA (mM)	88^{a}	81^{ab}	79^{ab}	78^{b}	79^{b}	91^{a}	82^{ab}	66^{c}	2.99	0.001	0.616	0.005	*L, Q*
Fatty acid (%)													
Acetate	62^{a}	56^{b}	55^{b}	53^{b}	67^{a}	62^{b}	61^{b}	58^{c}	1.31	0.001	0.002	0.575	*L, Q*
Propionate	18^{b}	21^{a}	21^{a}	23^{a}	15^{b}	18^{a}	18^{a}	19^{a}	0.76	0.001	0.001	0.973	*L*
Butyrate	12^{b}	13^{a}	14^{a}	13^{a}	9^{b}	11^{a}	11^{a}	12^{a}	0.61	0.019	0.011	0.745	*L, Q*
i-Butyrate	1.53^{b}	1.74^{a}	1.81^{a}	1.89^{a}	1.69^{c}	1.66^{c}	1.85^{b}	2.13^{a}	0.04	0.001	0.222	0.004	*L*
Valerate	3.45^{b}	3.98^{a}	4.03^{a}	4.07^{a}	3.56^{c}	3.76^{bc}	4.01^{b}	4.69^{a}	0.10	0.001	0.488	0.006	*L*
i-Valerate[2]	3.05^{c}	3.80^{b}	4.39^{a}	4.69^{a}	3.15^{c}	3.31^{c}	3.92^{b}	4.79^{a}	0.15	0.001	0.522	0.062	*L*
A:P	3.44^{a}	2.65^{b}	2.60^{b}	2.34^{b}	4.41^{a}	3.50^{b}	3.41^{b}	3.02^{c}	0.20	0.001	0.002	0.793	*L, Q*
Non-carboxyl SCFA carbon	135^{a}	125^{ab}	117^{b}	112^{b}	116^{b}	140^{a}	131^{a}	99^{c}	4.39	0.002	0.854	0.010	*L, Q*

[1] *Taken into account the DMI per animal. Moles of H_2 and CH_4 were calculated from the expelled gases.*
[2] *I-valerate (3-methyl butyrate) includes 2-methylbutyrate, which co-eluted.*
SEM, standard error of the mean.
[a–d] *Within a row treatment means for each diet without a common superscript differ, $P < 0.05$.*
Contrast: Significant ($P < 0.05$) linear (L) or quadratic (Q) effects of the response to incremental dose of CCD estimated by polynomial contrast.

of metabolites increased significantly ($P < 0.05$) for those animals fed the hay diet, (amino acids: serine, homoserine, asparagine, threonine, proline, valine, isoleucine, leucine, tyramine, and phenethylamine; the organic acids: malate, fumarate, malonate, and nicotinic acid; and the sugars: arabitol, fructose, and inositol). In animals fed the hay:concentrate diet the greatest increase ($P < 0.05$) was observed for the amino acids: homoserine, asparagine, proline, valine, isoleucine, glutamate ($P = 0.053$), and leucine ($P = 0.080$); and the sugars: ribose, arabitol, and inositol. A marked fold increase was observed in nucleic acid precursors/derivatives (inosine and hypoxanthine) in CCD treated animals compared with the control group, fed with hay ($P \leq 0.05$) or hay:concentrate ($P \leq 0.10$) diet, while no significant effect was observed on lactate concentrations (Supplementary Table S2).

The calculation of the [H] redirection showed a different pattern for the [H] into SCFA (**Figure 1A**) and formate/SCFA ratio (**Figure 1B**) for each diet. All the CCD doses showed a significant ($P \leq 0.001$) increase in [H] redirected into formate for the hay:concentrate diet, while for the hay diet, a measurable proportion of [H] was recovered in formate only at the highest CCD dose. On the other hand, a significant increase ($P \leq 0.05$) of [H] redirected into SCFA was observed with the low and mid dose for the hay diet, while no significant effect was detected for the grain:concentrate diet. Interestingly, a diet effect ($P \leq 0.05$) was observed for the H_2/CH_4 decrease ratio (**Figure 1C**), being more [H] recovered into H_2 per mole of CH_4 decrease for the hay:concentrate compared with the hay diet.

Microbial Community

The dose effect of CCD on the abundance of methanogens, protozoa, and fungi are shown in Supplementary Figure S1 for hay:concentrate and hay diet, respectively. The methanogen abundance decreased ($P < 0.05$) with increasing doses of CCD for both diets. The CCD doses tended to increse the protozoa abundance in animals fed with the hay:concentrate diet, whereas the anaerobic fungi were not significantly affected by the CCD doses for either diets.

The diversity analysis of the rumen microbiota showed that total microbial species richness was impacted with the administration of all CCD doses in animals fed the hay:concentrate diet, whilst only the highest dose of CCD resulted in a significantly altered for the hay diet. While the CCD caused an increase in observed and estimated speciess richness for hay:concentrate diet animals, a contraction in Shannon diversity was observed for mid and high doses on a roughage diet. The microbial species richness from the post-treatment period samples showed similar values to the highest dose of CCD on both diets (Supplementary Figure S2).

The composition of the microbiomes as determined by beta diversity analysis showed a clear separation between the control and CCD doses for both diets (Supplementary Figure S3). The post-treatment period was similar to the low CCD dose or intermediate between that dose and the control treatment. The greatest variance observed between control and CCD groups was 18 and 15% in hay:concentrate and roughage diets, respectively. Variation between animals explained the next level of variance

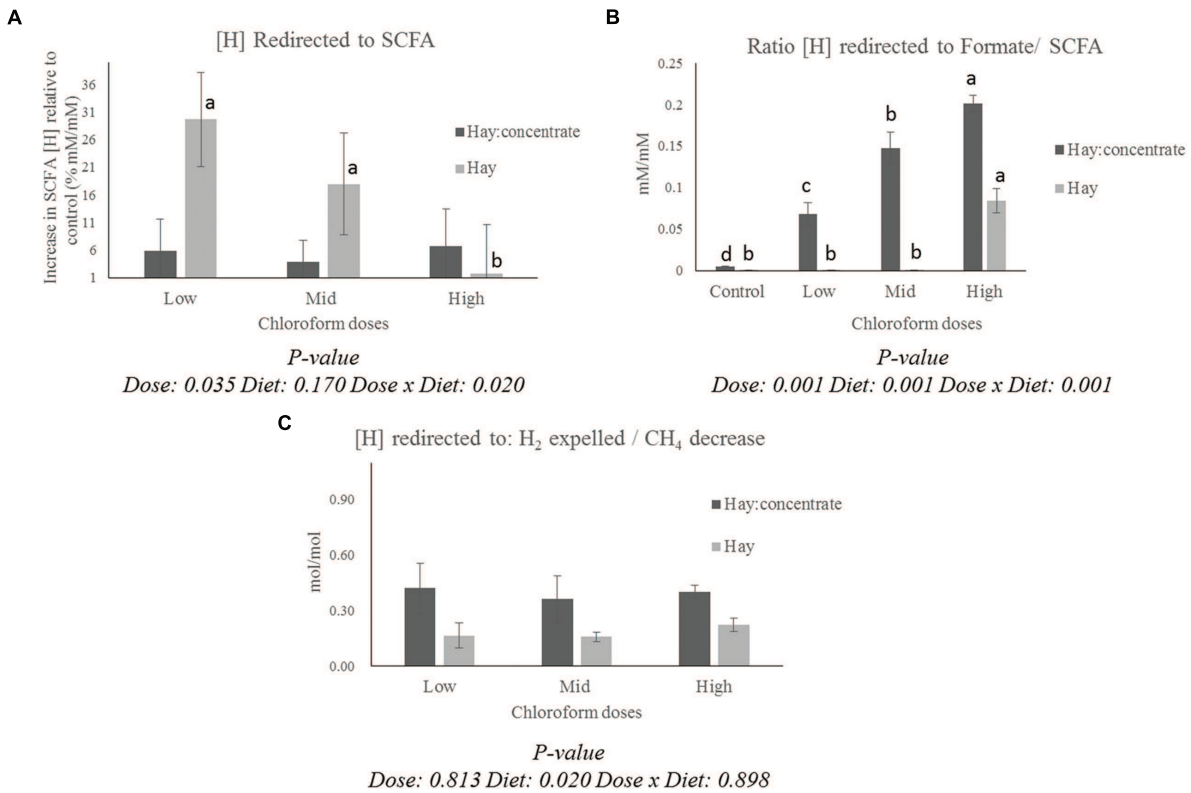

FIGURE 1 | Effect of CCD doses on (A) redirection of [H] to SCFA (Increase of [H] incorporated into SCFA relative to control for each diet, % mM/mM), (B) the ratio between [H] incorporated into formate and [H] incorporated into SCFA for each diet, and (C) the ratio between [H] redirected to H_2 expelled/CH_4 decrease with both diets. $^{a-d}$Letters denote significant differences between doses for each diet, bars that do not share the same letter are significantly different from each other in each diet ($P < 0.05$).

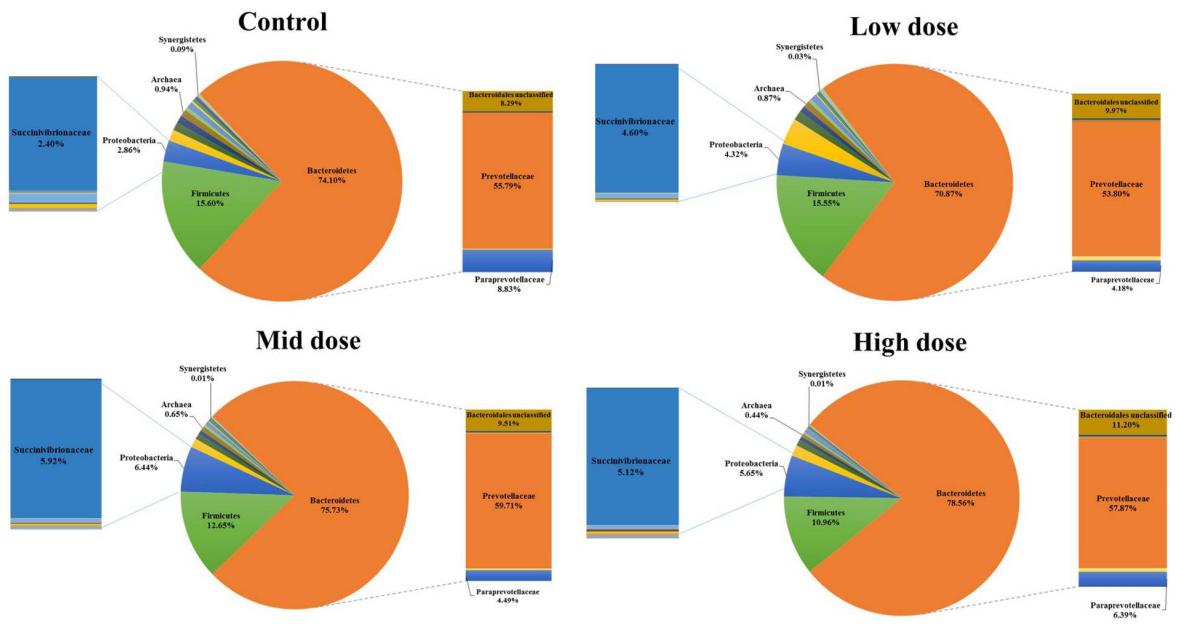

FIGURE 2 | Taxonomic composition of rumen microbiome at the phylum level (pie chart) and family level (bar chart) for control and CCD (low, mid, and high doses) in animals fed with hay:concentrate diet 3 h after feeding.

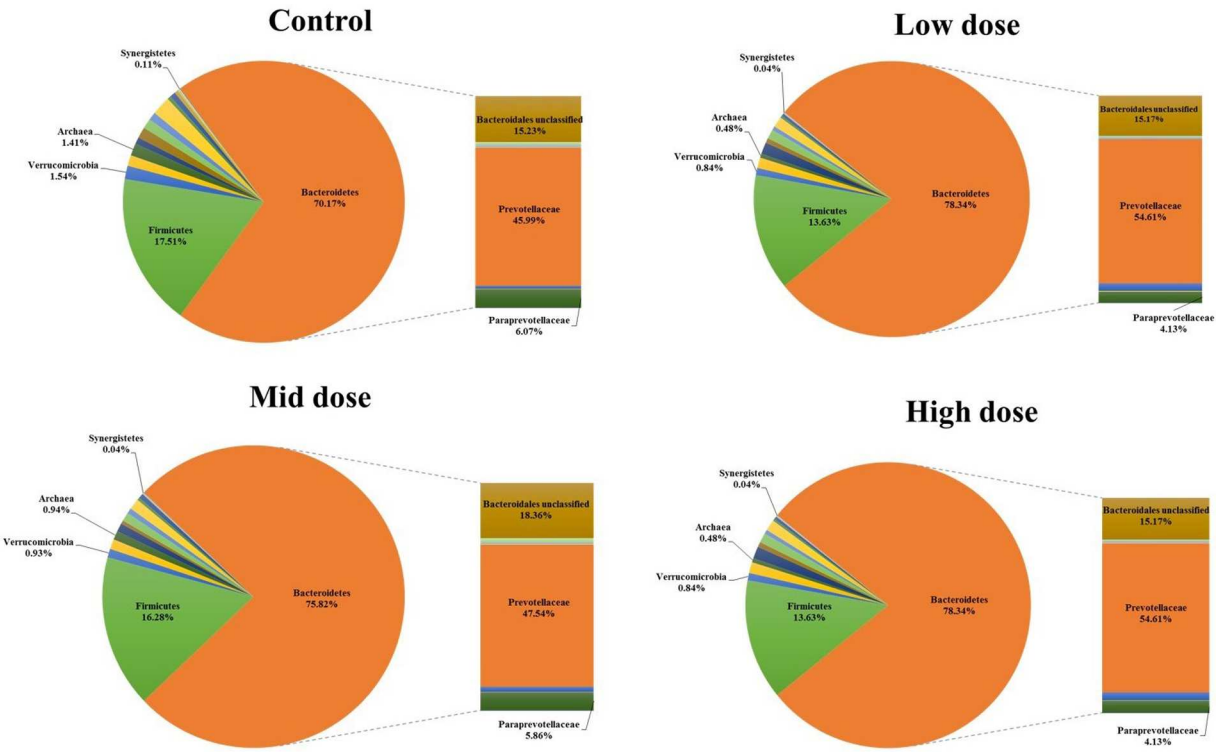

FIGURE 3 | Taxonomic composition of rumen microbiome at the phylum level (pie chart) and family level (bar chart) for control and CCD (low, mid, and high doses) in animals fed with hay diet 3 h after feeding.

irrespective of treatment due to one animal possessing a different microbial population compared to the other three animals on both diets. The variance between individual animals decreased at the high CCD dose and post-treatment period (Supplementary Figure S3).

Analysis of the rumen microbiome showed a shift in the relative abundance at the phylum level when the CCD dose was increased in both diets. An increase in the sequences assigned to the Bacteroidetes phylum and a decrease in Firmicutes, Synergistetes, Verrucomicrobia, and Archaea were observed in both diets (**Figures 2** and **3**). An increase in sequences assigned to the Proteobacteria phylum, mainly classified as Succinivibrionaceae family, were observed in the hay:concentrate diet animals upon treatment with CCD in contrast with hay diet. Consequently the Bacteroidetes:Firmicutes ratio increased with both diets when methanogenesis was inhibited (**Figure 4**). The ratios of sequences assigned to Archaea, Synergistetes, and Verrucomicrobia in relation to bacteria, decreased when CCD dose increased and methane was inhibited showing a dose-dependent effect (**Figures 4** and **5**).

Specific OTUs that were significantly increased with CCD treatment were classified in the *Prevotella* genus, for both diets. Specifically for the animals fed with the hay:concentrate diet, a few OTUs that increased in abundance with CCD were assigned to Moraxellaceae and Succinivibrionaceae family in the Proteobacteria phylum, and the fiber degrading microorganisms *Fibrobacter succinogenes* and *Ruminococcus* spp. Regarding the roughage hay fed animals, minor OTUs promoted by the CCD doses were within the Paraprevotellaceae family and *Butyrivibrio* genus for all the doses and at the highest dose, respectively (Supplementary Figures S4–S9).

The increasing level of CCD was negatively associated in both diets with the abundance of OTUs assigned to *Prevotella* genus compared with the control, which could suggest a shift within the Prevotella groups through both diets (Supplementary Figures S4–S9). In relation to the fibrolytic microorganisms minor OTUs assigned to *Fibrobacter* genus were decreased in both diets with the lowest dose but did not change or were promoted with the increasing doses of CCD, which could suggest a shift in those populations. OTUs assigned to the fibrolytic species *R. albus* did not change with the doses, although a single OTU classified as *R. flavefaciens* was suppressed at the highest dose of CCD with the hay diet.

The Archaea domain was negatively affected by CCD levels in both diets in accordance with the decrease observed in the Archaea:Bacteria (A:B) ratio. Specific OTUs assigned to the Methanobacteriaceae family and Methanoplasmatales order were decreased by the CCD levels (Supplementary Figures S4–S9).

DISCUSSION

This study established a model in cattle whereby methanogens were directly inhibited in a dose dependent manner and the

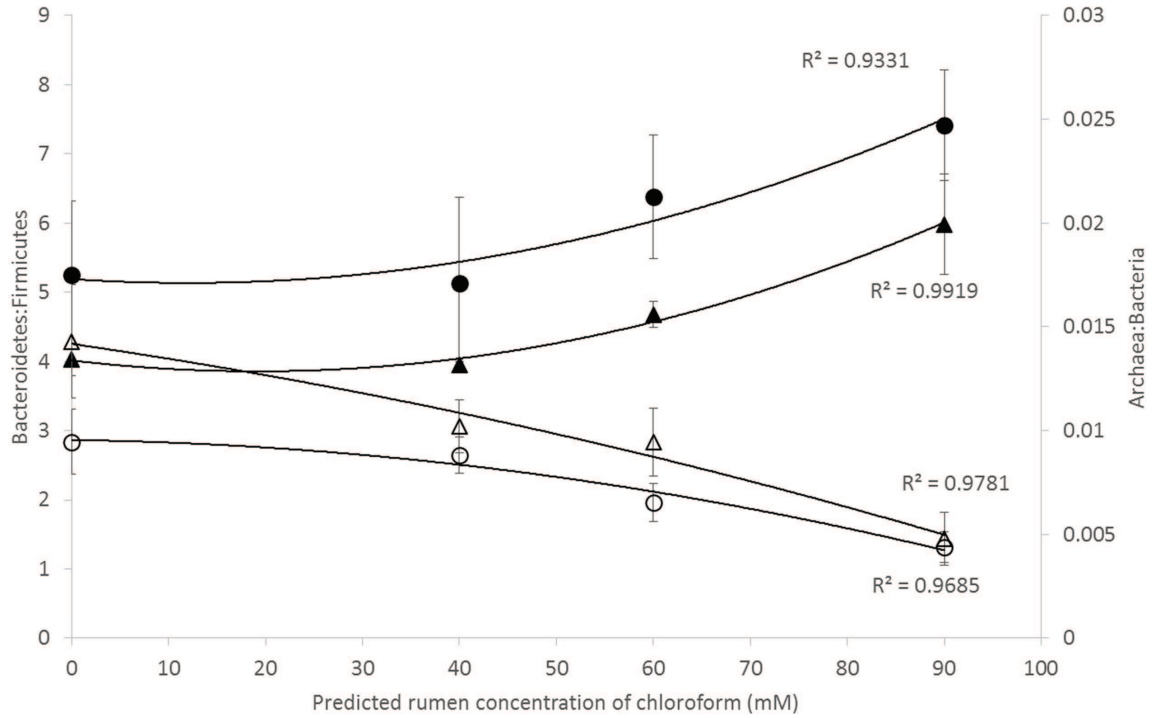

FIGURE 4 | Microbial ratios [Bacteroidetes:Firmicutes (B:F) and Archaea:Bacteria (A:B)] for increasing concentration of chloroform on animals fed with hay [B:F (▲) or A:B (△)] or hay:concentrate [B:F (●) or A:B (○)] diet 3 h after feeding. Predicted rumen concentration of chloroform: 40, 60, and 90 μM for low, mid, and high dose, respectively.

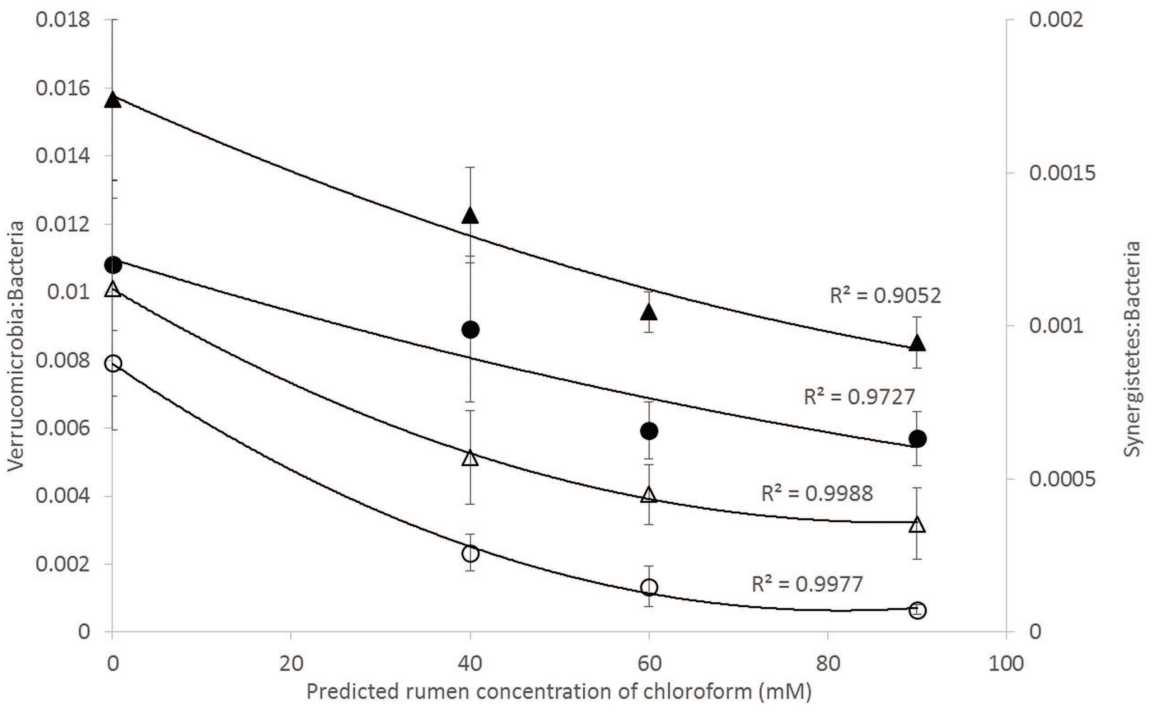

FIGURE 5 | Microbial ratios [Synergistetes:Bacteria (S:B) and Verrucomicrobia:Bacteria (V:B)] for increasing concentration of chloroform on animals fed with hay [V:B (▲) or S:B (△)] or hay:concentrate [V:B (●) or S:B (○)] diet 3 h after feeding. Predicted rumen concentration of chloroform: 40, 60, and 90 μM for low, mid, and high dose, respectively.

subsequent responses in rumen microbial metabolism were evaluated. The three levels of CCD, low, medium, and high, decreased CH_4 production by approximately 14, 37, and 55%, respectively, on average of both diets compared to the control period with no apparent effect on feed intakes. Conversely H_2 expelled by treated animals showed a dose-dependent increase as CH_4 decreased. A similar inverse relationship between CH_4 decrease and H_2 loss has been reported in dairy cows treated with nitrate or 3-nitrooxypropanol, and goats fed the CH_4 inhibitor BCM (Mitsumori et al., 2012; Hristov et al., 2015; Veneman et al., 2015). However, importantly this study showed that greater amounts of H_2 (1.7- to 2.9-fold; g/kg DMI) were expelled in animals supplemented with the hay:concentrate diet compared to the hay only diet. Furthermore the amount of H_2 expelled in the CH_4 inhibited animals was lower than the predicted amount of H_2 involved in hydrogenotrophic CH_4 formation (four moles of H_2/mole CH_4). This suggests that significant amounts of [H] were redirected into reduced end products other than CH_4 and H_2, and perhaps microbial protein in agreement with the meta-analysis of Ungerfeld (2015). Interestingly the rumen microbiota in the hay-fed animals appeared to utilize more H_2 that was available from the decrease in CH_4 formation than their hay:concentrate-fed counterparts, possibly due to the slower fermentation rate of the hay diet compare with the highly fermentable hay:concentrate diet which might produce a more consistent release of hydrogen. Two *in vitro* studies (Lin et al., 2013; O'Brien et al., 2013) observed a greater accumulation of H_2 when inhibiting methanogenesis in mixed roughage concentrate fermentations compared with the roughage substrates. Furthermore, a recently published article by Vyas et al. (2016) also shows increased expulsion of H_2 for methanogenesis-inhibited animals fed a high concentrate diet compared with a mixed forage:concentrate diet.

In relation to the redirection of [H], on both diets there was a shift in fermentation from acetate to fatty acids that were longer in length, particularly propionate that is a major gluconeogenic precursor in ruminants (Newbold et al., 2005). This pattern of fermentation along with an increase in branched chain fatty acids, has been reported previously in studies using the halogenated methane analog, BCM, as the methane inhibitor (Denman et al., 2007; Abecia et al., 2012; Mitsumori et al., 2012). In fact, the total non-carboxyl SCFA carbons increased at the low and mid dose with the hay diet, which might indicate that [H] is more effectively redirected into SCFA for that diet. The rumen microbiome analysis showed specific OTUs assigned to the *Prevotella* genus were promoted when methanogenesis was inhibited. Some of these *Prevotella* OTUs promoted by chloroform with the hay:concentrate diet (results not shown), were closely associated with the *Prevotella* group 7 which was increased in goats inhibited with BCM (Mitsumori et al., 2012). *Prevotella* species appear to increase propionate production via the randomizing pathway when methanogenesis is inhibited (Denman et al., 2015). The *Prevotella* OTUs, promoted in the CCD treated animals, may occupy the niche vacated by those *Prevotella* OTUs that declined as a result of increasing CCD concentration. OTUs assigned to the *Butyrivibrio* genus were positively affected by CCD in roughage fed animals, which might be a contributor to the increased butyrate in these animals.

Another potential [H] sink in absence of methanogenesis is acetate produced from reductive acetogenesis (Fonty et al., 2007). In the present study, the acetic acid concentration decreased with CCD treatments, which possibly indicates that reductive acetogenic bacteria have not contributed significantly to the redirection of [H]. However, it has been observed that chloroform might inhibit acetogens (Knight et al., 2011). The notion that acetogens would be promoted through an increase in availability of [H] in the methanogenesis-inhibited rumen remains unresolved and may be confounded by using chemical inhibitors that target methanogenesis but may inhibit reductive acetogenesis.

A particularly interesting observation was the gradual increase in formate concentration as methane formation declined and hydrogen accumulated. This has been observed previously when CH_4 analogs such as chloroform inhibited methanogenesis (Thiele and Zeikus, 1988). A meta-analysis of studies involving methane inhibition also identified that increased formate was a characteristic response to methane inhibition (Ungerfeld, 2015). Leng (2014) suggested that formate accumulates when methanogenesis is inhibited, and this helps to maintain a steady partial pressure of H_2 in the rumen fluid. Formate is produced by ruminal bacteria and fungi and it is mainly consumed by specific methanogens as a precursor for CH_4 formation (Hungate et al., 1970; Bauchop and Mountfort, 1981; Asanuma et al., 1998). Leng (2014) cited studies showing that some methanogens can produce formate when inhibited with halogenated hydrocarbons (Thiele and Zeikus, 1988; Bleicher and Winter, 1994). It is possible therefore that the increase of formate concentration might be due to a balance between an increase in production and a decrease in utilization, when methanogenesis is inhibited. Furthermore, formate has a greater coefficient of diffusion compared to H_2 (Boone et al., 1989). Thus, we hypothesize that more reducing equivalents might be released through pyruvate formate liases compared to pyruvate oxidoreductases when H_2 accumulates. Formate might help in the control of H_2 partial pressures in the rumen, playing a role as hydrogen sink and being an indicator of H_2 partial pressure. This is in accord with the present study where methanogenesis-inhibited animals fed with the hay:concentrate diet had higher concentrations of formate and H_2 release than those only fed with the roughage hay.

A shift in SCFA pattern and increase in formate were not the only significant changes, other metabolites such as amino acids and nucleic acids increased in rumen fluid as H_2 concentration rose. There were increases in amino acids and nucleic acids, which could be indicative of an increase in proteolysis and microbial growth. In our study, metabolites (such as hypoxanthine, inosine, or nicotinic acid) which are degradation products of microbial cells and diet, increased with CCD treatment. Nucleic acids can breakdown into inosine, xanthine, hypoxanthine, and uracil, and nicotinic acid can increase microbial protein synthesis (McAllan and Smith, 1973; Riddell et al., 1980). The amino acid profile showed an increase in valine, leucine, and isoleucine that could be due to greater digestion of protein in the rumen. Another important amino

acid which increased when methanogenesis was inhibited, was aspartate. This amino acid is the transamination product of oxaloacetic acid which is produced in the succinate–propionate (randomizing) pathway that is considered as a major route for propionate synthesis in the rumen (Baldwin et al., 1963; Joyner and Baldwin, 1966). An abundance of genes assigned to this pathway were found to be increased and predominately associated with *Prevotella* species in goats administered with the anti-methanogenic BCM (Denman et al., 2015). Other intermediates of the randomizing pathway, such as malate and fumarate, increased when methanogenesis was inhibited particularly with the roughage diet, supporting the redirection found toward propionate. The increase in amino acids in the rumen may be due to proteolytic activity associated with the relative increase in Bacteroidetes and *Prevotella*-related bacteria. Ungerfeld (2015) suggested that inhibition of methanogens, could stimulate amino acids and fatty acids synthesis and therefore the increase in microbial biomass would also be a [H] sink under these conditions. Also, some studies (Russell and Jeraci, 1984; Russell and Martin, 1984; Hino and Russell, 1985) have shown that methanogenesis suppression resulted in inhibition of deamination of amino acids. This might be due to the antimethanogenic compound used (ionophores and hydrogenase inhibitors) and the extent of methane decreased (almost total suppression). On the other hand, later *in vitro* and *in vivo* studies using halogenated compounds (BCM) or a more specific antimethanogenic compound (3-NOP) showed consistently an increase on branched-chain fatty acids when methane was decreased (between 30 and 50%), which might suggest that deamination of amino acids was not negatively affected (Denman et al., 2007; Mitsumori et al., 2012; Martinez-Fernandez et al., 2014, 2015; Romero-Perez et al., 2014; Haisan et al., 2016). These observations are further supported by the metabolomic data in the present study, which showed an increase in more reduced metabolites and likely greater fermentation of amino acids as shown by increasing concentration of isoacids with increasing CCD dose, particularly in the hay fed animals.

The Bacteroidetes:Firmicutes (B:F) ratio (∼75:17) observed in the untreated animals for both diets was substantially higher than has been reported in many other ruminant studies where temperate diets are common. Our study involving tropical adapted cattle fed a basal diet of tropical hay is in agreement with the high ratio reported by McCann et al. (2014) in Brahman cattle fed Coastal Bermuda-grass. In this study, a further increase in this ratio was observed in both diets when methane was decreased and is consistent with the redirection of hydrogen in the rumen. The Bacteroidetes are considered net H_2 utilizers whereas the Firmicutes phylum contains a higher number of known H_2 producers (Stewart et al., 1997). The same B:F shift was observed when methane was decreased *in vivo* and *in vitro* when methanogenesis was inhibited with BCM (Denman et al., 2015; Martinez-Fernandez et al., 2015). Cattle fed hay:concentrate, had a significant population of Proteobacteria (∼3%) compared with the hay only diet and within this phylum, the predominant family Succinivibrionaceae which are involved in propionate production, increased with the three levels of CCD. A higher abundance of this family in low-emission beef cattle compared with high emitters has also been observed (Wallace et al., 2015).

Bacteria affiliated with the Synergistetes and Verrucomicrobia phyla were negatively correlated with the increasing levels of CCD and increases in H_2, for both diets. A similar negative relationship for these groups of bacteria and increased of H_2 expelled has been previously reported in ruminants treated with BCM (Denman et al., 2015). In high and low methane emitting animals, Synergistetes were significantly more abundant in high emitters and there was a tendency for Verrucomicrobia to also be higher in these animals as well (Wallace et al., 2015). Collectively these data may indicate that Synergistetes and Verrucomicrobia bacteria are sensitive indicators of H_2 partial pressures in the rumen fluid. Recently, Leong et al. (2016) demonstrated that interspecies H_2 transfer between Synergistetes and methanogens enhanced the growth of the bacterium which may explain why these bacteria declined in abundance when methanogens were inhibited in the current study.

In relation to the rumen archaeal community, the A:B ratio decreased with increasing levels of CCD, which represented a fivefold to sevenfold decrease in the methanogen population and a 30–50% decrease in methane production. Furthermore the rumen microbiome analysis revealed that OTUs assigned to Methanobacteriaceae family and Methanoplasmatales order decreased with the increasing doses of CCD. Our results are in agreement with previous studies which observed a similar decrease using halogenated compounds to inhibit enteric methanogenesis in cattle (Denman et al., 2007). Mitsumori et al. (2012) observed that only a half-log reduction in the methanogen population was correlated with 50% decrease in methane when BCM was used. Previous studies (Wallace et al., 2015; Roehe et al., 2016) have also reported a higher proportion of A:B ratio from high methane emitting animals compared with low emitters. The decreasing A:B ratio observed when methanogenesis was inhibited may provide a simple index for relative methane production in ruminants.

It is assumed that H_2 accumulation in the rumen impairs fiber digestion and therefore would reduce productivity (Wolin et al., 1997; Janssen, 2010). However, a previous study showed that inhibition of methanogenesis by BCM in small ruminants dramatically increased H_2 without affecting DMI and feed digestibility (Mitsumori et al., 2012). In the present study, dry matter intakes were not affected and OTUs assigned to the fibrolytic species *R. albus* and *R. flavefaciens* did not change through the CCD low and mid doses compared with control period. Importantly, OTUs classified as *F. succinogenes* were positively associated with CCD in the hay:concentrate diet and were only negatively associated with the lowest dose in both diets. It is known that *F. succinogenes* might not be affected by H_2 accumulation (Wolin et al., 1997). In fact, *F. succinogenes* populations increased in the presence of H_2 that accumulated when BCM was used to inhibit methanogenesis but surprisingly *R. albus* abundance was unaffected even though it produces large amounts of H_2 in the presence of methanogens (Mitsumori et al., 2012). Our results suggest that the bacterial fibrolytic community was not affected by the increasing level of H_2, although the relative abundance of some particular OTUs changed. Further

analyses to study how those changes affect the fiber degradability in the rumen should be carried out in future experiments.

Protozoa and fungi also play a key role in fiber degradation and rumen metabolism, and can be affected by the H_2 accumulation in the rumen. No significant changes were observed in the abundance of those populations that suggests, in accordance with the microbial, metabolite and fermentation profiles, a non-detrimental effect on fiber digestion, and microbial and dietary proteolysis. However, further analyses using in-depth sequencing technology could be developed in future experiments to understand these important rumen community members.

CONCLUSION

The present study in cattle showed that a decrease in methane formation by 30–35% resulted in a redirection of [H] into more reduced microbial end-products and eructation of excess H_2 without an apparent adverse effect on DM intake, fibrolytic activity and general rumen function. The amount of expelled H_2 per mol of decreased methane was lower for the hay diet suggesting a more efficient redirection of [H] perhaps due to the slower fermentation rate and evolution of H_2 compared with the hay:concentrate supplemented animals. The metabolomics analysis showed increases in amino acids and nucleic acids concentration that may indicate an enhanced of the proteolysis and microbial protein synthesis in the rumen, particularly in the methanogenesis-inhibited cattle fed hay. These changes in metabolism were accompanied by a shift in the microbiota toward more Bacteroidetes and a decrease in Archaea and Synergistetes for both diets. Synergistetes among other bacteria groups, may be sensitive indicators of H_2 partial pressures in the rumen. Although there was a redirection of [H], dietary supplements or microbial treatments might be needed to drive the excess H_2 into energy-yielding substrates and consequently improve the energy supply to the animal.

AUTHOR CONTRIBUTIONS

CM, GM-F, and SD conceived and designed the experiments and analytical approaches; GM-F performed the animal trial; GM-F, JC, and CY analyzed biological samples. SD, MM, and GM-F analyzed the data. GM-F, CM, MM, and SD wrote the manuscript. All authors agree to be accountable for all aspects of the work.

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SUPPLEMENTARY MATERIAL

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ORIGINAL RESEARCH
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Phloroglucinol Degradation in the Rumen Promotes the Capture of Excess Hydrogen Generated from Methanogenesis Inhibition

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Strategies to manage metabolic hydrogen ([H]) in the rumen should be considered when reducing ruminant methane (CH_4) emissions. However, little is known about the use of dietary treatments to stimulate rumen microorganisms capable of capturing the [H] available when CH_4 is inhibited *in vivo*. The effects of the phenolic compound phloroglucinol on CH_4 production, [H] flows and subsequent responses in rumen fermentation and microbial community composition when methanogenesis is inhibited were investigated in cattle. Eight rumen fistulated Brahman steers were randomly allocated in two groups receiving chloroform as an antimethanogenic compound for 21 days. Following that period one group received chloroform + phloroglucinol for another 16 days, whilst the other group received only chloroform during the same period. The chloroform treatment resulted in a decrease in CH_4 production and an increase in H_2 expelled with a shift in rumen fermentation toward higher levels of propionate and formate and lower levels of acetate at day 21 of treatment. Bacterial operational taxonomic units (OTUs) assigned to *Prevotella* were promoted whilst Archaea and Synergistetes OTUs were decreased with the chloroform treatment as expected. The shift toward formate coincided with increases in *Ruminococcus flavefaciens*, *Butyrivibrio fibrisolvens*, and *Methanobrevibacter ruminantium* species. The addition of chloroform + phloroglucinol in the rumen resulted in a decrease of H_2 expelled (g) per kg of DMI and moles of H_2 expelled per mol of CH_4 decreased compared with the chloroform only treated animals. A shift toward acetate and a decrease in formate were observed for the chloroform + phloroglucinol-treated animals at day 37. These changes in the rumen fermentation profile were accompanied by a relative increase of OTUs assigned to *Coprococcus* spp., which could suggest this genus is a significant contributor to the metabolism of this phenolic compound in the rumen. This study demonstrates for the first time *in vivo* that under methanogenesis inhibition, H_2 gas accumulation can be decreased by redirecting [H] toward alternative sinks through the nutritional stimulation of specific microbial groups. This results in the generation of metabolites of value for the host while also helping to maintain a low H_2 partial pressure in the methane-inhibited rumen.

Keywords: rumen, microbial community, phloroglucinol, CH_4, H_2, 16S sequencing

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INTRODUCTION

Methane is a potent greenhouse gas and also represents a loss of gross feed energy for the ruminant, hence the increasing interest in strategies to manipulate CH_4 production (Johnson and Johnson, 1995; Gerber et al., 2013). Archaea produce CH_4 in the rumen mainly by reducing C_1 compounds with H_2 (among others substrates) thus maintaining a low hydrogen partial pressure (Janssen, 2010). Although methanogens are the main H_2 consumers, due to the thermodynamically favorable pathway of CH_4 formation, there are rumen bacteria, which are also able to use H_2 as substrate thus generating alternative end products (Leng, 2014). Thus, when CH_4 production is decreased, [H] might be redirected into alternative sinks nutritionally useful for the ruminant (Ungerfeld, 2015a). However, an excessive increase of the H_2 partial pressure in the rumen after methane inhibition might have detrimental effects on rumen function (Wolin et al., 1997). Therefore, management of H_2 accumulation in the rumen is an important strategy to consider when inhibiting CH_4 emissions from livestock.

A recent published study (Martinez-Fernandez et al., 2016), using chloroform as an antimethanogenic compound, has examined the [H] flow and rumen microbial community responses in cattle fed with hay or supplemented with concentrate. It showed that a CH_4 reduction of about 30% had no detrimental effects on rumen function in cattle. With both diets a decrease in CH_4 production was accompanied by an increase in H_2 expelled. Interestingly, the animals fed with the concentrate diet eructated relatively more H_2 than the hay diet suggesting inefficient redirection of [H] into other fermentation end products. The main changes observed in rumen fermentation metabolites with both diets were a shift toward propionate and branched-chain fatty acids; an accumulation of formate, particularly with the concentrate diet; and an increase in the concentration of amino acids, organic and nucleic acids. However, although a redirection of [H] was observed, it was predicted that dietary supplements would be needed to increase the capture of excess H_2 via alternative energy yielding metabolic pathways (Martinez-Fernandez et al., 2016). Failure to capture the excess H_2 released from the system should be considered as an energy loss to the animal.

Microbial metabolic processes, such as reductive acetogenesis, propionogenesis, reduction of nitrate and sulfate, formate formation, and an increase of microbial biomass production when CH_4 is inhibited, have been identified as nutritionally useful [H] sinks in the rumen (Newbold et al., 2005; van Zijderveld et al., 2010; Leng, 2014; Gagen et al., 2015; Ungerfeld, 2015b). Another [H] sink which has gained little attention is the reduction of phenolic compounds by rumen microorganisms. Phenolic compounds, such as flavonoids, are present in many of the forage plants consumed by ruminants. Rumen bacteria, in some cases are capable of transforming these compounds into energy yielding products for the host (Murdiati et al., 1992; McSweeney et al., 2001). Flavonoids undergoing microbial degradation in the rumen generally form phloroglucinol as an intermediate metabolite (Supplementary Figure 1). Several studies have shown that specific rumen bacteria are able to reduce phloroglucinol using H_2 or formate as electron donors thus yielding acetate as the terminal product (Tsai and Jones, 1975; Patel et al., 1981; Krumholz and Bryant, 1986). However, there is scarce information about the use of unconventional dietary treatments that may stimulate rumen microorganisms capable of capturing the [H] available when CH_4 is inhibited *in vivo*.

The aims of the present study were to analyze the effect of phloroglucinol on CH_4 production, [H] flows and subsequent responses in rumen fermentation and microbial community composition in cattle when methanogenesis is inhibited. It was hypothesized that less H_2 would be expelled by the chloroform + phloroglucinol-treated animals due to a shift in fermentation toward acetate by the reduction of the phenolic compound.

MATERIALS AND METHODS

The experimental protocol complied with the Australian Code for the Care and Use of Animals for Scientific Purposes (eighth edition, 2013) and was approved by the local Animal Experimentation and Ethics Committee (A08/2014).

Treatments

The antimethanogen compound used was a halogenated hydrocarbon (chloroform) entrapped in a β-cyclodextrin matrix (6–7% w/w chloroform) as described by Martinez-Fernandez et al. (2016). The phenolic compound used was phloroglucinol (Thermo Fisher Scientific, Scoresby, VIC, Australia; purity of 99%).

Experimental Design and Sampling

Eight rumen fistulated Brahman (*Bos indicus*) steers (live weight, 365 ± 12 kg) at Lansdown Research Station (Townsville, QLD, Australia) were randomly allocated in two groups (four animals per group), receiving a diet *ad libitum* with a ratio 60:40 forage:concentrate (Rhodes grass hay; chemical composition: dry matter (DM), 917 g/kg fresh matter; in g/kg of DM: organic matter, 814; crude protein (CP), 109; neutral detergent fiber (NDF), 651; acid detergent fiber (ADF), 351. Concentrate; Ridley AgriProducts Pty Ltd., Brisbane, QLD, Australia. Ingredients (g/kg): barley (458), sorghum (200), molasses (30), wheat (200), legume hulls (80), urea (2.5); concentrate chemical composition: DM, 887 g/kg fresh matter; in g/kg of DM: CP, 114; NDF, 199; ADF, 130; and fat, 24). Animals were adapted to the diet over 30 days and then placed into individual pens for the measurement of individual intakes (10 days) and treated through the cannula with cyclodextrin (2 g/100 kg LW). On days 9 and 10, animals were placed into open-circuit respiration chambers for measurement of CH_4 and H_2 production and collection of rumen samples. Following the initial control period animals received the chloroform through the rumen-cannula, increasing the dose progressively during 21 days (treatment period 1, 1.6 g/100 kg LW, divided into two doses per day) with the last 2 days confined in open-circuit respiration chambers for direct measurement of CH_4 and H_2 production and rumen fluid collection. After that period, one group received the chloroform + phloroglucinol for

16 days, whilst the other group received only the chloroform treatment (treatment period 2). Phloroglucinol was placed in the rumen through the cannula twice per day, progressively increasing the dose during the first 10 days up to 75 g/100 kg LW (estimated rumen concentration 40 mM). The last 2 days of that period rumen samples and CH_4 and H_2 measurements were taken as previously described.

Rumen fluid samples (approximately 60 ml per animal) were collected through the cannula of the animal using a probe (covered with two layers of cheesecloth) at 3 h post feeding during confinement in respiration chambers to determine the effect on rumen fermentation parameters and rumen microbial communities. Rumen samples were stored at −20°C for short chain fatty acid (SCFA) and NH_3-N analyses. Additionally, 20 ml were kept at −80°C prior to DNA extractions.

Four open circuit respiration chambers were used to determine CH_4 and H_2 production from individual steers as described by Martinez-Fernandez et al. (2016).

Chemical Analysis

The feed samples were dried in a forced-air oven at 65°C prior to grinding. Feed samples were ground through a 1-mm sieve before analysis. DM, ash, NDF, ADF, fat, and total nitrogen contents were analyzed by Symbio Alliance (Eight Mile Plains, QLD, Australia) following the accredited methods CF006.1, CF007, CF038.1, CF038.3, CF004, and CF003.2, respectively [AOAC International (2005) official methods: 925.10, 923.03, 920.39, 990.03, 2002.04, and 973.18]. The nitrogen values were converted to CP by a multiplication factor of 6.25.

Rumen fluid concentrations of SCFAs (acetate, propionate, *n*-butyrate, isobutyrate, isovalerate, and *n*-valerate) were measured by gas chromatography (GC) as described by Gagen et al. (2014). Isovalerate (3-methyl butyrate) includes 2-methyl butyrate, which co-elutes. The rumen NH_3-N concentration was determined by the colorimetric method of Chaney and Marbach (1962).

An UltiMate®3000 HPLC system (Dionex, Sunnyvale, CA, United States) with a dedicated Photodiode Array Detector and an Autosampler was used to determine the concentration of formic acid in rumen fluid as described by Gagen et al. (2014).

Calculation of [H] Redirection and Non-carboxyl SCFA Carbons

The differences between treatments regarding incorporation of [H] into SCFA (HUSr) and formate (HUFr) were estimated using the concentrations of metabolites as a proxy since actual production rates were not measured. The stoichiometry was calculated as described by Martinez-Fernandez et al. (2016). The CH_4 gas production (GP; mol/day) and H_2 gas production (GP; mol/day) was used to calculate the ratio between [H] redirected to H_2 expelled/CH_4 decreased (Martinez-Fernandez et al., 2016).

DNA Extractions

DNA extractions from rumen samples were performed using the cetyltrimethylammonium bromide (CTAB) method of Brookman and Nicholson (2005) with minor modifications as follows: samples were centrifuged (13,000 × *g* for 5 min), and the supernatant was removed before DNA extraction. Cells were homogenized with 200 mg of silica-zirconium beads (1:1 mixture of 0.1- and 1.0-mm beads; Biospec, Bartlesville, OK, United States) and 800 μl of CTAB buffer in a Mini-Beadbeater-8 (Biospec) on maximum speed for 2 min, twice. Samples were incubated at 70°C for 20 min and centrifuged at 10,000 × *g* for 10 min, and the supernatant was mixed with 500 μl of 25:24:1 phenol:chloroform:isoamyl alcohol (Fluka BioChemika, Buchs, Switzerland).

16S rDNA and Statistical Analyses

High throughput sequencing platforms, barcoding procedures for sample recognition and phylogenetic analysis of the 16S rDNA gene were used to characterize the microbial populations present in the rumen for the control and treatment periods. The V4 region of the 16S rRNA gene was targeted using specific primers (Kozich et al., 2013). Each individual DNA sample was amplified using the specific primers and a unique barcode combination. Afterward, amplification products were visualized by performing gel electrophoresis. Product quantities were calculated and an equal molar amount of each product was pooled. The pooled products were run in a 1% agarose gel and bands were visualized and excised under blue light transillumination. The amplicons were gel purified with QIAquick Gel extraction Kit (Qiagen, Hilden, Germany) prior to submission for Illumina Miseq (Macrogen Inc., South Korea).

Paired end short read sequence data generated on the Illumina Miseq was processed using the USEARCH package (Edgar, 2010). De-multiplexed paired end sequences were first merged prior to sequence quality filtering, followed by denoising (error correction) and chimera checking and clustering of sequences to operational taxonomic units (OTUs) of 97% similarity. Taxonomic assignment of sequences was performed against the Greengenes database (McDonald et al., 2012). Additional analysis of OTUs was performed in the R packages vegan, Phyloseq and DESeq2 and the ggplot2 graphics package (McMurdie and Holmes, 2013; Oksanen et al., 2013; Love et al., 2014; Wickham, 2016). The significances of grouping in the PCoA plots were tested by analysis of dissimilarity (ADONIS) with 999 permutations. The sequences obtained in this paper have been deposited in the European Nucleotide Archive under the accession number (PRJEB20458).

The effect of treatment was analyzed for CH_4/H_2 production, DMI, ADWG, and rumen fermentation metabolites. Chloroform only and chloroform + phloroglucinol groups were compared as a univariate model using the GLM procedure of SPSS (IBM, version 21.0), the treatment was considered the fixed effect with the animal as experimental unit. Data from the chloroform only treatment at day 21 and their respective controls (non-treated period) were analyzed separately as a repeated-measures analysis using the linear mixed-model of SPSS (IBM, version 21.0), with the animal as the experimental unit. Effects were declared significant at $P < 0.05$

and *P*-values between 0.05 and 0.10 were considered as a trend.

RESULTS

Ruminal Fermentation and Gas Production

No significant effects were observed on DMI, average daily weight gain, CH_4 and H_2 production (g/kg DMI), and fermentation parameters between experimental groups at either the control period or at day 21 of chloroform treatment before supplementation with phloroglucinol (Supplementary Tables 1, 2). A significant decrease ($P \leq 0.05$) in CH_4 production (g/kg DMI) (40% reduction) and increase in H_2 (1.2 H_2 g/kg DMI) were observed in both groups of animals treated with chloroform compared with their respective control period. The rumen fermentations parameters showed an increase ($P \leq 0.05$) of propionate, branched-chain fatty acids, and formate in chloroform-treated animals compared with the control period (Supplementary Table 3).

Animals that continued to receive only chloroform continued to maintain lower CH_4 emissions, but changes were observed in VFA profiles between days 21 and 37. With continued chloroform treatment there was a decrease in the molar percentage of propionate with concurrent increases in acetate and formate concentration (**Table 1** and Supplementary Table 3). Conversely those animals supplemented with chloroform + phloroglucinol at day 37 had a significant ($P \leq 0.05$) reduction in both the amount of H_2 expelled (g) per kg of DMI and moles of H_2 expelled per mol of CH_4 decreased compared with the chloroform only treated animals (**Table 1**). Rumen fermentation parameters showed a significant increase in acetate and reduction in formate concentration ($P \leq 0.05$) in animals treated with chloroform + phloroglucinol compared with the chloroform only group. Daily weight gain increased significantly ($P \leq 0.05$) in chloroform + phloroglucinol treated animals compared with the chloroform only treated group (1.38 vs 0.438 kg/day, respectively) at day 37.

The calculations of [H] flows showed no significant differences in the [H] redirection into SCFA between chloroform + phloroglucinol treated animals and the chloroform only group (**Figure 1A**). Whereas, the chloroform + phloroglucinol treated animals showed a significant ($P \leq 0.05$) decrease in [H] redirected into formate compared to the chloroform only treatment (**Figure 1B**). Furthermore, a significant decrease ($P \leq 0.01$) of [H] redirected into H_2 per mol of CH_4 decreased (**Figure 1C**) was observed for the chloroform + phloroglucinol group compared with the chloroform only treated group.

Microbial Community

The diversity analysis of the rumen microbiota showed that total microbial species richness and evenness (Shannon and Simpson, respectively) were not impacted significantly either by treatment or time (Supplementary Figure 2).

The structure of the microbiome as determined by non-phylogenetic beta diversity analysis (Bray–Curtis) showed the largest variance was due to time (treatment effect), with the control period more different than treatments at 21 and 37 days (Supplementary Figure 3). Time explained 14% of the variance from the centroid ($P < 0.001$), while the animal groups only explained 4% ($P = 0.03$), showing a separation between chloroform and chloroform + phloroglucinol treated animals at day 37 (beta dispersions; $P = 0.151$).

TABLE 1 | Chloroform and chloroform + phloroglucinol effects on DMI, CH_4, H_2, daily weight gain, and rumen fermentation parameters in animals at day 37.

	Chloroform + phloroglucinol	Chloroform	SEM	*P*-value
N (number of animals)	4	4		
DMI, kg	8.2	7.3	0.35	0.23
CH_4 (g/kg DMI)	11.8	13.8	0.61	0.11
H_2 (g/kg DMI)	0.44	0.89	0.116	0.042
mol H_2/mol CH_4 decreased	0.09	0.21	0.012	0.004
Daily weight gain, kg	1.38	0.453	0.211	0.012
Formate (mM)	0.83	11.3	2.76	0.046
pH	6.43	6.58	0.06	0.27
NH_3-N (mg/100 ml)	14.8	12.9	1.08	0.43
Total SCFA (mM)	94.9	90.4	4.32	0.63
SCFA (mol/100 mol)				
Acetate	65.2	60.7	1.03	0.012
Propionate	19.4	22.8	1.37	0.24
i-Butyrate	0.7	1.1	0.212	0.39
Butyrate	11.6	10.7	0.77	0.59
i-Valerate	0.9	1.2	0.282	0.56
Valerate	1.43	1.60	0.293	0.80
Caprionate	0.88	1.42	0.344	0.48
A:P	3.46	2.74	0.268	0.19

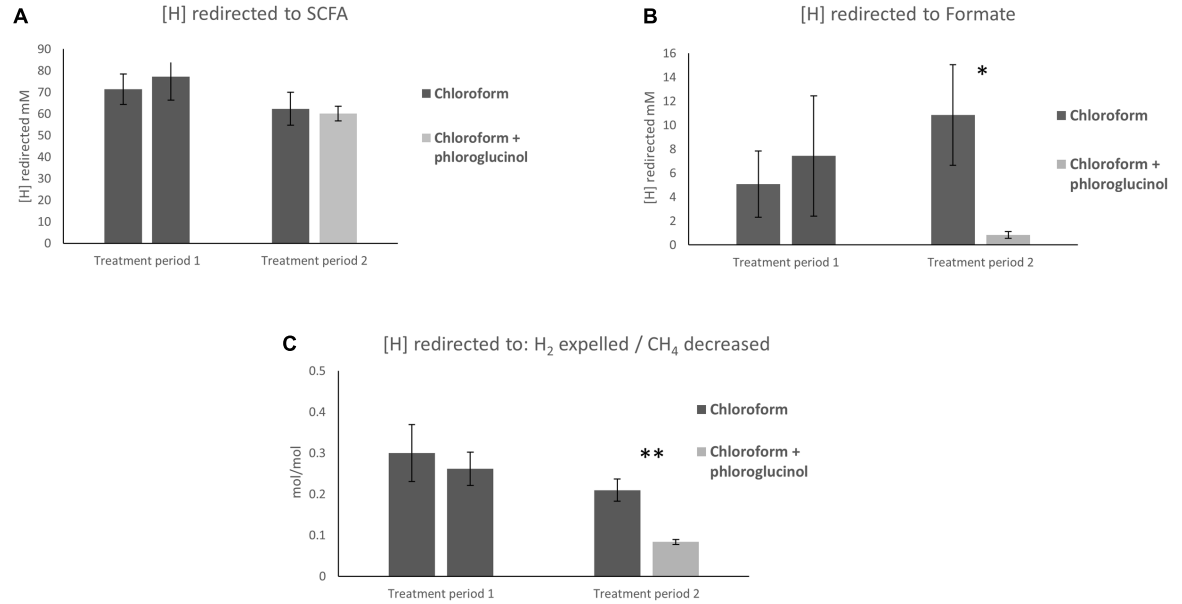

FIGURE 1 | Effect of treatments on **(A)** redirection of [H] to SCFA, **(B)** formate, and **(C)** the ratio between [H] redirected to H_2 expelled/CH_4 decreased. Asterisks "*" and "**" denote significant differences between treatments, $P < 0.05$ and $P < 0.010$, respectively.

The comparison of the rumen microbiome showed a shift in specific OTUs between the chloroform treated animals and the control period, with an increase of OTUs classified in the *Prevotella* genus and a decrease in OTUs assigned to the Archaea domain in the chloroform animals compared with the control period (Supplementary Figure 4). Animals that continued with the chloroform only treatment to day 37 showed decreases in OTUs assigned to *Prevotella* and increases for those assigned with *Ruminococcus*, *Butyrivibrio*, and *Methanobrevibacter* (Supplementary Figures 5–7). The chloroform + phloroglucinol treatment at day 37 was associated with an increase in OTUs affiliated with the genera *Prevotella*, *Ruminococcus*, *Fibrobacter*, CF231, YRC22, and *Coprococcus* compared to animals receiving only chloroform, with OTUs assigned to *Coprococcus* and *Prevotella* showing the greatest fold change (**Figure 2**). Compared to chloroform only treatment, the addition of phloroglucinol altered the methanogen rank abundance back to one more representative of the control animals (**Figure 3**). Within the *Methanobrevibacter* genus, *Methanobrevibacter gottschalkii* and Methanomassiliicoccaceae associated OTUs remained suppressed, while *Methanobrevibacter ruminantium* OTUs were significantly increased on day 37 (**Figure 3**).

The relative abundance of the *Coprococcus* genus was significantly ($P < 0.001$) increased in the chloroform + phloroglucinol treated animals at day 37 compared with the chloroform-only treatment at day 37 and the control period (**Figure 4**). A significant ($P < 0.001$) decrease in the Synergistetes phylum was observed with chloroform and chloroform + phloroglucinol treated animals at day 37 compared with the untreated animals at day 0 (Supplementary Figure 8). Synergistetes members were also more abundant ($P = 0.003$) in chloroform + phloroglucinol treated animals compared with the chloroform only group at day 37.

DISCUSSION

Inhibition of methanogenesis in ruminants using halogenated compounds has previously shown a redirection of [H] toward propionate and branched-chain fatty acids with increased H_2 eructation from the rumen (Mitsumori et al., 2012; Martinez-Fernandez et al., 2016). Likewise, the administration of chloroform to animals in this trial produced a similar response, thus providing a model system for testing the ability to alter rumen metabolic pathways through the capture of excess H_2. A number of alternative [H] sinks have been identified for when methanogenesis is inhibited in ruminant livestock such as: acetogenesis (Joblin, 1999; Fonty et al., 2007; Gagen et al., 2015), propionogenesis (Newbold et al., 2005), nitrate reduction to ammonia (Morvan et al., 1996; Anderson et al., 2003; van Zijderveld et al., 2010; Latham et al., 2016), sulfate reduction to hydrogen sulfite (van Zijderveld et al., 2010), formate formation (Leng, 2014), and increases of microbial biomass production (Ungerfeld, 2015b). However, there is scarce information about the amount of [H] redirected to these metabolic processes or emitted as H_2 gas under *in vivo* conditions. While the rumen microbiota can adapt to inhibition of CH_4 formation by redirecting a proportion of [H] into energy-yielding metabolites, an important proportion of the unutilized [H] accumulates and is eructated as H_2 gas, which still represents an energy loss to the animal. This study demonstrates for the first time *in vivo*

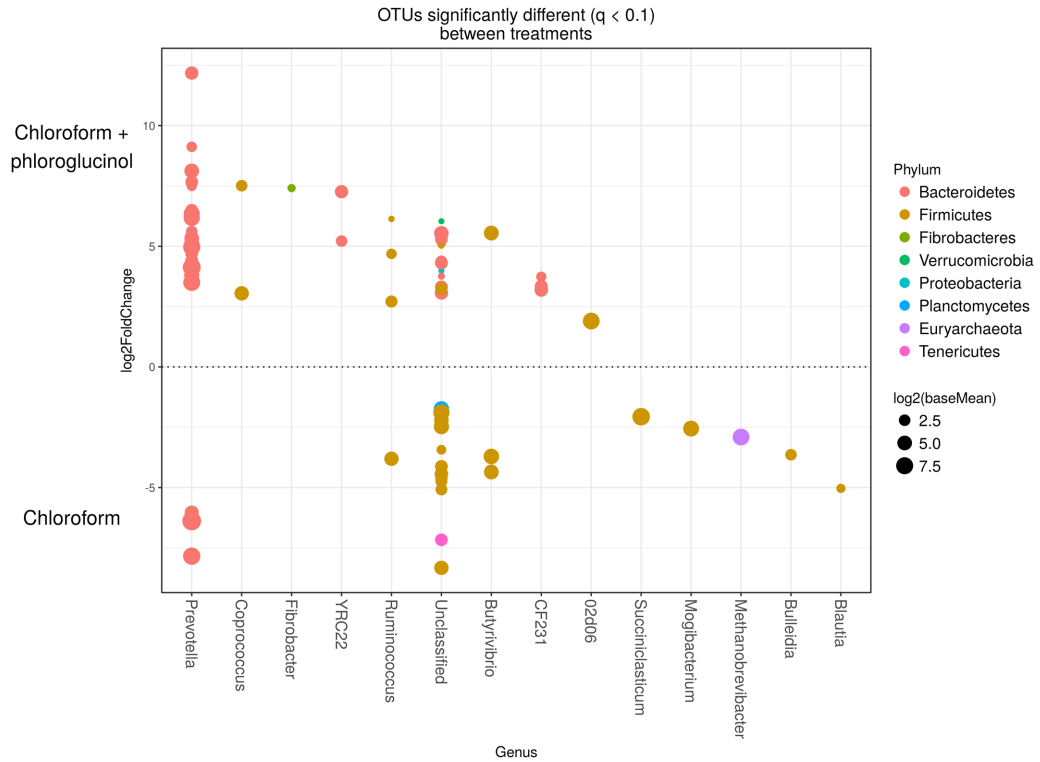

FIGURE 2 | OTUs significantly different ($q < 0.1$ FDR) between chloroform + phloroglucinol and chloroform treated-animals. Upper axis represents OTUs with a log2 fold positive difference for chloroform + phloroglucinol treatment relative to chloroform while the lower *y*-axis is the negative fold difference of the chloroform + phloroglucinol relative to chloroform. Each point represents a single OTU colored by phylum and grouped on the *x*-axis by taxonomic genus level, size of point reflects the log2 mean abundance of the sequence data.

that under methanogenesis inhibition, H_2 gas accumulation can be reduced by redirecting [H] toward alternative sinks through the nutritional stimulation of microbial groups that generate metabolites of value to the host. Therefore, the use of dietary treatments to promote key rumen microbial groups, may be a practical way of capturing [H] that would normally be lost as H_2 gas when a strategy of directly inhibiting methanogens in the rumen is employed to reduce CH_4 formation.

Interestingly, an increase in daily weight gain was observed for the chloroform + phloroglucinol treated animals, indicating that the [H] redirection toward an alternative sink facilitated the utilization of a substrate, which is not normally regarded as an energy-yielding nutrient for the host. Although the increase in weight gain is a promising result, it should be interpreted with caution due to the relatively short length of the trial and small number of animals used. A recent trial with dairy cattle using 3-nitrooxypropanol to reduce CH_4 emissions produced an 80% increase on the average body weight gain over the experimental period (Hristov et al., 2015). While this most likely indicates a shift in metabolism to end products of greater nutritional or metabolic value, the authors also observed a 64-fold increase in hydrogen emissions, suggesting that further efficiencies could be gained. However, to confirm this hypothesis further *in vivo* research on rumen digestibility and metabolism should be carried out.

In relation to the redirection of [H], the inclusion in the rumen of the phenolic substrate phloroglucinol resulted in greater acetate formation with less H_2 expelled per mole of CH_4 decreased, and decreased formate concentration in the rumen. This is in agreement with *in vitro* studies (Patel et al., 1981; Krumholz and Bryant, 1986) that have shown that rumen microorganisms were able to metabolize phloroglucinol to acetate by consuming H_2 or formate as reducing agents. It should be noted that the formula for calculation of the [H] used in SCFA formation (Martinez-Fernandez et al., 2016) does not account for the acetate produced through reductive processes, such as acetogenesis or reduction of phenolic compounds, which most likely explains why there was no difference in the calculated flow of [H] into these organic acids between the treatments. Chloroform + phloroglucinol treated animals expelled per day 1.42 mol of H_2 less than the chloroform only treated group, which is lower than the predicted moles of H_2 (2.18) used to degrade the amount of phloroglucinol provided per day (1 molecule phloroglucinol + 1 molecule H_2 = 2 molecules acetate + 2 molecules carbon dioxide (Tsai et al., 1976; Conradt et al., 2016) (Supplementary Figure 1). This discrepancy might indicate that phloroglucinol was not totally metabolized,

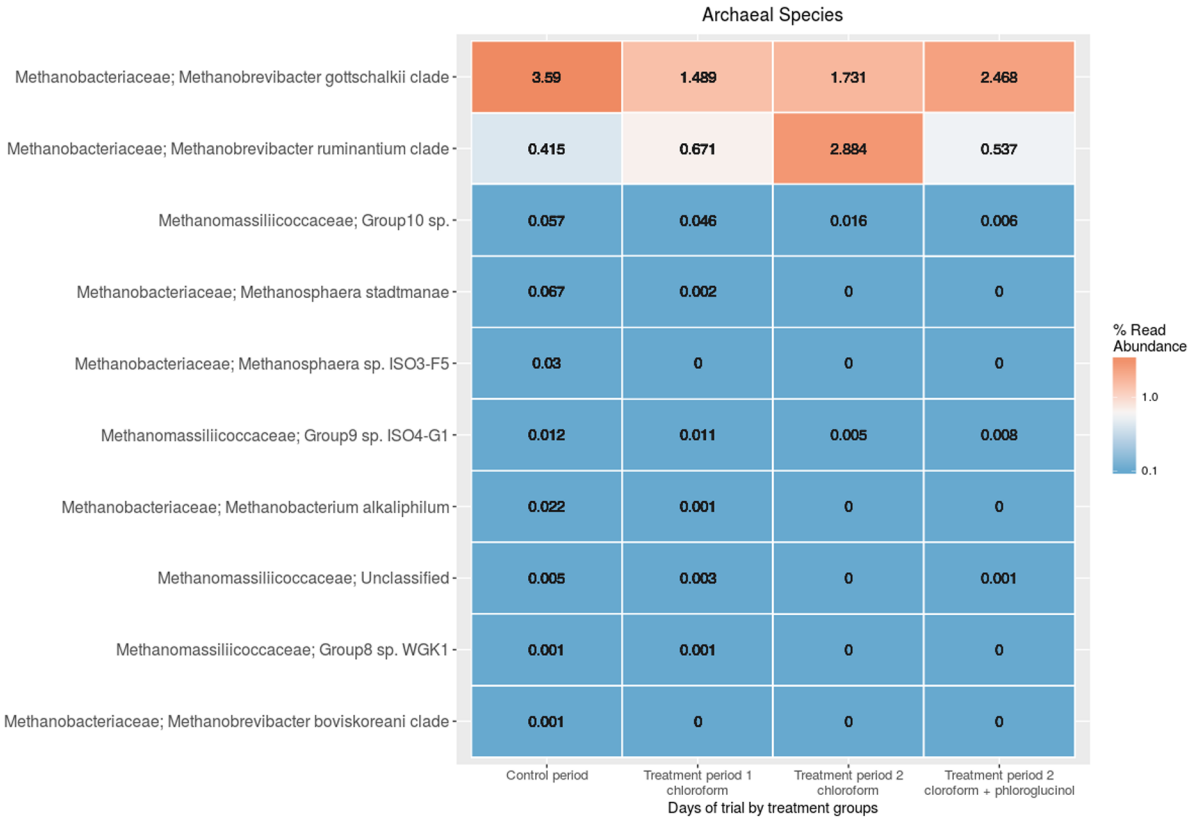

FIGURE 3 | Rumen methanogen community heat maps of the most abundant OTUs using regularized log transformed values from DeSeq2 at control period, treatment period 1 and treatment period 2.

however, near complete degradation of similar quantities of phenolic compounds have been reported previously (Murdiati et al., 1992). It is more likely that both formate and H_2 were used to reduce phloroglucinol based on the lower formate concentrations in the chloroform + phloroglucinol treated animals (0.83 vs 11.3 mM) compared with chloroform only. Formate is produced during normal rumen fermentation and is mainly consumed by methanogens to produce CH_4 (Hungate et al., 1970; Asanuma et al., 1998). Similar to the current study, an increase in formate has been reported previously in steers which were treated with chloroform alone to reduce methanogenesis, particularly with concentrate-supplemented diets (Martinez-Fernandez et al., 2016). Our results support the hypothesis that increased formate accumulation represents a response by the rumen microbiota to control H_2 partial pressures in the rumen, by acting as a hydrogen sink (Leng, 2014; Ungerfeld, 2015b; Martinez-Fernandez et al., 2016).

Members of the Synergistetes phylum are sensitive to H_2 partial pressures within the rumen and generally are reduced when the H_2 partial pressure increases or methanogenesis is restricted (Denman et al., 2015; Wallace et al., 2015; Martinez-Fernandez et al., 2016). Similar reductions in the Synergistetes phylum were observed in this study with the chloroform-only treatment and increased H_2 partial pressures.

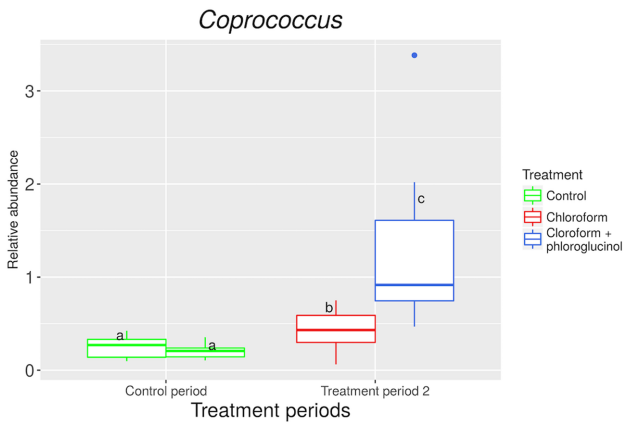

FIGURE 4 | Relative abundance of OTUs assigned to *Coprococcus* genus at control period (untreated animals) and treatment period 2 (chloroform + phloroglucinol and chloroform-treated animals). [a,b]Letters denote significant differences between groups, bars that do not share the same letter are significantly different from each other ($P < 0.05$).

The addition of phloroglucinol and redirection of [H] led to lower partial pressures of H_2 and probably resulted in increases in the Synergistetes phylum further supporting

their significance as a key indicator group around hydrogen transactions.

Of particular interest, was the greater increase in formate concentration when the animals were exposed to longer periods of chloroform, allowing for subsequent increases in the acetate:propionate ratio, presumably driven by the decrease in H_2 partial pressures. Fibrolytic *Ruminococcus* species are able to alter their fermentative pathways to provide alternative reducing equivalent sinks, including the production of formate and ethanol to relieve the inhibition of the reoxidation of NADH (Miller and Wolin, 1974; Shi et al., 1997). With the higher partial pressures of H_2 observed in the rumen for the chloroform treated animals, the shift to increased formate might be linked with increases of *Ruminococcus flavefaciens*, *Butyrivibrio fibrisolvens*, and *M. ruminantium* species. Methanogen species capable of utilizing formate are also able to produce formate from CO_2 and H_2 under conditions of high H_2 partial pressures and when inhibited with halogenated hydrocarbons (Thiele and Zeikus, 1988; Bleicher and Winter, 1994). The observed increase in the relative abundance of *M. ruminantium* over the predominant rumen methanogen *M. gottschalkii*, which is incapable of formate utilization, likely reflects the activation of this mechanism. By converting H_2 and CO_2 to formate while H_2 is in excess and methanogenesis is impeded may ensure survival until conditions become more favorable for producing CH_4, thus providing *M. ruminantium* with a competitive advantage over non-formate utilizing methanogens. The addition of phloroglucinol altered these conditions by redirecting [H] away from formate and reducing the H_2 partial pressure which allowed *M. gottschalkii* to outcompete *M. ruminantium* and once again become the dominant methanogen species in this system. The changes in the microbiota illustrate the ability of the rumen to approach a new equilibrium by adapting to the altered environmental conditions. Of course, this also means that these changes might not correspond to a fully adapted and stable rumen microbiome as is illustrated by the continued shift in the microbiome for the chloroform only group between days 21 and 37. Longer trial periods would need to be studied to fully answer if the rumen has reached a stable microbiota.

Phloroglucinol is a phenolic compound, formed through the degradation of complex plant molecules such as flavonoids, which are commonly present in the diet of the grazing ruminant (Tsai et al., 1976). It is also present at high levels in brown algae (*Ecklonia cava*) which is widely harvested for its medicinal effects (Park et al., 2012). In the rumen, microorganisms degrade this compound predominately to produce acetate and CO_2 as end-products. Our findings are in accordance with previous *in vitro* studies (Tsai et al., 1976; Patel et al., 1981; Krumholz and Bryant, 1986), which observed this increase in acetic acid concentration when phloroglucinol was metabolized by rumen microorganisms. Several rumen bacteria have been identified as phloroglucinol degraders and were classified as *Eubacterium oxidoreducens*, *Streptococcus bovis*, and *Coprococcus* spp. (Tsai et al., 1976; Patel et al., 1981; Krumholz and Bryant, 1986). Krumholz and Bryant (1986) reported that *E. oxidoreducens* required H_2 or formate to degrade phloroglucinol to acetate. The analysis of the rumen microbiome in this study did not identify *E. oxidoreducens* but did reveal several OTUs assigned to *Coprococcus* spp. increased within the chloroform + phloroglucinol treated animals, suggesting the genus is a significant contributor to the metabolism of this phenolic compound. Tsai et al. (1976) found that *Coprococcus* spp. were able to degrade 1 molecule of phloroglucinol to 2 molecules of acetic acid and 2 molecules of carbon dioxide. A more detailed study by Patel et al. (1981) showed that phloroglucinol is initially reduced to dihydrophloroglucinol by *Coprococcus* spp. using NADPH as the electron donor. This would help alleviate the increase in NADPH in the rumen generated by the inhibition of methanogenesis through its oxidation to $NADP^+$ (Chalupa, 1977). Furthermore, a recently published study (Conradt et al., 2016) identified three phloroglucinol reductases belonging to the family of NADPH dehydrogenases/reductases involved in the anaerobic degradation of phloroglucinol which involves hydrolytic ring cleavage to 3-hydroxy-5-oxohexanoic and then acetate formation. However, to confirm our hypothesis, further studies using metagenomics and transcriptomic approaches should be carried out to evaluate the functional genes in *Coprococcus* spp. associated with the NADPH-dependent reduction of phloroglucinol and the redirection of H_2 in the rumen. Furthermore, as the limitations of the trial did not allow for the inclusion of a phloroglucinol only treatment, we cannot ascertain if these effects may have also been possible in the presence of functional methanogenesis and lower H_2 partial pressures. Indeed, a calculated $\Delta G°$ of −158 kJ/mol phloroglucinol to acetate indicates this reaction is likely to be very favorable thermodynamically and may even occur in the presence of hydrogenotrophic methane formation (Kaiser and Hanselmann, 1982; Krumholz and Bryant, 1986).

The *Coprococcus* genus is also involved in other important metabolic pathways in the rumen. Recently, Shabat et al. (2016) found a greater proportion of *Coprococcus* species (*Coprococcus catus*) in the rumen of dairy cows with lower CH_4 emissions and higher feed efficiencies. In the more feed efficient cows there were an increase in abundance of genes aligned to the acrylate pathway, which involves the conversion of lactate to propionate, and were annotated as *C. catus*. In general, the acrylate pathway rather than the succinate pathway for propionate production was more dominant in the efficient animals (Shabat et al., 2016). Other metabolic pathways in which *Coprococcus* spp. are also involved, is the degradation of nitrotoxins in the rumen (Majak and Cheng, 1981) through reductive processes. These and our findings suggest that *Coprococcus* genus is involved in key metabolic pathways in the rumen, and they should be considered when developing strategies to promote microorganisms which might improve the rumen efficiency and metabolize plant toxins in ruminants.

CONCLUSION

The present study in cattle demonstrated that under anti-methanogenesis conditions the addition of

phloroglucinol into the rumen can stimulate microbial groups which utilize H_2 and formate as reductants in the metabolism of phloroglucinol to acetate and thus decrease the partial pressure of H_2 in the rumen and the amount of this gas eructated by the animal. This demonstrates that the negative impact of H_2 accumulation in the rumen under suppression of methanogenesis can be ameliorated by the provision of novel compounds that have nutritional value for the animal when degraded by reductive processes.

AUTHOR CONTRIBUTIONS

CM, SD, and GM-F conceived and designed the experiments and analytical approaches. GM-F performed the animal trial. GM-F and JC analyzed the biological samples. SD and GM-F analyzed the data. GM-F, CM, and SD wrote the manuscript. All authors agree to be accountable for all aspects of the work.

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Supersaturation of Dissolved Hydrogen and Methane in Rumen of Tibetan Sheep

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Hydrogen (H_2) is an essential substrate for methanogens to produce methane (CH_4), and also influences pathways of volatile fatty acids (VFA) production in the rumen. Dissolved H_2 ($H_{2\ (aq)}$) is the form of H_2 available to microbes, and dissolved CH_4 ($CH_{4\ (aq)}$) is important for indicating methanogens activity. Rumen $H_{2\ (aq)}$ concentration has been estimated by assuming equilibrium with headspace gaseous H_2 ($H_{2\ (g)}$) concentration using Henry's law, and has also been directly measured in the liquid phase in some *in vitro* and *in vivo* experiments. In this *in vivo* study, $H_{2\ (aq)}$ and $CH_{4\ (aq)}$ concentration measured directly in rumen fluid and their corresponding concentrations estimated from their gaseous phase concentrations, were compared to investigate the existence of equilibrium between the gas and liquid phases. Twenty-four Tibetan sheep were randomly assigned to two mixed diets containing the same concentrate mixed with oat grass (OG diet) or barley straw (BS diet). Rumen gaseous phase and contents were sampled using rumenocentesis and oral stomach tubing, respectively. Rumen $H_{2\ (aq)}$ and $CH_{4\ (aq)}$ concentration and VFA profile differed between sheep fed OG and BS diets. Measured $H_{2\ (aq)}$ and $CH_{4\ (aq)}$ concentration were greater than $H_{2\ (aq)}$ and $CH_{4\ (aq)}$ concentrations estimated using gas concentrations, indicating lack of equilibrium between gas and liquid phase and supersaturation of H_2 and CH_4 in rumen fluid. As a consequence, Gibbs energy changes (ΔG) estimated for various metabolic pathways were different when calculated using dissolved gases concentrations directly measured and when using dissolved gases concentrations assuming equilibrium with the gaseous phase. Dissolved CH_4, but not $CH_{4\ (g)}$, was positively correlated with $H_{2\ (aq)}$. Both $H_{2\ (aq)}$ and $H_{2\ (g)}$ concentrations were positively correlated with the molar percentage of butyrate and negatively correlated with the molar percentage of acetate. In summary, rumen fluid was supersaturated with both H_2 and CH_4, and $H_{2\ (aq)}$ was closely associated with the VFA profile and $CH_{4\ (aq)}$ concentration. The assumption of equilibrium between dissolved gases and gaseous phase affected ΔG estimation.

Keywords: dissolved hydrogen, dissolved methane, equilibrium, rumen, fermentation pathways, volatile fatty acids

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INTRODUCTION

Carbohydrates are mainly degraded through glycolysis to phosphoenolpyruvate and pyruvate in the rumen. Glycolysis and pyruvate oxidative decarboxylation to acetyl-CoA result in the release of reducing equivalents, which can eventually be transferred to protons, forming hydrogen (H_2). Hydrogen must be removed to facilitate rumen fermentation of feed to produce volatile fatty acids (VFA) (Russell and Wallace, 1997; McAllister and Newbold, 2008). Methane (CH_4) production by methanogenic archaea is the main electron sink in the rumen. However, the production of propionate and butyrate competes with methanogenesis for reducing equivalents, as the metabolism of glucose to propionate and to butyrate incorporates reducing equivalents or results in less reducing equivalents released per mol of hexose fermented compared to acetate production, respectively (Ellis et al., 2008; Janssen, 2010). Furthermore, Gibbs energy changes (ΔG) of CH_4 and VFA production are largely controlled by H_2 concentration (Janssen, 2010).

Concentration of dissolved H_2 ($H_{2\,(aq)}$) in the rumen is central to the thermodynamics of H_2-producing and H_2-utilizing reactions (Janssen, 2010). Hackmann (2013) reported that for several ecosystems there were large differences between ΔG of biochemical pathways calculated using dissolved gases concentrations directly measured and using dissolved gases concentration estimated by assuming equilibrium between the liquid and gas phases. In a recent meta-analysis, the assumption of equilibrium between $H_{2\,(aq)}$ and $H_{2\,(g)}$ likely underestimated the thermodynamic feasibility of H_2-incorporating pathways of *in vitro* rumen fermentation (Ungerfeld, 2015a). Dissolved H_2 concentration has been directly measured in a few experiments with rumen *in vitro* cultures (Hungate, 1967; Wang et al., 2014) and in the rumen *in vivo* (Robinson et al., 1981; Wang et al., 2016). It would be important to compare in the same experiment $H_{2\,(aq)}$ concentration directly measured in the rumen with its estimation assuming equilibrium between $H_{2\,(aq)}$ and $H_{2\,(g)}$, to understand how the assumption of equilibrium between $H_{2\,(aq)}$ and $H_{2\,(g)}$ could affect the estimation of ΔG in the rumen.

Hydrogen is produced as $H_{2\,(aq)}$ and then evolves to the $H_{2\,(g)}$ pool (Wang et al., 2013). The hypothesis for the present study was that rumen fluid is supersaturated in H_2 and CH_4 with respect to the gas phase of the rumen. Based on Hackmann (2013), we define supersaturation as a physical stage at which the concentration of a dissolved gas is above the expected concentration that would result from equilibrium with its gaseous phase. We investigated the relationship between gaseous and dissolved H_2 and CH_4 in the rumen of growing Tibetan sheep fed two different diets. Implications with regard to estimated ΔG of different rumen pathways is analyzed and discussed.

MATERIALS AND METHODS

Animal and Diets

This study was carried out in accordance with the recommendations of regulations of the Administration of Affairs Concerning Experimental Animals, the State Science and Technology Commission of P. R. China. The protocol was approved by the Laboratory Animal Ethical Commission of the Institute of Subtropical Agriculture, Chinese Academy of Sciences. The experiment was conducted at the research farm of the Academy of Agricultural and Animal Husbandry Sciences in Lhasa, Tibet, China (altitude = 3658 m, latitude = N29°30′, longitude = E91°15′, atmospheric pressure = 0.64 atm).

Twenty-four growing Tibetan sheep (body weight = 15.9 ± 1.92 kg) were blocked into two equal groups of males (n = 12) and females (n = 12). Sheep within each group were randomly and equally assigned to two experimental diets. The oat grass (OG) diet was formulated to meet the 1.3 times of maintenance metabolizable energy and crude protein requirements of sheep according to Zhang and Zhang (1998), and contained a pelleted concentrate mixed as a total mixed ration with oat grass at a 50:50 ratio (DM basis). The pelleted concentrate ingredients were (DM basis): 45 g/kg soybean meal, 470 g/kg corn, 424 g/kg wheat bran, 7 g/kg calcium carbonate, 9 g/kg palm oil, 9 g/kg sodium chloride and 36 g/kg minerals, and vitamins premix. The chemical composition of OG diet is shown in **Table 1**. The barley straw (BS) diet had the same pelleted concentrate of OG diet, and was formulated using barley straw to replace oat grass in the OG diet. The amount of BS diet allocated daily was set to be the same as that of OG diet. Both two diets were offered in two meals in equal proportions at 0900 and 1800. All sheep had free access to fresh water. The experiment comprised 30 d of adaptation to diets followed by a 2-days collection period. Feed refusals were recorded, and feed and refusals samples were collected, during the last 5 days (from days 26 to 30) of the adaption period.

The chemical composition of forage and total mixed ration is provided in **Table 1**. Contents of organic matter, gross energy and Kjeldahl N (CP = 6.25 × N) were determined according to AOAC (1995). Neutral detergent fiber (NDF) and acid detergent fiber were expressed inclusive of residual ash (Van Soest et al., 1991), and NDF assayed with the addition of a heat stable amylase, but without sodium sulfite. Starch content was determined after pre-extraction with ethanol (80%), and glucose released from starch by enzyme hydrolysis was measured using amyloglucosidase (Kartchner and Theurer, 1981).

Rumen Sampling

Samples of rumen gas and contents were collected before the morning feeding in the last 2 days of the collection period, with 12 sheep in each day (six males and six females randomly chosen within each diet). Rumen headspace gas was sampled using the rumenocentesis method of Moate et al. (1997) with a slight modification. A 150-mm, 14-g needle was inserted into the headspace of the rumen via the central area of the left paralumbar fossa, which had been trimmed of hair and then swabbed with 72% ethanol. A 50-mL syringe, fitted with a T-shaped tube, was attached to the 150-mm needle to collect 30 mL of headspace gas from the rumen. The collected gas was then injected into 10-mL evacuated tubes for subsequent determination of $H_{2\,(g)}$ and $CH_{4\,(g)}$ concentration.

Rumen contents were sampled using oral stomach tubing immediately after collecting rumen headspace gas. A flexible PVC tube (2 mm of wall thickness and 6 mm of internal

TABLE 1 | Chemical composition of diets and dry matter intake of Tibetan sheep.

DM	Forage		Diet[a]		SEM	*P*-value
	Oat grass	Barley straw	Oat grass	Barley straw		
	949	955	955	967	–	–
CHEMICAL COMPOSITION (g/kg DM)						
OM	952	923	932	918	–	–
CP	43.4	14.2	73.9	59.4	–	–
NDF	597	709	483	539	–	–
ADF	387	458	264	300	–	–
Starch	75.0	61.0	186	179	–	–
EE	62.0	61.0	69.4	65.7	–	–
NFC	250	139	306	254	–	–
ME[b] (MJ/kg DM)	1.41	1.11	7.76	7.13	–	–
DM intake (g/d)	–	–	609	582	13.3	0.32

DM, dry matter; OM, organic matter; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; EE, ether extract; NFE, non-fibrous carbohydrates, calculated by the equation of OM-CP-EE-CF.

[a]Forage plus concentrate diet (1:1). The concentrate contained (g/kg) soybean meal (45), corn (470), wheat bran (424), calcium carbonate (7), palm oil (9), sodium chloride (9), and premix (36).

[b]Metabolizable energy (ME) was estimated according to Zhang and Zhang (1998).

diameter) was warmed-up using hot water (about 50°C) and inserted to a depth of ∼120–150 cm via the esophagus. The first 100-mL of rumen contents were discarded to avoid saliva contamination, and the following 150 mL of rumen contents were rapidly collected for subsequent determination of rumen $H_{2\ (aq)}$ and $CH_{4\ (aq)}$ concentrations and fermentation end-products. Two 35-mL subsamples were immediately transferred to 50-mL plastic syringes for measuring $H_{2\ (aq)}$ and $CH_{4\ (aq)}$ concentration as explained below (see section Measured Dissolved Gases Concentration). The rumen pH was measured immediately after sampling using a portable pH meter (Starter 300; Ohaus Instruments Co. Ltd., Shanghai, China).

Analyses of Fermentation End Products

Two milliliters samples of strained rumen fluid were centrifuged at 15,000 g for 10 min at 4°C. One and a half milliliters of supernatant were transferred into tubes containing 0.15 mL of 25% (w/v) metaphosphoric acid. The mixture was vigorously hand-shaken and stored at −20°C for subsequent determination of fermentation end-products. After thawing, the acidified samples were re-centrifuged at 15,000 g for 10 min at 4°C, the pellet discarded, and volatile fatty acids (VFA) analyzed in the supernatant using gas chromatography (Agilent 7890A, Agilent Inc., Palo Alto, CA), according to the method described by Wang et al. (2014). Ammonia, lactic acid, and glucose were determined colorimetrically according to the methods of Weatherburn (1967), Taylor (1996), and Nelson (1944) respectively.

Determination of Headspace Gases Concentration

Concentration of $H_{2\ (g)}$ and $CH_{4\ (g)}$ in the collected rumen headspace gas were determined by gas chromatography (Agilent 7890A, Agilent Inc., Palo Alto, CA) using a thermal conductivity and a flame ionization detector, respectively. Hydrogen and CH_4 were separated using a Hayesep Q packed column (2.44 m × 1/8 in. × 2.0 mm ID). Carbon dioxide concentration in the rumen headspace gas was calculated as the difference between total gas concentration at the local atmospheric pressure, calculated in turn using the Ideal Gas Law (0.0262 M at 0.64 atm), and the sum of $H_{2\ (g)}$ and $CH_{4\ (g)}$ concentrations.

Estimation of Dissolved Gases Concentration Based on Headspace Gases Concentration

Concentrations of $H_{2\ (aq)}$ and $CH_{4\ (aq)}$ estimated as if they were at equilibrium with the gaseous phase were calculated based on rumen headspace $H_{2\ (g)}$ and $CH_{4\ (g)}$ concentrations, respectively. Wiesenburg and Guinasso (1979) proposed calculating gas solubility based on the Bunsen absorption coefficient, vapor pressure, gas concentration, atmospheric pressure, and relative humidity, according to:

$$Gas_{\ (aq)} = \alpha Gas_{\ (g)}(P_t - P_{VP}h/100) \quad (1)$$

where $Gas_{\ (aq)}$ is the concentration of the dissolved gas of interest in the liquid phase (mM), α is the Bunsen absorption coefficient of the gas of interest (L of dissolved gas/(L of liquid·atm), $Gas_{\ (g)}$ is the concentration of the gas of interest in the gas phase (mM), P_t is atmospheric pressure (atm), h is the relative humidity (%), and P_{vp} is the liquid vapor pressure (atm).

The vapor pressure is greatly affected by the concentration of dissolved salts (Wiesenburg and Guinasso, 1979). Our previous study indicated that Bunsen absorption coefficients were similar for pure water and the McDougall's buffer at the same temperature (Wang et al., 2014), therefore the factor including vapor pressure in the McDougall's buffer could be assumed to be the same as pure water, and then set to be zero in Equation (1) to calculate the dissolved gas in rumen fluid. Concentrations of $H_{2\ (aq)}$ and $CH_{4\ (aq)}$ estimated from $H_{2\ (g)}$ and

$CH_{4\ (g)}$ concentrations ($eH_{2\ (aq)}$ and $eCH_{4\ (aq)}$, respectively), in the rumen headspace were calculated as:

$$eGas_{\ (aq)} = Gas_{\ (g)}\alpha_{gas}P_t \quad (2a)$$

with:

$$\alpha_{H2} = \exp(-47.8948 + 65.0368(100/T) + 20.1709\ln(T/100)) \quad (2b)$$

$$\alpha_{CH4} = \exp(-68.8862 + 101.4953(100/T) + 28.7314\ln(T/100)) \quad (2c)$$

where $eGas_{\ (aq)}$ is the estimated concentration of the dissolved gas of interest (mM), $Gas_{\ (g)}$ is the corresponding gas concentration measured in the rumen headspace (mM), α_{gas} is the Bunsen absorption coefficient for each gas of interest (H_2 or CH_4) at 1 atm pressure (L of dissolved gas/(L of liquid·atm) calculated as function of absolute temperature T for a rumen temperature of 39°C (Wiesenburg and Guinasso, 1979), and P_t is 0.64 atm.

Measured Dissolved Gases Concentration

Rumen $H_{2\ (aq)}$ and $CH_{4\ (aq)}$ concentration were measured by establishing an equilibrium between the gas and the liquid phase in a sealed vessel containing a rumen fluid sample using the procedure decribed by Wang et al. (2014). Briefly, a 50-mL plastic syringe, containing 35 mL rumen fluid, was fitted with a T tube, which was closed immediately and cooled to room temperature. A 20-mL syringe was filled with 10 mL of N_2 gas, and connected to 50-mL plastic syringe via the T tube. The N_2 gas was then injected into the 50-mL syringe through the T tube, and the gases dissolved in the rumen fluid were extracted into the N_2 gas phase by vigorous hand shaking for 5 min. The volumes of gas and liquid phases were recorded using the scales in the small and large syringes, respectively. The room temperature was recorded as well. Gas samples from the 20-mL syringe were collected in evacuated tubes for the measurement of H_2 and CH_4 concentration using gas chromatography as above described (Agilent 7890A, Agilent Inc., Palo Alto, CA, USA).

Total H_2 or CH_4 before extraction is equal to that after extraction:

$$V_l Gas_{\ (aq)} = V_l Gas_{\ (g)}\alpha_{\ gas}P_t + V_{\ g}Gas_{\ (g)} \quad (3)$$

where V_l is the liquid volume (mL); $Gas_{\ (aq)}$ is the concentration of the dissolved target gas (H_2 or CH_4) in the original liquid sample (mM), $Gas_{\ (g)}$ is the target gas (H_2 or CH_4) concentration in the gas phase at equilibrium after extraction (mM), α_{gas} is the Bunsen absorption coefficient of each target gas (H_2 or CH_4) at room temperature, as calculated using Equations (2b) and (2c) (L of dissolved gas/(L of liquid·atm), P_t is the local atmospheric pressure equal to 0.64 atm, and V_g is the gas volume at equilibrium after extraction (mL).

Therefore, the rumen aqueous concentration of each dissolved target gas (H_2 or CH_4) is equal to:

$$Gas_{\ (aq)} = Gas_{\ (g)}(\alpha_{\ gas}P_t + V_g/V_l) \quad (4)$$

We could not use the method described by Wang et al. (2014) to determine the concentration of dissolved CO_2($CO_{2\ (aq)}$), because the release of CO_2 in solution toward the gas phase would displace the equilibrium from bicarbonate and carbonic acid toward additional $CO_{2\ (aq)}$ and finally extra $CO_{2\ (g)}$. For ΔG calculations, $CO_{2\ (aq)}$ concentration was calculated using Eq. 2 by assuming equilibrium with $CO_{2\ (g)}$ at the local atmospheric pressure of 0.64 atm. The Bunsen absorption coefficient for CO_2 in rumen fluid was set to be 0.234 volume/(volume atm) (Hille et al., 2016).

Calculation of the Saturation Factor

The saturation factor (Sf) was defined as the ratio between the measured dissolved gases ($H_{2\ (aq)}$ and $CH_{4\ (aq)}$) concentration, and the concentration of dissolved gases estimated based on headspace $H_{2\ (g)}$ and $CH_{4\ (g)}$ concentrations ($eH_{2\ (aq)}$ and $eCH_{4\ (aq)}$)(Wang et al., 2014):

$$Sf_{\ gas} = \frac{Gas_{\ (aq)}}{eGas_{\ (aq)}} \quad (5)$$

"$Sf_{gas} > 1$" and "$Sf_{gas} < 1$" indicate supersaturation and undersaturation of the dissolved gas in the liquid phase of the rumen, respectively.

Calculation of the Gibbs Energy Changes of Fermentation Pathways

Ingested dietary polysaccharides are hydrolyzed to hexoses in the rumen, which are fermented to VFA. Acetate production results in the release of reducing equivalents:

$$C_6H_{12}O_6 + 2\ H_2O \rightarrow 2\ \text{acetate} + 2\ CO_2 + 4\ [2H] + 2\ H^+ \quad \text{(Reaction 1)}$$

Interconversion between VFA has been shown to occur in the rumen (Ungerfeld and Kohn, 2006). Conversion of acetate to propionate and butyrate incorporates reducing equivalents:

$$\text{acetate} + CO_2 + 3\ [2H] \rightarrow \text{propionate} + 2\ H_2O \quad \text{(Reaction 2)}$$

$$2\ \text{acetate} + H^+ + 2\ [2H] \rightarrow \text{butyrate} + 2\ H_2O \quad \text{(Reaction 3)}$$

Fermentation shifts of acetate to propionate and acetate to butyrate then result in incorporation of reducing equivalents. Different examples of fermentation stoichiometries with differing [2H] release per mol of hexose fermented (Janssen, 2010), expressed as H_2 production in the following equations, can be generated by replacing the products of Reaction 1 with the reactants of Reaction 2 and 3:

$$C_6H_{12}O_6 \rightarrow 2/3\ \text{acetate} + 4/3\ \text{propionate} + 2/3\ CO_2 + 2/3\ H_2O + 2H^+ \quad \text{(Reaction 4)}$$

$$C_6H_{12}O_6 \rightarrow \text{acetate} + \text{propionate} + CO_2 + H_2 + 2\ H^+ \quad \text{(Reaction 5)}$$

$$C_6H_{12}O_6 \rightarrow \text{butyrate} + H^+ + 2\,CO_2 + 2\,H_2 \quad \text{(Reaction 6)}$$

$$C_6H_{12}O_6 + H_2O \rightarrow \text{acetate} + \tfrac{1}{2}\,\text{butyrate} + 2\,CO_2 + 3\,H_2 + \tfrac{3}{2}\,H^+ \quad \text{(Reaction 7)}$$

The amount H_2 production per mol of hexose fermented can thus vary widely.

Another two pathways of H_2 incorporation are reductive acetogenesis for acetate production and methanogenesis for CH_4 production:

$$2\,CO_2 + 4\,H_2 \rightarrow \text{acetate} + H^+ + 2\,H_2O \quad \text{(Reaction 8)}$$

$$CO_2 + 4\,H_2 \rightarrow CH_4 + 2\,H_2O \quad \text{(Reaction 9)}$$

The thermodynamic feasibility of these reactions was estimated through their ΔG. Gibbs energy changes of reactions at standard conditions (ΔG°) were calculated from standard Gibbs energy of formation (ΔG°_f) of reactants and products (Kohn and Boston, 2000):

$$\Delta G^\circ = \Delta G^0_{f\text{products}} - \Delta G^0_{f\text{reactants}} \quad (6)$$

Standard Gibbs energy changes so calculated for 298 K (0°C) were then adjusted to a rumen temperature of 312 K using the van't Hoff equation (Kohn and Boston, 2000). Gibbs energy changes estimated for actual rumen conditions were subsequently adjusted by the concentration of soluble metabolites and dissolved gases (Kohn and Boston, 2000):

$$\Delta G = \Delta G^\circ + RT\text{Ln}(\Pi_{i=1}{}^{i=n}[\text{Product}]_i{}^{\text{product i}} / \Pi_{i=1}{}^{i=n}[\text{Reactant}_i]^{\text{reactant i}}) \quad (7)$$

where $\Pi_{i=1}{}^{i=n}$ [Products] and $\Pi_{i=1}^{i=n}$ [Reactants] are the products of molar concentration of products and reactants in the liquid phase, respectively, each elevated to its corresponding stoichiometric coefficient, R is the gas constant equal to 8.314 J atm K^{-1} mol^{-1}, T is the rumen temperature in Kelvin, and ΔG° is the standard ΔG for the reaction adjusted to 39°C. Reactions are thermodynamically feasible if $\Delta G < 0$, at equilibrium if estimated $\Delta G = 0$, and unfeasible when $\Delta G > 0$.

Gibbs energy changes of various fermentation stoichiometries, as well as of methanogenesis, reductive acetogenesis, and VFA interconversions were calculated and compared using either directly measured $CH_{4\,(aq)}$ and $H_{2\,(aq)}$ concentrations, or concentrations of $CH_{4\,(aq)}$ and $H_{2\,(aq)}$ estimated from $CH_{4\,(g)}$ and $H_{2\,(g)}$ concentrations using Henry's Law by assuming equilibrium between gaseous and dissolved gases. Gibbs energy changes calculated using both methods shared the estimation of $CO_{2\,(aq)}$ and measured glucose and individual VFA concentration.

Statistics

The effect of diet on DM intake, rumen pH, and the concentrations and supersaturation indexes of gases, total VFA concentration, individual VFA molar percentages and ammonia concentration were evaluated through a one-way ANOVA.

Concentrations of $H_{2\,(g)}$, $CH_{4\,(aq)}$, $CH_{4\,(g)}$, and VFA molar percentages, were regressed against $H_{2\,(aq)}$ concentration as follows:

$$y = \text{intercept} + H_{2\,(aq)} + H^2_{2(aq)} + \text{diet} + H_{2\,(aq)} \times \text{diet} + \text{residual}$$

Statistical significance was set at $P < 0.05$ and tendencies at $0.05 \leq P \leq 0.10$. The main effect of the diet, the quadratic effect of $H_{2\,(aq)}$ and the interaction were removed from the models if their $P > 0.10$, and the reduced models re-fitted.

Gibbs energy changes for nine different pathways were analyzed as 2 × 2 factorials including the main effects of diet, method of estimation (calculation using $CH_{4\,(aq)}$ and $H_{2\,(aq)}$ concentration directly measured or using $eCH_{4\,(aq)}$ and $eH_{2\,(aq)}$ concentration estimated from $CH_{4\,(g)}$ and $H_{2\,(g)}$ concentration), and their interaction.

All statistical analyses were conducted with JMP® 12.1.0. (SAS Institute Inc.).

RESULTS

Sheep on the OG and BS diets had similar DM intake ($P = 0.32$; **Table 1**), rumen pH ($P = 0.20$), total VFA concentration ($P = 0.17$), and propionate ($P = 0.60$) and valerate ($P = 0.102$) molar percentages (**Table 2**), as well as $CO_{2\,(g)}$ ($P = 0.74$) and $CH_{4\,(g)}$ ($P = 0.76$) concentrations, and Sf_{H2} ($P = 0.67$; **Table 3**). Rumen glucose ($P = 0.002$), $H_{2\,(aq)}$ ($P = 0.017$), $CH_{4\,(aq)}$ ($P = 0.07$), $H_{2\,(g)}$ ($P = 0.001$), butyrate molar percentage ($P < 0.001$), and Sf_{CH4} ($P = 0.08$), were greater or tended to be greater in sheep fed the OG diet. Acetate ($P = 0.022$), *iso*-butyrate ($P = 0.053$), and *iso*-valerate ($P = 0.065$), and ammonia concentration ($P = 0.041$) molar percentages were greater or trended to be greater in sheep fed the BS diet (**Table 2**).

There was a positive quadratic relationship between $H_{2\,(g)}$ and $H_{2\,(aq)}$ ($R^2 = 0.91$; $P < 0.001$; **Figure 1**) without an interaction with the diet ($P = 0.90$). Measured $H_{2\,(g)}$ was roughly between one and two orders of magnitude smaller than $H_{2\,(g)}$ concentration predicted by assuming equilibrium with $H_{2\,(aq)}$ (**Figure 1**). There was a positive relationship between $CH_{4\,(aq)}$ and $H_{2\,(aq)}$ ($P < 0.001$) with a tendency to be quadratic ($P = 0.056$; **Figure 2**) and without an interaction with diet ($P = 0.95$). However, there was no relationship between $CH_{4\,(g)}$ and $H_{2\,(aq)}$ ($P = 0.40$; **Figure 2**), and no relationship between $CH_{4\,(g)}$ and $CH_{4\,(aq)}$ ($P = 0.91$; not shown). The saturation factors for H_2 and CH_4 were greater than unity ($P < 0.001$; **Table 3**) and positively correlated to dissolved gases concentrations (**Figures 3A,B**). The response of H_2 saturation factor to $H_{2\,(aq)}$ was affected by the diet (**Figure 3A**).

There were negative linear relationships between acetate molar percentage and $H_{2\,(aq)}$ ($P = 0.003$; **Figure 4A**) and $H_{2\,(g)}$ ($P = 0.003$; **Figure 4B**) without interactions with the

TABLE 2 | Fermentation end-productions in the rumen of Tibetan sheep feeding by two diets ($n = 12$).

	Diet		SEM	*P*-value
	Oat grass	Barley straw		
pH	6.85	6.94	0.047	0.20
Total VFA (mM)	109	81.0	5.51	0.17
INDIVIDUAL VFA MOLAR PERCENTAGE (%)				
Acetate	66.6	69.8	0.90	0.021
Propionate	19.5	18.9	0.78	0.60
Butyrate	10.5	7.75	0.41	< 0.001
Valerate	0.93	0.81	0.051	0.102
Iso-butyrate	1.40	1.62	0.077	0.053
Iso-valerate	0.97	1.12	0.055	0.065
Acetate:Propionate (mol/mol)	3.51	3.73	0.171	0.36
Lactate (mM)	1.30	1.25	0.14	0.81
Glucose (mM)	2.64	1.88	0.11	0.002
Ammonia (mM)	6.09	8.18	0.0678	0.041

BS, barley straw; OG, oat grass; VFA, volatile fatty acids.

TABLE 3 | Gaseous and dissolved gases concentration in the rumen of Tibetan sheep fed two different diets ($n = 12$).

	Diet		SEM	*P*-value
	Oat grass	Barley straw		
$H_{2\ aq}$ (μM)	6.49	2.34	1.13	0.017
$CH_{4\ (aq)}$ (mM)	0.932	0.680	0.0943	0.072
$H_{2\ (g)}$ (μM)	7.86	3.36	0.852	0.001
$CH_{4\ (g)}$ (mM)	4.15	4.11	0.111	0.76
$CO_{2\ (g)}$ (mM)	22.1	22.2	0.114	0.74
Sf_{H2}	42.7*	38.4*	6.90	0.67
Sf_{CH4}	7.88*	5.79*	0.81	0.08

BS, barley straw; $CH_{4\ (aq)}$, dissolved methane concentration in the original liquid sample; $CH_{4\ (g)}$, gaseous methane concentration in the rumen; $H_{2\ (aq)}$, dissolved hydrogen concentration in the original liquid sample; $H_{2\ (g)}$, gaseous hydrogen concentration in the rumen; OG, oat grass; Sf_{CH4}, CH_4 saturation factor; Sf_{H2}, H_2 saturation factor.
**Saturation factor significantly ($P < 0.05$) different from unity.*

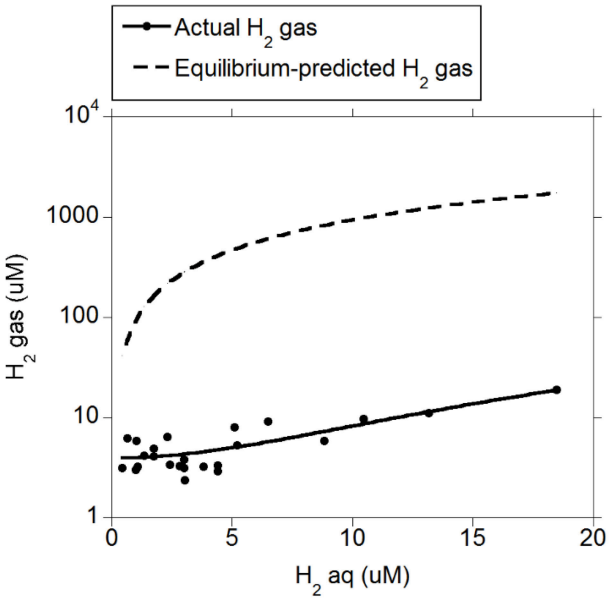

FIGURE 1 | Relationship between measured and equilibrium-predicted gaseous H_2 and dissolved H_2 concentration: $H_{2\ (g)} = 3.83$ (±0.43; $P < 0.001$) ± 1.20 (±0.28; $P < 0.001$) diet + 0.18 (±0.11; $P = 0.11$) ($H_{2(aq)}$) + 0.052 (±0.011; $P < 0.001$) $(H_{2\ (aq)} - 4.42)^2$; $R^2 = 0.91$ ($P < 0.001$); Equilibrium-predicted $H_{2\ (g)}$: $H_{2\ (aq)}/(\alpha \times P_t)$, where α is the Bunsen coefficient and P_t is the atmospheric pressure.

diet ($P > 0.71$). There was no relationship between propionate molar percentage and $H_{2\ (aq)}$ ($P = 0.24$; **Figure 5A**) or $H_{2\ (g)}$ ($P = 0.36$; **Figure 5B**) concentration. There were positive linear relationships between butyrate molar percentage and $H_{2\ (aq)}$ ($P = 0.001$; **Figure 6A**) and $H_{2\ (g)}$ ($P < 0.001$; **Figure 6B**) concentrations.

There were no interactions between diet and method of ΔG estimation, so only main effects are presented (**Table 4**). With both diets, ΔG estimated using measured dissolved gases was greater ($P < 0.001$) for H_2-producing reactions, and lesser for H_2-incorporating reactions, when compared to ΔG estimated using dissolved gases concentration estimated from their gaseous phase concentration (**Table 4**).

DISCUSSION

Effect of Forage Type on Rumen Fermentation End-Products

Different forages influence rumen fermentation and CH_4 production in ruminants, because dietary carbohydrate composition varies considerably with forage species and state of maturity (Chaves et al., 2006). Lower content of structural carbohydrates is associated with greater rate of digestion and fermentation, which in turn is associated with greater H_2 concentration (Janssen, 2010). In our study, the OG diet had lower NDF and ADF content than the BS diet, which would agree with sheep consuming the OG diet having higher $H_{2\ (aq)}$ and $CH_{4\ (aq)}$ and total VFA concentration in the rumen compared to those fed the BS diet. Such differences seem likely to be due to the greater content of fermentable carbohydrates in the OG diet. In an *in vitro* experiment, higher $H_{2\ (aq)}$ and total VFA concentration were also observed for incubated substrates that typically have higher rate and extent of degradation, although rumen degradation was not measured in that study (Wang et al., 2014). Other factors potentially affected by the diet, such as rumen passage rate and fractional rate of VFA absorption, also influence the VFA concentration in the rumen (Dijkstra et al., 1993).

Sheep fed the OG diet had lower molar percentage of acetate and higher molar percentage of butyrate than sheep fed the BS diet. Higher NDF content in the BS diet may account for greater molar percentage of acetate (Brask et al., 2013). Rumen

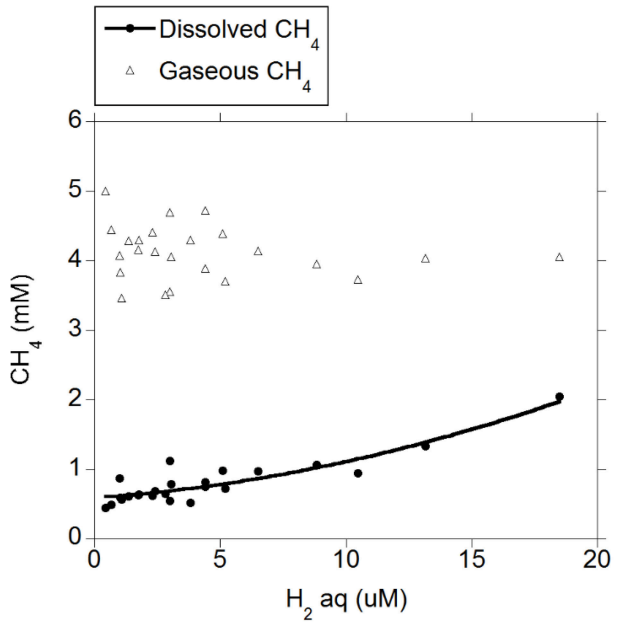

FIGURE 2 | Relationship of dissolved H_2 concentration with dissolved and gaseous CH_4: $CH_{4\ (aq)}$ = 0.54 (±0.049; P < 0.001) + 0.049 (±0.013; P < 0.001) $H_{2\ (aq)}$ + 0.0027 (±0.0013; P = 0.056) $(H_{2\ (aq)} - 4.42)^2$; R^2 = 0.82 (P < 0.001); Gaseous CH_4: $CH_{4\ (g)}$ = 4.20 (±0.11; P < 0.001) − 0.016 (±0.019; P = 0.40) $H_{2\ (aq)}$; R^2 = 0.032 (P = 0.40).

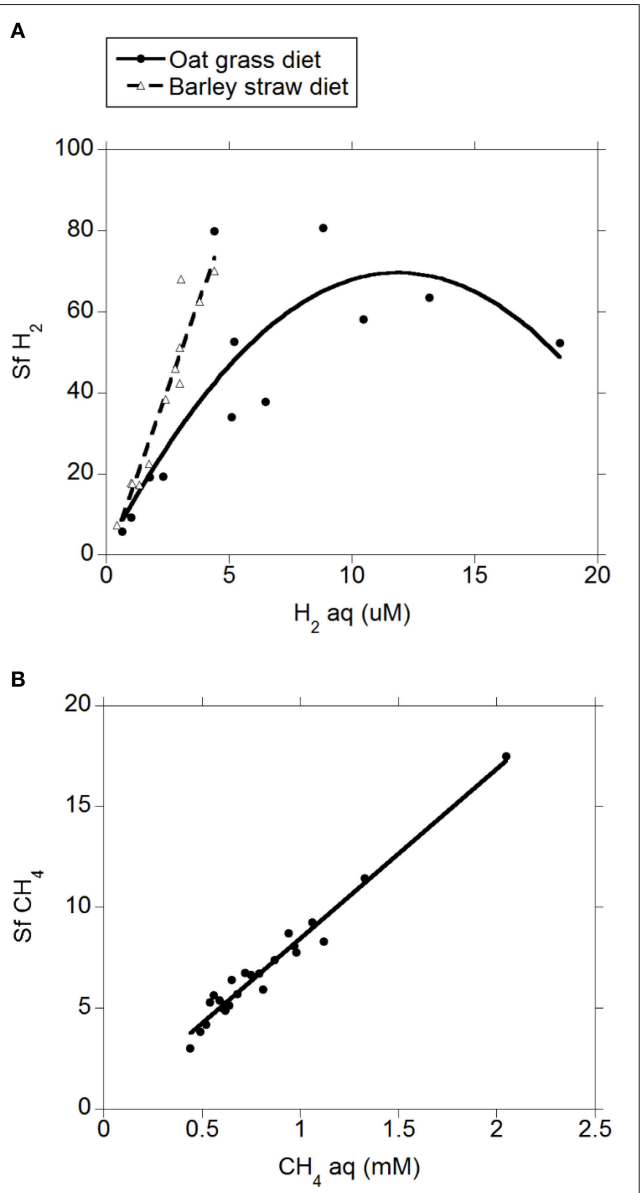

FIGURE 3 | Relationship between saturation factor and dissolved gases concentration. (A) *Sf* H_2 = 8.29 (±4.89; P = 0.11) ± 14.9 (±4.16; P = 0.002) diet + 11.2 (±1.57; P < 0.001) H_2 (aq) − 0.48 (±0.12; P < 0.001) $(H_{2\ (aq)} - 4.42)^2$ ± 3.936 (H_2 (aq) − 4.42) × diet; R^2 = 0.78 (P < 0.001); **(B)** *Sf* CH_4 = 0.048 (±0.30; P = 0.87) + 8.42 (±0.34; P < 0.001) $CH_{4\ (aq)}$; R^2 = 0.97; P < 0.001).

ammonia concentration was higher in the rumens of sheep fed BS diet, when compared with those fed the OG diet, even though the OG diet had more total N. Greater rumen ammonia concentration could suggest that incorporation of ammonia into carbon skeletons by rumen microbes was lower in sheep fed BS in comparison to the OG diet, perhaps as a result of lower supply of fermentable carbohydrates. Increased ammonia concentration and molar percentage of branched-chain VFA might also result from greater dietary and microbial protein degradation (Hassanat et al., 2014).

Supersaturation of H_2 and CH_4 in the Liquid Phase of the Rumen

In general, $H_{2\ (aq)}$ and $H_{2\ (g)}$ concentrations found in the present experiment are in the low end of $H_{2\ (aq)}$ and $H_{2\ (g)}$ concentrations ranges summarized by Janssen (2010). We attribute that in part to the low atmospheric pressure (0.64 atm) due to the elevation of the location where the experiment took place, as well as to the fact that sampling was conducted before the morning feeding, when $H_{2\ (aq)}$ concentration and H_2 production are at their lowest point during the day (Robinson et al., 1981; Rooke et al., 2014).

Concentrations of $H_{2\ (aq)}$ and $CH_{4\ (aq)}$ were directly measured by establishing an equilibrium between gas and liquid phase in a sealed vessel (Wang et al., 2014). Concentrations of $H_{2\ (aq)}$ and $CH_{4\ (aq)}$ so determined were considerably greater than those estimated by assuming an equilibrium between the liquid and the gas phase of the rumen. Thus, assuming equilibrium for H_2 or CH_4 between the gas and liquid phases seems to be inappropriate to understand the rumen fermentation. The saturation factor was greatly larger than unity for $H_{2\ (aq)}$ and $CH_{4\ (aq)}$ in the rumen, indicating that both H_2 and CH_4 were supersaturated in rumen fluid. Furthermore, H_2 supersaturation might have been even greater a few hours after feed was offered, when $H_{2\ (aq)}$ concentration is greatest (Robinson et al., 1981), as there was a positive relationship between the saturation factor and gases concentrations (**Figure 3**). The supersaturation of H_2 and CH_4 indicates mass-transfer

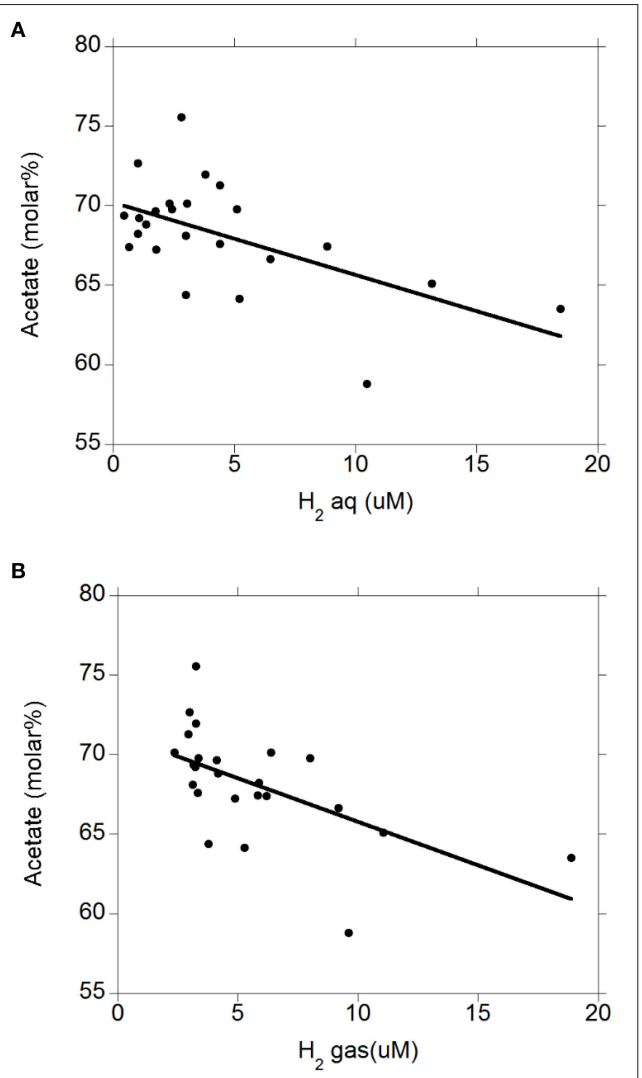

FIGURE 4 | Relationship between acetate molar percentage and concentration of dissolved and gaseous H_2. (A) acetate% = 70.2 (±0.84; $P < 0.001$) − 0.45 (±0.14; $P = 0.003$) $H_{2\ (aq)}$; $R^2 = 0.34$ ($P = 0.003$); **(B)** acetate% = 71.3 (±1.07; $P < 0.001$) − 0.55 (±0.16; $P = 0.003$) $H_{2\ (g)}$; $R^2 = 0.36$ ($P = 0.003$).

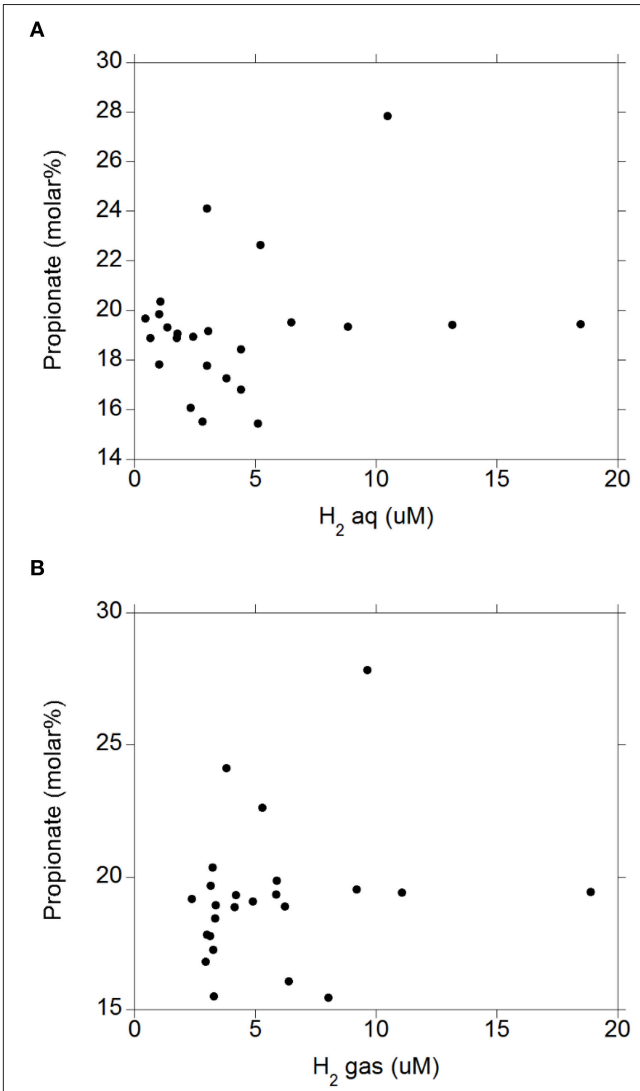

FIGURE 5 | Relationship between propionate molar percentage and concentration of dissolved and gaseous H_2. (A) propionate% = 18.6 (±0.78; $P < 0.001$) + 0.15 (±0.13; $P = 0.24$) $H_{2\ (aq)}$; $R^2 = 0.062$ ($P = 0.24$); **(B)** H_2: propionate% = 18.4 (±1.01; $P < 0.001$) + 0.14 (±0.15; $P = 0.36$) $H_{2\ (g)}$; $R^2 = 0.038$ ($P = 0.36$).

limitations to the movement of both H_2 and CH_4 from the liquid to gaseous phase in the rumen. Therefore, it does not seem appropriate to use $H_{2\ (g)}$ concentration to predict rumen $H_{2\ (aq)}$ concentration, which is the variable important to H_2-producing and H_2-utilizing microorganisms. Likewise, $CH_{4\ (aq)}$ concentration directly measured in the fluid appears as a more reliable indicator of methanogens activity than $CH_{4\ (g)}$ concentration.

Inhibiting methanogenesis in rumen *in vitro* batch and continuous mixed cultures consistently decreased the recovery of metabolic hydrogen in propionate, butyrate, CH_4 and H_2 (Ungerfeld, 2015b). In that analysis, metabolic hydrogen in H_2 was calculated taking into account only published data on $H_{2\ (g)}$, but $H_{2\ (aq)}$ was not reported in the studies used for the analysis by Ungerfeld (2015b) and was thus not considered. We now estimated $H_{2\ (aq)}$ for the experiments used for the analysis by Ungerfeld (2015b) using the reported concentrations of $H_{2\ (g)}$ based on the relationship between $H_{2\ (aq)}$ and $H_{2\ (g)}$ found in the present study (**Figure 1**). Adding reducing equivalents in $H_{2\ (aq)}$ to the calculation of metabolic H_2 recovery of the study by Ungerfeld (2015b) resulted in a marginal increase in the recovery of metabolic H_2predicted for 100% methanogenesis inhibition, of between 1 and 2% in batch culture and about 1% in continuous culture (calculations not shown). Reducing equivalents in estimated $CH_{4\ (aq)}$ were not calculated and added because in the present experiment $CH_{4\ (aq)}$ was unrelated to $CH_{4\ (g)}$, and therefore adding reducing equivalents in $CH_{4\ (aq)}$ would only affect the intercept of the relationship between metabolic

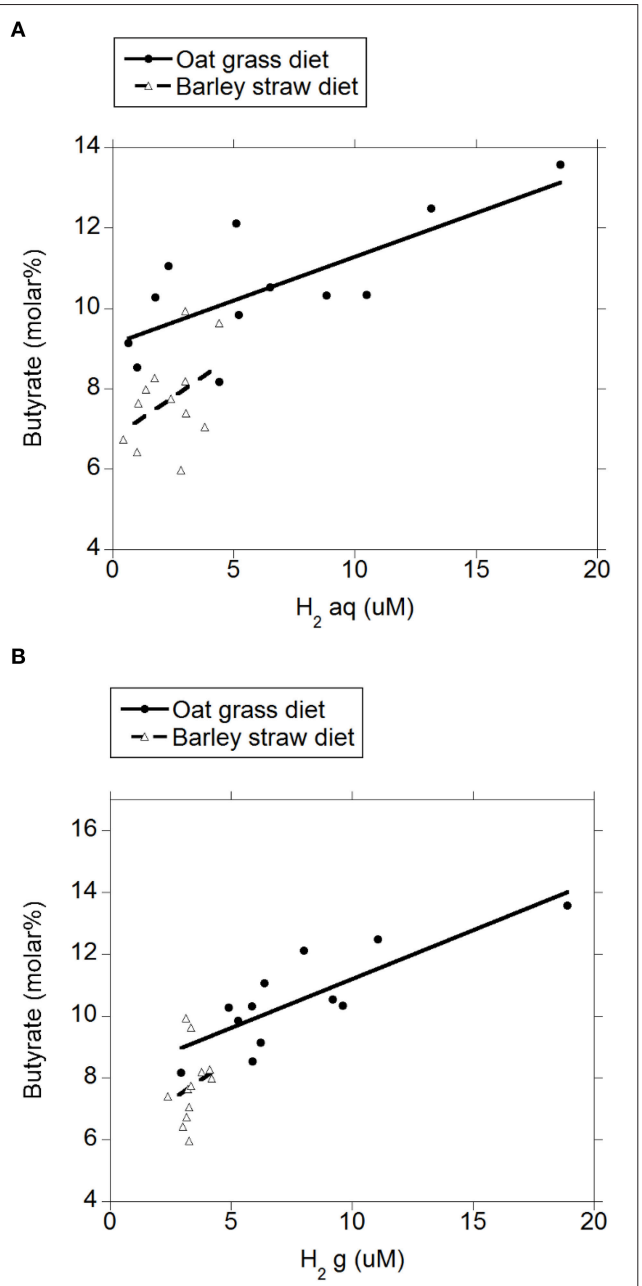

FIGURE 6 | Relationship between butyrate molar percentage and concentration of dissolved and gaseous H_2. (A) butyrate% = 8.14 (±0.35; $P < 0.001$) ± 0.92 (±0.26; $P = 0.002$) diet + 0.23 (±0.061; $P = 0.001$) $H_{2\,(aq)}$; $R^2 = 0.71$ ($P < 0.001$); **(B)** butyrate% = 7.35 (±0.48; $P < 0.001$) ±0.67 (±0.28; $P = 0.025$) diet + 0.32 (±0.077; $P < 0.001$) $H_{2\,(g)}$; $R^2 = 0.66$ ($P < 0.001$).

hydrogen recovery and the inhibition of methanogenesis but not the slope of their relationship.

Association of Dissolved and Gaseous H_2 with Rumen Fermentation Pathways

Rumen H_2 concentration affects H_2-producing and H_2-incorporating pathways in the rumen (Ellis et al., 2008), with lower $H_{2\,(aq)}$ concentration favoring acetate production, whereas greater $H_{2\,(aq)}$ concentration favors propionate and butyrate production (Janssen, 2010; Wang et al., 2014). In agreement, we observed that both measured and estimated $H_{2\,(aq)}$ concentration were negatively correlated with the molar percentage of acetate, and positively and linearly correlated with the molar percentage of butyrate, although there was no relationship with the molar percentage of propionate. Methanogens, as major H_2-utilizing microorganisms in the rumen, have a Monod relationship of growth with $H_{2\,(aq)}$ concentration, with the *Ks*-values (i.e., half the maximum growth rate) ranging from 4 to 9 uM of $H_{2\,(aq)}$ concentration (Janssen, 2010). As expected, we observed increased $CH_{4\,(aq)}$ concentration with greater $H_{2\,(aq)}$ concentration.

Gibbs energy changes of glucose fermentation in pathways producing H_2 (Reactions 1, 5–7) were greater (i.e., less favorable) using measured $H_{2\,(aq)}$ compared with $eH_{2\,(aq)}$ estimated from $H_{2\,(g)}$ concentration by assuming equilibrium. On the other hand, ΔG of H_2-incorporating reactions such as acetate conversion to propionate (Reactions 2) and to butyrate (Reactions 3), reductive acetogenesis (Reactions 8), and methanogenesis (Reactions 9), were lower using measured $H_{2\,(aq)}$ concentration compared to $eH_{2\,(aq)}$ estimated from $H_{2\,(g)}$. Furthermore, the thermodynamic ranking of glucose fermentation pathways changed when calculations were made using measured dissolved gases concentrations instead of dissolved gases concentrations estimated by assuming equilibrium with their corresponding concentrations in the gas phase. For example, Reaction 1 was the second to most favorable H_2-releasing pathway when using directly measured dissolved gases, but it was among the least favorable H_2-releasing pathway if using dissolved gases concentrations estimated from their corresponding concentrations in the rumen gaseous phase. It can be concluded that ΔG differed when calculations were made using measured instead of estimated dissolved gases concentration.

CONCLUSIONS

Measured $H_{2\,(aq)}$ and $CH_{4\,(aq)}$ were greater than $H_{2\,(aq)}$ and $CH_{4\,(aq)}$ concentrations estimated by assuming equilibrium with the gas phase, indicating that both H_2 and CH_4 were supersaturated in liquid phase of rumen. Thus, $H_{2\,(aq)}$ or $CH_{4\,(aq)}$ concentration estimated by assuming equilibrium with the gaseous phase do not seem appropriate for calculating ΔG of reactions that involve release of H_2 or CH_4, or H_2 incorporation, in the liquid phase of rumen. Concentration of $H_{2\,(aq)}$ was positively correlated with $CH_{4\,(aq)}$ concentration and the molar percentage of butyrate, and negatively correlated with molar percentage of acetate, confirming that changes in $H_{2\,(aq)}$ concentration are associated with shifts of rumen fermentation pathways and the extent of CH_4 generation. To our knowledge, this is the first *in vivo* study in which fluid and gaseous phase concentration of H_2 and CH_4 in the rumen were related. These relationships need to be studied within a much wider range of conditions, including different animals, diets,

TABLE 4 | Estimated Gibbs energy changes (kJ/reaction) of various rumen pathways.

σ	Diet		Method of ΔG estimation		SEM	*P*-value		Interaction
	Oat grass	Barley straw	*Gas* $_{(aq)}$	*eGas* $_{(aq)}$		Diet	ΔG estimation	
Reaction 1	−340	−348	−326	−362	1.64	< 0.001	< 0.001	0.93
Reaction 2	−6.42	−0.59	−17.0	10.0	1.04	< 0.001	< 0.001	0.92
Reaction 3	9.79	13.6	2.69	20.7	0.776	< 0.001	< 0.001	0.93
Reaction 4	−340	−341	−341	−341	0.575	0.27	>0.999	>0.999
Reaction 5	−418	−413	−406	−424	1.25	0.004	< 0.001	0.95
Reaction 6	−335	−330	−323	−341	0.876	< 0.001	< 0.001	0.93
Reaction 7	−335	−342	−325	−352	1.26	< 0.001	< 0.001	0.93
Reaction 8	−5.18	2.10	−19.6	16.5	1.35	< 0.001	< 0.001	0.92
Reaction 9	−34.2	−26.6	−46.0	−14.8	1.88	< 0.001	< 0.001	0.95

Reaction numbers correspond to sub-section 2.8 Calculation of the Gibbs energy changes of fermentation pathways: Reaction 1, glucose + 2 H_2O → 2 acetate + 2 H^+ + 2 CO_2 + 4 H_2; Reaction 2, acetate + CO_2 + 3 H_2 → propionate + 2 H_2O; Reaction 3, 2 acetate + H^+ + 2 H_2 → butyrate + 2 H_2O; Reaction 4, glucose → ⅔ acetate + ⅔ propionate + ⅔ CO_2 + ⅔ H_2O + 2 H^+; Reaction 5, glucose → acetate + propionate + CO_2 + H_2 + 2H^+; Reaction 6, glucose → butyrate + H^+ + 2 CO_2 + 2 H_2; Reaction 7, glucose + H_2O → acetate + ½ butyrate + 2 CO_2 + 3 H_2 + ½ H^+; Reaction 8, 2 CO_2 + 4 H_2 → acetate + H^+ + 2 H_2O; Reaction 9, CO_2 + 4 H_2 → CH_4 +2 H_2O.

eGas $_{(aq)}$, concentration of dissolved gases estimated from their concentration in the gas phase; Gas $_{(aq)}$, concentration of dissolved gases directly measured; ΔG, Gibbs energy changes.

and changes throughout the day, as well as locations situated at lower elevation, among other factors. The relationships of $H_{2\ (aq)}$ and $CH_{4\ (aq)}$ with microbial populations are also of much interest.

AUTHOR CONTRIBUTIONS

MW, ZT, and CZ designed research; MW, ZB, SA, and RW conducted research; MW and EU analyzed data; MW, ZT, and EU wrote the paper. All authors read and approved the final manuscript.

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Fermentation of Ammonia Fiber Expansion Treated and Untreated Barley Straw in a Rumen Simulation Technique Using Rumen Inoculum from Cattle with Slow versus Fast Rate of Fiber Disappearance

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The purpose of this study was to determine the effect of rumen inoculum from heifers with fast vs. slow rate of *in situ* fiber digestion on the fermentation of complex versus easily digested fiber sources in the forms of untreated and Ammonia Fiber Expansion (AFEX) treated barley straw, respectively, using an artificial rumen simulation technique (Rusitec). *In situ* fiber digestion was measured in a previous study by incubating untreated barley straw in the rumen of 16 heifers fed a diet consisting of 700 g/kg barley straw and 300 g/kg concentrate. The two heifers with fastest rate of digestion (Fast $\geq$ 4.18% h^{-1}) and the two heifers with the slowest rate of digestion (Slow $\leq$ 3.17% h^{-1}) were chosen as inoculum donors for this study. Two Rusitec apparatuses each equipped with eight fermenters were used in a completely randomized block design with two blocks (apparatus) and four treatments in a 2 $\times$ 2 factorial arrangement of treatments (Fast or Slow rumen inoculum and untreated or AFEX treated straw). Fast rumen inoculum and AFEX straw both increased ($P < 0.05$) disappearance of dry matter (DMD), organic matter, true DMD, neutral detergent fiber, acid detergent fiber, and nitrogen (N) with an interactive effect between the two ($P < 0.05$). Fast rumen inoculum increased ($P > 0.05$) methane production per gram of digested material for both untreated and AFEX straw, and reduced (interaction, $P < 0.05$) acetate: propionate ratio for untreated straw. Greater relative populations of *Ruminococcus albus* ($P < 0.05$) and increased microbial N production ($P = 0.045$) were observed in Fast rumen inoculum. AFEX straw in Fast inoculum had greater total bacterial populations than Slow, but for untreated straw this result was reversed (interaction, $P = 0.013$). These findings indicate that differences in microbial populations in rumen fluid contribute to differences in the capacity of rumen inoculum to digest fiber.

Keywords: fiber digestion, pre-treatment, real-time PCR, straw, microbiome, ammoniation

INTRODUCTION

Variation among beef cattle in residual feed intake (Koch et al., 1963; Herd et al., 2004), feed efficiency, feeding behavior, metabolic rate, and methane production (Nkrumah et al., 2006) has been well documented and thus it is logical to infer that variability in rumen fermentation occurs as well. It has recently been established that around the world, the rumen of cattle has the same core microbiome at the genus level (Jami and Mizrahi, 2012; Henderson et al., 2015), with abundance and types of microbial species varying among individual animals. Although there is variation among microbial species, there seem to be overall functional similarities of rumen microbial communities (Galbraith et al., 2004; Jami and Mizrahi, 2012). Weimer et al. (2010) reported that when >95% rumen contents were transferred between two cattle fed the same diet, with differing host-specific microbial populations, the populations reverted back to those possessed by the original host within 14 and 61 days. This suggests the existence of a hologenome, where interactions between host and microbial genetic components result in the establishment of a unique microbiota that helps regulate host physiological responses (Rosenberg et al., 2010). For example, Jami et al. (2014) reported that increased milk fat production in dairy cows was strongly correlated to an increase in the ratio of *Firmicutes* to *Bacteroidetes* in rumen contents. A decrease in *Bacteroidetes* relative to *Firmicutes* has been found in obese mice and is connected to an increase in blood and tissue fat (Turnbaugh et al., 2006). In line with that, greater feed efficiency has been reported in cattle with a less diverse rumen microbiome due to less complex metabolic pathways (Shabat et al., 2016). Exploration of the differences between cattle due to their inherent gut microbiomes and the potential differences in digestive capacity is of interest. There is a paucity of information that links individual variation in digestion efficiency and the rumen microbiome. Optimizing the ruminal microbiome of individual animals to improve digestive function could improve fiber digestion in the rumen and decrease cost of animal production.

Another potential avenue for mitigating feed costs is the use of less costly agricultural residues as ruminant feed sources. Straw is one such abundant byproduct, but its total digestible nutrient (TDN) content is low [40–46% of dry matter (DM); Kopp, 2003], limiting its use in ruminant diets. To this end, much research has examined the possible use of alkali pre-treatments such as ammoniation as a means of enhancing the digestibility of neutral detergent fiber (NDF) in the rumen (Hendriks and Zeeman, 2009; Alvira et al., 2010; Talebnia et al., 2010; Abdel-Aziz et al., 2015). Ammoniation of straw has been shown to disrupt hemicellulose-lignin bonds and cellulose crystallinity to allow enzymes access and increase hydrolysis of hemicellulose and cellulose. However, traditional ammoniation methods pose potential health hazards and a large portion of the ammonia is volatilized (Freney et al., 1983; Rasby et al., 1989). Efficiency of ammoniation treatment has been improved with the advent of ammonia freeze explosion (Dale and Moreira, 1982), later termed Ammonia Fiber Expansion (AFEX^{TM}). AFEX uses moisture and high pressure during ammonia treatment, with a subsequent pressure release and ammonia removal (Campbell et al., 2013). Bals et al. (2010) found that AFEX was far more effective as it increased digestion of late harvest switchgrass by 206% as compared to a 56% increase with traditional ammoniation methods. Using AFEX and untreated barley straw in the study allowed us to examine the effect of inoculum source on digestion of easily accessible fiber source and a more complex fiber source, while using the same feed source.

The *in situ* method (Ørskov and McDonald, 1979) is widely used to characterize fiber digestion in the rumen. As this method involves measuring fiber digestion at different time points, it is possible to estimate the rate of fiber digestion in the rumen. Rates of fiber degradation vary among animals and may be influenced by a number of host factors such as rate of passage, rumen capacity, and saliva production. Therefore in order to examine whether these differences in rate of digestion are related to differences in microbial populations, the rumen simulation technique (Rusitec; Czerkawski and Breckenridge, 1977) is well suited. The Rusitec affords strict control of saliva infusion, amount of feed, time of feeding, temperature, while allowing for measurement of rumen fermentation end products, such as methane (CH_4), volatile fatty acids (VFA), microbial populations, and pH. Controlling for physiological components such as saliva production and rate of passage allows for a focused investigation of differences in microbial populations (i.e., inoculum sources) while allowing multiple runs simultaneously, simulating multiple cows with the same inoculum.

The objective of this study was to use the Rusitec system to determine whether AFEX treatment improves the ruminal digestibility of barley straw, and whether the extent of this improvement varies among heifers with fast or slow rate of degradation of untreated straw NDF. It was hypothesized that AFEX treatment would increase digestibility of barley straw and that inoculum from heifers with fast rate of degradation would degrade both straws more completely in a 48 h time period than those with a slow rate of degradation.

MATERIALS AND METHODS

The experiment was conducted at Agriculture and Agri-Food Canada in Lethbridge, AB, Canada. The experiment was approved by the Lethbridge Research and Development Centre Animal Care Committee and cattle were cared for following the guidelines of the (Canadian Council on Animal Care [CCAC], 2009).

Experimental Design and Treatments

Two Rusitec apparatuses, each equipped with eight fermenters, were used ($n = 4$ fermenters per treatment) and the experiment was conducted over a period of 15 days with 8 days of adaptation and 7 days of sample collection. The experiment was a completely randomized block design with a 2 × 2 factorial arrangement of treatments; two sources of inoculum (slow or fast rate of NDF disappearance) and two substrates (untreated or AFEX treated barley straw diet). Inoculum from heifers with slow

and fast rate of NDF disappearance was obtained by pooling rumen inoculum from two heifers each chosen based on their rate of NDF disappearance (k_d) of barley straw measured *in situ*.

Inoculum donors were preselected by incubating untreated, ground (2-mm) barley straw in duplicate in the rumen of 16 cannulated Angus × Hereford beef heifers fed 700 g/kg untreated barley straw and 300 g/kg concentrate (DM basis) consisting of 666 g/kg dried distillers grains (DDGS), 267 g/kg canola meal, 57 g/kg supplement, and 10 g/kg urea. Barley straw was incubated in the rumen of each heifer for 0, 4, 8, 12, 24, 48, 96, and 120 h during a single incubation period. Bags used for incubation were 10 × 20 cm Ankom bags (R1020, ANKOM Technology, Macedon, NY, USA, 50 micron porosity) with 6.0 g (±0.05g) of feed per bag. Ten minutes prior to insertion into the rumen bags were submerged in 39°C water. Bags were inserted into the rumen 1 h after feeding, and removed after the appropriate amount of time. Duplicate Ankom bags were placed inside larger mesh bags (30 × 30 cm) which were placed into the rumen through the cannula and fully submerged. No microbial contamination correction was performed, as this contamination was assumed to be similar between heifers. Disappearance of NDF was calculated for each time point for each heifer and the rate of NDF disappearance in percent per hour (k_d) was estimated by fitting the data to the following model (McDonald, 1981):

$$P = a + b\,(1 - e^{-k_d(t-L)}),$$

where P is extent of degradation at time t, a is the soluble or washout fraction, b is the potentially digestible fraction, and L is the lag time. Lag time measurements are subject to error, and retention time varies by animals, therefore, k_d was chosen as the variable for animal selection. Heifers were then ranked from slow to fast based on k_d and the two animals with the fastest and the two with the slowest rates of disappearance were chosen for this study (Fast ≥ 4.18% h^{-1} vs. Slow ≤ 3.17% h^{-1}; **Table 1**).

Ammonia Fiber Expansion treatment was performed by Michigan Biotechnology Institute (Lansing, Michigan, USA) using a pair of packed bed AFEX reactors as described by Campbell et al. (2013). Briefly, barley straw was ground through a 30.5 mm screen and packed into stainless steel baskets at a density of 100 kg/m^2. Baskets were then inserted into a reactor tube where they were pre-steamed in order to displace air and raise the temperature to between 80–85°C. Vapor ammonia was applied at a rate of 80–100 g/min to a level of 1 kg ammonia per kilogram dry straw and a maximum pressure of 200 psi and left for 30 min to soak. Pressure was released, and residual ammonia was stripped by steam stripping and vaporized at atmospheric pressure before repressurized and charged to the next reactor by an ammonia compressor.

Substrate Processing

Substrates (untreated and AFEX barley straw; **Table 2**) were ground through a 4-mm screen using a Wiley mill (standard model 4; Arthur H. Thomas Co., Philadelphia, PA, USA) and particle size distribution was assessed by sieving 50 g of feed for 5 min on a Ro Tap particle separator (model RX-29; W.S. Tyler, Mentor, OH, USA) equipped with four screens (1,180, 850, 600, and 300 μm) and a bottom pan. Because AFEX straw had a greater percentage of smaller particles because it shattered more than untreated straw, the untreated straw was further ground through a 2-mm screen. To ensure that both substrates had the same particle size distribution each substrate was reconstituted from the sieved fractions to have the following particle size distribution: 100 g/kg > 1,180 μm; 200 g/kg < 1,180 μm and > 850 μm; 350 g/kg < 850 μm and > 600 μm; and 350 g/kg < 600 μm and > 300 μm. The fines (<300 μm) were removed from both substrates to prevent wash out from the bags in fermenters. The same concentrate that was fed to the heifers was ground through a 2-mm screen. Samples were mixed thoroughly and weighed separately into bags with a pore size of 50 μm. Bags used for concentrate were 5 × 10 cm (R510, ANKOM Technology, Macedon, NY, USA); bags used for straw were 10 × 20 cm (R1020, ANKOM Technology, Macedon, NY, USA).

Rumen Simulation Technique

Inoculum was collected 1 month after k_d was measured. Animals were maintained on the same diet of 700 g/kg barley straw and 300 g/kg pelleted concentrate (DM basis) in the interim. Inoculum was obtained from the four selected ruminally cannulated beef heifers 2 h after feeding. Rumen fluid and solid contents were pooled for the two heifers with fast and for the two with slow rates of NDF disappearance. Rumen fluid was filtered through four layers of cheesecloth into insulated thermoses and transported to the laboratory.

Treatments were randomly assigned to 900-mL fermenters so that both Rusitec systems had two replicates per treatment with four replicates per treatment overall. Each fermenter had a buffer input and effluent output port. Fermenters were maintained at 39°C by immersion in a water bath. Each fermenter was filled with 180 mL pre-warmed artificial saliva (pH = 8.2; McDougall, 1948) modified to contain 0.3 g/L of $(NH_4)_2SO_4$, and 720 mL of strained rumen fluid. Three labeled bags were placed in each fermenter, one containing 10 g of solid rumen digesta, one containing 7 g of barley straw (AFEX or untreated), and one containing 3 g of concentrate. The relative amounts of straw and concentrate were similar to that in the diets fed to the donor heifers. After 24 h, the bag containing rumen digesta was removed and replaced by two bags, one containing 7 g barley straw, and the other containing 3 g concentrate. Thereafter one bag containing concentrate and one bag containing straw were replaced at the same time daily so that each bag remained in the fermenter for 48 h. Bag were exchanged under a stream of O_2-free CO_2. The artificial saliva was continuously infused into the fermenters at a rate of 2.9%/h (replacing 70% of the fermenter volume each day). Effluent was collected in a 1 L flask, and gas was collected in a 2 L bag (Curity®; Conviden Ltd., Mansfield, MA, USA) attached to the effluent flask. Every day at the time of feed bag exchange, rumen fluid pH, total gas production, and effluent volume were measured.

TABLE 1 | *In situ* kinetics of NDF disappearance (NDFD) for Fast[1] and Slow heifers.

Animal	k_d[2] (%/h)	Lag (h)	a (%)[3]	b (%)	24 h NDFD[4]	48 h NDFD
Fast 1	4.32	2.80	0.90	58.5	38.6	49.1
Fast 2	4.18	1.67	0.90	56.7	35.7	48.5
Slow 1	3.17	4.13	2.60	56.1	27.4	46.7
Slow 2	2.88	1.11	0.90	61.1	24.4	48.4

[1]*Fast refers to inoculum from animals with fast rate of NDFD; slow refers to animals with slow rate of NDFD.*
[2]k_d *= rate of disappearance per hour based on disappearance of barley straw measured in sacco.*
[3]*'a' fraction is the percentage of washout from the initial substrate; 'b' is the percentage potentially degraded in the rumen over 120 h.*
[4]*24 h and 48 h observed NDFD.*

TABLE 2 | Ingredient and chemical composition of substrates.

Item (g/kg DM)	Ingredients		
	AFEX barley straw[1]	Untreated barley straw	Concentrate[2]
DM	935	924	905
OM	940	928	905
N	16	7	59
CP	99	43	366
NDF	666	804	357
ADF	488	456	145

[1]*Values for sieved and reconstructed AFEX and untreated straw.*
[2]*Comprised of 66.7% dried distillers grains solids, 26.6% canola meal, 5.7% supplement, 1% urea.*

Dry Matter and Organic Matter Disappearance

Dry matter disappearance (DMD) and organic matter (OM) disappearance (OMD) at 48 h were determined on days 9–11 and 13–15. Feed bags were removed from each fermenter, washed in cold running water until the water was clear, and dried at 55°C for 48 h. To ensure sufficient sample for chemical analysis, concentrate samples were pooled in groups of 3 days by fermenter for days 9–11 and 12–15. Forage and pooled concentrate samples were ground through a 1-mm screen prior to chemical analysis.

Fermentation Metabolites

Just prior to feed bag exchange, total gas production from each fermenter was measured daily on days 9–15 using a gas meter (Model DM3A, Alexander–Wright, London, UK). Gas samples (20-mL) were collected from the septum of the collection bags using a 26-gauge needle and transferred to 6.8-mL evacuated exetainers (Labco Ltd., Wycombe, Buckinghamshire, UK). Samples were stored at room temperature until the end of the experiment when they were analyzed for CH_4.

At the time of feed bag exchange, 2.5-mL subsamples of liquid were collected for VFA and NH_3N analysis from fermenters on days 11–14. Samples were placed in 5-mL scintillation vials containing 0.5 mL of 25% (w/w) metaphosphoric acid and immediately frozen at −20°C until VFA analysis. For NH_3N analysis, subsamples were placed in scintillation vials containing 0.5 mL of 1% sulfuric acid for NH_3N, and then frozen at −20°C until analysis. The concentrations of VFA and NH_3N (mmol/L) were multiplied by the outflow rate of fluid infused to the vessels (L/day) to determine VFA and NH_3N production (mmol/d).

Microbial Protein Synthesis

From day 7 until the end of the experiment, the McDougall's buffer was modified by replacing $(NH_4)_2SO_4$ with 0.3 g/L ^{15}N-enriched $(NH_4)_2SO_4$ (Sigma Chemical Co., St Louis, MO, USA; minimum ^{15}N enrichment 10.01 atom%; Pilgrim et al., 1970). On days 13–15, the 24 h accumulation of effluent in each flask was preserved with 20% (wt/vol) sodium azide (3 mL) and 40 mL of effluent was subsampled for isolation of bacteria associated with the liquid fraction. The 48 h bag residues were processed to obtain feed particle associated (FPA) and feed particle bound (FPB) bacterial fractions. Bags were removed from the fermenter, gently squeezed and then placed into a plastic bag with 20 mL of McDougall's buffer and processed for 60 s at 230 rpm in a Stomacher 400 laboratory paddle blender (Seward Medical Ltd., London, UK). Processed liquid was gently squeezed out, decanted and retained in a 50 mL falcon tube. Bags were washed twice more with 10 mL of buffer in each wash and each time the buffer was retained to estimate FPA bacterial fraction. Washed solid feed residues were considered to represent the FPB bacterial fraction.

The effluent liquid samples were then processed by centrifuging at 20,000 × *g* for 30 min at 4°C and the resulting pellet was centrifuged three times at 20,000 × *g* for 30 min at 4°C after washing with McDougall's buffer. Pellet was re-suspended in distilled water, frozen at −20°C, lyophilized and ball-ground for N and ^{15}N analysis. The FPA bacterial samples collected after stomaching were centrifuged (500 × *g*, 10 min, 4°C), with the resulting supernatant subsequently centrifuged (20 000 × *g*, 30 min, 4°C). The resulting pellet was washed three times as described for effluent pellets. The pellet was re-suspended in distilled water, frozen at −20°C, lyophilized and ball-ground for N and ^{15}N analysis and 16S rRNA quantification. The washed, solid feed residues, containing the FPB bacterial fraction were dried at 55°C for 48 h, weighed for DM determination and then ground and analyzed for N and ^{15}N concentrations.

Protozoa

Protozoa counts were determined in the fermenters on days 9, 12, and 15. Rumen fluid from each fermenter was collected

by gently squeezing the 48 h forage and concentrate bags. The fluid from the forage and concentrate bags were pooled by fermenter and 5.0 mL of the rumen fluid was preserved in 5.0 mL of methyl green formalin-saline solution (Ogimoto and Imai, 1981). Protozoa samples were stored in the dark at room temperature until counted. Protozoa were enumerated by light microscopy using a Levy-Hausser hemacytometer (Hausser Scientific, Horsham, PA, USA). Each sample was counted twice and if the duplicates differed by more than 10%, counts were repeated. Protozoa genera were not characterized as protozoa numbers were very low in the Rusitec making it difficult to accurately evaluate protozoa populations.

DNA Extraction and 16S rRNA Copy Quantification

DNA was extracted from all ground FPA samples using a Qiagen QIAmp Stool Mini kit (Qiagen Inc., Valencia, CA, USA), slightly modified to improve DNA extraction from Gram-positive bacteria. Briefly, 30 mg of sample was added to 1.4 mL Buffer ASL, stool lysis buffer, and vortexed until thoroughly homogenized (∼1 min). The solution was then pipetted into a new tube containing sterile zirconia beads (0.3 g, 0.1 mm; 0.1 g, 0.5 mm) and homogenized for 3 min at 30/s on a Qiagen Tissue Lyser II (Yu and Morrison, 2004). Samples were then mixed at 700 rpm while heated at 95°C for 5 min. Samples were vortexed briefly and centrifuged at 13,200 rpm for 1 min. The supernatant was separated, added to an inhibitEX tablet and the Qiagen Stool Mini Kit protocol was followed. Total DNA was quantified using PicoGreen with a NanoDrop 3300 fluorometer, normalized to 20 ng/μL, and run on a gel to check for quality.

Using previously described primers and annealing temperatures, qPCR was performed to determine the relative abundance of the following fibrolytic bacteria: *Ruminococcus albus* (Wang et al., 1997), *Fibrobacter succinogenes, Ruminococcus flavefaciens, Selenomonas ruminantium, Prevotella bryantii* (Tajima et al., 2001), and total bacterial 16S rRNA (Oss et al., 2016).

Sample analysis

Samples of feed and feed fermentation residues were analyzed for analytical DM by drying 1.0 g (± 0.05 g) of each sample for 2 h at 135° C using a forced air oven. Samples were ashed at 550° C for 5 h to estimate OM. NDF inclusive of ash (NDF) and acid detergent fiber (ADF) were analyzed by the sequential method with the ANKOM200 Fiber Analyzer using reagents as described by Van Soest et al. (1991). Sodium sulphite and α-amylase were used during NDF determination. Total N concentration and atom per cent excess (APE) of ^{15}N was determined using a mass spectrometer (Ribeiro et al., 2015). Concentration of CH_4 in the gas samples was determined using a Varian gas chromatograph equipped with a GS-Carbon-PLOT 30 m × 0.32 mm × 3 μm column and thermal conductivity detector (Agilent Technologies Canada, Inc. Mississauga, ON, Canada). The oven temperature was set at 35°C with an injector temperature of 185°C (1:30 split, 250 μL injector volume) a detector temperature of 150°C and helium (27 cm/s) as the carrier gas. Ammonia was analyzed using the modified Berthelot method as described by Rhine et al. (1998) and VFA were analyzed by gas chromatography as described by Wang et al. (2001).

Calculations and Statistical Analysis

True dry matter disappearance was determined as DMD adjusted for microbial DM: initial sample weight – (final sample weight−microbial DM)/initial sample weight.

Total effluent microbial N (MN) production (mg/day) was calculated using the N concentration (%) determined for the microbial pellet, multiplied by the microbial weight in the total effluent (mg/day). Microbial weight in the total effluent was calculated by multiplying daily effluent production (mL) by the microbial density (mg/mL) in the 40 mL subsample. Microbial N production from FPA fraction was calculated by multiplying the N concentration (%) in the FPA microbial pellet by the microbial weight of the FPA fraction (mg/day). FPB MN production (mg/day) from straw and concentrate fractions were calculated using the following equation:

$$MN = \frac{APE\ in\ RN}{APE\ in\ MN} \times RN$$

where APE in RN = the percent excess of ^{15}N in the fraction analyzed, and APE in FPA microbial pellet was used as the source of APE in MN. Total MN production (mg/day) was calculated as the sum of microbial production in the effluent, FPA, FPB of straw residues, and FPB of concentrate residues.

Totals presented in **Table 3** were calculated as [(concentrate + straw before incubation) – (concentrate + straw after incubation)]/(concentrate + straw before incubation).

Relative bacterial populations were calculated as (total copy number of species in a given fermenter on a given day/total bacterial copy number in the same fermenter on the same day) × 100.

All data were analyzed using the MIXED procedure of SAS (SAS Inc., Cary, NC, USA). Individual fermenter considered the experimental unit with day of sampling treated as a repeated measure. Straw, inoculum, straw × inoculum were considered fixed effects while apparatus was considered a random effect. For each parameter analyzed a covariance structure among compound symmetry, heterogeneous compound symmetry, autoregressive, heterogeneous autoregressive, Toeplitz, unstructured, and banded was chosen based on the lowest corrected Akaike information critical values. Significance was declared at $P < 0.05$ and a trend was considered at $0.05 \leq P \leq 0.10$. Differences among treatments were determined using Fisher's protected ($P < 0.05$) LSD test using the PDIFF option in SAS for straw × inoculum interactions.

RESULTS

Disappearance and Fermentation Characteristics

Ammonia Fiber Expansion treated straw had greater DMD, OMD, TDMD, NDFD, and ADFD ($P \pm 0.001$) than untreated

TABLE 3 | Effect of inoculum and ammoniation treatment (trt) of barley straw on DMD, OMD, TDMD, NDFD ADFD, N disappearance, and microbial N production.[1]

Item	Treatment[2]				SEM	*P*-value		
	Untreated		AFEX			Trt[3]	Inoculum	Int[3]
	Slow	Fast	Slow	Fast				
DMD (g/kg DM)								
Barley straw	461[c4]	464[c]	612[b]	636[a]	5.9	<0.001	<0.001	0.002
Concentrate	846	816	848	848	15.2	0.079	0.11	0.11
Total	624	618	720	733	8.2	<0.001	0.46	0.052
OMD (g/kg DM)								
Barley straw	466[c]	467[c]	615[b]	639[a]	4.7	<0.001	<0.001	<0.001
Concentrate	875	854	879	875	12.7	0.12	0.14	0.31
Total	586[b]	580[b]	694[a]	707[a]	7.7	<0.001	0.32	0.021
TDMD (g/kg DM								
Barley straw	500[c]	503[c]	633[b]	666[a]	5.6	<0.001	< 0.001	<0.001
Concentrate	860	828	858	859	14.0	0.081	0.12	0.092
Total	607[c]	601[c]	700[b]	725[a]	7.3	<0.001	0.015	<0.001
NDFD (g/kg DM)								
Barley straw	455[c]	451[c]	559[b]	593[a]	9.4	<0.001	<0.001	<0.001
Concentrate	785[a]	740[b]	769[ab]	785[a]	21.7	0.23	0.23	0.022
Total	507[c]	498[d]	599[b]	627[a]	10.8	<0.001	0.004	<0.001
ADFD (g/kg DM)								
Barley straw	427[c]	441[c]	534[b]	577[a]	12.3	<0.001	<0.001	0.008
Concentrate	689[a]	649[b]	674[ab]	685[a]	18.2	0.35	0.20	0.028
Total	460[c]	465[c]	551[b]	585[a]	12.4	<0.001	<0.001	<0.001
N Disappearance (g/kg DM)								
Barley straw	681	643	773	759	10.9	<0.001	0.029	0.27
Concentrate	931	915	925	940	16.8	0.33	0.94	0.13
Total	852	827	846	849	14.4	0.33	0.19	0.11
Microbial N production (mg/d)								
Effluent	31.1	34.5	29.7	36.2	2.50	0.94	0.048	0.52
FPA[5]	5.6	5.6	5.7	5.1	0.20	0.39	0.12	0.25
FPB straw	19.9	18.7	18.2	18.8	0.56	0.19	0.61	0.14
FPB concentrate	1.3	1.4	1.2	1.4	0.19	0.62	0.22	0.94
Total	57.6	60.2	54.8	63.3	2.73	0.95	0.045	0.27

[1]*DM, dry matter; DMD, dry matter disappearance; OMD, organic matter disappearance; NDFD, neutral detergent fiber disappearance; ADFD, acid detergent fiber disappearance; TDMD, true dry matter disappearance.*
[2]*Fast refers to inoculum from animals with fast rate of NDFD; slow refers to animals with slow rate of NDFD.*
[3]*Trt refers to straw treatment: AFEX or untreated; Int refers to interaction between treatment and inoculum.*
[4]*'a, b, c, and d' Values within a row with the same letter do not differ (P > 0.05).*
[5]*FPA, feed particle associated; FPB, feed particle bound.*

straw (**Table 3**). The straw × inoculum interactions ($P < 0.05$) for these variables indicate that Fast inoculum increased ($P < 0.05$) disappearance of AFEX straw, but had no effect on untreated straw. The NDFD and ADFD of concentrate was lowered with Fast inoculum with untreated straw ($P < 0.05$), but was not affected by the other treatments.

The N disappearance was greater ($P < 0.001$) for AFEX straw than for untreated straw (**Table 3**). N disappearance of untreated straw increased with Slow inoculum ($P = 0.008$), but inoculum source had no effect on N disappearance from AFEX. Microbial N production was greater for Fast inoculum in the effluent and overall ($P < 0.05$).

Untreated straw produced more CH_4 per gram of DMD then did AFEX straw ($P = 0.046$; **Table 4**). No other CH_4 variable was affected by straw or inoculum source. AFEX straw decreased pH compared to untreated straw ($P < 0.001$). AFEX straw and Slow inoculum promoted greater NH_3N production than untreated straw ($P < 0.001$) and Fast inoculum ($P = 0.015$), with no interaction between the two. AFEX straw resulted in more total VFA production than untreated straw ($P < 0.001$), and the straw × inoculum interaction indicated that more VFA was produced with AFEX straw incubated with Fast inoculum ($P = 0.035$) whereas Fast inoculum had no effect on VFA from untreated straw. Interactions were also observed for the proportions of acetate, butyrate, and caproate ($P < 0.05$). Fast inoculum decreased the proportion of acetate for untreated straw ($P < 0.001$) and caproate for AFEX straw ($P < 0.001$). Fast inoculum increased the molar proportion of butyrate for

TABLE 4 | Effect of inoculum and ammoniation treatment (trt) of barley straw on gas production and fermentation variables (pH, total VFA and individual VFA).

Item	Treatment[1]				SEM	*P*-value		
	Untreated		AFEX			Trt[2]	Inoculum	Int[2]
	Slow	Fast	Slow	Fast				
Gas production								
Total, L/d	1.54	1.61	1.59	1.60	0.135	0.87	0.76	0.79
CH_4, %	6.20	6.37	6.14	6.11	0.534	0.66	0.88	0.79
CH_4, mL/d	94.2	101	94.1	105	14.4	0.83	0.23	0.83
CH_4, mg/d	61.2	66.0	61.2	67.6	9.30	0.87	0.26	0.86
CH_4, mg/g incubated DM	6.66	7.19	6.65	7.32	0.973	0.91	0.26	0.88
CH_4, mg/g digested DM	11.7	12.9	9.67	10.8	1.49	0.046	0.22	0.98
pH	6.70	6.69	6.64	6.61	0.019	<0.001	0.095	0.34
Ammonia, mg/d	110	98.3	137	131	5.29	<0.001	0.015	0.45
Total VFA, mmol/d	50.6[c3]	49.0[c]	57.4[b]	59.6[a]	1.88	<0.001	0.67	0.035
VFA, mol/100 mol								
Acetate (A)	66.1[a]	63.9[b]	63.7[b]	64.1[b]	0.618	0.001	0.006	<0.001
Propionate (P)	22.6	23.8	25.9	26.2	0.603	<0.001	0.021	0.21
Butyrate	7.22[b]	8.28[a]	6.74[c]	6.17[d]	0.098	<0.001	0.025	<0.001
Valerate	1.62	1.66	1.40	1.34	0.042	<0.001	0.60	0.10
Isobutyrate	0.916	0.933	0.849	0.848	0.012	<0.001	0.55	0.48
Isovalerate	1.51	1.44	1.33	1.30	0.035	<0.001	0.19	0.52
Caproate ($\times 10^{-2}$)	4.52[c]	4.59[c]	5.19[a]	4.98[b]	0.011	<0.001	0.21	0.018
A:P ratio	2.93[a]	2.69[b]	2.47[c]	2.45[c]	0.090	<0.001	0.012	0.029

[1] *Fast refers to inoculum from animals with fast rate of NDFD; slow refers to animals with slow rate of NDFD.*
[2] *Trt refers to straw treatment: AFEX or untreated; Int refers to interaction between treatment and inoculum.*
[3] *'a, b, c, and d' Values within a row with the same letter do not differ ($P > 0.05$).*

untreated straw, yet it increased it for AFEX straw ($P < 0.001$), although proportions were greater for untreated than AFEX straw. AFEX increased ($P < 0.001$) the molar proportion of propionate, but reduced ($P < 0.001$) that of valerate, isobutyrate, and isovalerate. AFEX also reduced the A:P ratio ($P < 0.001$), with the effect of inoculum dependent on straw type; Fast inoculum reduced ($P = 0.029$) A:P ratio for untreated, but not AFEX straw.

Microbial Populations

Ammonia Fiber Expansion had no effect on total protozoa counts, nor was there an effect ($P > 0.10$) on bacterial populations (**Table 5**). Copy numbers of *R. albus* were increased ($P = 0.035$) for Fast inoculum. Total bacterial 16S rRNA after adaptation tended to be greater ($P = 0.10$) for AFEX straw, with Fast inoculum increasing copies for AFEX but not untreated straw (interaction, $P = 0.013$).

DISCUSSION

Ammoniation is known to increase DMD and N content of various straws including wheat (Horton, 1981; Herrera-Saldana et al., 1982; Givens et al., 1988; Kondo et al., 1992), oat (Horton, 1981; Givens et al., 1988), and barley (Horton, 1981; Hadjipanayiotou, 1982; Dryden and Kempton, 1983; Givens et al., 1988). AFEX is an advanced ammoniation technology and has been shown to increase digestibility of crop residues, compared to traditional ammoniation, by cleaving the hemicellulose-lignin ester linkages, or lignin carbohydrate complexes more efficiently (Chundawat et al., 2010). Ammoniation treatment disrupts the crystalline structure of cellulose I converting it to cellulose III (Mittal et al., 2011), which allows for much faster hydrolysation of β1–4 glycosidic bonds by microbial enzymes (Fan et al., 1980; Igarashi et al., 2007; Hall et al., 2010). Dale et al. (1997) found even low levels of enzymes digested AFEX to near theoretical yields. Thus, the 26% greater DMD and 21% greater NDFD of AFEX compared with untreated barley straw observed in the present study is consistent with the previous literature, and highlights the potential of AFEX technology to improve nutritive value of straw for feed. While AFEX may be impractical to implement on farms, Campbell et al. (2013) are working on developing this technology for regional depots, which would greatly increase access to this technology.

Observed differences between Fast and Slow inoculums may be attributed to differences in microbial populations within the inoculum, as the Rusitec system removes variation in physiological factors between ruminants such as saliva production, rumen fill, rumination time, rate of passage, and rate of absorption, that contribute to individual variability in fiber digestion observed *in vivo*. The increase in DMD, TDMD, and ADFD of AFEX straw when incubated with Fast inoculum in the Rusitec, with no effect on disappearance of

TABLE 5 | Effect of inoculum and ammoniation treatment (trt) of barley straw on rumen microbes.[1]

Item	Treatment[2]				SEM	*P*-value		
	Untreated		AFEX			Trt[3]	Inoculum	Int[3]
	Slow	Fast	Slow	Fast				
Total protozoa, $\times 10^3$ cells/mL	8.58	8.00	6.67	6.25	1.43	0.22	0.73	0.95
Total bacterial 16S rRNA copies after adaptation ($\times 10^9$)[4]	107.9[ab5]	85.9[b]	96.8[b]	130.0[a]	14.5	0.10	0.56	0.013
Fibrobacter succinogenes (%)	2.41	1.29	2.17	1.25	0.718	0.85	0.18	0.89
Ruminococcus albus (%)	0.0286	0.0433	0.0258	0.0522	0.0087	0.74	0.035	0.51
Ruminococcus flavefaciens (%)	0.0678	0.0538	0.0585	0.0610	0.0095	0.91	0.56	0.41
Selenomonas ruminantium (%)	0.0422	0.0685	0.0599	0.108	0.041	0.37	0.25	0.73
Prevotella bryantii (%)	0.0186	0.0011	0.0156	0.0323	0.0165	0.40	0.98	0.31

[1] *Populations calculated as percent of total bacterial 16S rRNA.*
[2] *Fast refers to inoculum from animals with fast rate of passage; slow refers to animals with slow rate of passage.*
[3] *Trt refers to straw treatment: ammoniated or untreated; Int refers to interaction between treatment and inoculum.*
[4] *All bacteria quantified using FPA samples from each fermenter.*
[5] *Letters 'a, b, c, and d' denote significant difference, values with the same letter are not significantly different than each other.*

untreated straw, indicates that heifers selected based on faster rate of NDF digestion of untreated barley straw were able to more thoroughly digest AFEX straw in 48 h compared to animals selected for slower rate of digestion. Rumen inoculum selected on the basis of a faster rate of NDF disappearance would likely contain greater populations and activity of microorganisms that degrade cell wall, which is consistent with the observation that *R. albus* was more abundant in Fast rumen inoculum than in Slow rumen inoculum. *R. albus* has long been known to be one of the most cellulolytic organisms in the rumen (Graham et al., 1985). The lack of effect of rumen fluid on the populations of the other four bacteria characterized may simply indicate that they were not responsible for the differences in digestibility observed. There are many fibrolytic bacteria such as *Butyrivibrio fibrisolvens*, *Clostridium longisporum*, *Clostridium lochheadii*, *Eubacterium cellulosolvens,* and *Prevotella spp.* (Stewart et al., 1997) that were not characterized in this study. This study did not look at interactions between bacteria, for example *Prevotella spp.* are known to be very effective at digesting hemicellulose in alfalfa, as well as contributing to increased digestion of cellulose when cultured with other cellulolytic bacteria (Dehority and Scott, 1967). There are also many, as of yet unculturable bacteria, that may also contribute to differences in ruminal degradation. Pooling the rumen fluid from two animals may have also eliminated some of the differences between individual Fast and Slow inoculum in relative population size of the selected bacteria due to potential antagonistic differences between bacteria from each donor animal. In addition, differences in methanogens, fungi, and protozoa species that may have contributed to differences in digestion observed were not characterized.

The increase in N disappearance of barley straw seen for AFEX was likely due to increased accessibility of cell wall contents due to enhanced NDFD (Graham and Åman, 1984). Ammoniated straw also contained more N than untreated barley straw because N from the ammoniation treatment is bound by the forage during treatment. While this excess is reduced by the ammonia recovery step in AFEX treatment (Chundawat et al., 2013), some of the N remains bound to the substrate and AFEX straw which accounts for the greater initial N content of AFEX compared with untreated straw (99 versus 43 g/kg DM). This agrees with the findings of Bals et al. (2010) who found increased N compared to untreated substrate for corn stover and switchgrass but reduced N compared to traditional ammoniation. The increase in N available for use in the rumen, along with the increase in degradability of AFEX straw, make it appealing as a potential feedstuff for cattle.

The reduction in CH_4 when expressed relative to digested DM for AFEX compared with untreated straw was likely due to greater propionate and decreased butyrate molar proportions, and lower acetate to propionate ratio. Propionate acts as an alternative hydrogen sink in the rumen diverting hydrogen away from CH_4 synthesis while the production of butyrate and acetate promote methanogenesis (Moss et al., 2000). The increase in VFA production caused by the AFEX treatment was likely responsible for the slight, but significantly lower pH measured in those fermenters compared with those fed untreated straw.

This study focuses on differences in rumen inoculum, but it is well known that other characteristics of individual animals, such as rumination time, saliva production, rumen fill, rate of passage, and rate of absorption also impact their ability to digest forage. As we continue to demystify the interactions between host animals and their microbiome, improving the ability of individual animals to digest forages will become more tenable.

CONCLUSION

Ammonia Fiber Expansion was found to increase digestibility of barley straw DM by more than 30%. As AFEX technology becomes more widely available, AFEX treatment of straw has potential to increase straw usage as feed. Further to this,

research is ongoing on microbiome contributions to variations in metabolic efficiency among animals (e.g., Hernandez-Sanabria et al., 2011; Khiaosa-ard and Zebeli, 2014). These differences may one day be exploited to improve individual efficiency. In working toward this, our study showed that rate of digestion due to rumen fluid source can be an important differentiating factor among ruminants, and contribute to significant differences in their ability to digest forage. This is likely due to differences in microbial populations, although this cannot be confirmed based on this study due to the limited number of bacterial species examined. In trying to improve the ability of ruminants to digest fiber it will be important to explore both physiological and microbiome characteristics of individual animals, and their interactions.

AUTHOR CONTRIBUTIONS

CG conducted all aspects of the study and drafted the manuscript; GR helped conduct the study, contributed to data analysis and revised the manuscript; and MO, TM and, KB provided advice on the protocol, data analysis, and final manuscript.

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Synergism of Cattle and Bison Inoculum on Ruminal Fermentation and Select Bacterial Communities in an Artificial Rumen (Rusitec) Fed a Barley Straw Based Diet

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This study evaluated the effect of increasing the proportion of bison relative to cattle inoculum on fermentation and microbial populations within an artificial rumen (Rusitec). The experiment was a completely randomized design with a factorial treatment structure (proportion cattle:bison inoculum; 0:100, 33:67, 67:33, and 100:0) replicated in two Rusitec apparatuses ($n = 8$ fermenters). The experiment was 15 d with 8 d of adaptation and 7 d of sampling. Fermenters were fed a diet of 70:30 barley straw:concentrate (DM basis). True digestibility of DM was determined after 48 h of incubation from d 13 to 15, and daily ammonia (NH_3) and volatile fatty acid (VFA) production were measured on d 9–12. Protozoa counts were determined at d 9, 11, 13, and 15 and particle-associated bacteria (PAB) from d 13 to 15. Select bacterial populations in the PAB were measured using RT-qPCR. Fermenter was considered the experimental unit and day of sampling as a repeated measure. Increasing the proportion of bison inoculum resulted in a quadratic effect ($P < 0.05$) on straw, concentrate and total true DM disappearance and on straw and total neutral detergent fiber (aNDF) disappearance, with greater disappearances observed with mixed inoculum. There were no effect of source or proportion of inoculum on ADF disappearance ($P > 0.05$). Increasing bison inoculum linearly increased ($P < 0.05$) concentrate aNDF disappearance, total and concentrate N disappearance as well as total daily VFA and acetate production. A positive quadratic response ($P < 0.05$) was observed for daily NH_3-N, propionate, butyrate, valerate, isovalerate and isobutyrate production, as well as the acetate:propionate ratio. Increasing the proportion of bison inoculum linearly increased ($P < 0.05$) total protozoa numbers. No effects were observed on pH, total gas and methane production, microbial N synthesis, or copies of 16S rRNA associated with total bacteria, *Selenomonas ruminantium* or *Prevotella bryantii*. Increasing bison inoculum had a quadratic effect ($P < 0.05$) on *Fibrobacter succinogenes*, and tended to linearly ($P < 0.10$) increase *Ruminococcus flavefaciens* and

decrease ($P < 0.05$) *Ruminococcus albus* copy numbers. In conclusion, bison inoculum increased the degradation of feed protein and fiber. A mixture of cattle and bison rumen inoculum acted synergistically, increasing the DM and aNDF disappearance of barley straw.

Keywords: bacteria, barley straw, bison, *in vitro,* cattle, protozoa, rumen

INTRODUCTION

Animal agriculture must find alternative and cost-effective feed ingredients to remain profitable in a projected future of increased food demand and costs (Thornton, 2010). Lignocellulosic crop residues could fulfill this need if their digestion could be optimized. Ruminants are unique in their ability to utilize lignocellulosic feeds as the rumen microbial consortia is considered to be one of the most efficient microbial systems at degrading lignocellulosic biomass (Flint et al., 2008). However, even in light of this efficiency, often less than 50% of carbohydrates in low quality forages such as straw are digested by cattle (Sari et al., 2015). Comparing the rumen microbial community of ruminants that are more effective at degrading lignocellulose to those that are less efficient may expand our understanding of the key microbes and their roles in plant cell wall deconstruction.

The American buffalo or bison (*Bison bison*) and the domestic bovine (*Bos taurus*) are ruminant species that evolved in different environments (Koch et al., 1995), a factor that may account for the tendency of bison to graze lower quality forages than cattle (Peden et al., 1974). Some studies have suggested that bison are more efficient than cattle at digesting poor-quality forages (Richmond et al., 1977; Hawley et al., 1981a,b). Proposed mechanisms for this heightened efficiency include a reduction in the rumen particulate passage rate, an increase in nitrogen (N) recycling, as well as differences in ruminal microbial populations (Hawley et al., 1981b). However, relatively little is known about ruminal fermentation characteristics and microbial populations in bison. Compared to cattle, bison possess a greater percentage of *Fibrobacter succninogenes*, *Ruminococcus albus,* and *Ruminococcus flavefaciens* in rumen contents (Varel and Dehority, 1989). Higher ruminal ammonia (NH_3) concentrations and total protozoal numbers, and a differing species density (greater *Dasytricha* spp., *Eudiplodinium maggii*, *Eudiplodinium bursa*, and *Epidinium* spp.) was also observed for bison compared to cattle when both were fed poor-quality hay (Towne et al., 1988).

The manipulation of the ruminal microbial community to improve fiber digestion has been largely unsuccessful (Weimer, 2015). In a classic study, despite massive inoculation of highly efficient cellulolytic bacteria strains to nearly empty rumens, the inoculated bacteria failed to colonize the rumen and were washed out within 24 h (Varel et al., 1995). There is evidence suggesting that the rumen microbiome may be host-specific, possibly raising barriers to the establishment of introduced microbes across different hosts (Weimer et al., 2010). A possible reason for this is that each individual animal possess a microbial community that is able to reconstitute itself even after serious perturbation, reflecting the ecological principles of inertia and resilience (Westman, 1978). The alternative use of the *in vitro* semi-continuous rumen simulation system (Rusitec) allows testing the effect of different rumen inoculums (i.e., cattle vs. bison) on fiber digestion under more standardized environmental conditions (i.e., temperature, pH, passage rate) as a step toward defining the importance of host specificity.

Therefore, we hypothesized that ruminal inoculum from bison would promote greater degradation of lignocellulose in the Rusitec as compared to ruminal inoculum from cattle. Thus, the objective of this study was to evaluate the effect of increasing the proportion of bison rumen inoculum on fermentation parameters, microbial populations and the digestion of barley straw using the Rusitec.

MATERIALS AND METHODS

The present experiment was conducted at the Agriculture and Agri-Food Canada Research and Development Centre in Lethbridge (LRDC), Alberta, Canada. Donor animals used in the experiment were cared for in accordance with the guidelines of the (Canadian Council on Animal Care, 2009) and protocols were approved by the Lethbridge Research and Development Centre Animal Care Committee.

Experimental Design and Treatments

The experiment was a completely randomized design with four treatments (ruminal inoculum) carried out in 16 Rusitec fermenters ($n = 4$/treatment) as described by Czerkawski and Breckenridge (1977). The duration of the experiment was 15 d. The Rusitecs were allowed to reach steady state over the first 8 d, followed by a 7 d sampling period (d 9 to 15). Treatments consisted of increasing replacement of ruminal inoculum from cattle (*Bos taurus*) with contents from bison (*Bison bison*) with the following proportions of cattle:bison inoculum in the fermenters: (1) 100:0; (2) 67:33; (3) 33:67; and (4) 0:100. Rusitecs were fed a diet of barley straw (700 g/kg DM) and concentrate mix (300 g/kg DM basis; 667 g/kg of corn distiller's dried grains with solubles (DDGS), 266 g/kg of canola meal, 57 g/kg of mineral/vitamin supplement and 10 g/kg of urea; **Table 1**). Feeds were ground through a 4-mm screen (Arthur H. Thomas Co., Philadelphia, PA, USA), with the straw and concentrate placed in separate polyester bags (for concentrate: 50 × 100 mm; pore size = 50 μm; for straw: 100 × 200 mm; pore size = 50 μm; ANKOM; Ankom Technology Corp.).

Experimental Apparatuses and Incubations

Each Rusitec apparatus was equipped with eight 920 mL fermenters. Each fermenter had an inlet for the infusion of buffer and an out port for the collection of effluent. Fermenters were immersed in a water-bath maintained at 39°C. The four

TABLE 1 | Chemical composition of barley straw and concentrate used as diet.

Item	Ingredients		Diet
	Barley straw[a]	Concentrate[b]	
DM, g/kg	922	898	915
OM, g/kg DM	915	896	909
N, g/kg DM	9	56	23
CP, g/kg DM	58	352	146
aNDF, g/kg DM	729	309	603
ADF, g/kg DM	473	131	370

[a] *70% on DM basis.*
[b] *30% on DM basis; 66.7% of DDGS, 26.6% of canola meal, 5.7% of supplement and 1% of urea.*

treatments were randomly assigned to duplicate fermenters within each Rusitec apparatus (four replications per treatment). The experiment was started by filling each fermentation vessel with 180 mL of warmed McDougall's buffer (McDougall, 1948) modified to contain 0.3 g/L of $(NH_4)_2SO_4$ and 720 mL of strained rumen fluid as described by Ribeiro et al. (2015).

Cattle inoculum was obtained before feeding from a pool of 16 ruminally cannulated heifers fed the same diet as the Rusitec. Bison inoculum was obtained from a pool of 32 bison rumens collected at a local slaughterhouse. The bison were fed a forage diet containing barley silage and oats (75:25 DM basis). The time from slaughter of the bison to initiation of fermentation in the Rusitec was less than 2 h. Rumen contents from the bison were pooled in a large heated vessel, a representative sample of fluid was collected and filtered through four layers of cheesecloth into an insulated thermos and immediately transported to the laboratory. Approximately 400 g (200 g from cattle, 200 g from bison) of ruminal solid digesta was also collected for the initial inoculation of the fermenters. All fermenters were inoculated at the same time and a large number of each ruminant species was deliberately sampled to ensure that the sample collected represented the core microbiome of the herd as opposed to an individual.

On day 0, a solid digesta bag (1 bag, 20 g wet wt) according to inoculum proportions for each treatment, along with two additional bags containing either barley straw (7 g DM) or concentrate (3 g DM) were included in each fermenter. After 24 h (day 1), the solid rumen digesta was replaced with two bags, one containing straw, and another containing concentrate. From day 1 onwards, fermenters now containing four bags were opened daily to replace the two bags that had been incubated for 48 h. Artificial saliva was continuously infused into the fermenters at a dilution rate of 2.9%/h. During bag exchange, each fermentation vessel was flushed with O_2-free CO_2 to maintain anaerobic conditions. Effluent was collected in a 2.0 L Erlenmeyer flask and measured daily during feed bag exchange.

Measurements

True DM Disappearance

True DM disappearance at 48 h was determined from day 13 to 15. Feed bags (forage and concentrate) were removed from each fermenter, processed together in a paddle blender (Stomacher 400; Seward Ltda, Worthing, UK) to obtain feed particle-associated (FPA) and feed particle-bound (FPB) bacterial fractions, and dried at 55°C for 48 h. Upon removal from fermenters, both straw and concentrate bags were gently squeezed to expel excess liquid. Bags were placed together in a plastic bag with 20 mL of buffer (McDougall, 1948) and processed in the paddle blender for 60 s at 230 RPM. The processed liquid was exuded, poured off and retained. Feed residues were then washed twice with 10 mL of McDougall's buffer in each wash. The wash buffer was retained and pooled with the fluid obtained after paddle blending to obtain the FPA bacterial fraction, and total volume was recorded. Bacteria attached to the washed solid feed residues were considered to represent the FPB bacterial fraction. The true DM disappearance was calculated by the difference between feed initially added to the bag and feed residues minus FPB (i.e., minus microbial mass).

Fermentation

Fermentation gas was collected daily into reusable 2 L gas-tight vinyl collection bags (Curity®; Conviden Ltd., Mansfield, MA, USA) that were attached to each of the effluent vessels. Gas bags were clamped before opening the fermenters or effluent collection. Just before feed bag exchange, daily total gas production (d 1 to d 15) from each fermenter was measured using a gas meter (Model DM3A, Alexander-Wright, London, England, UK). From d 9 to d 15, before total gas measurements, gas samples (20 mL) were collected from the septum of the collection bags using a twenty-six-gauge needle (Becton Dickinson, Franklin Lakes, NJ, USA) and transferred to evacuated 6.8 mL Exetainer vials (Labco Ltd., Wycombe, Bucks, UK) for CH_4 analysis. The CH_4 concentration in gas samples was determined as described by Avila-Stagno et al. (2014) using a Varian gas chromatograph equipped with GS-Carbon-PLOT 30 m × 0.32 mm × 3 μm column and thermal conductivity detector (Agilent Technologies Canada Inc., Mississauga, ON, Canada).

The pH of the fluid from each fermenter was recorded (Orion model 260A, Fisher Scientific, Ottawa, ON, Canada) daily (d 1 to d 15) at the time of feed bag exchange. The amount of effluent produced daily was measured with a graduated cylinder. To determine VFA concentration in fermenter effluent, subsamples (2.5 mL) were collected directly from the effluent flasks containing 20 mL of 3.66 *M* H_2SO_4 (20%, vol/vol) (Giraldo et al., 2007) at the time of feed bag exchange. Samples were placed in screw-cap vials, preserved with 500 μL of 25% (w/w) metaphosphoric acid, and immediately frozen at −20°C until analyzed. At the same time, 2.5 mL subsamples of effluent were also collected, placed in screw-cap vials and preserved with 500 μL of H_2SO_4 (1%, vol/vol) for determination of NH_3-N. The concentrations of VFA and NH_3-N (mmol/L) were multiplied by daily effluent production (L/d) to determine VFA and NH_3-N production (mmol/d).

Protozoa

Protozoa were enumerated on d 3, 9, 11, 13, and 15 from each fermenter. Bags were gently pressed to expel fermentation

fluid and a 2.5 mL subsample of rumen fluid was obtained and preserved using 2.5 mL of methyl green formalin-saline solution (Ogimoto and Imai, 1981). Protozoa samples were stored in the dark at room temperature until enumerated by light microscopy using a Levy–Hausser counting chamber (Hausser Scientific, Horsham, PA, USA).

Microbial Protein Synthesis

Bacteria in the fermenters were labeled using ^{15}N. On day 7, 0.3 g/L $(NH_4)_2SO_4$ in McDougall's buffer was replaced with 0.3 g/L ^{15}N-enriched $(NH_4)_2SO_4$ (Sigma Chemical Co., St. Louis, MO, USA; minimum ^{15}N enrichment 10.01 atom%) until the end of the experiment. On d 13, 14, and 15, daily effluent samples were preserved with 3 mL of a sodium azide solution (20%; wt/vol) and 40 mL were subsampled for isolation of liquid-associated bacteria.

To determine ^{15}N concentration, effluent liquid samples were centrifuged (20,000 × g, 30 min, 4°C) and the resulting pellets were washed using de-ionized water and centrifuged 3 times (20,000 × g, 30 min, 4°C). The pellets were then re-suspended in distilled water and frozen at −20°C until lyophilized. The FPA bacterial samples collected after stomaching were centrifuged (500 × g, 10 min, 4°C) to remove large feed particles and the supernatant was decanted and centrifuged (20,000 × g, 30 min, 4°C) to isolate a bacterial pellet which was washed 3 times as previously described. The pellet was then resuspended in distilled water and stored at −20°C. Washed feed residues (FPB fraction) were dried at 55°C for 48 h, weighed for DM determination, ball ground (MM 400; Retsch Inc., Newtown, PA, USA), and analyzed for total N and ^{15}N by combustion analysis using a mass spectrometer (NA 1500, Carlo Erba Instruments).

Quantification of Microbial 16S rRNA Marker Genes by qPCR

Samples prepared from FPA were also used to estimate density of selected bacterial populations using real-time polymerase chain reaction (qPCR) (Narvaez et al., 2013). The 48-h incubated residues from d 2, 13, 14, and 15 were processed as described above to obtain the FPA and to quantify microbial populations before (d 2) and after adaptation (d 13, 14, and 15).

Total DNA was extracted from FPA samples using a Qiagen QIAmp DNA Stool Mini Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions, using a slight modification to enhance the yield of DNA from Gram-positive bacteria. Briefly, approximately 30 mg of each sample was suspended in 1.4 mL of ASL buffer (Stool lysis buffer; Qiagen Inc., Valencia, CA, USA) with 0.4 g of sterile zirconia beads (0.3 g of 0.1 mm and 0.1 g of 0.5 mm), and homogenized for 3 min at maximum speed (30/s) using a Qiagen Tissue Lyser II (Qiagen Inc., Valencia, CA, USA). The suspension was heated at 95°C and mixed gently in a thermomixer (Eppendorf-Thermomixer comfort, Eppendorf Ltd, Mississauga, ON, Canada) for 5 min before processing according to the manufacturer's protocol. Total DNA was eluted in 200 μL of Buffer AE (Elution buffer; Qiagen Inc., Valencia, CA, USA) and quantified using a Quant-iT™PicoGreen® dsDNA Assay Kit (Invitrogen Canada Inc., Burlington, ON, Canada) with a NanoDrop 3300 fluorometer (Thermo Scientific, Wilmington, DE, USA). Real-time PCR was used to quantify 16S rRNA copies for total bacteria and sequences specific to *Fibrobacter succinogenes* subsp. S85, *Prevotella bryantii* B_14, *Ruminococcus albus* 7, *Ruminococcus flavefaciens* C94 and *Selenomonas ruminantium* subsp. Lactilytica (ATCC 19 205). Primers and annealing temperatures were as described previously for *F. succinogenes*, *P. bryantii*, *R. flavefaciens, S. ruminantium* (Tajima et al., 2001) and *R. albus* (Wang et al., 1997). Detailed information on PCR cycling conditions, plasmid standards and reference bacterial strains are as described by Wang et al. (2009). Universal 16S primer sequences: 16S-F: CTCCTACGGGAGGCAGCAGT and 16S-R: TTACCGCGGCTGCTGGCAC were used to detect total bacteria using amplification conditions: one cycle at 95°C for 3 min, 30 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. A total of 25 μL of PCR mixture containing 150 nM of each 16S primer was used. For PCR, all samples were normalized to contain 40 ng/μL and 2 μL of extracted DNA template per reaction.

Chemical analysis

Forage and concentrate bag residues were dried at 55°C for 48 h and pooled over 3 days (d 9, 10 and 11, and d 13, 14, and 15 for each fermenter) to ensure that there was sufficient sample for chemical analysis. Samples were ground through a 1 mm screen in a Wiley mill (standard model 4; Arthur H. Thomas Co., Philadelphia, PA, USA) before chemical analysis. Substrates were analyzed for DM (method no. 930.15) and ash content (method no. 942.05) according to AOAC (2006). The concentration of neutral detergent fiber (aNDF) was assayed with a heat stable amylase and sodium sulphite and expressed inclusive of residual ash (Van Soest et al., 1991). The concentration of acid detergent fiber (ADF) was determined according to method 973.18 (AOAC, 2006). The concentration of total N (method no. 990.03; AOAC, 2006) was determined by combustion analysis using a mass spectrometer (NA 1500, Carlo Erba Instruments). Concentrations of VFA and NH_3-N in the liquid effluent were analyzed by gas chromatography (Wang et al., 2001) and the modified Berthelot method (Rhine et al., 1998), respectively.

Calculations

Disappearance of aNDF, ADF and N was determined by the difference between the amount of those components in the substrates before and after incubation. Total disappearances (forage + concentrate) were calculated by difference between the amount substrate (forage + concentrate) incubated and the residue after incubation (forage + concentrate).

Total daily effluent microbial N (MN) was calculated using the N concentration (%) determined for the microbial pellet (harvested from the 40 mL effluent subsample) multiplied by the microbial weight in the total effluent (mg/day). Microbial weight in the total effluent was calculated by multiplying daily effluent production (mL) by the microbial density (mg/mL), which was determined in a 40 mL subsample. The microbial N production (mg/day) from the FPA fraction was calculated by multiplying the N concentration (%) in the FPA microbial pellet by the microbial weight of the FPA fraction (mg/day). The MN

production (mg/day) from the FPB fraction was estimated using the following equation:

$$MN = \frac{\text{APE in RN}}{\text{APE in MN}} \times RN$$

where atom per cent excess (APE) in residue nitrogen (RN) is the percent excess of ^{15}N in solid residue, APE in MN is the percent excess of ^{15}N in the microbial N fraction of the FPA and RN is the total N (mg) in the residue. Total daily MN production (mg/day) was calculated as the sum of microbial production in the effluent, FPA, FPB of straw residues and FPB of concentrate residues (Ribeiro et al., 2015).

True DM disappearance was calculated by subtracting the microbial mass from feed residues. Microbial mass in feed residues was calculated by multiplying MN production (mg) in feed residues by the microbial mass per mg of MN (g of DM of microbial pellet/mg of MN). Microbial mass per mg of MN was determined in FPA bacterial pellets. Ammonia-N and daily VFA production were calculated by multiplying the concentration of the fermentation end product in the effluent by the daily production of effluent. The total bacterial and specific bacteria *16S rRNA* used an absolute quantification method with comparison to cloned standards of known quantity as described (Wang et al., 1997). Copies of *16S rRNA* were analyzed individually for the five bacterial species and expressed as a percentage of total bacterial *16S rRNA* present in the FPA fraction.

Statistical Analysis

Data were analyzed using the MIXED procedure of SAS (SAS Inc., Cary, NC, USA). The model included the fixed effects of inoculum, day and inoculum × day interactions with the day of sampling from each fermenter treated as a repeated measure with individual fermenter considered as the experimental unit. The minimum values of Akaike's Information Criterion was used to select the covariance structure among compound symmetry, heterogeneous compound symmetry, autoregressive, heterogeneous autoregressive, Toeplitz, unstructured and banded for each parameter. The effect of day and its interactions were removed from the model when we had just 1 day of sampling or when different day samples were combined for analysis. Outliers were identified and excluded from the dataset when the studentized residual was outside the range of −2.5 to 2.5 (Neter et al., 1996; Kaps and Lamberson, 2004). Orthogonal polynomial contrasts were performed to determine if replacing cattle inoculum with increasing concentrations (0, 33, 67, and 100%) of bison inoculum resulted in a linear or quadratic effect on measured parameters. Significance was declared at $P \leq 0.05$, and trend was discussed when $0.05 < P < 0.10$.

RESULTS

Dry Matter and Nutrient Disappearance and Fermentation Characteristics

Increasing the proportion of bison inoculum resulted in a positive quadratic effect ($P < 0.05$) on total (straw + concentrate), straw and concentrate true DM disappearance, and on straw and total aNDF disappearance (**Table 2**). For disappearance of straw aNDF, five data points from a total of 32 were identified as having studentized residual outside the range of −2.5 to 2.5 and were removed before contrast analysis. Concentrate aNDF disappearance increased linearly ($P = 0.008$) with increasing proportions of bison inoculum. The relative proportions of cattle:bison rumen inoculum did not affect ($P > 0.22$) total or strawADF disappearance, but concentrate ADF disappearance increased linearly with increasing proportions of bison inoculum ($P = 0.02$). Increasing the proportion of inoculum from bison also resulted in a linear increase in total ($P = 0.01$) and concentrate ($P = 0.04$) N disappearance, but strawN disappearance was unaffected.

The source of rumen inoculum did not affect ($P \geq 0.10$) pH, total gas production, methane production, or microbial N production (total, FPB, FPA or Effluent; **Table 3**). Increasing the proportion of inoculum from bison resulted in a linear increase in total daily VFA ($P = 0.04$) and acetate ($P = 0.03$) production. Increasing levels of bison inoculum had a quadratic effect ($P < 0.05$) on daily NH_3-N, propionate, butyrate, valerate, isovalerate and isobutyrate production and acetate:propionate ratio (C2:C3). Increasing the proportion of inoculum from bison also resulted in a linear increase in numbers ($P < 0.001$) of total protozoa on d 3, and after adaptation on d 9, 11, 13, and 15 ($P < 0.001$).

Microbial Population

Prior to adaptation, qPCR analysis revealed a linear decrease in 16S rRNA copies of total bacteria ($P = 0.03$) and in the percentage of *F. succinogenes* ($P = 0.004$) with increasing proportions of

TABLE 2 | Effect of differing proportions of cattle and bison rumen inoculum on true DM, aNDF, ADF, and nitrogen (N) disappearance (g/kg) of a barley straw diet in the rumen simulation technique (Rusitec).

Item	Cattle:Bison Inoculum				SEM	Linear	Quadratic
	100:0	67:33	33:67	0:100			
TRUE DM DISAPPEARANCE[a]							
Total	503	520	528	503	5.2	0.73	0.002
Barley straw	401	408	419	391	5.6	0.44	0.01
Concentrate	747	787	790	771	11.1	0.15	0.02
aNDF DISAPPEARANCE[b]							
Total	360	382	381	367	6.0	0.54	0.003
Barley straw	326	330	337	316	5.1	0.37	0.02
Concentrate	566	639	653	652	2.3	0.01	0.09
ADF DISAPPEARANCE[b]							
Total	328	332	329	323	7.7	0.60	0.47
Barley straw	310	310	307	297	7.4	0.21	0.52
Concentrate	492	533	545	545	17.0	0.02	0.22
N DISAPPEARANCE[b]							
Total	617	695	692	697	21.7	0.01	0.08
Barley straw	388	400	417	398	19.8	0.59	0.43
Concentrate	725	811	826	815	31.6	0.04	0.11

[a] *Samples from days 13 to 15.*
[b] *Samples from days 9 to 15.*

TABLE 3 | Effect of differing proportions of cattle and bison rumen inoculum on pH, ammonia-N, volatile fatty acid (VFA), total gas, methane (CH_4), microbial N production and protozoa for a barley straw diet using the rumen simulation technique (Rusitec).

Item	Cattle:Bison Inoculum				SEM	Linear	Quadratic
	100:0	67:33	33:67	0:100			
pH[a]	6.80	6.77	6.80	6.80	0.015	0.96	0.32
Ammonia-N, mmol/d[b]	6.35	7.46	7.53	7.65	0.163	<0.001	0.007
Total VFA, mmol/d[b]	37.1	38.9	39.1	40.1	0.90	0.04	0.63
Acetate (C2), mmol/d[b]	23.5	24.4	24.8	26.1	0.78	0.03	0.86
Propionate (C3), mmol/d[b]	10.0	10.6	10.3	9.7	0.17	0.12	<0.001
Butyrate, mmol/d[b]	2.3	2.3	2.4	2.7	0.04	<0.001	<0.001
Valerate, mmol/d[b]	0.59	0.69	0.66	0.67	0.011	0.001	<0.001
Isovalerate, mmol/d[b]	0.46	0.60	0.60	0.61	0.022	<0.001	0.003
Isobutyrate, mmol/d[b]	0.30	0.36	0.35	0.37	0.011	<0.001	0.04
C2:C3[b, c]	2.36	2.31	2.42	2.70	0.048	<0.001	<0.001
Gas, L[a]	1.55	1.46	1.39	1.39	0.134	0.36	0.74
CH_4, mg/d[a]	37.9	37.3	37.2	37.8	4.63	0.97	0.93
CH_4, mg/g incubated DM[a]	4.1	4.1	4.1	4.1	0.51	0.97	0.93
CH_4, mg/g degraded DM[a]	8.0	7.6	7.6	7.8	1.03	0.88	0.80
Production of microbial N, mg/d[d]							
Total	55.9	56.5	57.6	56.3	1.48	0.73	0.54
FPB[e] Concentrate	2.1	2.2	1.7	2.2	0.29	0.91	0.48
FPB Barley straw	20.0	17.4	17.8	17.7	1.23	0.26	0.33
FPA[f]	5.6	5.4	5.6	5.5	0.26	0.82	0.97
Effluent	30.6	31.4	32.5	30.9	1.03	0.64	0.23
Protozoa (× 10^3/mL), d 3	16.0	25.5	59.0	71.0	9.10	<0.001	0.89
Protozoa (× 10^3/mL)[g]	7.2	7.9	14.9	17.0	1.83	<0.001	0.70

[a] *Samples from days 9 to 15.*
[b] *Samples from days 9 to 12.*
[c] *Acetate: propionate ratio.*
[d] *Samples from days 13 to 15.*
[e] *Feed particle-bound.*
[f] *Feed particle-associated.*
[g] *Samples from days 9, 11, 13 and 15.*

inoculum from bison (**Table 4**). A positive quadratic effect was observed for the percentage of *R. albus* ($P = 0.05$) whereas increasing the proportion of inoculum from bison did not affect the percentage of *S. ruminantium* or *P. bryantii*.

After adaptation, the source of inoculum did not affect *16S rRNA* copies of total bacteria or the percentages of *S. ruminantium* or *P. bryantii* in FPA fractions (**Table 4**). However, increasing the proportion of bison inoculum had a positive quadratic effect on *F. succinogenes* ($P = 0.05$), a linear decrease on *R. albus* ($P = 0.03$), and a tendency of a linear increase on *R. flavefaciens* ($P = 0.09$) proportion.

DISCUSSION

In the present study the bison used as rumen inoculum donors were fed a diet that differed from that of cattle, as we were unable to convince commercial producers to feed bison an extremely low quality straw diet. However, bison were fed a forage based diet consisting of barley silage and oats (75:25 DM basis), but even with this difference solid ruminal digesta from bison possessed similar crude protein, NDF and ADF concentrations to that of cattle. Fermenters containing inoculum from both host species were allowed to adapt to the concentrate—straw diet for a period of 8 d, a period that is 6 d longer than the 48 h that have been recently reported to be sufficient to allow microbial populations in the Rusitec to stabilize (Lengowski et al., 2016). Although, there is a possibility that diet may have influenced the nature of the microbial populations within the initial inocula, if it was an overriding factor one would predict that the aNDF disappearance in fermenters inoculated with digesta from cattle should be far higher than those inoculated with bison. The fact that the aNDF disappearance was highest in fermenters containing a mixture of cattle and bison contents suggests that some of this synergy arose from the mixing of microbiomes from different ruminant hosts.

DM and Nutrient Disappearance and Fermentation Characteristics

Bison inoculum alone did not improve total DM or ADF disappearance compared to cattle inoculum. However, mixtures of bison and cattle rumen inoculum improved straw and total DM and aNDF disappearances as compared to either bison or cattle rumen inoculum alone. These results suggest

TABLE 4 | Effect of differing proportions of cattle and bison rumen inoculum on total *16S rRNA* bacterial gene copies and on relative abundance of marker genes for specific bacterial species (% of total bacterial *16S rRNA*) in the feed particle-associated (FPA) fraction of barley straw diet, before and after adaptation in the rumen simulation technique (Rusitec).

Item	Cattle:Bison Inoculum				SEM	Linear	Quadratic
	100:0	67:33	33:67	0:100			
BEFORE ADAPTATION (d 2)							
Total bacterial 16S rRNA copies[a] ($\times 10^{10}$)	75.4	72.3	49.8	48.8	9.09	0.03	0.91
SPECIFIC MICROBIAL POPULATION (% $\times 10^{-1}$)							
F. succinogenes	4.22	2.54	0.87	0.34	0.888	0.004	0.49
R. flavefaciens	0.40	0.55	0.58	0.38	0.098	0.99	0.10
R. albus	1.09	1.24	1.79	1.07	0.197	0.57	0.05
S. ruminantium	4.68	1.81	5.17	2.17	0.516	0.11	0.90
P. bryantii	0.52	2.40	3.04	1.70	1.259	0.44	0.18
AFTER ADAPTATION (d 13, 14, 15)							
Total bacterial 16S rRNA copies[a] ($\times 10^{10}$)	48.4	41.4	47.4	49.6	3.114	0.49	0.16
SPECIFIC MICROBIAL POPULATION (% $\times 10^{-1}$)							
F. succinogenes	4.59	6.29	5.93	3.51	0.992	0.42	0.05
R. flavefaciens	0.31	0.34	0.30	0.43	0.033	0.09	0.16
R. albus	0.46	0.35	0.34	0.35	0.032	0.03	0.10
S. ruminantium	0.34	0.18	0.32	0.25	0.086	0.75	0.63
P. bryantii	0.12	0.04	0.11	0.03	0.044	0.38	0.99

[a] *Total bacterial 16S rRNA gene copies in FPA were the total amount estimated from daily residual outputs of each fermentation vessel.*

possible synergistic effects of the mixture of cattle and bison inoculum on DM and aNDF degradation of straw based diets. Synergy has been previously reported in pure cultures when a non-fibrolytic *Prevotella ruminicola* strain was co-cultured with either *F. succinogenes* or *R. flavefaciens*, as fiber digestion was improved compared to that of the fibrolytic species alone (Osborne and Dehority, 1989; Fondevila and Dehority, 1994). Also, *F. succinogenes* acted synergistically with the fungal species, *Caecomyces*, to degrade perennial ryegrass (*Lolium perenne*) stem fragments, suggesting that these prokaryotic and eukaryotic species may have complementary fibrolytic activities (Joblin et al., 2002). Although synergies among ruminal microorganisms within consortia from different ruminant species are likely far more complex, these laboratory studies do demonstrate that such synergies do occur.

Corn DDGS and canola meal, the main concentrate ingredients in this study, are common protein supplements fed to cattle and both have high proportion of rumen-undegradable protein (RUP; Santos et al., 1998). In spite of this fact, N disappearance from the concentrate increased with increasing proportion of bison inoculum. As the urea portion of the concentrate can be considered completely degraded or washed out of the fermenters for all treatments, the increased N disappearance is most likely a result of a specific effect of the bison inoculum on RUP. Furthermore, the increase in N disappearance from the concentrate corresponded to an increase in NH_3-N. High ruminal NH_3-N concentration has been associated with large numbers of protozoa in rumen contents (Veira et al., 1983; Towne et al., 1988), presumably a reflection of the predatory activity of these eukaryotes against bacteria (Belanche et al., 2011, 2012) and their ability to degrade feed protein (Ushida et al., 1986). Previous studies demonstrated greater feed protein degradation of diets with low protein solubility in faunated ruminants compared to protozoa-free ruminants (Ushida and Jouany, 1985, 1986; Ushida et al., 1986). The present study is in agreement with these findings, as the linear increase in total and concentrate N disappearance and daily NH_3-N production observed with increasing proportions of bison inoculum is consistent with the increase in total protozoa numbers.

The greater number of protozoa in the Rusitec with increasing proportion of bison inoculum likely reflects the greater protozoa numbers in the rumen of bison than cattle *in vivo*. Greater total protozoa numbers in the rumen of bison compared to cattle have been previously reported when both were fed similar diets (Towne et al., 1988).

The linear increase in total VFA and the quadratic increase in valerate, isovalerate and isobutyrate production observed with increasing proportions of bison inoculum is likely a reflection of the linear increase in CP degradation (N disappearance) and the catabolism of branched-chain amino acids (Lindsay and Reynolds, 2005). Lack of effect on total gas and CH_4 production is somewhat surprising given the observed linear or quadratic changes in DM and CP degradation and the differences in C2:C3 ratio. The observed increase in C2:C3 ratio with increasing proportion of ruminal inoculum from bison was expected to correspond to an increase in CH_4 production (Moss et al., 2000), particularly because production of microbial N (total, FPB concentrate or forage, FPA and effluent) was not affected as microbial synthesis can serve as a hydrogen sink. However, the lack of an effect on microbial N is consistent with the similar total copies of bacterial 16S rRNA observed across treatments after fermenters were adapted.

Some studies have suggested that bison have a superior ability to digest low-quality forages as compared to cattle (Peden et al., 1974; Richmond et al., 1977; Hawley et al., 1981a), but no studies have compared possible synergy between the two sources of inoculum. Hawley et al. (1981b) observed that the *in vivo* digestion coefficients of DM, CP, aNDF and ADF from sedge hay were greater in bison than in Hereford steers. They suggested that digestibility of DM, CP and fiber is superior in bison when poor-quality, low-protein diets are fed. Richmond et al. (1977) also observed in an *in vivo* experiment, greater digestibility of sedge and grass hays (7–8% CP) in bison than cattle, but the digestibility of alfalfa (19% CP) was similar between these two species. These authors theorized that the superior ability of bison to digest poor-quality, low protein forages as compared to cattle, was due to their greater ability to recycle ruminal N, a reduced rate of ruminal particulate passage and differences in ruminal microbial populations. These first two factors would not be responsible for differences in digestion in the artificial rumen as feed bags were retained in fermenters for the same period of time, and recycling of N did not occur.

Microbial Population

The reduction in total copies of bacterial 16S rRNA observed with increasing bison rumen inoculum at the beginning of the Rusitec study (d 2), with a lack of a difference after adaptation, may reflect the fact that cattle were more adapted to the diet fed to the fermenter, whereas the bison were not. Another possibility is that there were carryover effects of the differing composition of the diets fed to the donor animals that influenced initial bacterial populations that were introduced into the Rusitec. Alternatively, it might indicate that the longer delay between obtaining the inoculum and initiating the fermenters for bison compared with cattle resulted in lower bacterial populations. The lack of differences in total copies of bacterial 16S rRNA in fermenters is a reflection that adaptation occurred and in part likely reflects that the dilution rate and substrates provided in all fermenters was identical.

The quadratic effect observed for *F. succinogenes* after adaptation is consistent with the quadratic response observed for total aNDF and DM disappearance. This result indicates that *F. succinogenes* was positively affected by cattle and bison rumen inoculum mixture. It is possible that the disturbance in the microbial community created by the mixture of rumen inoculum from the different species created favorable conditions for the growth of *F. succinogenes*.

The interactions among major fibrolytic species in the rumen (*F. succinogenes, R. flavefaciens* and *R. albus*) are complex, and some studies have documented antagonistic responses in cellulose digestion among these species *in vitro* (Odenyo et al., 1994; Shi et al., 1997). Our results are suggestive of this antagonistic behavior as we observed a linear decrease in the *R. albus* population, and a tendency for a linear increase in *R. flavefaciens* and a quadratic effect on *F. succinogenes* population with increasing proportions of bison inoculum. There is evidence that *R. albus* and *R. flavefaciens* are capable of producing inhibitors (bacteriocin-like factors) that suppress the growth of *R. flavefaciens* and *F. succinogenes,* respectively (Chen and Weimer, 2001).

The Rusitec eliminates any potential differences between cattle and bison in chewing efficiency, passage rate, N recycling, and nutrient absorption, and thus enables a comparison of the inoculum alone. According to a recent global rumen census (Henderson et al., 2015), differences in the rumen microbial community are predominantly attributable to diet, with the host being less influential. A recent study by Witzig et al. (2015) evaluated the effect of different donor animal species (cattle vs. sheep) and diet fed to the donor animals (hay-concentrate vs silage-based diets) on the microbial community established in the Rusitec. Their results suggest that the donor animal diet had a substantial impact on composition of the microbial community within the Rusitec and that the effect of the donor animal species itself was of minor consequence. In our study, bison and cattle were fed different diets, but of similar aNDF content and most measurements were made after adaptation. Thus, composition of the diet fed to host animals may have influenced fermentation immediately after the Rusitec started, but diet was likely not a factor once the system was adapted. We recognize that the Rusitec system is a simplified microbiome reflecting the starting cultures and that microbial interactions may be more complex *in vivo*, but nevertheless the technique may aide in the identification of strategies that improve digestion efficiency.

CONCLUSIONS

To our knowledge, this is the first *in vitro* continuous culture experiment examining cross species inoculation of fermenters. Overall, bison inoculum more readily degraded feed protein than cattle inoculum, with a mixture of inoculums synergistically increasing the DM and aNDF disappearance of a barley straw based diet. Ruminal inoculum from bison alone did not promote greater fiber digestion as compared to that obtained from cattle. Rumen inoculation across ruminant species may be a means of increasing ruminal fiber and protein degradation, but this still needs to be investigated *in vivo*. In addition, microbiome studies are needed to explore whether the higher digestibility observed in mixed inocula correlates with greater bacterial or protozoal biodiversity.

AUTHOR CONTRIBUTIONS

DO, GR, and TM designed the experiment; DO and GR conducted the research; GR, KB, and RF analyzed the data and DO, GR, TM, KB, and MM wrote the manuscript; TM, KB, and MM provided experimental resources; GR, KB, and TM critically reviewed the manuscript. All authors read and approved the final manuscript.

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Nutritional Models of Experimentally-Induced Subacute Ruminal Acidosis (SARA) Differ in Their Impact on Rumen and Hindgut Bacterial Communities in Dairy Cows

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Effects of subacute ruminal acidosis (SARA) challenges on the bacteria in rumen fluid, cecal digesta, and feces of dairy cows were determined using 16S rRNA gene pyrosequencing and real-time quantitative PCR. Six non-lactating Holstein cows with cannulas in the rumen and cecum were used in a 3 × 3 Latin square arrangement of treatments. During the first 3 wk of each experimental period, cows received a control diet containing 70% forages on a dry matter (DM) basis. In wk 4 of each period, cows received one of three diets: (1) the control diet; (2) a diet in which 34% of the dietary DM was replaced with pellets of ground wheat and barley (GBSC); or (3) a diet in which 37% of dietary DM was replaced with pellets of ground alfalfa (APSC). Rumen fluid, cecal digesta and feces were collected on d 5 of wk 4 of each period and the composition of the bacterial community was studied. Rumen fermentation responses were reported in a companion study. Both SARA-inducing challenges resulted in similar digesta pH depressions (as shown by the companion study), and reduced bacterial richness and diversity in rumen fluid, but GBSC had the larger effect. None of the challenges affected these measures in cecal digesta, and only GBSC reduced bacterial richness and diversity in feces. Only GBSC reduced the abundance of Bacteroidetes in rumen fluid. Abundances of limited number of bacterial genera identified by 16S rRNA gene sequencing in the rumen, cecum and feces were affected by the GBSC. The APSC did not affect any of these abundances. Both challenges increased the abundances of several starch, pectin, xylan, dextrin, lactate, succinate, and sugar fermenting bacterial species in the rumen, cecum, and feces as determined by qPCR. Only GBSC increased that of *Megasphaera elsdenii* in the rumen. Both challenges decreased the abundance of *Streptococcus bovis,* and increased that of *Escherichia coli,* in cecal digesta and feces, with GBSC having the larger effect. These results showed that the SARA challenges caused moderate and reversible changes of the composition of the bacteria in the foregut and hindgut, with the greater changes observed during GBSC.

Keywords: subacute ruminal acidosis, rumen, cecum, bacteria, 16S rRNA gene sequencing, real-time quantitative PCR

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INTRODUCTION

In order to meet their production potential, high yielding dairy cows require high-energy diets. These diets commonly contain high inclusion rates of grains. These high inclusion rates affect conditions for microorganisms in the rumen and large intestine, including the acidity, osmolality, and the contents of fermentable substrates (Tajima et al., 2001; Plaizier et al., 2008, 2012). Increases of the dietary grain content and, as a result, the dietary starch contents can affect the rumen and hindgut bacteria, but these effects vary greatly among animals (Khafipour et al., 2009c; Mao et al., 2013; Petri et al., 2013). Feeding high-grain diets to cows creates the risk of subacute ruminal acidosis (SARA), a metabolic disorder characterized by a reversible rumen pH depression for extended periods each day (Plaizier et al., 2008; Kleen and Cannizzo, 2012). Also, experimentally induced SARA by feeding high-grain diets increases concentrations of free bacterial lipopolysaccharide endotoxin (LPS) both in the rumen and hindgut digesta, and triggers an immune response in dairy cows (Li S. et al., 2012; Plaizier et al., 2012). The induction of SARA also reduces bacterial richness and diversity in the rumen and leads to a decline in Bacteroidetes and an increase in Firmicutes abundance in the rumen (Khafipour et al., 2009b,c; Mao et al., 2013; Petri et al., 2013). Grain-induced SARA can also affect the bacteria and increase fermentation in the hindgut, most likely via increasing by-pass starch that escapes rumen fermentation and small intestine digestion, which increases acidity and volatile fatty acid (VFA) concentrations of digesta in the hindgut and the feces (Khafipour et al., 2009c; Mao et al., 2012; Petri et al., 2013). Feeding diets that contain ground forages, such as pellets of ground alfalfa hay, can induce SARA without increasing the starch contents of digesta in the hindgut and feces (Khafipour et al., 2009b; Plaizier et al., 2012). Also, Khafipour et al. (2009b) and Li S. et al. (2012) observed that replacing alfalfa hay with pellets made of ground alfalfa hay resulted in a rumen pH depression representative of SARA, without causing the innate immune response that occurs during grain-induced SARA (Plaizier et al., 2008, 2012). These differences between the two models of SARA induction may be the result of differences in the impact of these challenges on the bacteria in the rumen and the hindgut (Khafipour et al., 2009c; Plaizier et al., 2012). This may be expected, as the composition of digesta and the conditions and availability of substrates for the bacteria in the digestive tract vary between these two SARA-induction models (Khafipour et al., 2009a,b). It has been hypothesized that the differences between the two-SARA induction models may be caused by the increase in rumen by-pass starch during grain-induced SARA that results in an increase in the lysis and shedding of LPS by gram-negative bacteria in the hindgut that triggers the immune response (Khafipour et al., 2009a,b; Plaizier et al., 2012). These hypotheses challenge the current definition of SARA, which is only based on a rumen pH depression.

Several studies on the impact of grain-induced rumen acidosis on rumen bacteria of dairy cows have been conducted using culture-based, quantitative PCR (qPCR), and fragmentation techniques, such as terminal restriction fragments length polymorphism (Nagaraja et al., 1978; Russell and Hino, 1985; Khafipour et al., 2009c). Recent advances in sequencing technology, however, offer rapid, low-cost molecular-based methodologies that can investigate bacterial communities with high resolution and as a whole (Krause et al., 2013). Using these techniques, Mao et al. (2013) and Li et al. (2013) reported that grain-induced SARA increased the abundance of Firmicutes and decreased that of Bacteroidetes in the rumen. However, there were discrepancies in the proportion of lower-abundance phyla, such as Proteobacteria, Actinobacteria, Spirochaetes, and Tenericutes and the shift in their proportions due to induction of SARA. These authors also investigated the impact of grain-induced SARA on the fecal bacteria of dairy cows and reported associations among the abundances of several species of Bacteroidetes and Firmicutes and the concentrations of VFA in the feces, which suggests that such associations exist in hindgut digesta also. The conflicting and inconsistent results among studies may be caused by the complexity of the bovine gut microbiota, the difference between experimental approaches and sequencing techniques, the small scale of these studies, and the variation among cows in the susceptibility to SARA. Studies that compared the effects of different models of SARA induction on the bacteria of the rumen and the large intestine within the same experiment have not yet been conducted. Such studies are needed to enhance the understanding of the relationship between the SARA and the gut bacteria of dairy cows, as this will lay the foundation for the development of strategies to prevent this disorder.

This report is part of a larger experiment in which both a grain and a finely ground alfalfa hay SARA challenges were induced in dairy cows. The companion study of Li S. et al. (2012) described the effects of those challenges on the pH and the concentrations of VFA and free LPS of digesta in the foregut and hindgut. The current report describes the effects of this challenge on the microbiota in these digesta that occurred in the same experiment.

We hypothesized that SARA induced by high-grain feeding and SARA induced by feeding pellets of ground forage alter the bacteria of rumen digesta, cecum digesta and feces, but that these effects differ between the two models. In this study, we used pyrosequencing technology and qPCR to investigate and compare the impact of these two models on the bacteria of digesta in the rumen and the cecum, and in the feces of non-lactating dairy cows.

MATERIALS AND METHODS

Animals Models and Experimental Treatments

The design of the study was described earlier in the companion manuscript of Li S. et al. (2012) that described effects of the experimental treatments on fermentation and endotoxins in the rumen and the hindgut. The study was pre-approved by the Fort Garry Campus Animal Care Committee of the University of Manitoba in accordance with the Canadian Council for Animal Care guidelines (Canadian Council on Animal Care (CCAC), 2009). In brief, six non-lactating, multiparous Holstein cows with cannulas in the rumen and cecum were used. The animals had

live weights of 620 ± 45.7 kg (mean ± SD). They were randomly allocated to three treatments in a 3 × 3 Latin square design experiment which consisted of three periods of 4 weeks.

In the first 3 weeks of each experimental period, all cows received a basal diet with a forage-to-concentrate ratio of 70:30. Starting the Friday of the 3rd week to Monday of the 4th week of each experimental period, the diets of three groups of cows were changed as follows: (1) the basal diet remained unchanged (control), (2) the grain pellets consisting of 50% ground wheat and 50% ground barley gradually replaced with 34% of the dry matter of the basal diet (Grain-based SARA challenge, GBSC); and (3) alfalfa pellets were added up to 37% of the basal diet DM to replace alfalfa hay (alfalfa-pellet SARA challenge, APSC). Three diets were then fed to all cows in the remainder of 4th week. Experimental diets were described in detail by Li S. et al. (2012). A summary of the chemical composition of these diets is given in **Table 1**.

Cows were housed in individual stalls in the large animal metabolism facility of the Glenlea Research Station, University of Manitoba, and were cared for in accordance with the Canadian Council for Animal Care guidelines (Canadian Council on Animal Care (CCAC), 2009). Cows were fed *ad libitum* once daily at 0900 h, allowing for between 5 and 10% of feed refusals, and had free access to fresh water.

Rumen Fluid and Cecum Digesta Sampling

Rumen fluid, digesta in the cecum, and feces were sampled on d5 of wk 4 of each experimental period at 6 h after feed delivery. Rumen fluid was collected from the ventral sac of the rumen and strained through 4 layers of sterile cheesecloth. Cecal digesta was collected via the cecal cannula and fecal samples were collected from the rectum. All samples were then aliquoted into 10 ml sterile tubes or Whirl-Pak 60 g bags (NASCO, WI, USA) before they were snap frozen in liquid nitrogen and stored in −80°C until further analysis.

DNA Extraction

Rumen fluid and digesta samples were thawed at room temperature and subsequently kept on ice. A total of 1 ml of rumen fluid was centrifuged at 15,000 × g to collect the sediment. Subsequently, DNA was extracted from the sediment using a ZR fecal DNA kit (D6010; Zymo Research Corp., Orange, CA, USA) that included a bead-beating step for disrupting bacterial cells. The extraction of DNA of cecal and fecal samples was conducted on 200 mg of sample using the same kit. At the last step of the procedure, DNA was eluted from the column with elution buffer, and DNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). The DNA samples were normalized to 20 ng/μl for pyrosequencing and to 2 ng/μl for qPCR. All DNA were quality verified by PCR amplification of the 16S rRNA gene using universal primers 27F (5′-GAAGAGTTTGATCATGGCTCAG-3′) and 342R (5′-CT GCTGCCTCCCGTAG-3′) as described (Khafipour et al., 2009c). Amplicons were verified by agarose gel electrophoresis. For qPCR analyses, DNA samples were aliquoted into 10 μl/vials, which was sufficient for testing one set of primers, in order to avoid repeated freeze-thaw cycles. All DNA samples were stored at −80°C.

TABLE 1 | Chemical composition of experimental diets.

Item	Nutrient composition		
	Control	APSC	GBSC
Dry matter, %	54.3	69.0	61.6
Crude protein, % DM	16.1	16.0	16.0
Neutral detergent fiber, % DM	35.6	34.5	22.9
Starch, % DM	14.5	15.9	33.7

Control, Control; APSC, alfalfa-pellet SARA diet; GBSC, grain-based SARA challenge diet.

16S rRNA Gene Sequencing and Bioinformatics

A total of 54 DNA samples from rumen fluid samples were pyrosequenced using the bacterial tag-encoded GS FLX-Titanium amplicon as described by Dowd et al. (2008b). In brief, a mixture of Hot Start, HotStar high fidelity Taq polymerases, and Titanium reagents were used to perform a one-step PCR (35 cycles) with primer 28f (5′-GAGTTTGATCNTGGCTCAG-3′) and 519r (5′-GTNTTACNGCGGCKGCTG-3′), which covered the hypervariable regions V1-V3 of the bacterial 16S rRNA genes (Dowd et al., 2008a). The pyrosequencing procedures were carried out at the Research and Testing Laboratory (Lubbock, TX; http://www.Researchandtesting.com). The raw data are presented in Supplementary Data Sheet 1.

Sequence Editing, Classification, and Building of the Phylogenetic Tree

Pyrosequencing data were binned using sample-specific barcode sequences, and filtered using QIIME 1.7 (Caporaso et al., 2010b). All sequences <200 bp, with ambiguous nucleotide bases, or a homopolymer length longer than 7 bp were removed from downstream analyses. Chimeric sequences were detected using the UCHIME algorithm (USEARCH 6.1) and sequences were assigned to Operational Taxonomic Units (OTU) using the QIIME implementation of UCLUST (Edgar et al., 2011). In total, 71,029 sequences (6733 unique observations) from 18 ruminal samples, 113,734 sequences (10,363 unique observations) from 18 cecal samples and 104,277 sequences (10,784 unique observations) from 18 fecal samples were generated in this step. An open reference-based OTU picking approach was implemented with the QIIME algorithm and usearch61 method with default parameters (Edgar et al., 2011) were used to cluster the sequences at the 97% sequence similarity level using the Greengene database (Version 13_5) (McDonald et al., 2012). Those sequences that failed to cluster were subsampled for de novo OTU picking. All picked OTUs were subsequently aligned by PyNAST (Caporaso et al., 2010a), and a phylogenetic tree was built using FastTree method (Price et al., 2009) to calculate UniFrac distances (Lozupone et al., 2011) within QIIME. Taxonomy was assigned to OTUs using RDP classifier via QIIME with a confidence threshold of 0.8 (Wang et al., 2007).

Alpha- and Beta-Diversity Analyses

Samples were rarefied for alpha-diversity calculations and generation of rarefaction curves (**Figure 1**), in order to eliminate the bias caused by the different sample sizes (Roesch et al., 2007). Standard alpha-diversity indices were determined. The α parameter of Fisher's log-series was used as a diversity index (Fisher et al., 1943). Richness indices included the Chao1 index and the abundance based coverage estimation (ACE) richness indices. Diversity estimators included the Shannon and Simpson indices (Hill, 1973).

The dataset was also subsampled to the median (de Cárcer et al., 2011) for beta-diversity analysis using Phyloseq (McMurdie and Holmes, 2013). UniFrac-based principal coordinates analysis (PCoA; **Figures 2–4**) were conducted with Phyloseq. PCoA plots were generated based on both weighted and unweighted UniFrac distance matrix. In addition, permutational multivariate analysis of variance (PERMANOVA; Anderson et al., 2011) based on the same similarity matrix were used to test the effect of the treatments.

Partial Least Square Discriminant Analyses

Partial least square discriminant analysis (PLS-DA; SIMCA P+ 13.0, Umetrics, Umea, Sweden) was performed on the proportional data at the genus level to test the effects of treatments (Li R. et al., 2012) (Supplementary Figures 1–3). The PLS-DA is a particular case of partial least square regression analysis in which Y is a set of binary (0 vs. 1) variables describing the categories of a categorical variable on X. In this case, X

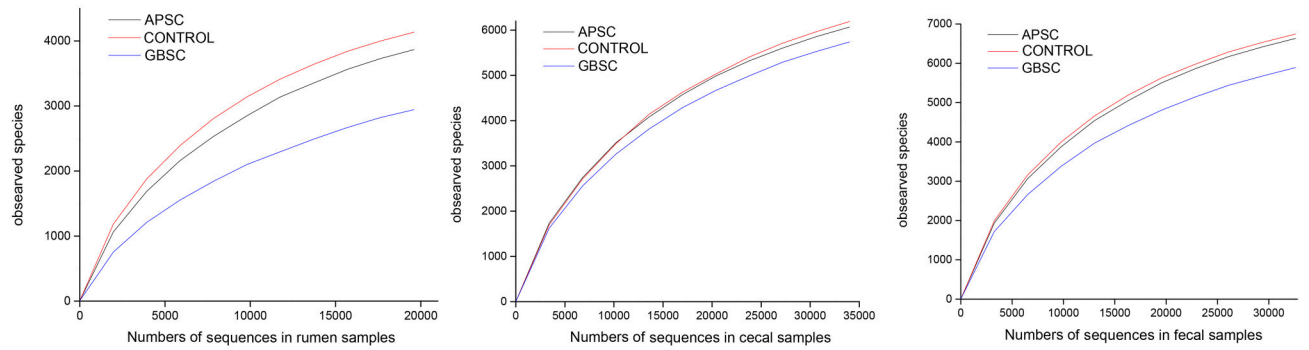

FIGURE 1 | Rarefaction curves indicating the observed number of operational taxonomic units (OTUs) at a genetic distance of 3% in rumen bacterial communities of dairy cows under control feeding, an alfalfa-pellet SARA challenge (APSC) or a grain-based SARA challenge (GBSC) conditions. The number V1–V3 sequences of 16S rRNA gene in the pyrosequencing library was the pooled reads across individual samples (6 samples).

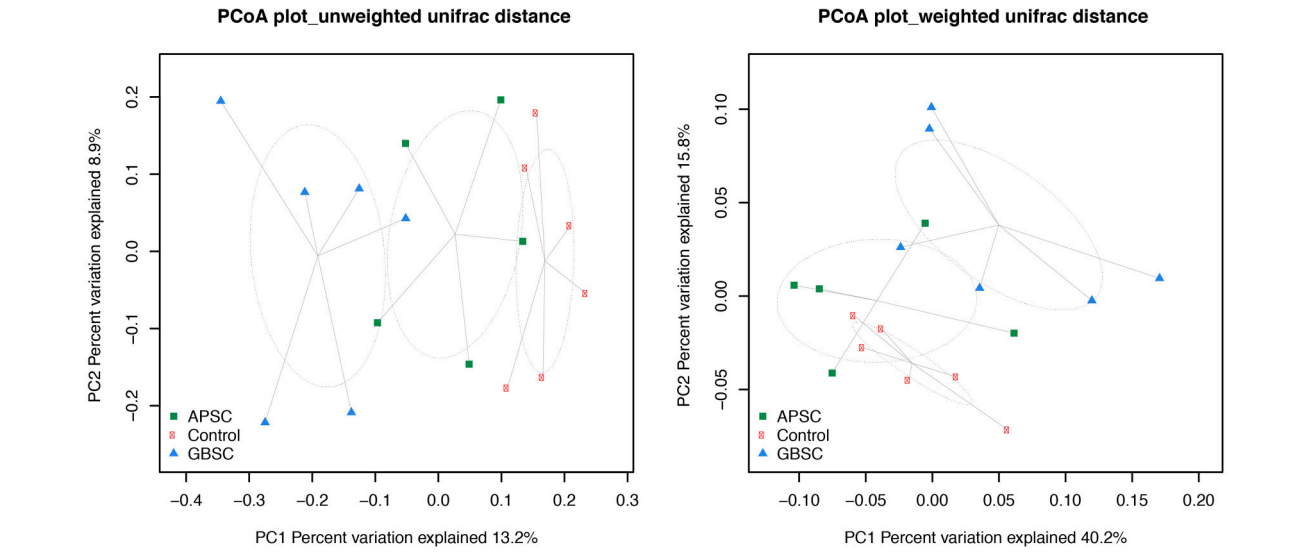

FIGURE 2 | Two-dimensional PCoA plots based on the unweighted and weighted UniFrac distance matrix illustrates variation in rumen fluid bacterial communities as affected by different subacute ruminal acidosis (SARA) challenge conditions. The ellipses were drawn with standard errors of the points at 0.95 confidence limit. Labels are placed at means centers of each site and are linked to each sample of the corresponding site. Abbreviations in figure: APSC, alfalfa-pellet SARA challenge; GBSC, grain-based SARA challenge. Significance levels unweighted analysis, APSC vs. Control $P = 0.01$; GBSC vs. Control $P < 0.01$; GBSC vs. APSC $P = 0.15$. Significance levels weighted analysis, APSC vs. Control $P = 0.22$; GBSC vs. Control $P < 0.01$; GBSC vs. APSC $P = 0.06$.

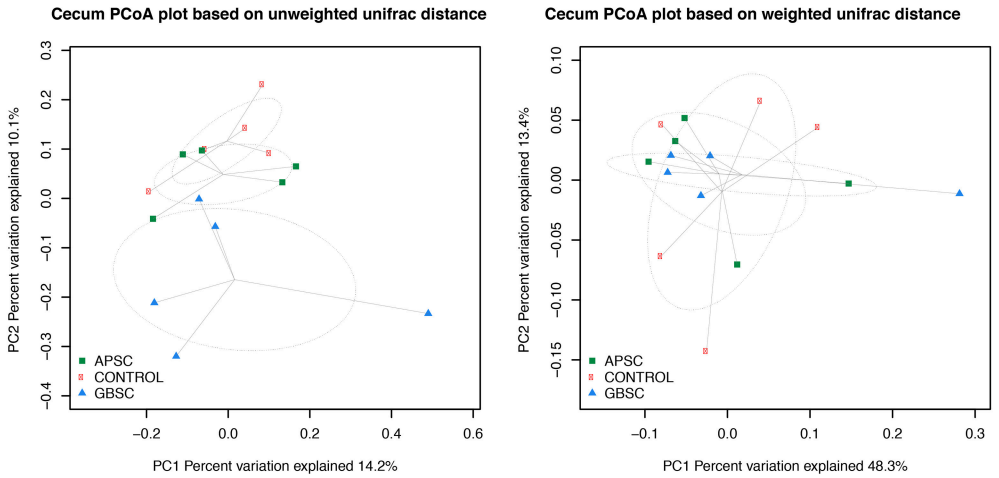

FIGURE 3 | Two-dimensional PCoA plots based on the unweighted and weighted UniFrac distance matrix illustrates variation in cecal bacterial communities as affected by different subacute ruminal acidosis (SARA) challenge conditions. The ellipses were drawn with standard errors of the points at 0.95 confidence limit. Labels are placed at means centers of each site and are linked to each sample of the corresponding site. Abbreviations in figure: APSC, alfalfa-pellet SARA challenge; GBSC, grain-based SARA challenge. Significance levels unweighted analysis, APSC vs. Control $P = 0.53$; GBSC vs. Control $P = 0.05$; GBSC vs. APSC $P = 0.26$. Significance levels weighted analysis, APSC vs. Control $P = 0.75$; GBSC vs. Control $P = 0.56$; GBSC vs. APSC $P = 0.77$.

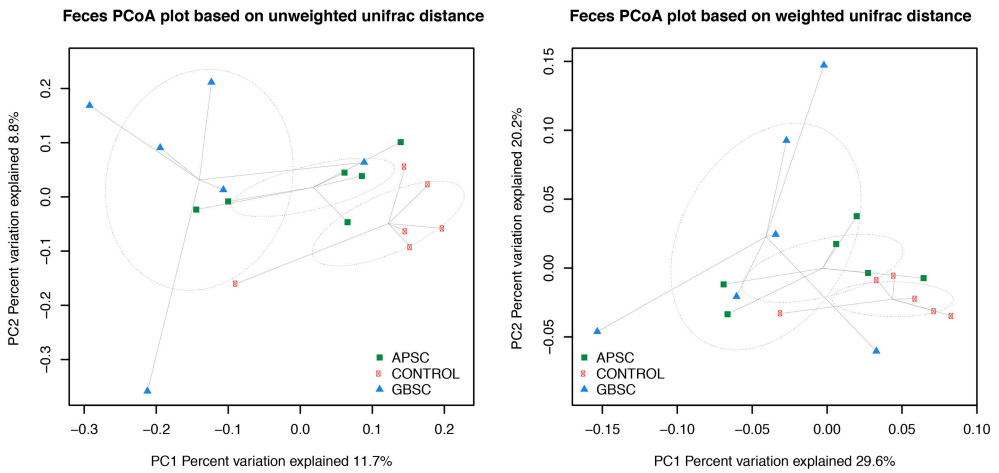

FIGURE 4 | Two-dimensional PCoA plots based on the unweighted and weighted UniFrac distance matrix illustrates variation in fecal bacterial communities as affected by different subacute ruminal acidosis (SARA) challenge conditions. The ellipses were drawn with standard errors of the points at 0.95 confidence limit. Labels are placed at means centers of each site and are linked to each sample of the corresponding site. Abbreviations in figure: APSC, alfalfa-pellet SARA challenge; GBSC, grain-based SARA challenge. Significance levels unweighted analysis, APSC vs. Control $P = 0.01$; GBSC vs. Control $P < 0.01$; GBSC vs. APSC $P = 0.15$. Significance levels weighted analysis, APSC vs. Control $P = 0.05$; GBSC vs. Control $P < 0.01$; GBSC vs. APSC $P = 0.28$.

variables were bacterial genera and binary Y was observations of control, GBSC, and APSC. For this analysis, data were scaled using Unit Variance in SIMCA. Cross-validation then was performed to determine the number of significant PLS components and a permutation testing was conducted to validate the model. R^2 estimate then was used to evaluate the goodness of fit and Q^2 estimate was used to evaluate the predictive value of the model. The PLS-regression coefficients were used to identify genera that were most characteristic of each treatment group. The significant shifts of taxa were determined when the error bars of each component was above or below x axis of coefficient plot (Wang et al., 2016). The results of PLS-DA were visualized by PLS-DA loading scatter plots (Supplementary Figures 1–3).

Quantitative PCR Analysis

Quantitative PCR was carried out in 96-well optical plates on an AB 7300 system (Applied Biosystems, Foster City, CA, USA) as described previously (Khafipour et al., 2009c). The primers listed in Supplementary Table 1 were synthesized by University Core DNA Services (University of Calgary, Calgary, AB, Canada).

Each reaction mixture was run in triplicate in a volume of 15 μl in optical reaction plates (Applied Biosystems, Foster City, CA, USA) sealed with optical adhesive film (Applied Biosystems, Foster City, CA, USA). Amplification reactions were carried out with 7.5 μl Power SYBR green PCR master mix (Applied Biosystems, Foster City, CA, USA) mixed with the selected primer set (Supplementary Table 1) with a final concentration of 450 nM. Amplification consisted of one cycle of 95°C (10 min) to activate AmpliTaq Gold polymerase, followed by 40 cycles of denaturation at 95°C (15 s), and annealing/extension at 60°C (1 min). Final melting analysis was obtained by slow heating from 65° to 95°C in order to assess the specificity of the prime set. The efficiency of the amplification of each primer set was calculated from the slope of the standard curve generated with pool DNA samples. The change in the quantity of target species in a tested samples relative to the same target species in a calibrator sample was calculated after all real-time data were normalized for Eubacteria using bacteria 16S RNA gene primer sets, which detect all bacterial strains (Khafipour et al., 2009c).

Statistical Analyses

The effects of treatment on alpha-diversity indices, bacterial abundances at the phylum and lower taxonomical levels, and species relative ratios were analyzed with SAS version 9.3 (SAS Institute Inc., 2011). The UNIVARIATE procedure was used to test the normality of error terms. Non-normally distributed data were transformed using the Box-Cox power transformation implemented within TRANSREG procedure that iteratively tests a variety of λ and identifies the best power transformation. Normalized data were used to assess the effect of treatment using MIXED procedure of SAS (SAS Institute Inc., 2011), with treatment and experimental period as fixed factors. The effect of cow was treated as random in the model. Pairwise comparisons between the treatments were conducted with Tukey's honestly significant difference test corrected for multiple comparisons. Statistical differences were declared as significant and highly significant at $P < 0.05$ and $P < 0.01$, respectively. Trends toward significance were discussed at $0.05 < P < 0.10$.

RESULTS

Bacterial Richness and Diversity

The rarefaction curves of the observed number of OTUs are given in **Figure 1**. Effects of the treatments on measures of bacterial richness and diversity in rumen fluid, cecal digesta and feces are reported in **Table 2**. Both SARA induction models reduced the bacterial richness and diversity in the rumen, but GBSC lead to the greatest reductions. The GBSC treatment reduced the richness of species, as indicated by the reduction of the numbers of OTUs classified at 97% distance of amplified 16S rRNA gene sequences and tended to reduce the effective number of species calculated from Simpson's reciprocal. Moreover, the GBSC treatment reduced the Fisher Richness Index and tended to reduce the Chao1 and ACE indices. In contrast, the APSC only tended to reduce the numbers of OTUs and the Fisher index. When both evenness and richness of the bacterial communities were considered, the GBSC treatment also had a greater impact than the APSC treatment, as indicated by the lower Shannon index of the GBSC. In the cecum, both SARA challenges did not affect the measures of the bacterial richness and diversity. The GBSC treatment reduced the Fisher and Shannon indices, and tended to reduce the number of OTUs and the effective number of species in feces. In contrast, APSC did not affect the richness and diversity in the feces.

TABLE 2 | Summary statistics of sequences in rumen fluid, cecum digesta and feces of dairy cows during Control, an alfalfa-pellet SARA challenge (APSC) or a grain-based SARA challenge (GBSC) treatment, including number of OTU[1] (97% distance), Fisher index, richness indices (Chao1 and ACE), diversity indices (Shannon and Simpson), and effective number of species (Simpson's reciprocal).

	Control	APSC	GBSC	SEM	*P*-value
RUMEN FLUID					
Number of OTU[1] (97% distance)	1031^{aA}	714^{abB}	618^{bC}	95	0.03
Fisher[2]	854^{aA}	450^{abB}	338^{bC}	107	0.02
Chao1	2540^{A}	1514^{AB}	1363^{B}	345	0.06
ACE	2725^{A}	1630^{AB}	1579^{B}	387	0.07
Shannon	6.41^{a}	5.86^{ab}	5.07^{b}	0.28	0.03
Simpson	0.99	0.99	0.96	0.02	0.13
Simpson's reciprocal	302^{A}	136^{AB}	56^{B}	73	0.09
CECAL DIGESTA					
Number of OTU[1] (97% distance)	1973	1782	1679	97	0.17
Fisher[2]	1102	972	952	65	0.29
Chao1	3569	2948	3722	580	0.63
ACE	3728	3275	3739	498	0.77
Shannon	6.81	6.65	6.47	0.29	0.72
Simpson	1.00	0.99	0.99	0.01	0.83
Simpson's reciprocal	312	265	348	83	0.73
FECES					
Number of OTU[1] (97% distance)	2188^{A}	2056^{A}	1773^{B}	102	0.06
Fisher[2]	1375^{a}	1237^{ab}	908^{b}	103	0.03
Chao1	3986	3213	3085	560	0.5
ACE	4104	3470	3132	408	0.28
Shannon	7.21^{a}	7.11^{ab}	6.77^{b}	0.1	0.03
Simpson	1.00	1.00	1.00	0.01	0.21
Simpson's reciprocal	737^{A}	560^{AB}	392^{B}	95	0.08

[a,b] Treatments that do not share a letter had significantly different results by Tukey's Honest Significant Difference (HSD) test at a $P < 0.05$, corrected for multiple comparisons.
[A,B,C] Treatments that do not share a letter had significantly different results by Tukey's honestly significant difference (HSD) test at a $P < 0.10$, corrected for multiple comparisons.
[1] OUT, operational taxonomic units.
[2] The α parameter of Fisher's log-series was used as a diversity index (Fisher et al., 1943).

Phylogenetic–Based Sample Clustering

The results of PCoA analysis of the rumen, cecum, and feces data are given in **Figures 2–4**, respectively. Based on the weighted and unweighted UniFrac distances the control and GBSC treatments separated ($P < 0.01$) into distinct clusters in the rumen, whereas only the unweighted UniFrac distances showed that APSC clustered separately ($P < 0.01$) from the control treatment. The

GBSC and APSC treatments only tended to cluster differently ($P = 0.06$) in the rumen when their weighted UniFrac distances were tested. In the cecum, only the GBSC and control treatment clustered separately based on their unweighted UniFrac distances ($P = 0.05$). Based on their unweighted UniFrac distances in feces, GBSC and APSC clustered differently ($P < 0.01$) from control. Based on the weighted UniFrac distances, a difference ($P < 0.01$) in clustering was observed between GBSC and control. The APSC and control clusters only tended ($P = 0.05$) to differ. In feces, GBSC and APSC only tended to cluster differently ($P = 0.05$) based on their unweighted UniFrac distances. Based on their weighted UniFrac distances, the GBSC and APSC treatments did not cluster differently in feces.

Bacterial Community Composition

Phylum Level

The vast majority (>99%) of the sequences of V1–V3 region of 16S rRNA gene were assigned to seven dominant phyla (abundance above 0.1% of the total community) in the rumen, cecum, and feces, including Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes, Proteobacteria, Spirochaete, and Tenericutes (**Table 3**). Bacteroidetes and Firmicutes were most abundant phyla in all three compartments, and comprised about 90% of each community. The relative abundances of Bacteroidetes and Firmicutes were equal in the rumen, whereas Firmicutes dominated in the cecum and feces.

Bacteroidetes and Tenericutes were less prevalent in rumen liquid digesta during the GBSC treatment compared to the APSC and control treatments. Compared to the control treatment, the GBSC treatment tended to decrease the relative abundance of Cyanobacteria. The abundance of Cyanobacteria differed between the GBSC and APSC treatments. The APSC treatment also tended to lower the abundance of SR1, but this reduction was greater during the GBSC treatment. In the cecum, only the GBSC treatment reduced the abundance of Lentisphaerae. In addition, the effect of the two SARA challenges on Verrucomicrobia was in the opposite direction with a slightly higher abundance in APSC. In the feces, the APSC only tended to reduce the abundance of Lentisphaerae and did not affect the abundance of Cyanobacteria, and, the GBSC treatment had lower Lentisphaerae and Cyanobacteria compared to the Control treatment.

Genus Level

In rumen fluid samples, 71,029 sequences passed the quality check and were used for downstream bioinformatics analysis. A total of 36,178 sequences were classified into 60 genera. The APSC treatment did not affect the abundances of these genera. In contrast, the GBSC treatment increased the abundance of *Sharpea* and tended to increase those of *Ruminococcus, Megasphaera,* and *Shuttleworthia,* while it decreased those of *CF231*and *BF31* (Supplementary Table 2). In cecal digesta, 27,100 sequences of 94,252 sequences were classified into 106 genera, of these, only that of *Sharpea* was increased by the GBSC treatment (Supplementary Table 2). In the feces, 18,903 sequences of 104,277 sequences were classified into 73 genera. The GBSC treatment tended to increase the abundances the *CF231* and *YRC22* in feces and tended to decrease those of *Paludibacter* and *Epulopiscium* (Supplementary Table 2). The APSC treatment did not affect the abundances of any of the identified genera on the feces.

TABLE 3 | Relative abundance of phyla (above 0.1% of community) in rumen fluid, cecum digesta and feces of dairy cows fed a control diet or on cows given an alfalfa-pellet SARA challenge (APSC) or a grain-based SARA challenge (GBSC).

Phyla in each compartment	Percentage of sequences in:			SEM	*P*-value
	Control	APSC	GBSC		
RUMEN					
Bacteroidetes	48.9^{a}	49.6^{a}	41.9^{b}	2.3	<0.01
Firmicutes	43.0	41.8	52.2	3.9	0.13
Spirochaetes	3.8	3.3	0.9	2.1	0.19
Tenericutes	1.1^{a}	0.9^{a}	0.4^{b}	0.1	<0.01
Proteobacteria	0.56	0.73	0.30	0.21	0.16
Actinobacteria	0.37	0.26	3.24	1.96	0.58
Fibrobacteres	0.35	0.59	0.32	0.11	0.24
SR1	0.25^{aA}	0.14^{abB}	0.02^{bB}	0.05	0.02
Cyanobacteria	0.18^{abA}	0.32^{aA}	0.01^{bB}	0.08	0.01
TM7	0.10	0.06	0.14	0.06	0.29
CECUM					
Firmicutes	69.9	71.4	66.6	8.66	0.93
Bacteroidetes	22.7	21.5	25.8	5.36	0.85
Fusobacteria	3.8	2.8	4.9	3.96	0.35
Spirochaetes	0.56	1.22	1.03	0.45	0.74
Proteobacteria	0.35	0.48	0.23	0.12	0.31
Tenericutes	0.47	0.30	0.27	0.16	0.36
Lentisphaerae	0.42^{a}	0.25^{a}	0.05^{b}	0.10	0.01
Verrucomicrobia	0.17^{AB}	0.66^{A}	0.07^{B}	0.10	0.06
Actinobacteria	0.13	0.28	0.22	0.14	0.53
Cyanobacteria	0.12	0.20	0.03	0.05	0.29
Fibrobacteres	0.05	0.11	0.18	0.12	0.22
FECES					
Firmicutes	77.7	75.1	74.7	3.02	0.67
Bacteroidetes	18.4	20.8	21.0	2.63	0.65
Proteobacteria	0.48	0.38	0.50	0.13	0.83
Spirochaetes	0.43	1.30	0.92	0.29	0.17
Lentisphaerae	0.55^{aA}	0.44^{abA}	0.11^{bB}	0.14	0.03
Cyanobacteria	0.46^{a}	0.25^{ab}	0.13^{b}	0.09	0.05
Tenericutes	0.48	0.64	0.40	0.17	0.79
Verrucomicrobia	0.27	0.25	0.58	0.25	0.96
Actinobacteria	0.14	0.14	0.53	0.24	0.51

^{a,b}Treatments that do not share a letter had significantly different results by Tukey's honestly significant difference (HSD) test at a $P < 0.05$, corrected for multiple comparisons.

^{A,B}Treatments that do not share a letter had significantly different results by Tukey's honestly significant difference (HSD) test at a $P < 0.10$, corrected for multiple comparisons.

Classical Species Quantified by Quantitative PCR

The results of the qPCR of 16 bacterial species and a group of *Lactobacillus* spp. in rumen liquid, cecal digesta, and

feces are given in **Figure 5**. In the rumen, both the GBSC and APSC treatments increased *Prevotella albensis, Prevotella bryantii, Succinivibrio dextrinosolvens, Anaerovibrio lipolytica, Selenomonas ruminantium*, but reduced *Streptococcus bovis*. Only the GBSC increased *Megasphaera elsdenii*. In the cecum, both the GBSC and APSC challenges increased *P. albensis, Prevotella brevis, Prevotella ruminicola, and Lactobacillus* spp., and decreased *S. bovis*. However, only the GBSC treatment increased *Escherichia coli*, and only the APSC treatment increased *Treponema bryantii*. In the feces, *P. albensis*, *S. dextrinosolvens*, *Fibrobacter succinogenes* and *Lactobacillus* spp. were increased and *S. bovis* was decreased by both the GBSC and APSC treatments. Only the GBSC treatment increased *P. ruminicola*, and *Ruminococcus flavefaciens*. In addition, both the GBSC and APSC treatments increased the abundance of *E. coli,* although the magnitude of the increase caused by GBSC was greater than that by APSC.

DISCUSSION

Changes of the Gut Environment Due to the APSC and GBSC Challenges

Among the factors affecting bacteria in the gut environment, the most significant ones arguably are the capacity to utilize available nutrients and high growth rates to avoid washout and appease a reaction-ready immune system (Ley et al., 2006). In dairy cows, experimentally-induced SARA, either by feeding high-grain diets or by feeding pellets of ground forage, adversely impacts the environmental conditions in the rumen and the hindgut that can affect their bacteria (Plaizier et al., 2008, 2012; Khafipour et al., 2009a,b,c). The pH and concentrations of VFA and free LPS obtained in this experiment were reported in the companion paper from Li S. et al. (2012). In brief, the GBSC increased the duration of the rumen pH below 5.6 from 56.4 to 298.8 min/d, and free LPS in rumen fluid from 10,405 to 168,391 EU/ml. In addition, this challenge increased the concentration of free LPS in cecal digesta from 12,832 to 93,154 EU/ml, and the starch content of this digesta from 2.8 to 7.4% of DM. The GBSC also reduced the pH of cecal digesta from 7.07 to 6.79. The APSC caused reductions of the pH of rumen fluid, cecal digesta, and feces that were similar to those caused by the GBSC. In contrast to the GBSC, the APSC did not affect the free LPS and starch contents of cecal digesta, and only increased the free LPS content of rumen fluid to 12.832 to 30.715 EU/ml.

The above shows that in terms of the decrease in pH of digesta, both SARA challenges did not differ substantially (Li S. et al., 2012). The main differences between these challenges as reported in the companion manuscript of Li S. et al. (2012) were in starch and LPS contents of digesta in the foregut, hindgut and feces that were higher during the GBSC, and in agreement with the increase in starch feeding during this SARA challenge (Plaizier et al., 2008, 2012). These differences between the two SARA challenges suggests that the bacterial communities in the digestive tract could differ between these challenges, with larger populations of starch fermenting and LPS-shedding bacteria (gram-negatives) during the GBSC. The size of rumen pH depressions obtained by both challenges suggests that the induced acidosis was subacute and that no lactic acidosis and accumulation of lactate in rumen digesta occurred.

Effects of SARA Challenges on Bacterial Alpha- and Beta-Diversities

Both SARA challenges reduced the richness, diversity, and evenness of bacteria in the rumen, with higher magnitudes

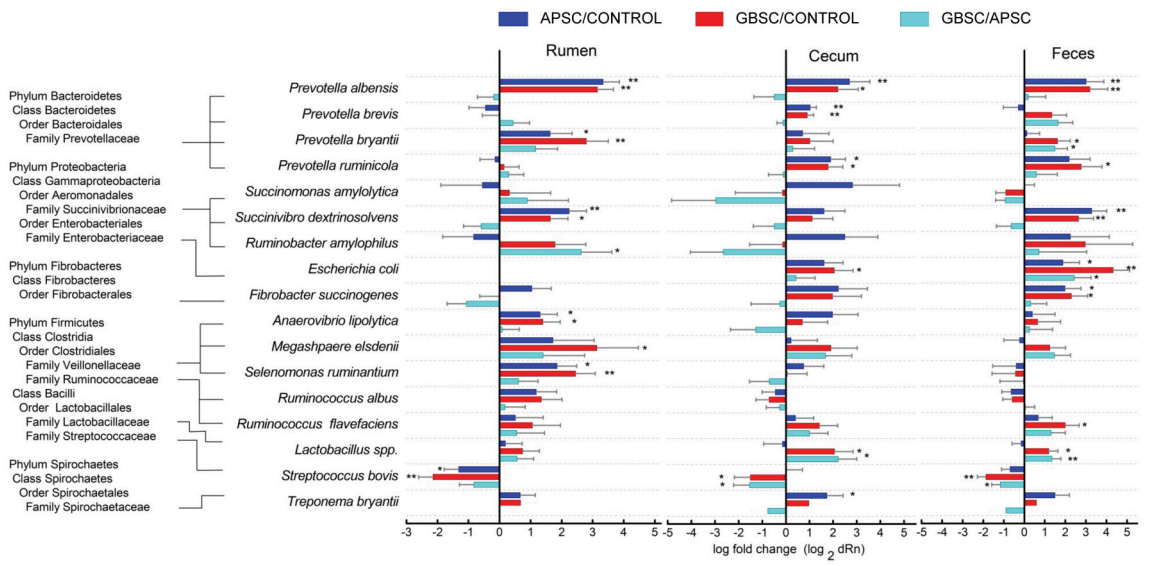

FIGURE 5 | Changes (log 2) in the abundances of 16 classical bacterial species and a group of Lactobacillus spp. during a grain-based SARA challenge (GBSC) and an alfalfa-pellet SARA challenge (APSC) in rumen liquid, cecal digesta, and feces determined by qPCR. Symbols "*" and "**" indicate significance levels at $P < 0.05$ and $P < 0.01$, respectively.

observed during GBSC. In addition, only the GBSC challenge reduced bacterial richness and diversity in the feces. In agreement, Fernando et al. (2010), Petri et al. (2013), and Mao et al. (2013) also reported that increasing the grain and starch contents of diets lowered the bacterial richness and diversity in the rumen of cattle. In these studies, these effects of high-grain feeding were more pronounced than in our study, which may be resulting from the higher increases in grain feeding in those studies.

It has been suggested that the bacterial communities that are high in species-richness and evenness use resources more efficiently, as species differ in their functionality and specialization to use fractions of the limiting substrate resources in the digestive tract (Levine and D'Antonio, 1999). Hence, the reduction in species richness and diversity during the SARA challenges, and especially during the GBSC, suggest that the rumen bacteria were transformed into less functional and desirable state.

Based on the UniFrac distances, the bacterial communities of the control and GBSC treatments clustered separately in rumen fluid, cecal digesta and feces, indicating significant impacts of high-grain feeding on gastrointestinal bacteria of dairy cows. These results agree with many earlier studies (Khafipour et al., 2009c; Fernando et al., 2010; Mao et al., 2013). The higher bacterial distance between the GBSC and control clusters than that between the control and the APSC clusters indicates that the magnitude of impacts does not solely depend on the reductions in the pH of digesta that these challenges cause, as these pH reductions did not differ between GBSC and APSC. Bacterial clustering based on the unweighted UniFrac distance provided a clearer separation than that based on the weighted UniFrac distance. Hence, the separation was reduced when the presence and abundance, rather than only the presence of bacteria were considered (Lozupone et al., 2012), which suggests that less abundant bacterial taxa are more affected by the SARA challenges than the more abundant ones.

Changes of Bacteria at Different Taxonomic Levels

Similar to other mammals, the bacteria of rumen fluid, cecal digesta and feces in the dairy cows of our study were dominated by members of two bacterial phyla, i.e., Bacteroidetes and Firmicutes (Ley et al., 2008). The GBSC reduced the relative abundance of Bacteroidetes and, thereby, increased the Firmicutes to Bacteroidetes ratio in the rumen. However, the abundance of Bacteroidetes was not affected by the APSC. Mao et al. (2013) and Khafipour et al. (2009c) also studied the effect of a grain-based SARA challenge on the abundance of Bacteroidetes. The reductions in this abundance reported by Mao et al. (2013) and that observed by Khafipour et al. (2009c) in cows with severe SARA were greater than that found in our study. However, Khafipour et al. (2009c) reported a smaller reduction in cows with mild SARA. It still needs to be determined whether an increase in the Firmicutes to Bacteroidetes ratio, such as that observed in our study, is unfavorable for the functionality of the bacteria in the digestive tract of cattle. White et al. (2014) concluded that Firmicutes differ from Bacteroidetes in how they degrade plant biomass in the rumen, as Firmicutes degrade cell surfaces and the degradation of Bacteroidetes is mainly periplasmic and intracellular. El Kaoutari et al. (2013) concluded that, on average, Firmicutes encoded fewer glycan-cleaving enzymes than Bacteroidetes. This functional difference between these two phyla may explain and support the consideration that an increase in the Firmicutes to Bacteroidetes ratio in the rumen is undesirable.

In agreement with earlier studies (Petri et al., 2012, 2013; Mao et al., 2013), *Prevotella* was the most abundant genus of Bacteroidetes and *Ruminococcus* was the most abundant genus of Firmicutes in the rumen. Our study shows that the abundances of most bacterial genera that were classified in our study in the rumen, cecum, and feces were not affected by the SARA challenges, which agrees with the findings of Petri et al. (2012, 2013) and Mao et al. (2013).

The effects of the SARA challenges at the individual species level were determined by qPCR, as 16S rRNA gene sequencing based bacterial community profiling do not have sufficient resolution to determine treatments effects at the species level (McCann et al., 2014). The populations of amylolytic bacteria were expected to increase during the GBSC treatment, as this treatment increased availability of substrates for these bacteria in digesta in the rumen and the large intestine. In agreement, the GBSC increased the populations of *P. albensis, P. bryantii and S. ruminantium* in rumen liquid digesta, the population of *P. ruminicola* in cecal digesta, and that of *P. albensis* in feces. In contrast, the GBSC reduced the population of amylolytic *S. bovis* in the rumen, cecum, and feces. Despite not increasing the starch content of the diet, the APSC increases the populations of several amylolytic bacteria in the rumen, cecum and feces. Hence, changes in the availability of starch cannot only explain changes in populations of these bacteria. In order to explain the effects of APSC on these bacteria, effects of this treatment on rumen metabolomics and competition among various bacteria for substrates may be required.

Next to increasing *S. ruminantium*, GBSC increased, *S. dextrinosolvens,* and *A. lipolytica* in the rumen. A higher availability of pectin, dextrins, and sugars in the rumen resulting from the increase GBSC would be the reason for increased *S. dextrinosolvens* (Russell and Rychlik, 2001). *A. lipolytica* utilizes sugars, and the increase in their abundance during GBSC may, therefore, be explained by increased availabilities of these substrates.

A reduction in the dietary fiber content and in the rumen pH reduce the relative abundances of cellulolytic bacteria in the rumen (Shi and Weimer, 1992). Despite of this, the GBSC had no effects on the populations of cellulolytic *F. succinogenes*, *R. albus*, and *R. flavefaciens.* This finding may be due to a potential limitation in our study in which only rumen liquid digesta was analyzed. Cellulolytic bacteria are more associated with the solid than with the liquid digesta fraction (Petri et al., 2012, 2013). Hence, the impact of a SARA challenge on cellulolytic bacteria may not be evident when only liquid digesta is analyzed. A surprising finding was that both SARA challenges increased cellulolytic *F. succinogenes* in feces. However, as the rumen pH depressions during both SARA challenges may have reduced digestion of cellulose in the rumen, they may have increased the

amount of cellulose, and, thereby, the substrates for cellulolytic bacteria in the hindgut.

As *S. bovis* is a starch utilizer and pH tolerant bacterium (Russell and Hino, 1985), the grain-based SARA challenges would be expected to increase its abundance. However, both the GBSC and the APSC reduced its abundance in rumen fluid, and GBSC reduced its abundance in cecal digesta and feces. Tajima et al. (2001) and Petri et al. (2013) also reported similar findings in the rumen during excessive grain feeding to cattle. An explanation for this may be that the abundance of this bacterium only increases during severe and lactic rumen acidosis (Khafipour et al., 2009c), and that the pH and lactic acid concentrations of rumen fluid in our study did not indicate that lactic acidosis was induced. The increase in the abundance of *M. elsdenii* in the rumen fluid during the GBSC also confirms the increased production of lactate and sugars and that the induced SARA was not severe (Russell and Rychlik, 2001; Khafipour et al., 2009c). Increases in the population of *E. coli* due to high-grain feeding, such as that seen in our study, have been described earlier (Diez-Gonzalez et al., 1998; Khafipour et al., 2011). These authors did not only observe an increase in the population of this species, but also in the population of more acid-resistant and virulent *E. coli* strains.

Relationships between Bacterial Populations and LPS

The companion study by Li S. et al. (2012) reported that the GBSC increased the concentration of LPS in rumen fluid by 16.0-fold. In our study, the GBSC decreased the relative abundance of Bacteroidetes in the rumen by 16% (from 48.9% to 41.9% of the community) but did not change the abundance of Proteobacteria and Fibrobacteres. In the cecum and feces, the abundances of Bacteroidetes and Proteobacteria were not affected by the GBSC, whereas the LPS concentration in cecal digesta and feces increased during this treatment. Treatments effects on the abundances of several bacterial species were observed, but as only a selection of gram-negative species were monitored by PCR, changes in the abundances of these species may not be the sole cause of the observed changes in the LPS content of digesta. The limulus amoebocyte lysate assay used in the parallel study (Li S. et al., 2012) is a bioassay that is based not on the concentration of LPS, but on the bioactivity of this LPS, which varies among bacterial species (Plaizier et al., 2012). This assay also does not indicate the source of the LPS (Plaizier et al., 2012). This information is important, as the toxicity of LPS varies among gram-negative bacterial species (Plaizier et al., 2012). Hence, changes in the populations of these bacteria do not have to cause changes in the concentration of LPS as they were reported by Li S. et al. (2012).

Functional changes in bacteria, such as the changes in the growth and lysis rates of several species of gram-negative bacteria, are likely to be responsive for the effects of both SARA challenges on LPS concentrations in digesta (Plaizier et al., 2012), but the resolution of the sequencing used in our study was not sufficient to assess these the effects of treatments on bacterial species (McCann et al., 2014). This shows that in order to assess the effects of dietary changes on the functionality of bacteria, techniques that can determine changes in the metagenome, such as whole genome shotgun sequencing, need to be used. The relationship between concentration of free LPS and the populations of LPS containing bacteria in digesta, therefore, remains not fully understood.

CONCLUSIONS

The APSC and the GBSC both reduced the bacterial richness and diversity in rumen fluid, but the GBSC had a larger effect. The bacterial community of GBSC also clustered differently from control feeding in rumen fluid, cecal digesta and feces. The bacterial community of APSC also clustered differently from control feeding in rumen fluid and feces, but not in cecal digesta. Despite of this, only GBSC reduced bacterial richness and diversity in feces. The abundances of Bacteroidetes and Tenericutes in rumen fluid were decreased by GBSC, but not by APSC. Effects of the GBSC on the abundances of bacterial genera in the rumen, cecum and feces were also limited. The APSC did not affect any of these abundances. Both challenges increased the abundances of several bacteria that utilize non-structural carbohydrates and their metabolites in the rumen, cecum, and feces to a larger extent than the genera and phyla to which they belong, but both challenges decreased the abundance of *S. bovis*. Only GBSC increased the abundance of *M. elsdenii* in the rumen. Differences in the starch content of rumen and hindgut digesta between the GBSC and the APSC as reported in the companion manuscript from Li S. et al. (2012) may have contributed to the dissimilarities in the gut bacteria with regard to the differing SARA-induction challenges.

ETHICS STATEMENT

The study were approved by the University of Manitoba Animal Care Committee according to guidelines of the Canadian Council on Animal Care (1993).

AUTHOR CONTRIBUTIONS

JP, EK, and SL conceived and designed the experiment. SL performed the experiment. EK and SL performed lab analyses. EK, SL, HT, and JP analyzed the data. All authors drafted the manuscript. All authors carefully read and approved the final version of the manuscript.

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The Contribution of Mathematical Modeling to Understanding Dynamic Aspects of Rumen Metabolism

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All mechanistic rumen models cover the main drivers of variation in rumen function, which are feed intake, the differences between feedstuffs and feeds in their intrinsic rumen degradation characteristics, and fractional outflow rate of fluid and particulate matter. Dynamic modeling approaches are best suited to the prediction of more nuanced responses in rumen metabolism, and represent the dynamics of the interactions between substrates and micro-organisms and inter-microbial interactions. The concepts of dynamics are discussed for the case of rumen starch digestion as influenced by starch intake rate and frequency of feed intake, and for the case of fermentation of fiber in the large intestine. Adding representations of new functional classes of micro-organisms (i.e., with new characteristics from the perspective of whole rumen function) in rumen models only delivers new insights if complemented by the dynamics of their interactions with other functional classes. Rumen fermentation conditions have to be represented due to their profound impact on the dynamics of substrate degradation and microbial metabolism. Although the importance of rumen pH is generally acknowledged, more emphasis is needed on predicting its variation as well as variation in the processes that underlie rumen fluid dynamics. The rumen wall has an important role in adapting to rapid changes in the rumen environment, clearing of volatile fatty acids (VFA), and maintaining rumen pH within limits. Dynamics of rumen wall epithelia and their role in VFA absorption needs to be better represented in models that aim to predict rumen responses across nutritional or physiological states. For a detailed prediction of rumen N balance there is merit in a dynamic modeling approach compared to the static approaches adopted in current protein evaluation systems. Improvement is needed on previous attempts to predict rumen VFA profiles, and this should be pursued by introducing factors that relate more to microbial metabolism. For rumen model construction, data on rumen microbiomes are preferably coupled with knowledge consolidated in rumen models instead of relying on correlations with rather general aspects of treatment or animal. This helps to prevent the disregard of basic principles and underlying mechanisms of whole rumen function.

Keywords: dynamic models, rumen digestion, rumen microbiota, volatile fatty acids, rumen regulatory mechanisms

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INTRODUCTION

A large part of human edible protein is produced from human inedible resources by ruminants. Ruminant production systems can range from extensive pasture-based systems with low external inputs, to intensive production systems with high external inputs and intensive farm management. Invariably, rumen function is key to ruminant performance, to the level of production intensity and efficiency of nutrient use achieved, and to the level and type of emissions into the environment. A quantitative understanding of rumen function is a prerequisite of 'engineering' rumen function and, given its boundaries, to predict how to make best use of available resources.

The present paper delivers an overview of how mathematical modeling aids in understanding the variation in whole rumen function and addresses several concepts concerning the dynamics of rumen fermentation. It describes how a quantitative integration of the sources of information that have become available from rumen research (varying from intra-microbial processes to that of the ruminant host) can be achieved, and what further modeling efforts are foreseen. It is postulated that there is merit in adopting a dynamic rumen modeling approach to understand and predict the more dynamic aspects of rumen microbial metabolism and rumen fermentation conditions. This holds for concepts generally used in current rumen models. First, representing the effect of feeding frequency on rumen metabolism requires the dynamics of rumen microorganism and feed substrate degradation to be accounted for. Second, next to the importance of solids turnover for rumen function, there can also be a profound impact of fluid turnover on microbial metabolism in the gastrointestinal fermentation compartments, requiring fluid dynamics to be taken into account. Third, the impact of the dynamics of the nitrogen exchange between the host and the rumen fermentation compartment needs to be accounted for to predict when rumen nitrogen levels are approached that start to limit microbial protein synthesis. Fourth, predicting of end-products of fermentation requires not only the dynamics of microbial growth to be taken into account, but also the impact of rumen fermentation conditions on production of volatile fatty acids (VFA; and hydrogen and methane). Fifth, adaptive processes in the rumen wall have an important role when quantifying response to dietary changes and are to be accounted for.

Dynamics of Substrate Degradation and Microbial Growth

Microbial fermentation in the rumen is generally simplified by assuming that the rumen is a chemostat with ideal mixing of contents and continuous inflow of feed substrate and subsequent outflow of rumen contents. Therefore, the basic approach to representing rumen microbial activity in mathematical terms corresponds to that used for chemostat cultures (Dijkstra et al., 1998). The quantity of substrate (to be utilized by micro-organisms) and the mass of micro-organisms present in the rumen are variables in more complex rumen models. In mechanistic rumen models, these quantities are calculated by the use of differential equations that describe the dynamics of interactions between the processes of inflow of feed substrate, microbial substrate degradation, microbial growth, and outflow of undigested feed and microbial matter. Some processes can be assumed to follow mass action kinetics but many are, in essence, of a non-linear nature, and this requires complex models comprising non-linear differential equations to be developed (**Figure 1A**).

Despite their typically non-linear nature, representation of these processes is often simplified in rumen models. Usually, in simplified rumen models, mass action kinetics are assumed. This allows the non-linear differential equations to become linear without, however, any interaction between the micro-organisms and substrates (**Figure 1B**). In which case, the formulation reduces into that of a static model (excluding the need to represent rumen concentrations and time as model variables). The outflow rates of substrate and micro-organisms can then basically be calculated directly from ratios of the fractional rate of substrate degradation and rumen outflow (**Figure 1C**). With some exceptions, the latter is the basic approach adopted in most models and protein evaluation systems for ruminants applied in practice (e.g., Danfaer et al., 2006; Tylutki et al., 2008; Van Duinkerken et al., 2011; Volden, 2011). These static rumen models are useful as they enable key nutritional characteristics required for diet optimization to be calculated. Such characteristics include the amount of microbial protein synthesized, rumen bypass protein and rumen N availability of different feedstuffs. However, from a mathematical as well as a microbiological viewpoint (i.e., the rumen following the principles of chemostat theory), these static models cannot predict the same accuracy of response to dietary changes as non-linear dynamic models.

Non-linear dynamic representations are used to explain more complex aspects of observed variation in feed substrate degradation and microbial growth. Variation in observed degradation cannot be fully explained without representing the variation in the intrinsic physical and chemical characteristics of feed substrates. Therefore, Dijkstra et al. (1992) adopted the concept of using *in situ* degradation characteristics derived under standardized conditions in the rumen to generate model inputs, which are the same inputs as those used in static rumen models. The earlier dynamic rumen model of Baldwin et al. (1987) initially adopted a different approach using fixed intrinsic degradation characteristics of fiber for specific forage classes (grasses, legumes, maize), of starch, and of degradable protein, leaving all observed variation in substrate degradation to be explained by the dynamic model through a combined representation of microbial activity and particle dynamics (particle size distribution, particle comminution and outflow). The use of intrinsic degradation characteristics as model inputs may be perceived as making modeling efforts partially self-fulfilling instead of having the mechanisms explain the physical and biological processes that underlie variation in substrate degradation rate (Baldwin, 1995). In a later version of the model, intrinsic *in situ* degradation characteristics were introduced to improve the model's capacity to explain observed variation in feed degradation (Hanigan et al., 2013). Hence, a large part of the observed variation in substrate degradation

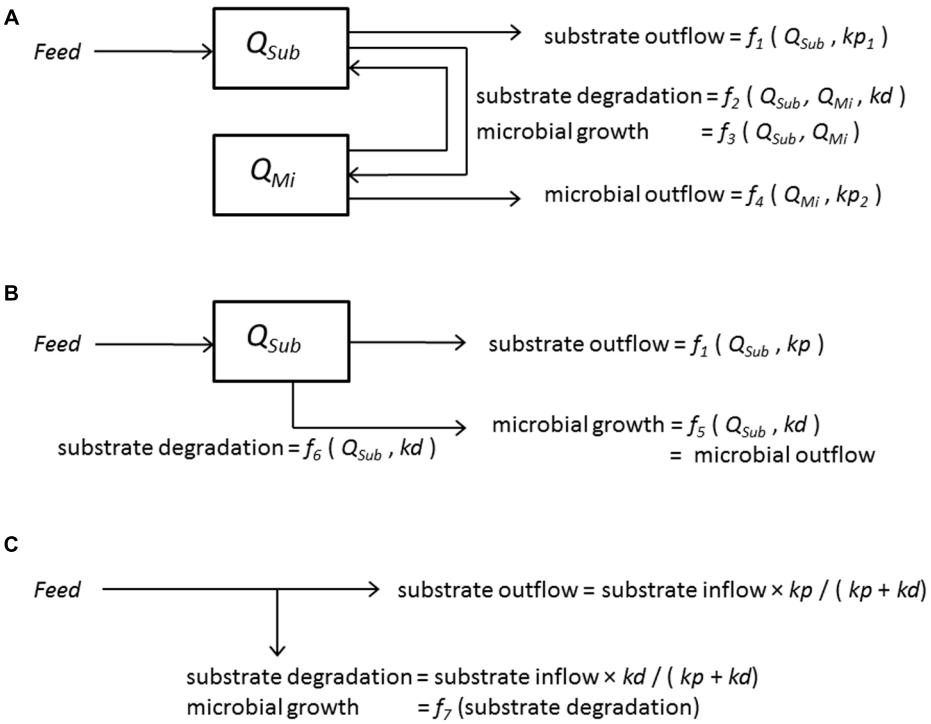

FIGURE 1 | Modeling approaches to quantify microbial activity and substrate degradation in the rumen (adapted from Bannink et al., 2006a). Boxes indicate state variables of rumen substrate (*Sub*) (Q_{Sub}, quantity of rumen substrate, g or mol); rumen micro-organisms (*Mi*) (Q_{Mi}, quantity of rumen micro-organisms, g) determined by inflows and outflows indicated by arrows (g/d or mol/d) which are defined by feed intake (*Feed*, g/d), fractional rate of substrate degradation (*kd*, /d) and fractional rate of rumen passage (*kp*, /d). **(A)** Representation of a dynamic model including interrelationships between substrate and micro-organisms. **(B)** Representation of a dynamic model without interrelationships between substrate and micro-organisms. A dynamic model that represents all in- and out-flows by mass action forms (using *kd* and *kp*) can be simplified into a static model (depicted in **C**). **(C)** Representation of a static model.

remains dependent on degradation characteristics that relate to the chemical and physical properties of feeds and are truly intrinsic, independent of the mechanisms or calculation rules represented in both static and dynamic rumen models. Therefore, they are required as inputs to virtually all rumen models.

Apart from substrate degradation characteristics, further aspects and details about factors influencing microbial activity are included in rumen models, depending on the specific modeling aims. With the aim of predicting microbial protein supply, Dijkstra et al. (1998) compared various conceptual approaches and concluded that it is highly relevant to represent the mechanisms that underlie the interactions between micro-organisms and substrates. Because a substantial, but variable, proportion of gross microbial protein synthesis is recycled within the rumen without flowing to the duodenum, the interactions between different functional classes of micro-organisms were included. These interactions include predation (of bacteria and protozoa) by protozoa and microbial death (lysis), competition for substrates in the rumen, and differentiation of substrate requirement for growth and maintenance, of susceptibility to rumen outflow and of sensitivity to rumen pH. Although the importance of these microbial interactions is evident and has been reviewed frequently (e.g., Dijkstra et al., 1998; Firkins et al., 2007), there are hardly any attempts to include them in model representations of rumen fermentation dynamics. For example, in the Cornell Net Carbohydrate and Protein System (CNCPS), protozoal predation of bacteria is accommodated by a fixed 20% decrease in maximum growth yield of bacteria, independent of dietary or rumen characteristics (Russell et al., 1992). An exception to this is the model of Dijkstra (1994) which adopts a multispecies approach by representing the different functionalities of amylolytic bacteria, fibrolytic bacteria and protozoa, including their interactions. Such an approach is fundamentally different from the approaches used in the aforementioned static models, which may represent multiple microbial classes but lack a representation of dynamic interactions, making the final modeling outcome for the whole rumen the arithmetic summation of the outcomes of the representations for the individual functional classes. Recently, other modeling efforts have been undertaken to improve the representation of interactions between functional classes of micro-organisms (review Tedeschi et al., 2014). For example, a new version of the rumen submodel in CNCPS was constructed that includes more detailed aspects of growth and activity of Holotrichs and Entodiniomorphid protozoa (Higgs, 2014). Also, the AUSBEEF model is developed further based on the earlier work of Nagorcka et al. (2000), based again on Dijkstra (1994).

Dynamics of Rumen Metabolism

The importance of dynamic representation of the interactions between substrate and micro-organisms becomes apparent when investigating the effect of inflow rate of a single starch source on its ruminal degradation *in vivo*. Starch, as an example, is more illustrative in this respect compared to other substrates for the following reasons. In the rumen, almost all fiber is present in large particles, which become exposed to microbial degradation and rumen outflow after comminution and rumination, following a substantial delay, which is not or far less the case with starch (given processed starch sources are used). Fiber also has a substantial undegradable fraction which confounds the observed interaction between degradable fiber and fibrolytic micro-organisms, again in contrast to starch which in principle is fully degradable. Sugars typically have too small a rumen pool size and too high a digestibility to serve as an illustrative example. Also, an extensive exchange of N with the rumen wall and intra-ruminal N recycling (protozoal activity) complicates the case of N. The benefit of adopting a dynamic approach to explain rumen starch digestion does not readily become apparent from *in vivo* observations because effects on rumen starch digestion are masked by variation in dry matter (DM) intake, origin of starch, starch content and rumen outflow rate among treatments. Larsen et al. (2009) evaluated four experiments in which the effects of starch source and processing were examined. The results do not indicate that rumen starch digestibility of various cereal starch sources was affected by the level of starch intake. The depicted data for ground cereal starch do suggest an increased starch digestibility with increase of starch intake, but different cereal starch sources are compared here, level of DM intake varied, and starch intakes were always in the higher range (>4.5 kg/d). Some rumen digestion trials allow a direct comparison between starch treatments because an (almost) identical starch source was used across treatments (e.g., Herrera-Saldana et al., 1990; Poore et al., 1993; Joy et al., 1997). These studies indicate increased apparent rumen digestibility of starch source with increased intake of that source. Under the assumption that these effects were not caused by differences in fractional outflow rate, they might be explained by a larger pool of amylolytic micro-organisms in the rumen, and a larger capacity to degrade starch being present. Dynamic rumen models (**Figure 1A**) accommodate such an interaction between starch substrate and amylolytic microbial activity. A static model (**Figure 1C**) or a dynamic model with constant coefficient values which can be solved analytically and can be reduced to a static model description (**Figure 1B**) *a priori* do not. Another aspect to take into account with modeling is the contribution of microbial synthesis of storage polysaccharides with measurements of rumen starch digestion. These storage polysaccharides are included in analysis of rumen starch outflow, hence lowering apparent rumen starch digestion. Larsen et al. (2009) deliver strong empirical support for this effect of microbial starch. Their evaluation indicated that observed apparent ruminal digestibly underestimates true ruminal digestibility at low starch intake, due to a relatively higher contribution of microbial sources to total duodenal starch flow compared to rumen escape feed starch.

Observations on the effect of feeding frequency on rumen fermentation are a further illustration of the dynamics invoked. When a representation of meal intake pattern and a mechanism for particle dynamics are introduced into a dynamic rumen model (we use the rumen model of Dijkstra et al. (1992), for the sake of demonstration), a significant increase in rumen starch digestion and decrease in duodenal starch flow is simulated (**Figure 2A**) when moving from single to twice daily feeding.

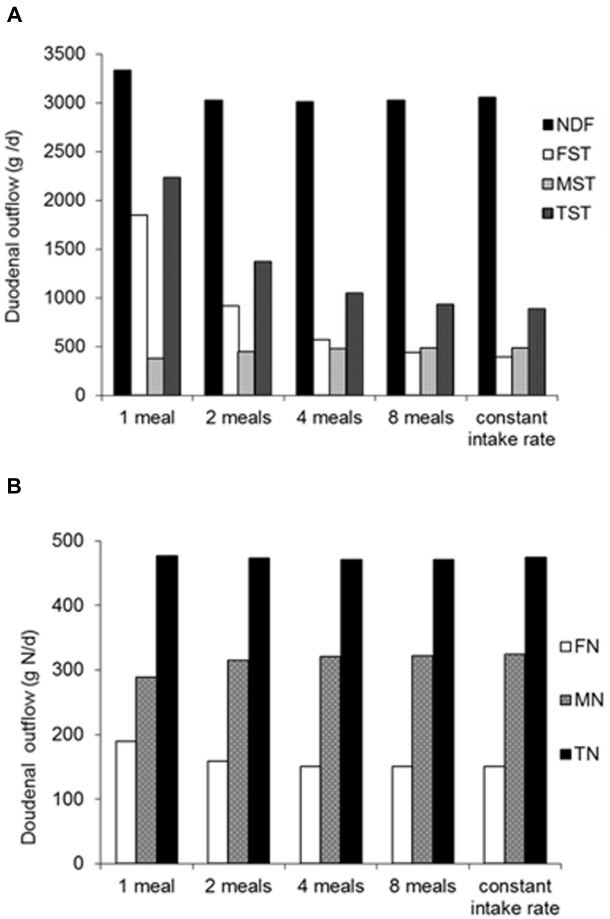

FIGURE 2 | Effect of feeding frequency on rumen digestion. Simulations for different feeding frequencies were performed with identical diet composition (average Dutch dairy diet in 2004 with 13% grass herbage, 34% grass silage, 24% maize silage, 26% concentrates and 3% wet by-products in dietary DM). Further assumptions were a DM intake of 20 kg DM/d, a fractional passage rate of 1.0/d for particulate matter and 3.0/d for fluid, a mean rumen pH of 6.0 and a proportion of 0.35 of protozoa in total amylolytic microbial mass. An adapted version of the rumen model of Dijkstra et al. (1992) was used, including a representation of distinct meals, a mechanism for particle dynamics (distinction between large and small particles; a 0.4 proportion of small particles in feed was assumed and a fractional rate of comminution of large to small particles of 3.5/d). The contribution of individual meals to *in situ* degradation characteristics of rumen substrate pools was continuously monitored during the simulation runs. **(A)** Simulated duodenal outflow (g/d) of fiber (NDF), feed starch (FST), microbial starch (MST; engulfed starch granula as well as microbial polysaccharide synthesized), total starch (TST). **(B)** Simulated duodenal flow (g N/d) of feed N (FN), microbial N (MN) and total N (TN).

Lower starch degradation when feeding once daily corresponds to the results of Froetschel and Amos (1991), who showed an increase in fractional turnover rate of rumen starch of 75% with increase in feeding frequency from once to twelve times daily with a ground corn and sorghum silage diet, whereas the increase was limited to 24 and 1% for rumen DM and NDF, respectively. Le Liboux and Peyraud (1999) tested twice versus six times daily feeding of identical diets of (dehydrated) whole-crop maize and either ground or chopped alfalfa. More starch was ingested with six times feeding due to a higher DM intake, which resulted in a trend for increased starch digestion. Similar to these observations, the model simulations indicate a moderate effect of such an increase in feeding frequency from twice to four times daily on rumen starch digestion (**Figure 2A**). Le Liboux and Peyraud (1999) also observed that rumen digestion of digestible NDF increased with increase in feeding frequency, probably due to less fluctuation in rumen pH and improved rumen fibrolytic activity. This will not have played a role in starch digestion as it tends to be rather unaffected by pH. The present model simulations showed hardly any effect of feeding frequency on rumen digestion of NDF (**Figure 2A**) and N (**Figure 2B**), as model assumptions did not account for changes in rumen pH and fractional passage rate.

The stimulatory effect of feeding frequency on rumen digestibility observed by Le Liboux and Peyraud (1999) contrasted with a consistently negative quantitative effect observed for digestibility of the feed determined at the level of fecal excretion (i.e., fecal digestibility). This may be explained by less substrate inflow and hence a smaller microbial population in the large intestine, resulting in a reduction in the contribution of the large intestine to OM and NDF digestion which was larger in size than the increased contribution by the rumen. Unless caused by bias introduced with measurement methodology, such effects can also be seen as illustrative of the previous discussion on the dynamics involved with interactions between substrate and micro-organisms in the rumen. This warrants fermentation in the large intestine to also be represented in a dynamic model. Therefore, we introduced a dependency of the fractional outflow rate of digesta on VFA concentration, assuming an increase of 10 mM is associated with a 5.6% increase of fractional outflow rate up to a maximum of 80 mM (taken from the results of López et al., 2003), into the dynamic model of the large intestine described by Mills et al. (2001). The consequence of this model adaptation (**Figure 3A**) was a marked decline in predicted fibrolytic activity with an increased inflow of rapidly fermentable carbohydrates into the large intestine. A similar response of a declined contribution of the large intestine to NDF digestion with an increased ileal starch flow has been reported in several studies (Knowlton et al., 1998; Yang and Beauchemin, 2005; Van Vuuren et al., 2010). Although the rather theoretical model simulations require confirmation, these *in vivo* results illustrate the dynamic aspects of how substrate availability and activity of amylolytic and fibrolytic micro-organisms impact NDF digestion beyond the rumen, when feeding large amounts of starch relatively resistant to rumen degradation. Also, the importance of addressing these dynamic aspects was highlighted in a recent modeling study, where Ellis et al. (2014) evaluated

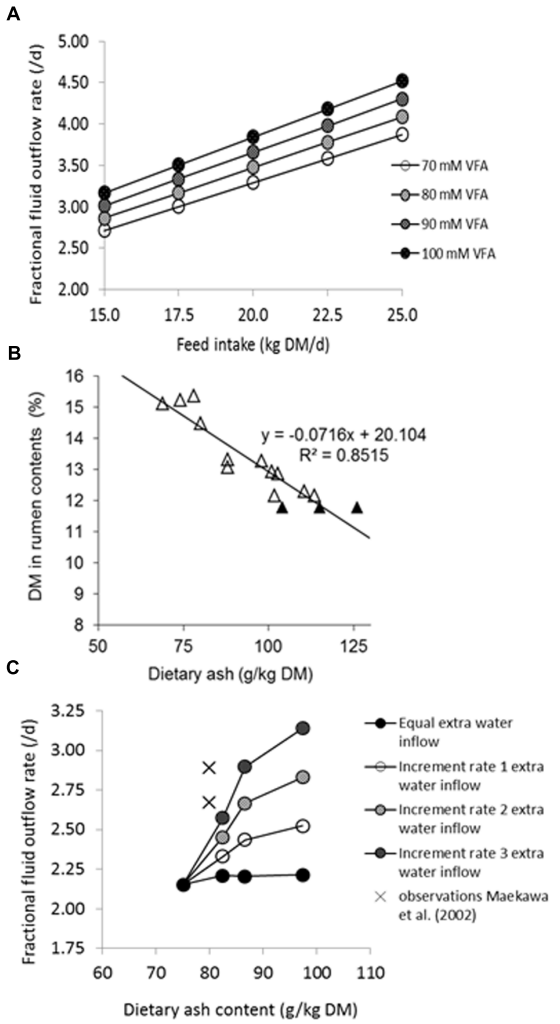

FIGURE 3 | Effects of dietary ash on rumen fluid dynamics. **(A)** Simulated effect of DM intake (kg/d) on the fractional rate of fluid outflow as simulated by the model of Mills et al. (2001), increased by 5.6% per 10 mM increase in rumen VFA concentration as established for the sheep rumen by López et al. (2003) in the range of 50–80 mM VFA, and presumed to be applicable to a physiologically more realistic range for the dairy cow rumen of 70–100 mM VFA. **(B)** The effect of dietary ash content (g/kg DM) on proportion of DM (% of total weight) in whole rumen contents [open symbols for data from De Visser et al., 1992, 1993; Chilibroste et al., 2001; Bruinenberg et al., 2004; closed symbols independent data (average, and average ± standard deviation) from Reynolds et al., 2004]. **(C)** Effect of observed water intake with increase of dietary ash (salt) content (g/kg DM; according to Spek et al., 2012), adopting a rumen water volume based on the relationship depicted in **(B)**, and four scenarios of extra water inflow (an equal extra water inflow of 100 L/d, or an incremental rate 1, 2, or 3 of extra water intake of 1.4, 2.7, or 4.1 L/d per %DM increase in dietary ash content, respectively) on calculated fractional outflow rate of rumen fluid. Observed values for primiparous and multiparous lactating cows derived from Maekawa et al. (2002) are indicated by crosses.

predictions of digestion and enteric methane (CH_4) emissions in beef cattle against *in vivo* observations. For realistic predictions they first had to make changes to the rumen and large intestinal model of Mills et al. (2001), which was developed for lactating

cows, to accommodate the specific enteric conditions observed in beef cattle fed low-roughage diets. The dynamics of digesta volume and the fractional outflow rates for the rumen and large intestine had to be modified, as well as the fractional outflow rate of rumen protozoa, and large intestinal fibrolytic activity, to achieve realistic simulations of fibrolytic activity in both the rumen and large intestine. Gregorini et al. (2015) recently revisited representation of digesta outflow in the mechanistic model of Baldwin (1995), addressing effects of osmolality of rumen fluid (calculated from solutes including VFA) on rumen fluid dynamics.

Influencing Factors

Other factors, as well as the characteristics of substrates and rumen micro-organisms, have a profound impact on rumen function and can cause seemingly contrary results for starch degradation to those discussed earlier. For example, the results of Sutton and Oldham (reported by Mills et al., 1999) fail to confirm the positive effect of higher intake of a single source of barley or maize starch on rumen starch digestion. In this case, rumen starch digestion was reduced rather than increased for maize and remained the same for barley. This outcome may be related to the low proportion of roughage in dietary DM with the high starch level compared to the low level, as studies mentioned in the previous section always tested diets where roughage accounted for >35% of dietary DM. The discrepancy illustrates that other mechanisms must override the effect of an increased starch inflow on rumen starch digestion, with turnover or outflow rate of rumen contents as the most likely candidate. The same was suggested by the findings of Ellis et al. (2014) for representing the digestion of low-roughage diets in beef cattle. Also in contrast, Kreikemeier et al. (1990) observed an increased *in situ* fractional rate of starch degradation with increased roughage in a wheat-based finishing steer diet, in spite of a reduced starch intake, a lower rumen starch pool, and a higher fractional outflow rate of labeled starch. Given that bacterial and protozoal numbers were not affected by increased dietary roughage, Kreikemeier et al. (1990) explained this result by an increased fractional liquid passage rate that improved the efficiency of microbial growth.

The need for an integrative approach to understand these phenomena of rumen function becomes more apparent when attempting to represent the aforementioned dynamics of substrate degradation and microbial metabolism, and the modulating effect of rumen conditions. For example, fiber degradation rate is affected not only by the fractional outflow rate of particulate matter but also by the acidity of rumen contents with pH under 6.3. The influences of rumen fluid and particle dynamics, of rumen fluid volume and rumen fill, of rumen fluid pH and of VFA absorption are represented in essence in every dynamic rumen model, albeit in a very different manner. Such differences are due to the different assumptions made or due to the different empirical bases used to parameterize the dynamic (Baldwin et al., 1987; Dijkstra et al., 1992) or static models which cover these aspects (Danfaer et al., 2006; Tylutki et al., 2008; Volden, 2011). Volume and fractional outflow rate of rumen fluid have a major effect on rumen concentrations of substrates and micro-organisms (Argyle and Baldwin, 1988). Next to saliva production, several additional aspects are important to quantify dietary impact on rumen fluid dynamics which will be demonstrated here using some theoretical calculations. **Figure 3A** illustrates the hypothetical effect of VFA accumulation in rumen fluid on net fluid exchange in the direction from blood to the rumen, driven by osmotic pressure, as demonstrated in sheep by López et al. (2003). **Figure 3B** illustrates the effect of dietary ash content on rumen fluid volume (derived from *in vivo* observations of DM% in total rumen content). This higher rumen fluid volume is accompanied by extra water intake, affecting fluid dynamics and concentrations of substrate and substrate fermenting micro-organisms in the rumen ecosystem. **Figure 3C** illustrates the impact of several scenarios for incremental extra water intake with increased dietary ash content on calculated fractional outflow rate of rumen fluid. A comparison with observations by Maekawa et al. (2002) seems to indicate that the highest incremental rate of extra water intake is realistic for lactating cows. Aspects that have received less attention in efforts to model the rumen are rumen fill, water holding capacity, fluid osmotic value, viscosity of rumen contents, rumen retention of fluid and its consequences for the different functional classes of feed particles and micro-organisms, and buffering capacity of rumen contents. These aspects significantly impact fractional outflow rate and rumen pH, both main drivers of rumen function in all rumen models (dynamic as well as static). They have received most attention in the modeling work of Argyle and Baldwin (1988; overview by Baldwin, 1995) which was recently revisited by Gregorini et al. (2015). Next to scientific purpose for proposing a mechanism and increase understanding, the integration of this type of aspect in rumen models widens the domain of their use. The alternative of not integrating them would be to reparameterize rumen models by a fully empirical approach to allow them accommodate the various rumen conditions or nutritional strategies.

Classes of Microbial Function

Reasons to distinguish functional classes of micro-organisms in rumen models are differences in the type of substrate fermented (type of carbohydrate in particular), the particular rumen niche they occupy with its own physical-chemical characteristics, e.g., retention time and acidity, and the specific role they exert that is related to the modeling aim; e.g., bacterial predation by protozoa and storage of polysaccharides (Dijkstra et al., 1992; Dijkstra, 1994; **Figures 4A,B**) and rumen lactate metabolism (Mills et al., 2014). Mechanistic rumen models distinguish fibrolytic and amylolytic microbial activity because such a classification is well documented and in line with the distinct carbohydrate substrates for which intrinsic degradation characteristics are available. This is exclusively a distinction based on type of substrate and far less inspired by rumen-ecological arguments which would require representation of other types of variation. Such a representation may include variation in specific niches, diversifying the current distinction between particulate and fluid associated micro-organisms. It may include variation in specific rumen fermentation conditions, introducing intra-rumen compartmentation or gradients with respect to substrate availability and concentration of fermentation end-products. Or

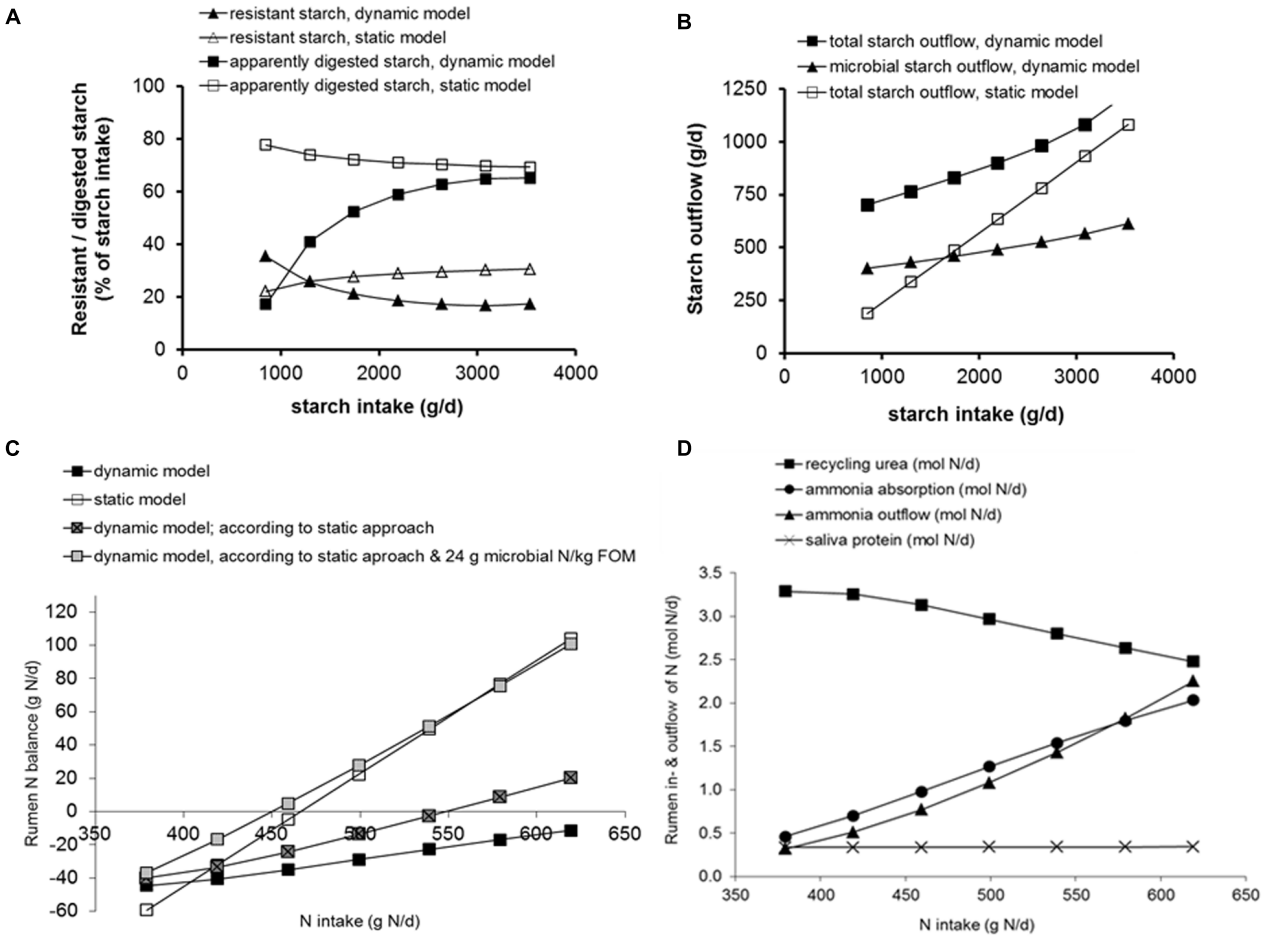

FIGURE 4 | Effect of substitution of maize silage for grass silage (up to 70%) in a diet containing 80% roughages and 20% concentrates on a DM basis, assuming a DM intake of 20 kg DM/d, simulated by the model of Dijkstra et al. (1992) as well as by a current protein evaluation systems (Van Duinkerken et al., 2011), as examples of a dynamic and a static rumen model, respectively (results derived from Bannink et al., 2006a). (A) Resistant or apparently digested starch as % of starch intake simulated with the dynamic and the static rumen model. **(B)** Rumen outflow of microbial starch (microbial polysaccharides synthesized, and starch engulfed by micro-organisms) and total starch simulated with the dynamic and the static rumen model. **(C)** Rumen nitrogen balance simulated with the dynamic and the static rumen model, with the dynamic rumen model for the concept of rumen protein balance represented in the static model, and with the dynamic rumen model for the concept of rumen protein balance represented in the static model with a constant 24 g microbial N synthesized per kg rumen fermented OM (FOM) as assumed in the static model. **(D)** Simulation results for N flows to and from the rumen compartment with the dynamic rumen model (urea recycling, ammonia outflow, ammonia absorption, and saliva protein inflow). These flows as such are not represented in the static model.

it may include variation in some unique role or metabolic state that rumen micro-organisms can achieve. Representing the latter may be the introduction into the model of metabolism of specific nutrients or metabolites or the identification of different metabolic states or responses of micro-organisms. These different states or responses might be due to a changed energy requirement for maintenance functions (Baldwin, 1995), or to regulatory mechanisms driven by changed availability of reduced co-factors (Van Lingen et al., 2016). With respect to representing the dynamics of inter-microbial relationships between various classes of micro-organisms, amongst the best documented and detailed models are the rumen protozoa metabolism model of Dijkstra (1994) and the rumen lactate metabolism model of Mills et al. (2014). Some other additions to rumen models can be considered as model parameterization rather than addition of a functional class of micro-organisms if they do not include representation of the dynamics of this functional class and its inter-relationships with other classes. For example, Nagorcka et al. (2000) introduced a consensus stoichiometry of VFA production derived from microbiological studies on individual bacteria genera and types of protozoa into the model of Dijkstra (1994), but no new dynamics were introduced. They used general microbiological data and not *in vivo* observations to describe the profile of rumen VFA in relation to substrates degraded. Whether the extra detail introduced by such a consensus outweighs the benefit of an empirical approach based on *in vivo* measurements in the target animal (Bannink et al., 2006b; Ghimire et al., 2014) remains unclear. Other aspects that have been incorporated in the various rumen models reported in literature include effective fiber, peptides, distribution of particles, fats and fatty acids, and

sulfate (review Tedeschi et al., 2014), which have their own merit when aiming to explain rumen response to related factors.

A reason to introduce more detail on microbial activity into future rumen models, instead of representing a single type of micro-organism, might be the different niches that different species or classes of micro-organisms occupy. Another reason might be the differences in metabolic characteristics and growth capacity of micro-organisms, instead of making generic assumptions about amylolytic bacteria, fibrolytic bacteria and protozoa. Also differences in the sensitivity of microbial activity to conditions in the rumen environment instead treating it as fully independent, might be a reason. Currently, we are unaware of modeling efforts or new approaches to further distinguish microbial classes in order to increase understanding of whole rumen function. Although new molecular techniques in principle enable measurement up to the level of detail of the individual species or genus, such information would be particularly useful if it could be related to the functional aspects of rumen fermentation already represented in whole rumen models [i.e., rates of substrate degradation, microbial growth rates, formation rates of end-products of fermentation, and rumen fermentation conditions (fractional outflow rates, pH, and VFA)]. For example, effects of subclinical acidosis on the rumen microbiome have been observed by Mao et al. (2013). Also Petri et al. (2013) were able to establish clear relationships between the observed microbial profile and dietary treatment and acidotic challenge. Others, however, could not establish a relationship between observed rumen fermentation parameters (including severity of acidosis) and shifts in the rumen microbiome, and a large variation existed between individual animals and measurement periods (Mohammed et al., 2012). Having *in vivo* rumen measurements or data on microbial physiology alongside data from modern molecular techniques and the wealth of sequence-based information seems pivotal in relating such data to whole rumen function and using them to make progress in rumen modeling. A completely statistical approach can be adopted to relate this level of detailed information directly to observed rumen function without representing the underlying mechanisms. Although useful to mark the consequences of different rumen states, e.g., by deriving microbiome profiles marking a specific rumen (or even animal) state (Chaucheyras-Durand and Ossa, 2014), the challenge remains to go beyond a fully empirical approach (Bannink et al., 2011; Weimer, 2015) and use this information to contribute to concepts that can be applied to the rumen modeling approaches discussed herein (and vice versa).

Details of a specific functional microbial class can be added to a model to allow prediction of the associated functional response. For example, Mills et al. (2014) integrated a representation of lactate metabolism within the rumen model of Dijkstra (1994) by separating a single class of amylolytic bacteria into lactate-utilizing and lactate-producing bacteria. Except under strongly acidic conditions where lactobacilli become prominent, *Streptococcus bovis* is a major contributor to lactate production in the rumen (Nagaraja and Titgemeyer, 2007). In the model, growth parameters for lactate-utilizing bacteria were based on those reported for *Megasphaera elsdenii*, the major lactate utilizer in the rumen. The capability of the model to simulate peaks in rumen lactate post-feeding can be considered encouraging. When combined with an improved representation of rumen acid-base chemistry and sensitivity of the microbial classes to rumen pH, the model should become a useful tool for increasing our understanding of how nutritional factors (non-structural carbohydrate feeding, feed intake patterns) lead to lactate and VFA accumulation in the rumen, which can be highly detrimental to rumen function. Due to saliva production and its buffering capacity in the rumen, prediction of pH from rumen VFA concentration in current empirical relationships is inaccurate. In the case where saliva is absent, prediction of pH from VFA concentration is far more accurate, as shown for pH of fresh cow feces by Dijkstra et al. (2012). Therefore, there is much scope to model rumen acid-base chemistry and improve pH prediction under a wide range of rumen conditions (Imamidoost and Cant, 2005).

In addition to microbial classes in the rumen ecosystem, further distinction of specific microbial metabolic functions can also be relevant. The models of Dijkstra et al. (1992) and Dijkstra (1994) attempt to capture the variation in microbial composition as a key aspect of microbial metabolism. Amylolytic bacteria and protozoa are able to store polysaccharides. This functionality is important when predicting sugar or starch outflow to the intestine as glucogenic precursors. It may also be an important buffering mechanism acting against sudden, large loads of rapidly fermentable carbohydrate and temporal rumen acidification, as the carbohydrates stored will not generate rumen VFA during these moments. Later on, part of these storage carbohydrates re-enter fermentation with lysis of micro-organisms and contribute to VFA production, and part flows out of the rumen with microbial matter (Dijkstra et al., 1992; Dijkstra, 1994). In **Figures 4A–D**, outcomes of such microbial composition dynamics are illustrated and compared to outcomes from static modeling approaches for the dietary substitution of maize silage for grass silage. With the static approach, a small decline in percentage of starch digested (and increase of starch resisting digestion) is predicted with an increasing proportion of maize silage in the diet (**Figure 4A**). This outcome is the combined result of an increasing proportion of maize silage starch at the expense of concentrate starch in the diet, and a lower rumen degradation rate for maize silage compared to concentrate starch. With the dynamic approach a different trend for starch digestion was predicted due to representation of microbial storage of carbohydrates. Microbial storage of polysaccharides contributes to measured rumen starch outflow and as a result, at a very low starch intake, the percentage of apparently digested starch remains much lower and the percentage of resistant starch higher compared to outcomes with the static approach. Because the represented microbial capacity of storage of polysaccharides is limited in size, with increasing starch intake the impact of microbial polysaccharides on predicted results declines and predictions of apparently digested starch approach those obtained with the static approach. Due to the contribution of microbial polysaccharide storage to resistant starch (as

measured in rumen digestion trials), predicted daily starch outflow remains higher with the dynamic compared to the static approach, even at high levels of starch intake (**Figure 4B**). Such contrasting results from both modeling approaches can be evaluated against observed phenomena on rumen starch digestion. Although any expected variation in (the biosynthesis of) components of microbial matter would have major impact on the quantification of microbial synthesis and predicted rumen metabolism, no other modeling examples other than for microbial polysaccharides are known that take this aspect into account.

A further example of adding specific functionality to a rumen model is the work on modeling rumen fat metabolism by Dijkstra et al. (2000) and Moate et al. (2004). A comparison of both studies illustrates how different modeling aims lead to different modeling approaches, although they both strive to represent the key details of rumen fat metabolism. The former model is dynamic with a simplified representation of fat source, by only distinguishing saturated from unsaturated fat and hydrolyzed from non-hydrolyzed fatty acids. It does, however, include a representation of how fatty acid concentrations in the rumen interact with microbial activity and fiber digestion. The latter model is static and aims to represent details of the type and fate of dietary fatty acids. However, it excludes a representation of interactions with microbial activity and substrate degradation. Such modeling work is of considerable interest in studying the effects of dietary fat supplementation on milk fat composition and rumen fatty acid metabolism. With current interest in using milk fatty acid profiles as a proxy for enteric CH_4 emissions (e.g., Van Lingen et al., 2014), details of rumen fat metabolism seem an essential link in being able to interpret such profiles. Accounting for the modulating effect of fat supplementation on rumen microbial activity and VFA production requires the prediction of rumen fatty acid concentrations, favoring a dynamic modeling approach. A combination of both approaches seems most promising in predicting all relevant effects (microbial metabolism, methanogenesis, fatty acid outflow).

Non-microbial End-Products

In addition to the representation of microbial protein synthesis, reviewed by Dijkstra et al. (1998), and which may include representations of various functional microbial classes, further details can be added to explain the variation in rumen microbial metabolism. Such details would have to be integrated at the whole organ level to address the aims of rumen models, i.e., to generate predictions of microbial protein outflow to the intestine, and production of VFA, ammonia and CH_4. The VFA as end-products of rumen fermentation are of particular importance because they deliver the majority of metabolizable energy to the ruminant. It is noted here that modeling of VFA production has been strongly hampered by much of this work being based on molar proportion of VFA found in rumen fluid instead of actually measured VFA production rates. The production of hydrogen (H_2) is strongly associated with that of VFA, as it is used by methanogens to produce CH_4. Recent attempts to further improve the description of microbial metabolism and predict the consequences for VFA and CH_4 production are discussed here.

Microbial Metabolism

Hydrogen is an important metabolite in the fermentation of glucose to VFA in the rumen (Baldwin and Allison, 1983; Hegarty and Gerdes, 1999). Levels of H_2 in the rumen depend on the amount of substrate fermented and type of VFA produced (mainly acetate, propionate and butyrate). Glycolysis, the primary pathway of glucose fermentation by rumen microbes, yields two equivalents of pyruvate and reduces two equivalents of NAD^+ to NADH. This NADH must be oxidized back to NAD^+ to maintain glycolysis. For thermodynamic reasons, this oxidation proceeds spontaneously at low concentrations of H_2 and is decreased or even ceases at higher concentrations of H_2. In other words, the H_2 concentration in the anaerobic environment dictates the redox state of the cofactor (Zhang et al., 2013). Rumen methanogens may increase CH_4 production when the H_2 concentration is increased to utilize the accumulated H_2 and, as a consequence, favor the re-oxidation of the reduced cofactor in bacterial cells. Based on the H_2 emission profile shown in Rooke et al. (2014), this condition may occur directly after a meal, in particular after one containing a large proportion of rapidly degradable carbohydrates. Various microbial species are still able to oxidize NADH by reducing intracellular metabolites to ethanol, lactate or succinate (Stams and Plugge, 2009). The formation of lactate and succinate, being precursors of propionate, explains why elevated levels of propionate are associated with increased levels of H_2 (Janssen, 2010; Van Lingen et al., 2016).

Janssen (2010) and Ungerfeld (2013) evaluated the thermodynamic effect of H_2 concentration on various fermentation pathways in terms of Gibbs energy change (ΔG). Negative values of ΔG indicate a reaction will proceed in the forward direction, positive values indicate the reverse direction, and $\Delta G = 0$ indicates equilibrium between the forward and reverse reactions. Janssen (2010) showed the ΔG of several fermentation pathways becomes less negative at increasing concentrations of H_2 and argued that an increase in H_2 concentration shifts fermentation away from H_2 releasing pathways, resulting in increased propionate production. Ungerfeld (2013) showed the ΔG of the acetate to propionate conversion to become more negative at increased levels of H_2. These thermodynamic investigations determine the energetic favorability of fermentation pathways at different H_2 partial pressures in the rumen. However, ΔG is not a direct measure of the rate at which a reaction takes place, nor does it provide a direct measure of the extent to which changed rumen conditions affect VFA production rates. A further aspect that deserves attention is microbial biosynthesis, and modeling rumen microbial metabolism under various conditions would benefit from investigations into energetic costs of various microbial (biosynthetic) functions, but it is not a focus of current rumen modeling work. Some modeling work has been undertaken to improve the accuracy of the prediction of rumen VFA profiles based on these thermodynamic principles.

Initially by Ungerfeld and Kohn (2006), and later by Ghimire et al. (2014), interconversion rates of VFA were predicted by combining general laws of reaction kinetics with reaction quotients of chemical equilibria. These approaches go beyond previous ones with a strictly empirical description derived from rumen observations (to be discussed in the next section). However, these new approaches might still not be fully consistent with both reaction kinetics and thermodynamic control of reaction rates. Thermodynamic control on a particular reaction pathway is assessed from equations that have been derived from kinetic rate laws, with stoichiometric coefficients used as exponent values of reactant concentrations in these equations. However, physical chemistry text books such as that of Atkins and de Paula (2006) indicate that experimentally determined exponent values instead of stoichiometric ones have to be used for reactant concentration in kinetic rate laws; thermodynamic control on reactions might be realistically assessed by correcting the kinetic rate laws for thermodynamic effects (Van Lingen et al., 2016).

Ghimire et al. (2014) fitted rate constants for VFA interconversion from *in vivo* observations of rumen VFA interconversion with control treatments that were expected not to affect thermodynamic control (i.e., a roughage diet and saline infusion), and assumed a constant partial pressure of H_2. They subsequently applied their fitted rate constants to predict VFA production for the other treatments which were expected to have a different thermodynamic control (i.e., a low roughage diet and intra-ruminal propionate infusion), and concluded that the model did not perform well in predicting ruminal VFA production rates due to lack of data on thermodynamic control factors other than pH and rumen VFA concentrations. Also, for an evaluation of thermodynamic control of rumen fermentation pathways, H_2 partial pressure is best varied, as opposed of being kept constant. Prediction inaccuracy may also result from some limitations in their quantitative approach. Bannink et al. (2006b), who estimated coefficients for VFA molar proportions using a fully empirical approach, recommended inclusion of the dynamics of cofactor redox state as a controlling factor of VFA production rate and VFA molar proportions (in non-steady-state conditions). Mostly NAD^+/NADH is considered, but for a more detailed discussion on this presumption the reader is referred to Van Lingen et al. (2016).

Offner and Sauvant (2006) introduced the dynamics of reduced cofactors in their model to predict end products of rumen fermentation, adopting a similar modeling approach as Ghimire et al. (2014). However, they chose their rate constants arbitrarily which might have caused unrealistically high (as high as 15) acetate to propionate ratios at certain moments when simulating the transient diurnal response. Better representation of thermodynamic controlled factors should improve the prediction of rumen VFA profiles by future mechanistic rumen models. This also holds for modeling CH_4 production, considering VFA production and associated H_2 are the main drivers for methanogens. However, this type of modeling work has rarely been reported. Also no examples are known of rumen models that describe the variation in microbial composition and biosynthetic functions (and consequences for metabolism; Hegarty and Gerdes, 1999) in response to rumen fermentation conditions, and its consequences for the activity of specific types or classes of rumen micro-organisms. Recently, Vetharaniam et al. (2015) introduced a representation of the dynamics of H_2 and methanogens (represented as a constant fraction of rumen microbial pool size) to facilitate future consideration of thermodynamic control of CH_4 production in their rumen model.

Fully Empirical Approaches

Since the early work of Koong et al. (1975) there have been numerous studies to derive the stoichiometry of rumen VFA production from rumen observations on substrate digestion and molar VFA proportions using empirical approaches (Murphy et al., 1982; Bannink et al., 2006b; Sveinbjörnsson et al., 2006; Nozière et al., 2011). Instead of using rumen VFA observations, Nagorcka et al. (2000) adopted a different approach by using microbiological data on VFA formed, for the first time separating VFA formed by bacteria (comparable to the other studies) from VFA with a relatively high amount of butyrate formed by protozoa. Nagorcka et al. (2000) reported an improved prediction of rumen VFA in lactating cows, heifers and growing cattle, compared to predictions obtained with the stoichiometry of Murphy et al. (1982) which was derived from steer observations. This outcome might partly be due to the stoichiometry of Murphy et al. (1982) being less applicable to the evaluation data set used by Nagorcka et al. (2000) due to differences in level of DM intake, diets and rumen fermentation conditions. The stoichiometry derived by Bannink et al. (2006b, 2008) was based strictly on lactating cow data resulting in VFA predictions that were shown by Morvay et al. (2011) to deviate substantially from those obtained using the stoichiometry of Murphy et al. (1982), and to deliver an improved prediction when evaluated against lactating cow data. This particular study evaluated the stoichiometries of Murphy et al. (1982), Argyle and Baldwin (1988), Friggens et al. (1998), Bannink et al. (2006b, 2008) and Sveinbjörnsson et al. (2006) against an independent data set for lactating cows. Predictive power for propionate and butyrate differed strongly among the alternative representations of VFA stoichiometry, and linear relationships between rumen pH and VFA production did not appear to improve prediction of VFA profile. Another evaluation study of enteric CH_4 in beef cattle by Ellis et al. (2014) confirmed that inclusion of linear pH-dependency in VFA stoichiometry worsened CH_4 predictions (reflecting VFA profile) whereas non-linear dependency did not. The calculations on the VFA stoichiometry of Nozière et al. (2011) were not available to Morvay et al. (2011), but were used in a follow-up evaluation study on lactating cow data by Alemu et al. (2011). This study showed that both the stoichiometries of Bannink et al. (2006b) and Nozière et al. (2011) outperformed those of Murphy et al. (1982) and Sveinbjörnsson et al. (2006) in predicting VFA molar proportions. The stoichiometry of Nagorcka et al. (2000) was recently evaluated by Gregorini et al. (2013) following enhanced representation of the digestive

elements in the model of Baldwin et al. (1987) by Hanigan et al. (2013). Prediction of CH_4 emissions did not improve however, which probably reflects VFA prediction did not improve either.

When additives known to modify the VFA profile of the rumen are included in the diet, the limitations of empirical approaches in predicting VFA (i.e., their lack of relation to microbial metabolism) become even more evident. As an example, with monensin there is typically a reduction in ruminal gram-positive bacteria population size and activity, and this shift in bacterial population results in an increase in propionate production. Ellis et al. (2012) modeled the change in VFA profile with monensin dose in feedlot beef cattle and showed improved VFA predictions with this modified VFA stoichiometry. Combining the equations derived by Ellis et al. (2012) to correct predicted VFA profile for the effect of monensin in beef cattle diets also improved CH_4 predictions when using pH-independent, but not when using pH-dependent VFA stoichiometry. However, the authors noted that the extent of VFA profile change will differ between dairy and feedlot beef cattle, which might be due to differences in the typical basal diet and rumen microbial population.

As discussed in the previous section, there is renewed interest in modeling the thermodynamic aspects of variation in VFA production empirically (Ghimire et al., 2014). So far, this does not appear to have improved our understanding of the variation in VFA stoichiometry, and mean squared prediction errors across studies typically remain around 10, 15, and 20% of observed proportions of acetate, propionate and butyrate, respectively. A lower concentration or molar proportion of individual VFA results in a higher mean squared prediction error, as model simulations by Bannink et al. (2006b) demonstrate. Although unclear which approach (mechanistic or fully empirical) unravels the driving factors of change in rumen VFA profile best, more elementary approaches to modeling metabolic aspects of rumen micro-organisms and thermodynamic control of their fermentation pathways (including the dynamics of cofactor redox state; Mosey, 1983) are welcome. Rumen pH is a less useful explanatory variable as it is likely not closely associated enough with these driving factors. If pursuing a fully empirical approach (i.e., based on rumen observational data only), future efforts would be best focused on introducing the effects of the intensity at which micro-organisms ferment substrates (reflecting reduced co-factor dynamics), with the effect of amount of substrate fermented as the main driver.

The Rumen Organ

Almost all rumen modeling efforts have been directed at representing the chemical and physical processes that take place in the lumen. However, lumen conditions in the host are closely regulated by exchange of water and solutes, rumen motility and outflow, and rumination (Baldwin, 1995). Thereby the ruminant host has a profound impact on rumen microbial activity. Fully integrated models of rumen metabolism that include the functions of organ tissues are currently lacking. Two aspects in particular deserve to be discussed because of their importance in regulating rumen fermentation conditions: urea-N recycling and VFA absorption.

Urea-N Recycling

Conservation of N through urea-N recycling by the kidney and by the rumen wall is an important mechanism for ruminants to prevent N from limiting microbial growth with low N intake (Reynolds and Kristensen, 2008). This mechanism is represented differently in static and dynamic rumen models. Use of both types of model to evaluate the effect of N supply on rumen metabolism is demonstrated by comparing outcomes of the dynamic model of Dijkstra et al. (1992) with a static protein evaluation system currently applied in practice (Van Duinkerken et al., 2011). Simulations were performed for an exchange of maize silage for grass silage in a dairy cow diet, resulting in a continuous decline in N supply (**Figure 4**). The response of the dynamic model shows urea recycling to become saturated at the highest level of maize silage inclusion (i.e., 11.9% crude protein in dietary DM; **Figures 4C,D**), whereas the static model shows a linear decline in rumen degradable protein balance (reaching an ill-advised negative value at 14.4% crude protein in dietary DM; **Figure 4C**). *In vivo* measurements confirm the merit of adopting a more dynamic approach as it demonstrates the limiting effect of N supply on rumen fiber digestion at similar crude protein contents in dietary DM. A crude protein level of 11.5% in maize silage diets was tested by Spek et al. (2013) and a level of 10.3% in grass herbage diets was tested by Warner et al. (2015), which in both studies resulted in a rumen ammonia concentration below 2 mM which is considered to be well below critical levels for ensuring optimal microbial activity (Dijkstra et al., 1998). The static model calculated a rumen protein balance of around zero at this low protein level (Spek et al., 2013), hence falling within recommendations.

VFA Absorption (Adaptation)

Maintaining rumen pH between narrow bounds is important for optimal fibrolytic activity. Regulatory mechanisms involve the buffering of rumen contents and removal of VFA (saliva secretion, VFA transport, VFA outflow). To our knowledge none of the published whole rumen models takes account of the changes in these buffering mechanisms with dietary and physiological transitions. *In vivo* studies with repeated measurements in transition cows reveal that the rumen wall adapts extremely quickly to changes in dietary regime through changes in morphology and histological characteristics (Bannink et al., 2012; Dieho et al., 2016). The faster of the two regimes for incrementing concentrate allowance after calving more than doubled the rate of VFA production as measured using the stable isotope technique, but the rumen wall epithelia appeared to adapt within 2–3 weeks. This adaptive response moderated the effect of the rapid increase in VFA load after calving to only modest changes in rumen VFA concentration and rumen pH (**Figure 5A**). Based on observed dynamics of rumen papillae size and changes in epithelial tissue, Bannink et al. (2008) proposed a model containing lumen, epithelial tissue and arterial blood compartments and described the rate of increase in rumen epithelial mass and surface area in response

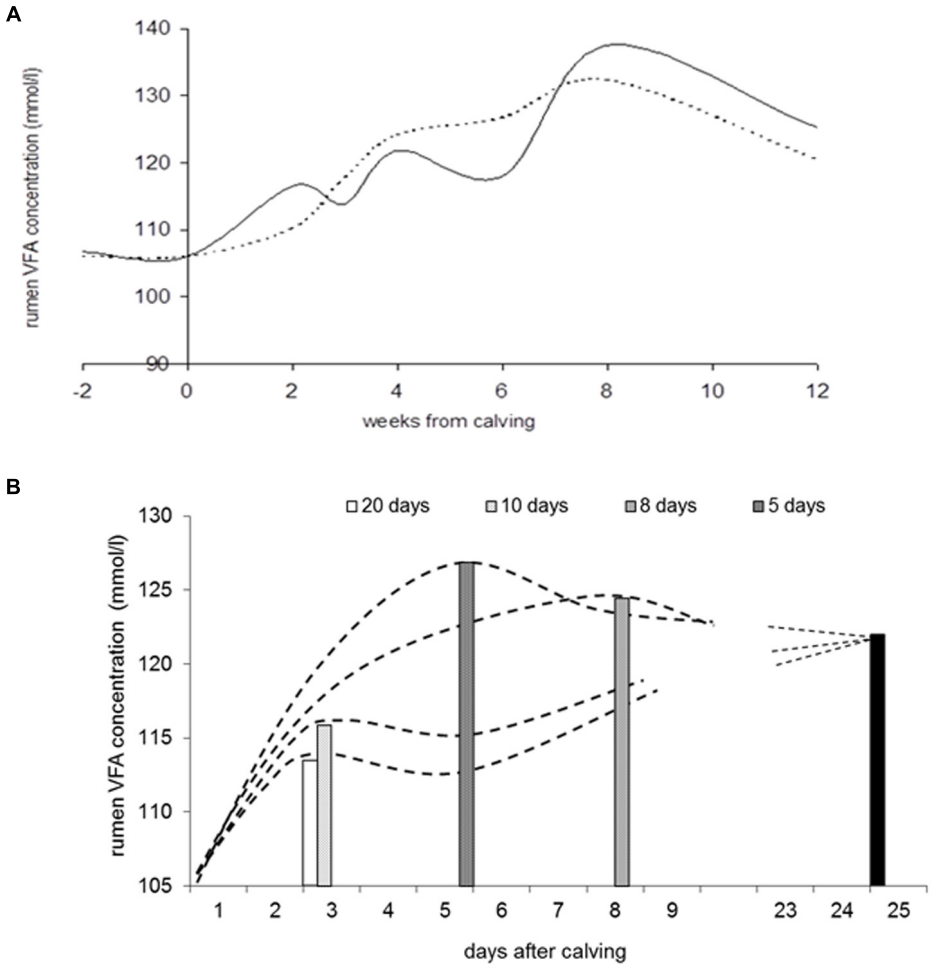

FIGURE 5 | Response in rumen VFA concentration with increment of concentrate allowance after calving (results reproduced from Bannink et al., 2012). (A) Observed development in rumen VFA concentration with two rates of daily increment in concentrate allowance after calving to a maximum of 12 kg concentrate DM/d (in 10 days, solid curve; in 20 days, dashed curve). **(B)** Predicted development in rumen VFA concentration when increasing concentrate allowance to a maximum of 12 kg DM/d in 5, 7.5, 10, or 20 days after calving. The model describes rumen VFA absorption, growth of epithelial mass and surface area, epithelial VFA metabolism and VFA transport to portal blood in response to rumen VFA production. The model was calibrated using the trial for which results are depicted in **(A)**, including estimated rumen VFA production rate and observations on rumen epithelial tissue development. Bars indicate the moment maximum VFA concentration is achieved during the various strategies of increment of concentrate allowance; the black bar indicates steady-state VFA concentration achieved at 25 days after calving.

to the increased VFA load epithelia are exposed to after calving. Predicted VFA absorption followed observed phenomena (e.g., López et al., 2003) when assuming a far higher VFA transport rate at the serosal side (reflecting extensive epithelia protrusions and higher VFA exchange capacity) compared to the mucosal side of the epithelia. In support of this, Storm et al. (2012) demonstrated a profound effect of epithelial blood flow on rumen VFA absorption rate by modeling observations of rumen VFA absorption and epithelial blood flow. They also concluded that properties of rumen epithelial membranes, epithelial metabolism and epithelial blood flow should be represented in future models of ruminal VFA kinetics. Furthermore, assuming epithelial adaptation as modeled by Bannink et al. (2012), prevention of an accumulation of VFA during the increment of concentrate allowance after calving was predicted for a wide range of strategies (**Figure 5B**). This result seems to contrast with the risk of development of (subclinical) rumen acidosis during early lactation due to a rumen wall that was not yet fully adapted. It invites further *in vivo* testing of hypotheses on the implications of rumen adaptive capacity on rumen function.

Engineering Rumen Metabolism

The ever increasing capacity to analyze detailed information on the rumen metabolome allows researchers to study rumen microbial metabolism in far more detail than ever before. Assignment of rumen function to rumen microbial genes is required to link the rumen microbiome to the nutritional and productive state of ruminants (Morgavi et al., 2013). Recently, Henderson et al. (2015) established a varying microbiome across

a wide range of host species and dietary conditions worldwide, showing a more prominent effect of diet than of host. Metabolic interactions also appeared non-selective rather than specific, indicating a high functional redundancy and flexibility of the rumen microbial community at the level of detail that was examined (Henderson et al., 2015). There is a gap, however, between the information at the level of the rumen microbiome (i.e., microbial diversity, abundancy and gene expression) and the level of rumen microbial metabolism represented in whole rumen models (microbial population present, enzymatic degrading capacity, microbial growth characteristics) that needs to be bridged. Many studies lack measurement of rumen functionality and give the rumen microbiome or metabolome as an ordinal response to a pre-selected set of conditions or animal phenotypes. McCann et al. (2014) discussed the apparent absence of an association between residual feed intake of cattle and the rumen microbiome because it is either too subtle to discern, or the microbiome is biologically less significant in explaining genetic diversity in this phenotypic trait, or this trait varies with diet and feeding strategy. It may also be that the degree of detail by which the microbiome is characterized is still inadequate. Ross et al. (2012) concluded a method of untargeted massively parallel sequencing can be applied to characterize the relationship between rumen microbial community metagenome and economically or environmentally important traits (e.g., feed conversion efficiency and methane emission), but with the important constraint that rumen samples are taken at the same time and cows are fed the same diet. On top of this, standardization of the rumen sampling procedure is prerequisite as this strongly affects results. Yáñez-Ruiz et al. (2015) reviewed the development of the rumen in the context of early life development of rumen function determining host-microbiome specificity at the adult stage. They concluded the rumen needs to be understood in terms of the interplay between anatomical/functional and microbial aspects during early life. Despite these host-microbiome specificities and possibility of microbiome programming, the diet remains the most important driver of the rumen microbiome at the adult stage (McCann et al., 2014; Henderson et al., 2015).

Methodological aspects are also important for analysis of the relationship between rumen microbiome and rumen or animal function. Jami et al. (2014) reported the ratio of rumen Firmicutes to Bacteroidetes explains 51% of variation in milk fat yield by dairy cows on the same dietary treatment, and suggested the rumen microbiome modulates milk composition. However, it remains unclear whether other factors than the rumen microbiome might explain such observations just as well; milk fat content and DM intake explained only very small proportions of observed variation in milk fat yield (15 and 3%, respectively). Furthermore, it is noted that analysis of a rumen sample does not directly reflect whole rumen function in terms of gene expression, enzymatic capacity, microbial growth, VFA production or methanogenesis. A rumen sample can only deliver a measurement of concentration, expression or activity in that particular sample of rumen digesta. Even when the sample is taken from evacuated rumen contents, and is therefore representative of whole rumen contents, the sample will still not deliver an estimate of the total quantity, expression or activity present in the rumen as a whole. In this respect, there is a clear parallel with VFA measurements in rumen fluid samples not being representative of treatment effects or VFA production rate (Hall et al., 2015). In a recent review, Weimer (2015) concluded that advances in determining community composition and diversity have outpaced the ability to factor out the physiological and ecological roles of individual phylotypes that impact rumen function and animal performance. A high redundancy, strong resilience and host specificity of the rumen microbiome (Weimer, 2015) complicates the delineation of these precise roles, however, which remains a major challenge at present. To go beyond details of the variation in the rumen microbiome, observations of important drivers of whole rumen function and rumen functionality must be made (Bannink et al., 2011; McCann et al., 2014; Weimer, 2015). As discussed in the present paper, likely candidate rumen traits to observe are rumen volume, rumen outflow, the rumen microbiome, rumen substrate degradation, rumen VFA profiles and rumen fermentation conditions (e.g., pH). Insight into the interplay between these factors, to a sufficient depth or precision to allow them to improve upon current model parameterization, is required to account for these factors in rumen models and to make goal-directed use of variation in microbiome and in rumen functionality. It may, however, prove to be very difficult to gather all such details in conjunction with data on the rumen microbiome, and furthermore it puts stringent constraints on experimental designs.

CONCLUSION

Use of mathematical models to advance our understanding of rumen metabolism requires appraisal of (i) the representation of metabolic factors that govern microbial metabolism and associated fermentation pathways, (ii) the diversity of functional classes or unique niches for micro-organisms needing to be distinguished, and (iii) the rumen functioning as an organ which includes the physiological mechanisms that regulate the rumen environment and exchanges with the ruminant host. Depending on the modeling aim, there is merit in representing the dynamic aspects of interactions between substrate degradation and microbial metabolism, and of interactions between the different functional classes of micro-organisms. The present review demonstrates how such dynamical aspects need to be represented in rumen models to explain observed phenomena in rumen metabolism. These phenomena include those observed with changes in feeding frequency and rumen fluid dynamics, changes in the profile of VFA produced, the regulatory mechanisms involved with N exchange between host and the rumen on low protein diets, and adaptation processes in rumen wall tissues to regulate rumen fermentation conditions.

Areas requiring more attention are the representation of (i) rumen fluid (and particle) dynamics (i.e., delineation

of the processes contributing to fluid dynamics), (ii) the profile of VFA produced (i.e., representation of the impact of microbial metabolism on VFA profile), (iii) clearance of VFA from the rumen environment (i.e., representation of rumen VFA absorption capacity, including adaptive response of the rumen wall), (iv) the functional classes of micro-organisms needing to be distinguished. Emphasis should be placed on translating outcomes of rumen research in functional terms that can be integrated with efforts to model whole rumen function. These terms include substrate degradation rate, microbial metabolic costs for maintenance and synthetic purposes, composition of microbial mass, shifts in fermentation pathways, and interactions between classes of micro-organisms that one needs to distinguish from the perspective of their differing ecological roles in the rumen.

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All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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